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Control of NF- κ B DNA binding

by

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A Thesis presented for the Degree of
Doctor of Philosophy
in
The Faculty of Science
at
University of St Andrews

September 2000



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ABSTRACT

Many genes controlling cell cycle, immune responses, inflammation etc are regulated by the activation of NF- κ B/Rel transcription factors. NF- κ B is kept as an inactive cytoplasmic form due to its binding to I κ B, and is induced by a series of extracellular stimuli. The mechanism of activation leads to the nuclear translocation of NF- κ B, DNA binding and transactivation.

The objectives of this work were to understand the molecular interactions which lead to the formation of NF- κ B•DNA and NF- κ B•I κ B complexes, as well as to clarify the role of the controversial I κ B molecule Bcl-3 in NF- κ B driven transcription. More precisely, it was achieved to identify residues that play critical role in the stabilisation of the complexes mentioned above, whose role was not revealed by crystallographic analyses.

A few independent lines of evidence lead us to investigate the hypothesis that a lysine residue, conserved among all NF- κ B molecules and located in the tip of the C-terminal part of NF- κ B p50 L1 loop, could interact with the phosphate backbone of the DNA target and with the C-terminus of the ankyrin repeat domain of I κ B γ .

For that reason, 6 p50 mutants were constructed where the three neighbouring lysine residues (K77, K79 and K80) of L1 loop C-terminus were mutated to alanines. The binding activity of the wild type and the mutants was tested by Surface Plasmon Resonance analysis. What was revealed is that K80 is critical for the interaction of p50 with both its DNA target and its protein inhibitor I κ B γ . K80 stabilises p50•DNA complex and together with K77, it interacts with I κ B γ leading to the dissociation of the p50•DNA complex.

The mechanisms of NF- κ B•I κ B binding and those which play a role in the inhibition of NF- κ B DNA binding were also investigated. We demonstrated that Bcl-3 effectively interacts with p50 through p50 NLS, that the ankyrin repeat 6 of I κ B α effectively interacts with p65 and that p50 K77 and K80 interact with I κ B γ . Finally, we showed how Bcl-3 can repress/co-activate via p50 in a DNA sequence specific way and we proposed a mechanism for I κ B-driven NF- κ B DNA binding inhibition.

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I am thankful to my fiancée Christianna Kargadouri, my sister Katerina Michalopoulou and my friend Dr Harris Mantzaridis for their invaluable moral support. Finally, thanks are also due to parents to whom I owe everything.

ABBREVIATIONS

ANK	ankyrin repeat
ARD	ankyrin repeat domain
ATP	adenosine 5'-triphosphate
bp	base-pair
BSA	bovine serum albumin
C-	carboxy-
°C	Celsius degrees
Ci	Curie
CTP	cytidine 5'-triphosphate
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddATP	2',3'-dideoxyadenosine 5'-triphosphate
ddCTP	2',3'-dideoxycytidine 5'-triphosphate
ddGTP	2',3'-dideoxyguanosine 5'-triphosphate
ddTTP	2',3'-dideoxythymidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
g	gramme
G-MEM	Glasgow modified Earle's minimal essential medium
GTP	guanosine 5'-triphosphate
h	hour
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
HIV-1	human immunodeficiency virus type 1

I κ B	inhibitor κ B
IL-1	interleukin 1
IL-2	interleukin 2
IPTG	isopropyl β -D-thiogalactopyranoside
IRE	interferon response element
IRAK	interleukin-1 receptor associated protein
Da	dalton
KOAc	potassium acetate
l	litre
LIF	lymphocyte inhibitory factor
LPS	lipopolysaccharides
LTR	long terminal repeat
M	molar
MHC	major histocompatibility complex
min	minute
MOPS	3-(N-morpholino) propanesulphonic acid
mRNA	messenger ribonucleic acid
N-	amino-
NRD	NF- κ B/Rel/Dorsal
NF- κ B	nuclear factor κ B
NLS	nuclear localisation signal
Nonidet P40	octylphenoxypolyethoxyethanol
O/N	overnight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline type A
PKC	phosphokinase C
PMSF	phenylmethylsulphonyl fluoride
RHR	rel homology region
RNA	ribonucleic acid

RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
ss	single strand
STE	sodium chloride, Tris-HCl, EDTA buffer
TBE	Tris-H ₃ BO ₃ , EDTA buffer
TE	Tris-HCl, EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TNF α	tumour necrosis factor α
Tris	tris (hydroxymethyl) aminomethane
TTP	thymidine 5'-triphosphate
U	unit
v/v	volume to volume ratio

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1 INTRODUCTION

1.1 Eukaryotic transcription

In eukaryotes, the transcription of the nuclear genes is catalysed by three distinct DNA-dependent RNA polymerases. RNA pol I is sequestered in the nucleoli and transcribes ribosomal RNA (rRNA) which is constitutive part of the 40S and 60S subunits of the ribosomes, RNA pol II transcribes heterogeneous nuclear RNA (hnRNA) which is subsequently processed to messenger RNA (mRNA) which codes for the amino acid sequences of the proteins, and RNA pol III catalyses the synthesis of transfer RNA (tRNA) which is the carrier molecule of the activated amino acids in protein synthesis (Kedinger et al., 1970; Roeder and Rutter, 1969; Sklar et al., 1975). None of these three enzymes can directly recognise its class promoters (the DNA sequences which determine the starting point and the rate of transcription). Instead, the RNA polymerases rely on general transcription factors (Conaway and Conaway, 1993; Conaway and Conaway, 1997) and DNA sequence specific transcriptional activators.

Protein-coding genes are transcribed by RNA pol II (Reinberg et al., 1998; Roeder, 1998). Gene expression can be regulated in many levels: Transcription, RNA splicing (Hodges and Bernstein, 1994), RNA stability (reviewed in Ross, 1996), translation (Kaufman, 1994) or post-translationally. The most important stage of the regulation of gene expression is the first level (transcription). The steps of transcription are: initiation, elongation and finally termination. The critical step in transcription is the first one (initiation). The three main phases of transcriptional initiation are: assembly of the preinitiation complex (the reversible association to promoter DNA, of the multiprotein complex containing RNA pol II, transcription factors and transcription activators), isomerisation (irreversible unwinding of the DNA sequences proximal to the transcriptional initiation site) and promoter clearance (where RNA pol II leaves the promoter, starting the elongation of the transcript). Once more, the first phase is the critical one (preinitiation complex assembly). The preinitiation complex contains

the basal transcription unit. This consists of RNA pol II and several basal transcription factors which recognise core promoter elements. The basal transcription unit interacts with *trans*-acting protein factors which recognise distant to promoters DNA sequences (enhancers) for efficient transcription. Absence of these transactivators results in minimal level transcription. Thus, the transactivators play the most important role in gene expression. That is why a lot of research is focused on the assembly of the preinitiation complex and especially the role of transcription activators on the stabilisation of these complexes.

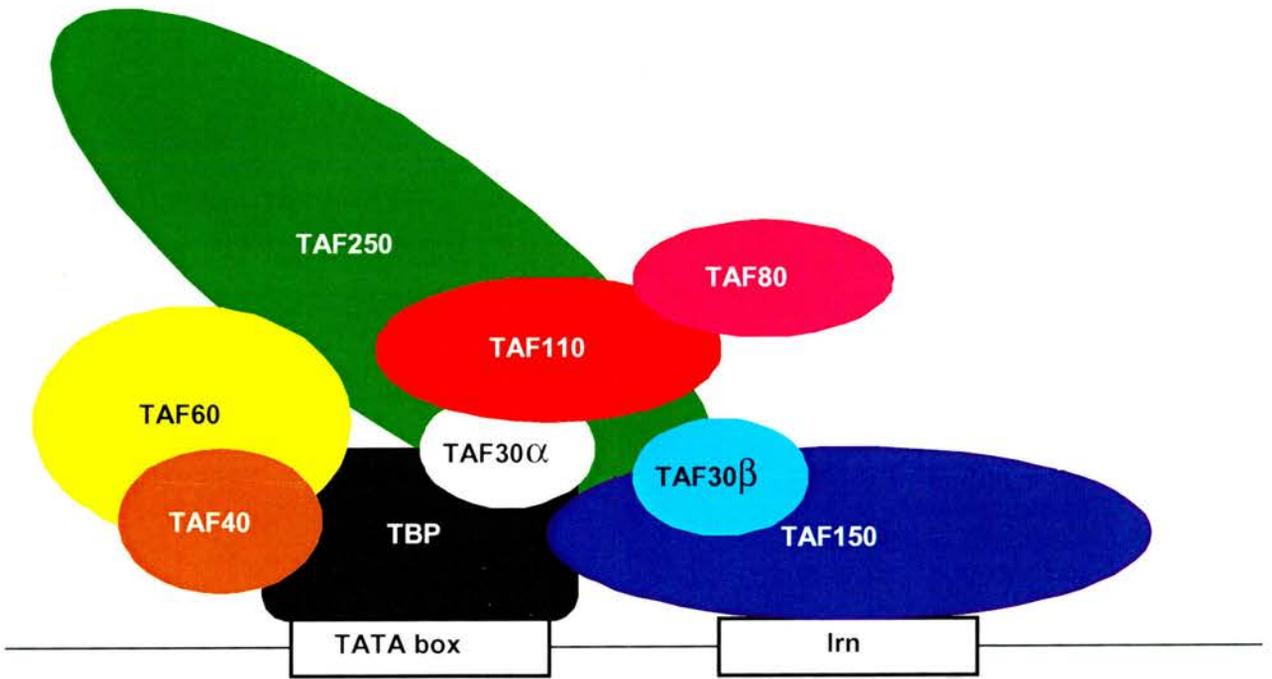
1.1.1 Basal transcription unit

The components of the protein-DNA complex of the basal transcription unit are biochemically characterised, cloned and in some cases structurally solved (Patikoglou and Burley, 1997). The general initiation factors which can be comprised of multiple subunits, are TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH and are assembled in a specific order to form the preinitiation complex (Orphanides et al., 1996).

The most important DNA sequence elements that function independently or synergistically as determinants for the preinitiation assembly in RNA pol II promoters are the TATA box, an A+T rich DNA sequence with consensus 5'-TATAWAWA-3' (where W is either A or T), located 25 bp upstream of the transcription start site, and the pyrimidine-rich initiator (Inr), a DNA sequence with consensus 5'-Y⁻²Y⁻¹A⁺¹N⁺²W⁺³Y⁺⁴Y⁺⁵-3' (where Y is pyrimidine, W is either A or T, N is any nucleotide and numbering is from the start site) (Smale and Baltimore, 1989; Weis and Reinberg, 1992). Promoter sequence analysis has shown that promoters may have both these two elements, only one of them or even none of them.

The only transcription factor which possesses sequence specific DNA binding activity is TFIID (Shen and Green, 1997). TFIID complex is comprised of a TATA box-binding protein (TBP) subunit and a series of TBP-associated factors (TAFs) (Burley and Roeder, 1996) (Figure 1). TBP is responsible for the specific recognition of TATA box sequence by TFIID. TAFs bind TBP directly (TAF30 α , TAF40, TAF60, TAF150, and TAF250) or indirectly (TAF30 β , TAF80, and TAF110) via contacts

Figure 1: TFIID complex: The transcription factor TFIID is responsible for the recognition of the promoter. TATA binding protein (TBP) binds to TATA box, while TBP associated factors (TAFs) interact with TBP and/or other TAFs. TAF150 binds to Inr sequence, contributing to the specificity of TFIID binding.

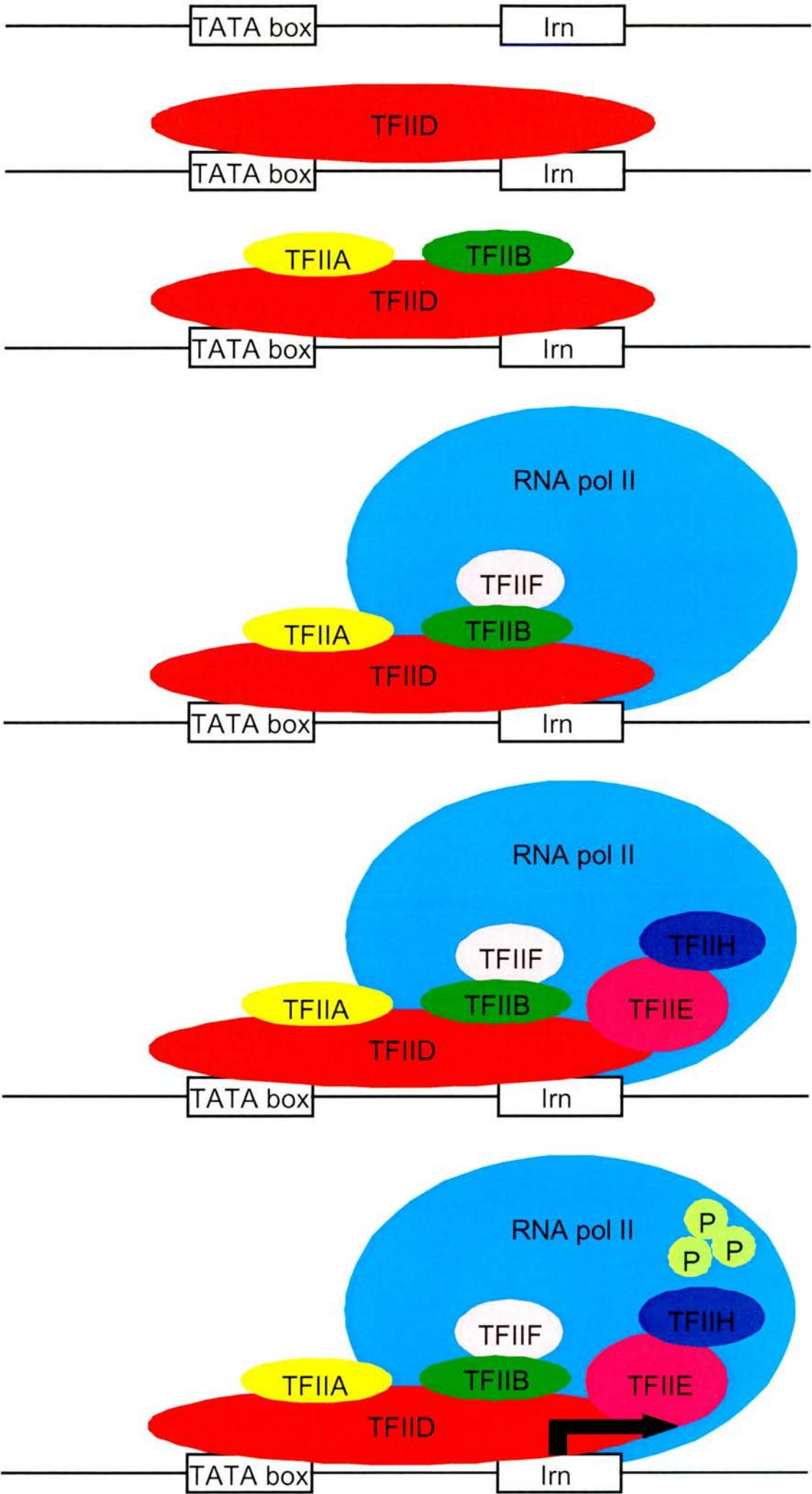


with other TAFs. TAF150 is responsible for the recognition of Inr in the presence or absence of a discernible TATA box. The structure of some TBPs bound to TATA boxes is now solved. These studies have revealed that TBP binds the minor groove and the phosphoribose backbone of TATA box and induces a strong DNA bending (Burley, 1998; Leuther et al., 1996; Patikoglou et al., 1999). The fact that the TATA consensus has the minimal amount of total hydrogen bonds between complementary bases facilitates both the minor groove contact and the distortion of the double helix.

After TFIID (TBP) recognition of TATA box, the next transcriptional factors which bind the complex are TFIIA and TFIIB (TFIIA might bind at any stage of the assembly, after TBP recognition). Structural studies of the TFIIA•TBP•DNA (Nikolov et al., 1995) and TFIIB•TBP•DNA (Geiger et al., 1996; Tan et al., 1996) complexes, enabled the building of a possible model of the TFIIA•TFIIB•TBP•DNA (Patikoglou and Burley, 1997), where both TFIIA and TFIIB recognise the preformed TBP•TATA box complex, by binding the N- and C-terminal tails of TBP and the phosphoribose backbone of the distorted double helix of the TATA box.

The TFIIA•TFIIB•TFIID•DNA is then recognised by a complex of RNA pol II and TFIIF (pol/F). TFIIF functions as a precise spacer/bridge between TBP and RNA pol II which determines the transcription start site (Li et al., 1994; Pinto et al., 1994). In the presence of nucleoside triphosphates, the preinitiation complex is finally assembled, when TFIIE and TFIIH are recruited. The recruitment of TFIIE and TFIIH signals critical events for the initiation of transcription: DNA strand separation in the start site forming, in the presence of NTPs, an open complex (isomerisation), and hyperphosphorylation of the C-terminus of the large subunit of RNA pol II, which causes the initiation of transcription and the release of the RNA pol II from the promoter (promoter clearance) (Figure 2). During elongation, TFIID remains bound to the promoter in order to support the reassembling of a new preinitiation complex which will lead to rapid reinitiation of transcription (Zawel et al., 1995).

Figure 2: Formation of the preinitiation complex (PIC): TFIID recognises the promoter. Once TFIID binds to the promoter, TFIIA and TFIIB bind to it. This complex is then recognised by a complex of RNA pol II and TFIIF and consequently TFIIIE and TFIIH are recruited, to signal RNA pol II hyperphosphorylation which initiates transcription.

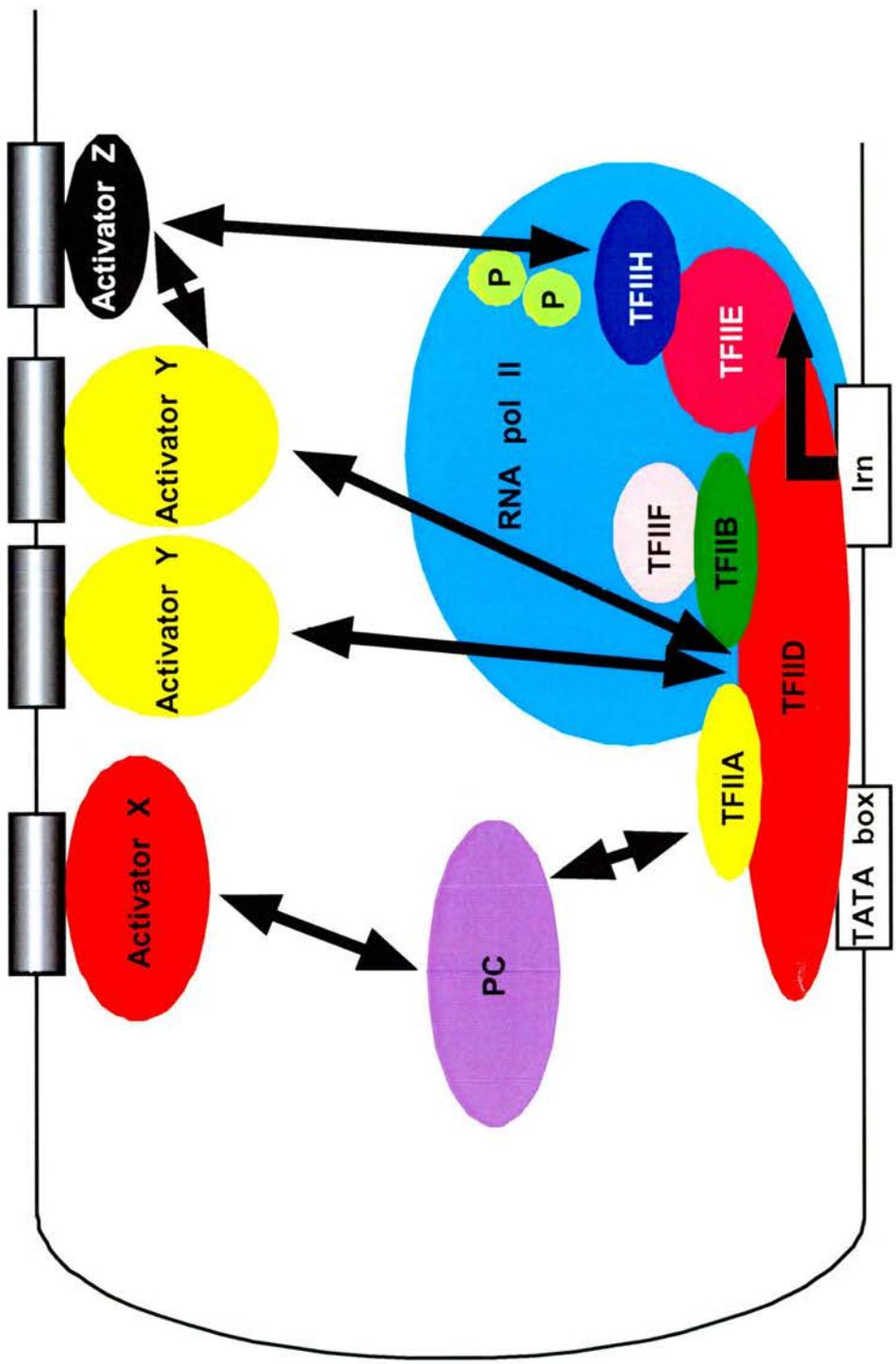


1.1.2 Transactivators/Co-activators/Repressors

Transcriptional activators (*trans*-activators) are proteins which specifically bind DNA in distal sites from the transcription initiation site. They stimulate transcription by enhancing the transcriptional initiation rate. The exact mechanisms are yet to be clarified, but we know that they involve favourable interactions between transactivators and members of the preinitiation complex (TBP, TAFs, TFIIB, TFIID, TFIIF) both directly and indirectly (via co-activators, i.e. proteins which serve as molecular bridges between transactivators and the components of the machinery of basal transcription) (Bagga et al., 1998; Blackwood and Kadonaga, 1998). On the other hand, other sequence specific DNA binding proteins called repressors, inhibit transcription by interacting with the preinitiation complex.

There are a lot of target surfaces for transactivators, co-activators and repressors in the preinitiation complex. The interactions of the repressors with the components of the basal machinery may lead to inhibition of its assembly or its function. Transactivators interacting with the proteins of the basal complex, may cause displacement of repressor proteins or nucleosomes. That refutes the inhibitory role of the repressors and enables these transactivators or other proteins to bind crucial for transcription DNA sites that were previously occupied by the repressors. Moreover, the interaction with the constitutive parts of the basal transcriptional machinery may induce favourable conformational changes or other modifications to them which could also introduce novel or altered interactions between these components. All these interactions can stabilise and/or enhance the rate of the formation of the preinitiation complex. The result of this multistep process is the enhancement of transcriptional activity. The effect of the various transactivators on transcription is synergistic (i.e. the level of transcription caused by the simultaneous action of all transactivators is higher than the sum of the transcriptional levels caused by the action of all the transactivators individually). This co-operative effect is possible because the transactivators which participate in a promoter/enhancer region may have distinct targets in the basal machinery (e.g. TFIIB, TBP, TAF40, TFIID, PC4, etc) (Figure 3).

Figure 3: The synergistic effect of various transactivators on transcription because the transactivators which recognise a promoter/enhancer region may have distinct targets in the basal machinery (general transcription factors, co-factors, etc).



1.1.3 Mediator

Recently, the role of the Mediator polypeptide complex as a common in all eukaryotes important interface between activators and RNA pol II, was revealed (Björklund and Kim, 1996; Flanagan et al., 1991). The mediator complex transduces regulatory information from enhancers to promoters (Kornberg, 1999). The human homologue of mediator which was initially found in *Saccharomyces cerevisiae*, is an 1.5 MDa complex termed Srb and Med-containing cofactor complex (SMCC) or thyroid hormone receptor-associated protein (TRAP) (Ito et al., 1999). Electron microscopy showed that addition of the core RNA pol II induces a conformational change in the mediator complex, which then acquires a crescent-like shape, to cover a large portion of the polymerase (Figure 5).

1.2 Chromatin

When we study eukaryotic transcription and transcription factors, we should always keep in mind that the substrate of transcription is not a naked DNA molecule. Instead, eukaryotic DNA is packed into chromatin, which acts as a repressor of transcription. The basic repeating unit of chromatin is the nucleosome which is comprised of an 146 bp DNA superhelix around a histone protein octamer. The structural and functional analysis of nucleosome particles and the discovery that acetyltransferases and deacetylases act as transcriptional coactivators and corepressors respectively, demonstrated that the nucleosome was not only a structure necessary for packing of chromatin but also, an integral part of the dynamic mechanisms of the regulation of gene expression.

1.2.1 Nucleosome structure

1.65 turns of stretched DNA superhelix is wrapped around an octameric histone protein core. This DNA•protein complex consists of 2 molecules of histones H2A, H2B, H3 and H4, and 145-147 bp of DNA and is known as the nucleosome core particle. Millions of nucleosome particles participate in genome condensation, since the length of human DNA is 1.74 cm. Chromatin, as it was revealed by electron microscopy, has a "worry-beads"-structure. Repeating nucleosome core particles, seen by electron microscopy as dots, are linked by DNA strands, seen as curved lines. These short stretches of DNA between consecutive core particles have variable length and are known as linker DNA. In most nucleosomes, this linker DNA is associated with one molecule of a linker histone H1 (or its variant H5). A nucleosome consists of a nucleosome core particle (the histone octamer wrapped by DNA) and a molecule of linker histone bound to linker DNA.

The crystal structure of the core particle revealed the mechanisms of histone octamer assembly and DNA wrapping (Luger et al., 1997) (Figure 4). A tetramer which consists of a stable complex of two H3•H4 heterodimers, is bound to two distinct H2A•H2B heterodimers. The protein architectural motif of the H3•H4 and H2A•H2B

heterodimers is common and is known as histone fold, and consists of a structured C-terminal domain (3 helices connected with loops) and an unstructured positively charged N-terminal tail (H2A has also a C-terminal basic tail domain). All the histone-fold domains organise 121 bp of DNA in total: Each heterodimer (H3•H4 and H2A•H2B) is associated with 27-28 bp of DNA, with 4 bp unbound linker DNA between them. Histones predominantly interact with the phosphate backbone of DNA. The average diameter of the DNA superhelix is 41.8 Å.

1.2.2 Nucleosome transcription

Although nucleosome core particles are to some extent obstacles of RNA polymerases, these enzymes can still transcribe through these particles, since absolute displacement of the nucleosomes is not required for transcription. The mechanisms that are necessary for the assembly of the preinitiation complex and the transcript elongation in the presence of nucleosomes are yet to be clarified. What is generally accepted is that RNA pol II can not initiate transcription, when a promoter is inside a nucleosome, but it can elongate the transcript through the nucleosome with high efficiency, leading to the production of full length transcripts, when the promoter is out of the nucleosomes (Felsenfeld, 1996; Kornberg and Lorch, 1995). The ability of RNA pol II to traverse through nucleosomes easily, is based on a series of spontaneous uncoiling events which occur on DNA. Transcriptional elongation accumulates positive DNA supercoiling in front of RNA pol II and negative supercoiling behind it. Positive DNA supercoiling destabilises the nucleosome, whereas negative one stabilises it (Clark and Felsenfeld, 1991; Liu and Wang, 1987). That favours a transfer of the nucleosome from ahead of to behind the RNA pol II (Studitsky et al., 1994; Studitsky et al., 1997; reviewed in Widom, 1997).

1.2.2.1 DNA sequence/nucleosome dependent gene regulation

Previously we have discussed how DNA sequences can regulate the gene expression, by recruiting transcription factors, activators, coactivators or repressors that modulate the rate of the assembly of the preinitiation complex. There is another way DNA

Figure 4: Nucleosome core particle structure: Totally 146 bp are wrapped around H2A, H2B, H3 and H4 histone octamer. The serpentine structures are the histone N- and C-terminal tails.

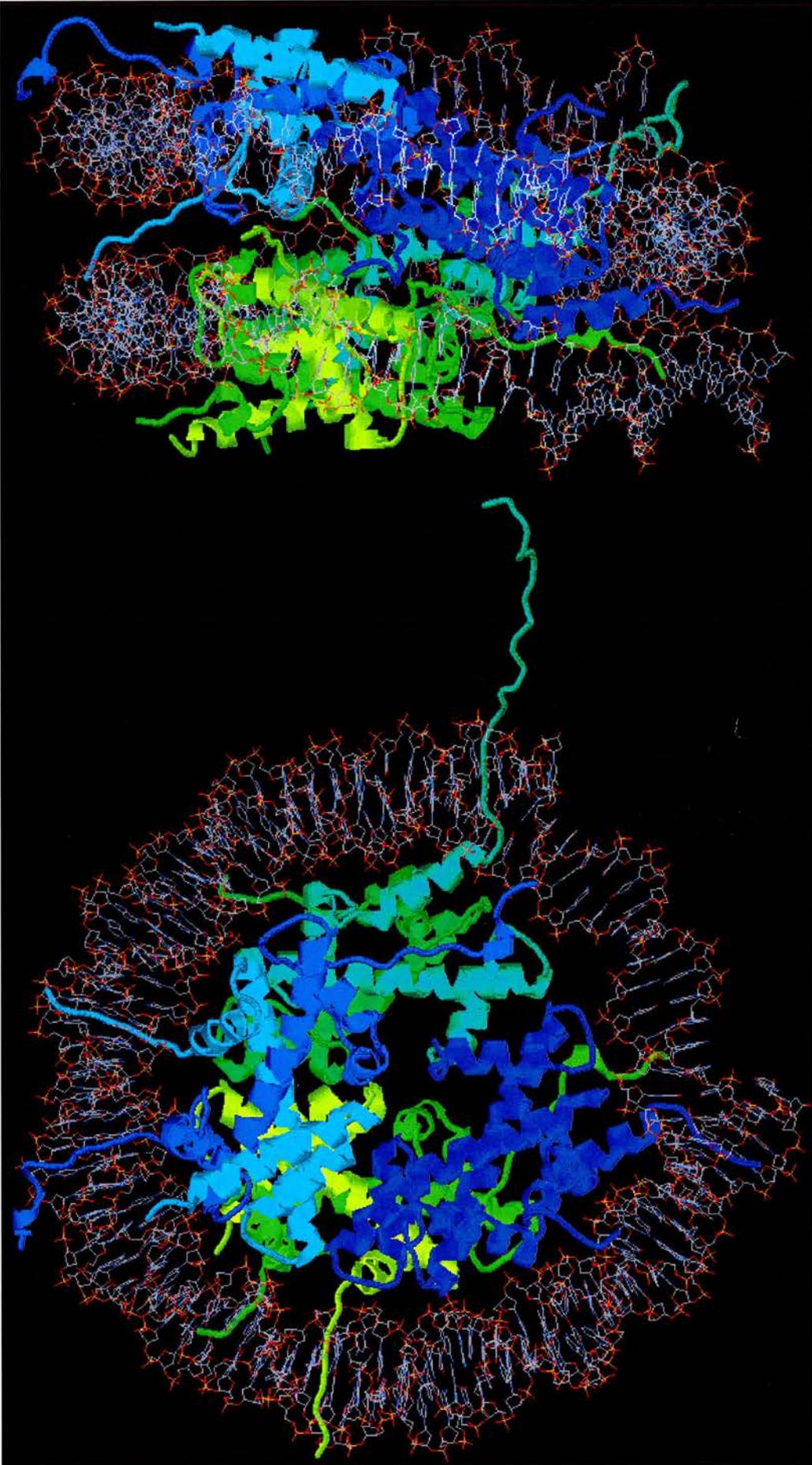
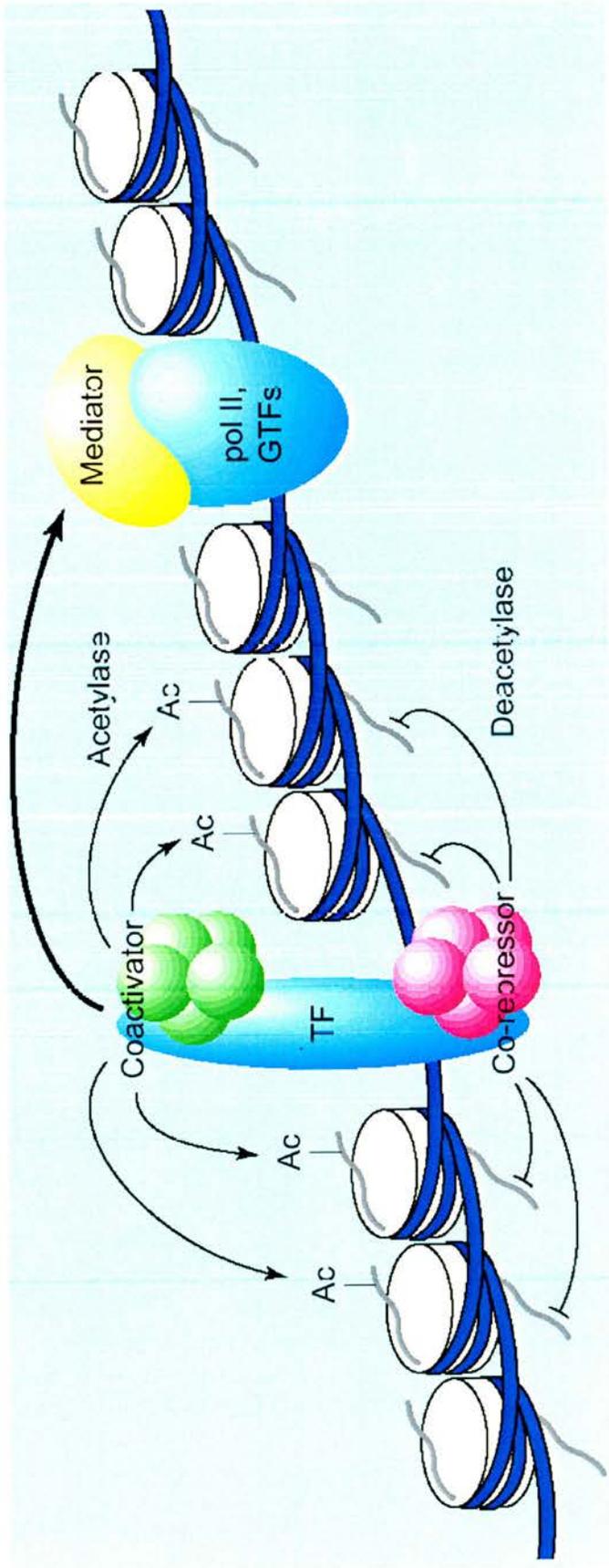


Figure 5: Dual mode of action of a transcription factor (TF which is either an activator or a repressor) in eukaryotic transcription: TF is bound on a regulatory element. An activator induces transcription by associating with a coactivator that recruits an acetylase to acetylate the histone tail and by directly interacting with the Mediator which is bound to the complex between RNA pol II (pol II) and the general transcription factors (GTFs). A repressor associates with a corepressor, which recruits a histone deacetylase (Kornberg, 1999).



sequences can regulate gene expression, exploiting the properties of nucleosomes to repress transcription.

Nucleosomes do not bind to DNA in a precisely sequence specific way, as transcription factors do, but they can have strongly biased positioning caused by some combination of forces dependent on the DNA sequence. So, the nucleosome biases the time-averaged accessibility of regulatory sites, greatly contributing to gene regulation.

Moreover, a 15-30 bp polypurine sequence found in many promoters, seems to contribute to gene expression almost as much as the gene activating proteins (Iyer and Struhl, 1995). This element is normally located close to a binding site of a protein essential for gene expression. A nucleosome is averagely positioned so that the binding site of the regulatory protein is in the middle of it, dramatically reducing the accessibility of the binding site. The polypurine element which is in close proximity to the transactivator binding site, increases the accessibility of the adjacent regulatory sequence, by a mechanism based on the free energy of the interactions between histones and different DNA sequences.

1.2.2.2 Histone acetylation/deacetylation & chromatin remodelling

The reversible acetylation of the ϵ -amino groups of lysine residues of the N-terminal tails of the core histones H3 and H4 neutralises the positive charges of these residues. In general, this hyperacetylation results in more transcriptionally active chromatin. It was initially hypothesised that acetylation could weaken the electrostatic interactions between the histone and the DNA backbone, reducing the affinity of histones for DNA. According to that model, acetylation-mediated activation was caused by the higher accessibility of transcriptional activators and the basal transcription machinery to the DNA area that is covered by the loose nucleosome (Grunstein, 1997). Nevertheless, the uncovering of the nucleosome structure showed that the positively charged N-terminal tails of the core histones extend unstructured from the core nucleosomal particle without interacting with DNA (Luger et al., 1997), thus, these

tails do not contribute to the organisation or stability of the nucleosome. Furthermore, the structural analysis revealed that hypoacetylated histone tails mediate contacts between adjacent nucleosomes. Therefore, acetylation is likely to affect higher order repressive chromatin structure. A possible scenario is that the N-terminal tails directly participate in the binding of certain transcriptional activators (Lee et al., 1993; Vettese-Dadey et al., 1996) or chromatin-remodelling complexes (Georgel et al., 1997) (Figure 5).

The enzymes that catalyse the acetylation of the histones are known as histone acetyltransferases (HATs). Many of them are transcriptional coactivators. TAFII250 (reviewed in Mizzen et al., 1996), p300/CBP (Ogryzko et al., 1996), P/CAF (p300/CBP-associates protein) (Yang et al., 1996), and ACTR (Chen et al., 1997), are known HATs. Interestingly, the last three HATs all interact with each other (Chen et al., 1997). Even if it is still unclear why multiple HATs are required, there is evidence that P/CAF is crucial for p300/CBP-dependent gene activation. Furthermore, although the substrate specificity of p300 is far broader than that of P/CAF (P/CAF acetylates H3 K14 and H4 K8, while p300 acetylates H2A K5, H2B K5, K12, K15 and K20, H3 K14, K18 and K23 and H4 K5, K8 and K12 (Schiltz et al., 1999)), it is P/CAF acetylase activity, and not that of p300, that is required for myogenic transcription and differentiation (Puri et al., 1997). Therefore, additional mechanisms, other than acetylation are probably involved.

The enzymes that deacetylate histones are called histone deacetylases (HDACs) and they are active components of transcriptional corepressor complexes (Ayer, 1999). There are at least three classes of HDACs. These molecules require additional factors to *in vivo* deacetylate histones in complex chromatin structures (Emiliani et al., 1998; Hassig et al., 1998; Zhang et al., 1998b). Therefore, they are enzymatically active components of multiprotein complexes. Mammalian HDAC1 and HDAC2 are found in mSin3A and NuRD complex. mSin3A was initially identified as part of the DNA-binding heterodimeric repressor Mad-Max (Ayer et al., 1995; Schreiber-Agus et al., 1995). HDAC1 and HDAC2 are stably associated with mSin3A and their enzymatic

activity is necessary for full transcriptional repression by Mad-Max (Hassig et al., 1997; Laherty et al., 1997; Zhang et al., 1997). That suggests the model of “targeted repression” where a HDAC complex is tethered to the promoter by the transcriptional repressor. Other corepressors mSin3-HDAC complex interacts with are N-CoR and SMRT which were initially identified as corepressors for unliganded nuclear receptors (Horlein et al., 1995). HDAC1/HDAC2 are also components of another distinct complex, the NuRD complex (Tong et al., 1998; Wade et al., 1998; Xue et al., 1998; Zhang et al., 1998a).

Additional to histone hypoacetylation, DNA methylation is also related to transcriptional silencing, e.g. the Barr body (inactive X chromosome of female mammals) is both hypoacetylated and hypermethylated (Jeppesen and Turner, 1993; reviewed in Riggs and Pfeifer, 1992). MeCP2, which specifically recognises methylated CpG dinucleotides, functions as a potent repressor. Its DNA binding and its repression domain are distinct. MeCP2 associates with Sin3A and HDAC in a very similar way Mad tethers these molecules to DNA (Nan et al., 1997). Therefore, hereditary methylation-dependent transcriptional repression is Sin3A/HDAC-dependent (Ng and Bird, 1999).

1.3 NF- κ B

1.3.1 *Why NF- κ B?*

Nuclear Factor κ B (NF- κ B) is a transcription factor. Although it was discovered in 1986, its importance was acknowledged in the beginning of the last decade. Only in 1991, the publications related to NF- κ B were more than all the previous publications on this issue from the day of its discovery. Since 1991, the annual rate of publications related to NF- κ B increases literally exponentially. Around 10,000 NF- κ B relevant studies were published in total so far, while the current rate is around 5 publications per day. The reasons of the increasing interest of the international scientific community on NF- κ B are:

- As a transcription factor, NF- κ B plays a pivotal role in a plethora of physiological processes, and involves in a series of pathological conditions.
- There are quite a few NF- κ B molecules with similar but not identical properties. There are also a few similar inhibitor proteins of the NF- κ B molecules which demonstrate different preferences and modes of inhibitory action.
- A lot of different stimuli trigger the NF- κ B signal cascade through a variety of cellular and viral converging mechanisms and molecules.
- There is a great deal of different approaches of the study of NF- κ B.
- The therapeutic role of NF- κ B is very important, as it offers many potential targets for intervention in the mechanisms which cause many diseases.

1.3.2 *NF- κ B*

NF- κ B is a DNA binding protein dimer which can act as a transcriptional activator. It was originally identified as a protein that bound to a specific decameric DNA sequence (5'-GGGACTTCC-3') within the intronic enhancer of the immunoglobulin κ light chain in mature B- and plasma cells, but not pre-B cells. Thus, it was hypothesised that it was a tissue-specific enhancer-binding protein involved in the activation of κ light-chain gene expression in mature B lymphocytes (Sen and Baltimore, 1986a). However, further studies demonstrated that NF- κ B is a ubiquitous

transcriptional factor whose DNA binding activity can be induced in cell types other than B-cells, in response to a variety of exogenous stimuli, such as inflammatory cytokines (TNF α and IL-1), lipopolysaccharides (LPS) and phorbol esters (PMA) by a mechanism that is independent of *de novo* protein synthesis (Sen and Baltimore, 1986b), suggesting the existence of an inactive form in the pre-B cells.

Before the structural analysis of NF- κ B which revealed its mechanisms of DNA binding, it was hypothesised that its binding sites (termed κ B sites) have an approximately 10-bp consensus sequence (5'-GGGRNNYYCC-3') (where R is purine and Y is pyrimidine) (Kunsch et al., 1992; Parry and Mackman, 1994). κ B sites are found in a variety of promoter/enhancer regions of more than 60 cellular and viral genes such as the regulatory sequences of Ig κ , cytokines, cytokine receptors, c-Myc, and HIV-1. These genes are involved in the regulation of acute-phase response, inflammation, apoptosis, and cell growth or differentiation (Lenardo and Baltimore, 1989).

NF- κ B proteins are held in an inactive form in the cytoplasm by inhibitor proteins (I κ Bs) that mask the Nuclear Localisation Signals (NLS) (Beg et al., 1992; Henkel et al., 1992; Matthews et al., 1993b) of the NF- κ B proteins. After exposure of cells to signals which activate NF- κ B, specific I κ B family members are rapidly phosphorylated, ubiquitinated and degraded. Proteolysis of I κ B proteins thus releases the active DNA binding heterodimer of NF- κ B which translocates to the cell nucleus (Finco and Baldwin, 1995).

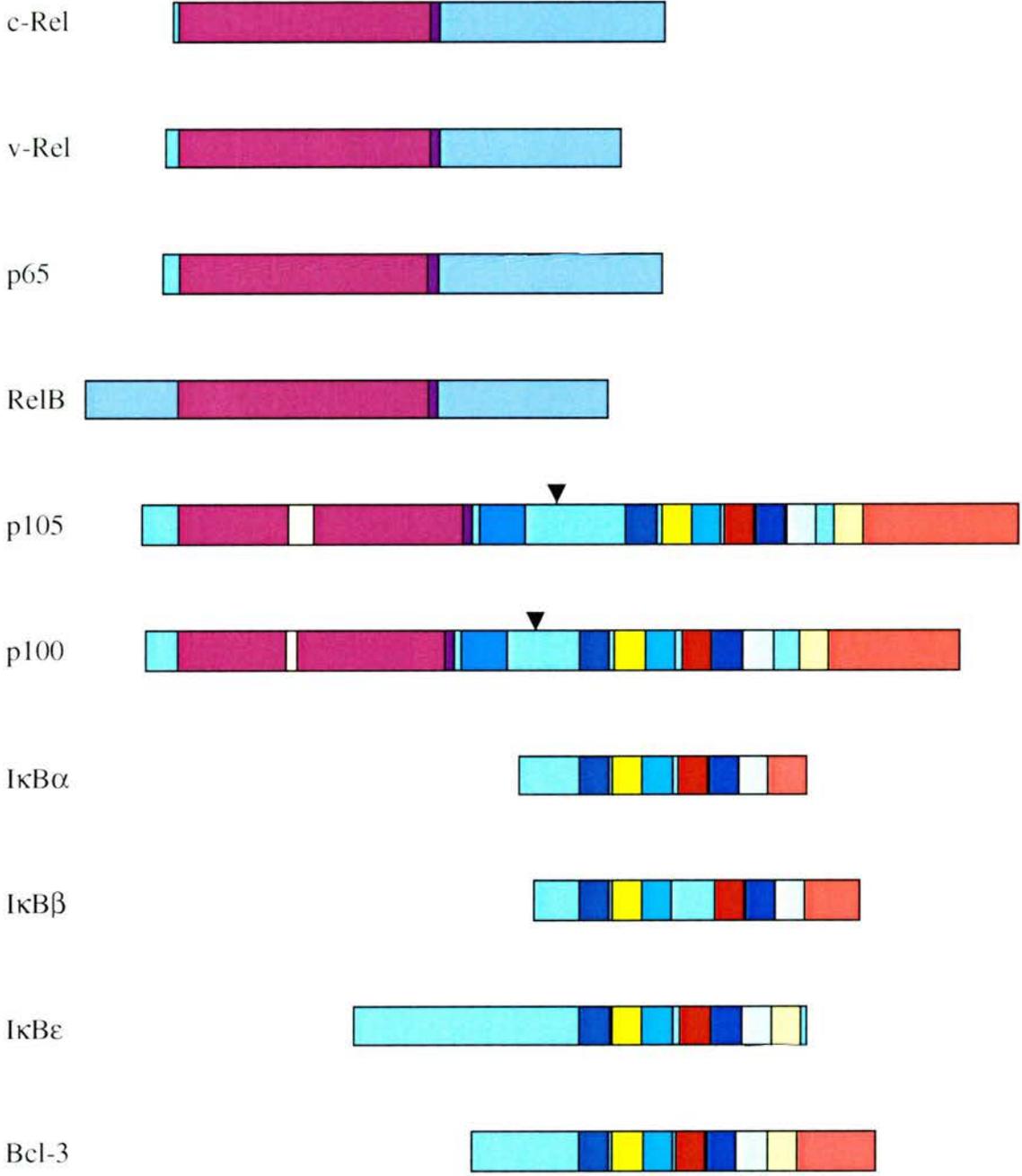
As originally identified, NF- κ B was composed of two polypeptide species of 50 kDa (p50) and 65 kDa (p65 or RelA) molecular weight (Baeuerle and Baltimore, 1989) although the p50 subunit could also be found as a homodimeric protein. cDNAs encoding each of these subunits have been isolated, but it is now clear that these genes are part of a larger multigene family (Rel/NF- κ B family of proteins). All members of this family possess a highly conserved region of ~300 amino acid in their N-termini which is responsible for DNA binding, dimerisation, and nuclear localisation of these proteins (Beg et al., 1992; Haskill et al., 1991) and is known as Rel homology region

(RHR) (Grimm and Baeuerle, 1993) (Figure 7). These proteins can form different homodimers or heterodimers, such as (p50)₂, (p65)₂, p50•p65 etc. Each dimer possesses a unique DNA binding specificity, since they bind to various κB motifs with different affinities (e.g. (p50)₂ preferentially binds to symmetrical 11-mers like MHC-κB, (p65)₂, to symmetrical 9-mers and p50•p65 to asymmetrical 10-mers like IκB).

Rel/NF-κB protein family can be conveniently divided into two subgroups: p50-related and Rel-related. The proteins that belong to p50-related subfamily are p50 and p52 (Bours et al., 1992; Schmid et al., 1991). They are synthesised as precursor molecules (p105 and p100 for p50 and p52 respectively) which contain 7 "ankyrin repeats" in their C-terminus, are cytoplasmically located and inactive in DNA binding (Ghosh et al., 1990; Kieran et al., 1990). The proteins that belong to Rel-related subfamily are the gene product of the *rel* proto-oncogene (Brownell et al., 1989; Wilhelmsen et al., 1984), RelA (p65) (Nolan et al., 1991; Ruben et al., 1992), RelB (Ryseck et al., 1992), the maternal morphogen Dorsal (Steward, 1987) which establishes dorsal-ventral polarity in the early *Drosophila* embryo, and Dif (Ip et al., 1993) which coordinates the *Drosophila* "immune" response by regulating the production of antibacterial peptides in response to infection (Figure 6). These proteins, in addition to the DNA binding and dimerisation domain also contain C-terminal regions that allow them to function as transcriptional activators (p50 related proteins lack such regions).

The members of the IκB protein family contain a homologous region of 6 or 7 tandem ankyrin repeats (Ghosh et al., 1990; Kieran et al., 1990). The ankyrin repeats are 33 amino acid motifs which are present in proteins that control the cell cycle (Breedon and Nasmyth, 1987) and in erythrocyte ankyrin (Lux et al., 1990), and are characteristic structures for protein-protein interactions (see section 4.3.1). Their consensus sequence is: -G-TPLH-AA--GH---V--LL--GA--N---- (Figure 8). IκB proteins function by binding to NF-κB proteins and thereby modify their localisation and DNA binding properties (some IκBs not only inhibit DNA binding of

Figure 6: The Rel/NF- κ B/I κ B family of proteins. All the dimensions are in scale. RHR: Rel Homology Region; TD: Transactivation Domain; GRR: Gly-Rich Region; UFR: Undefined Function Region. The arrows indicates the final p105 and p100 amino acid which is cleaved during proteasomal proteolytic processing of these molecules for the creation of p50 and p52 respectively. I κ B γ and I κ B δ are the C-termini of p105 and p100 respectively (starting from the area between the NLS and GRR).



	RHR		GRR
	NLS		ARD
	TD		TD
	IR		UFR

Table 1: I κ B specificities: I κ B proteins interact with different affinities to various NF- κ B molecules: I κ B α , I κ B β and I κ B ϵ generally prefer dimers than contain Rel related proteins while I κ B γ and Bcl-3 prefer p50 related protein homodimers.

IκB Species	Specificity	Ankyrin Repeats
IκBα	(p65) ₂ , p50•p65, p52•p65, (c-Rel) ₂ , p50•c-Rel, p52•c-Rel, p65•c-Rel	6
IκBβ	(p65) ₂ , p50•p65, p52•p65, (c-Rel) ₂ , p50•c-Rel, p52•c-Rel, p65•c-Rel	6
IκBγ	(p50) ₂ , (p52) ₂	7
IκBδ	(p52) ₂	7
IκBε	(p65) ₂ , p65•c-Rel	7
Bcl-3	(p50) ₂ , (p52) ₂	7

Figure 7: NF- κ B/Rel family of proteins share an area with strong sequence homology and structural similarity, termed Rel Homology Region (RHR). β -sheets (in orange) are connected through flexible loops, five of which (L1-L5) contact DNA. The N-terminal domain (where β -sheets are termed with capital letters) is separated from the C-terminal domain (where β -sheets are termed with small letters) by L3 loop. The N-terminal domain contains one or two α -helices (in pink). Finally, positively charged NLS (in red) is located after the C-terminal domain.

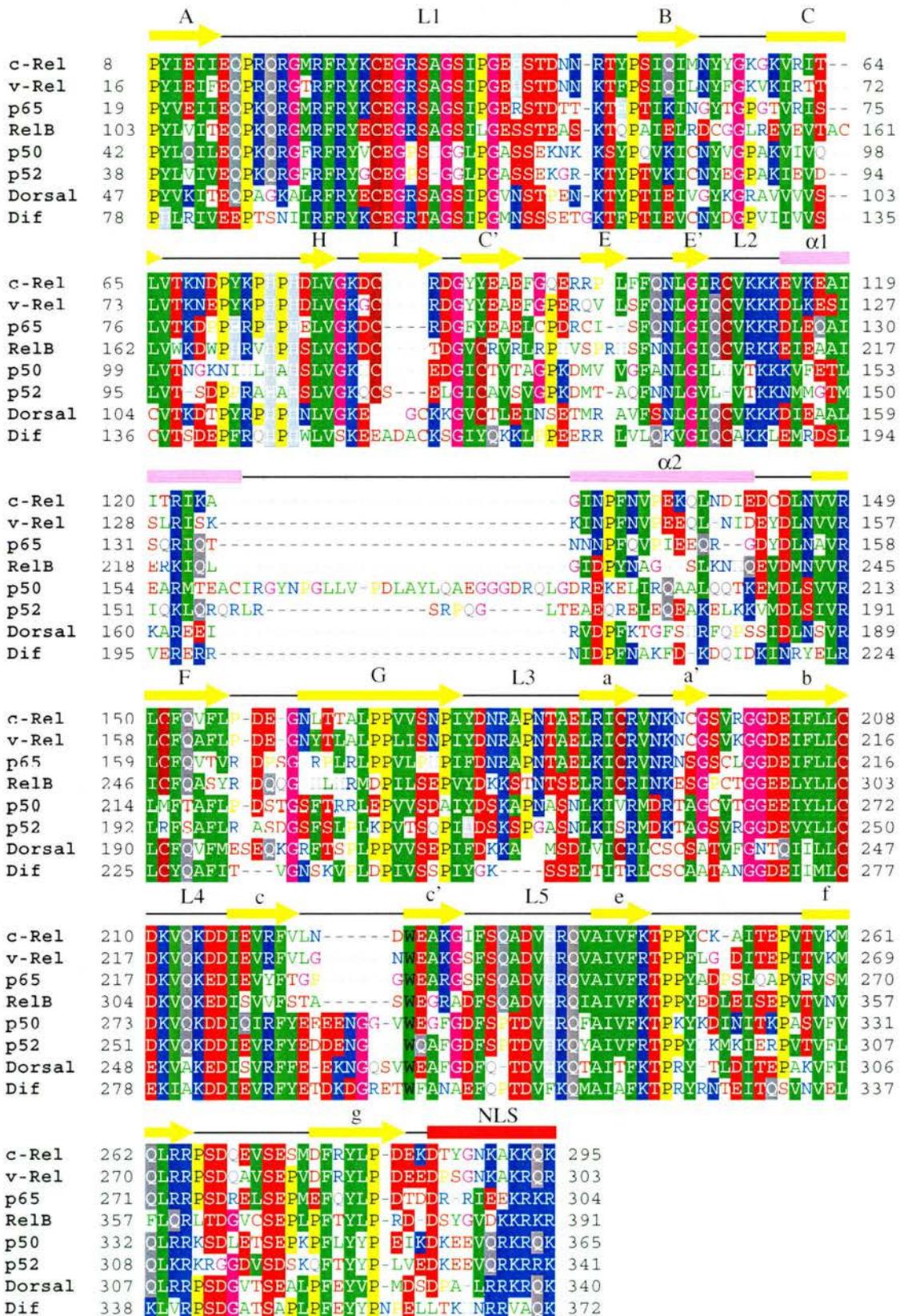


Figure 8: Homology of I κ B ARD. I κ B α and I κ B β proteins contain 6 while I κ B γ , I κ B δ , I κ B ϵ and Bcl-3 contain 7 repeats. Each ankyrin repeat is 33 amino acid long and consists of a β -loop and two antiparallel α -helices. In some cases, insertion occur between the outer (second) α -helix and the β -loop of the adjacent ankyrin repeat.

NF- κ B, but even induce its dissociation from DNA). At least five different proteins, I κ B α (Davis et al., 1991; Haskill et al., 1991), I κ B β (Thompson et al., 1995), I κ B γ (Inoue et al., 1992), I κ B ϵ (Whiteside et al., 1997), Bcl-3 (Franzoso et al., 1992; Hatada et al., 1992; Ohno et al., 1990; Wulczyn et al., 1992), and Cactus (Geisler et al., 1992; Kidd, 1992) possess I κ B activity (Figure 6) and appear to have distinguishable affinities and specificities with respect to inhibition of the various homo- and heterodimers of the members of the Rel/NF- κ B protein family (Table 1). cDNAs for each of these proteins have been isolated revealing that all contain ankyrin repeats and that I κ B γ , in fact, represents the C-terminal half of p105 (because of the translation of an alternatively spliced mRNA which encodes p105). I κ B δ is supposed to be the C-terminal form of p100, but its role *in vivo* has yet to be determined. I κ B proteins interact with different affinities and specificities with the members of the Rel/NF- κ B protein family (Table 1).

1.3.3 NF- κ B signal cascade

1.3.3.1 Inducers of NF- κ B

NF- κ B activation is possible by various stimuli (Baeuerle and Henkel, 1994). Cytokines, viruses, bacterial and viral components, proapoptotic/necrotic stimuli, phorbol esters etc are among the NF- κ B inducers (Table 2).

1.3.3.2 Genes induced by NF- κ B

NF- κ B activation results in the induction of the expression of a series of cellular genes (cytokines, haematopoietic growth factors, acute phase proteins, transcription factors, cell adhesion molecules, immunoreceptors, viruses, etc) and viruses (Baeuerle and Henkel, 1994) (Table 3).

1.3.3.3 NF- κ B Stimuli Receptors

Most of the receptors of the NF- κ B inducers are transmembrane proteins located in the cell membrane. Binding of an extracellular stimulating molecule on the extracellular domain of its specific receptor results in conformational changes of the intracellular domain of the receptor. That abolishes some of the already existent

Table 2: Various factors induce NF- κ B; viruses or viral components, bacteria or bacteria components, parasites, stress, drugs etc.

	Class	Inducing condition
Bacteria		<i>Shigella flexneri</i>
Bacterial components		<i>Mycobacterium tuberculosis</i> Lipopolysaccharide Muramyl peptides (G(Anh)MTetra) Staphylococcus enterotoxin A and B Exotoxin B Toxic shock syndrome toxin 1 (Cholera toxin)
Viruses		HIV-1 HTLV-1 HBV HSV-1 HHV-6 CMV Human herpes virus 6 Newcastle disease virus Sendai virus EBV Adenovirus 5
Viral components		dsRNA sphingomyelinase HTLV-1 Tax HBV HBx HBV MHBs EBNA-2 EBV LMP
Eukaryotic parasite Inflammatory cytokines		<i>Theileria parva</i> TNF α TNF β Lymphotoxin IL-1 α IL-1 β IL-2 Leukotriene B4 LIF
T cell mitogens		Antigen Lectines (PHA, ConA) Anti- $\alpha\beta$ T cell receptor Calcium ionophores Anti-CD2 Anti-CD3 Anti-CD28 Phorbol esters DAG
B cell mitogen Fibroblast mitogen Protein synthesis inhibitors		Anti-surface IgM Serum Cycloheximide Anisomycin Emetine
Physical stress		UV light X and γ radiation (Photofrin plus red light) (Hypoxia)
Oxidative stress		H ₂ O ₂
Drugs		Butyl peroxide Okadaic acid Calyculin A Pervanadate Ceramide Dibutyl c-AMP Forskolin

Table 3: NF- κ B can induce a large variety of genes which are related to inflammation, acute phase response, immune response, or cell cycle. A substantial amount of viruses exploit NF- κ B activation to effectively replicate.

	Class	Induced condition
Viruses		HIV-1 HIV-2 SIV _{mac} CMV Adenovirus HSV-1 JCV SV40
Immunoreceptors		Igκ T cell receptor α T cell receptor β MHC I MHC II β ₂ -Microglobulin Invariant chain I _i
Cell adhesion molecules		Tissue factor-1 ELAM-1 VCAM-1 ICAM-1
Cytokines and haematopoietic growth factors		IFN-β GM-CSF G-CSF M-CSF IL-2 IL-6 IL-8 TNFα TNFβ Proencephalin MPC-1/JE
Acute phase proteins		Angiotensinogen Serum amyloid A precursor Complement factor B Complement factor C4 Urokinase-type plasminogen activator
Transcription factors and subunits		c-Rel p105 IκBα c-myc IRF-1 (IRF-2) Interferon regulatory factor 1 A20
Others		NO Synthetase (Apolipoprotein CIII) (Perforin) Vimentin (Decorin)

interactions of this domain with cytoplasmic proteins and also allows new interactions to occur. Three different stimuli which trigger signal cascades that all lead to NF- κ B activation, have been extensively studied (Figure 9):

1.3.3.3.1 IL-1 induction

The extracellular cytokine IL-1 binds to IL-1 receptor (IL-1R). Upon stimulation, the intracellular domain of IL-1R recruits the serine/threonine IL-1 receptor associated kinases I and II (IRAK-1 and IRAK-2 respectively) and MyD88, a death domain-containing adapter molecule (Muzio et al., 1997). IRAK activates TRAF-6 (O'Neill and Greene, 1998). This event consequently activates (phosphorylates) MEKK1.

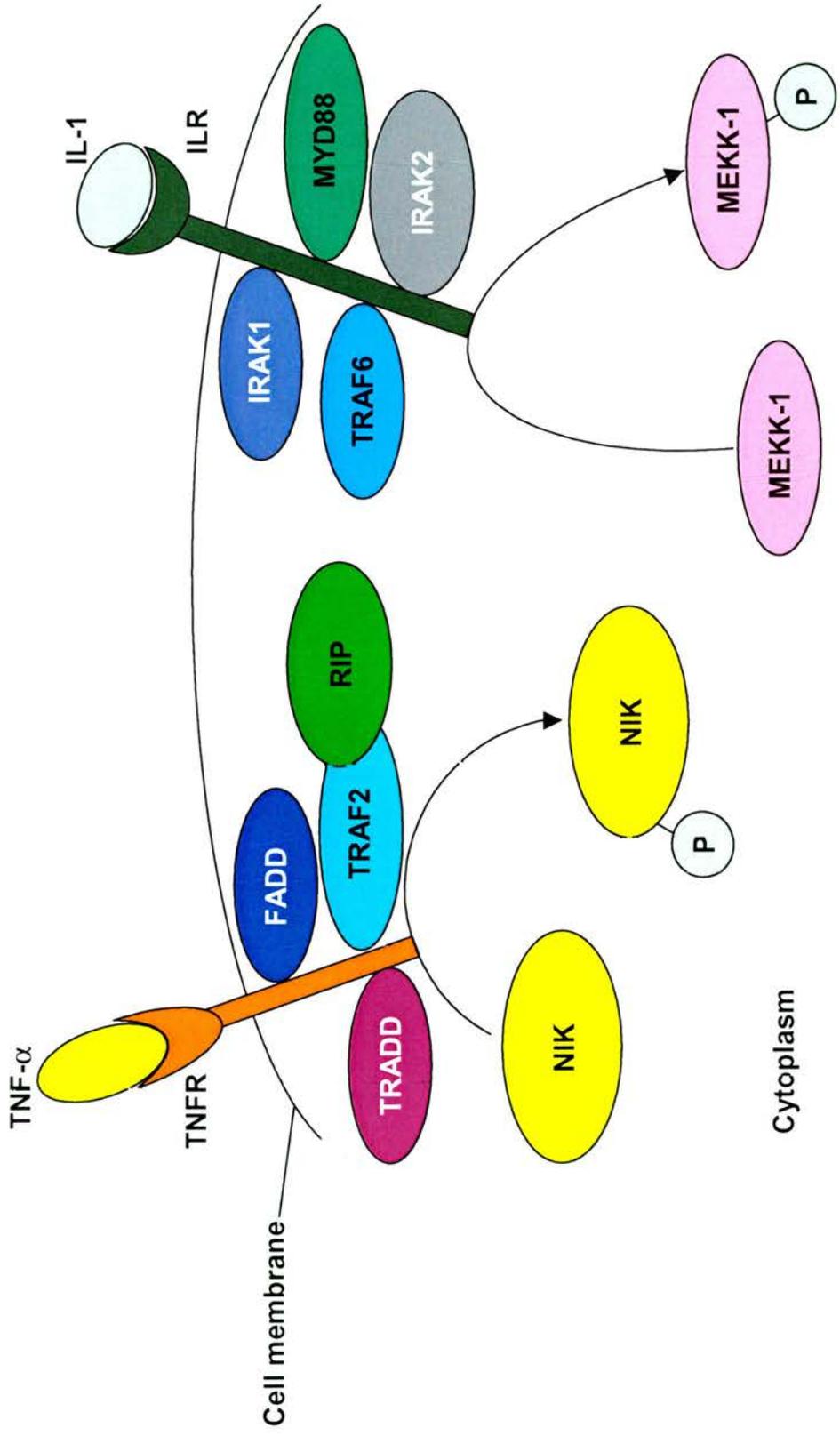
1.3.3.3.2 LPS induction

The cell wall of Gram- bacteria contains lipopolysaccharides which are exposed on the bacterial surface. Serum LPS-binding protein (LBP) binds specifically to the cell wall, through interactions with LPS. The LPS•LBP complex is presented to the surface CD14 transmembrane protein which is LPS receptor (LPSR) (Schumann et al., 1990; Wright et al., 1990). The human homologue of *Drosophila* Toll TLR2 (Medzhitov et al., 1997) associates with CD14 which leads to LPSR oligomerisation. (Yang et al., 1999). Receptor oligomerisation is a common mechanism to activate cytokine receptors (Lemmon and Schlessinger, 1994; Lemmon and Schlessinger, 1998). Oligomerisation recruits IRAK-1 and IRAK-2. This is where LPS activation of NF- κ B converges with IL-1 induction.

1.3.3.3.3 TNF α induction

When the cytokine TNF α is released in the extracellular space, it binds to the external domain of the transmembrane TNF α receptor I (TNFR1 or p55). The binding leads to trimerisation of the receptor. The TNFR1 intracellular domain is a death domain that after the trimerisation interacts with TNF α receptor associated protein with death domain (TRADD), another death domain containing protein (Hsu et al., 1996b; Shu et al., 1996). Then other molecules are recruited: the serine/threonine ring finger interacting protein (RIP), and the TNF α receptor associated factor II (TRAF2) (Hsu et

Figure 9: NIK and MEKK-1 activation: Once TNF- α is bound its membrane receptor (TNFR), various proteins (TRADD, FADD, TRAF2 and RIP) are associated to the cytoplasmic part of TNFR. This recruitment activates NIK, by phosphorylation. In a similar mode, MEKK-1 is activated, by the recruitment of IRAK1, IRAK2, TRAF6 and MYD88 to the cytoplasmic domain of the IL receptor, following the binding of IL1 to its receptor.



al., 1996a; Hsu et al., 1996b; Shu et al., 1996). The association of TRADD with FADD results in activation of the caspase family of proteases (initially with Caspase-8) which leads to apoptosis (Muzio et al., 1996). The interaction of TRADD with TRAF2 and RIP activates NF- κ B inducing kinase NIK (Liu et al., 1996; Malinin et al., 1997; Natoli et al., 1997; Song et al., 1997).

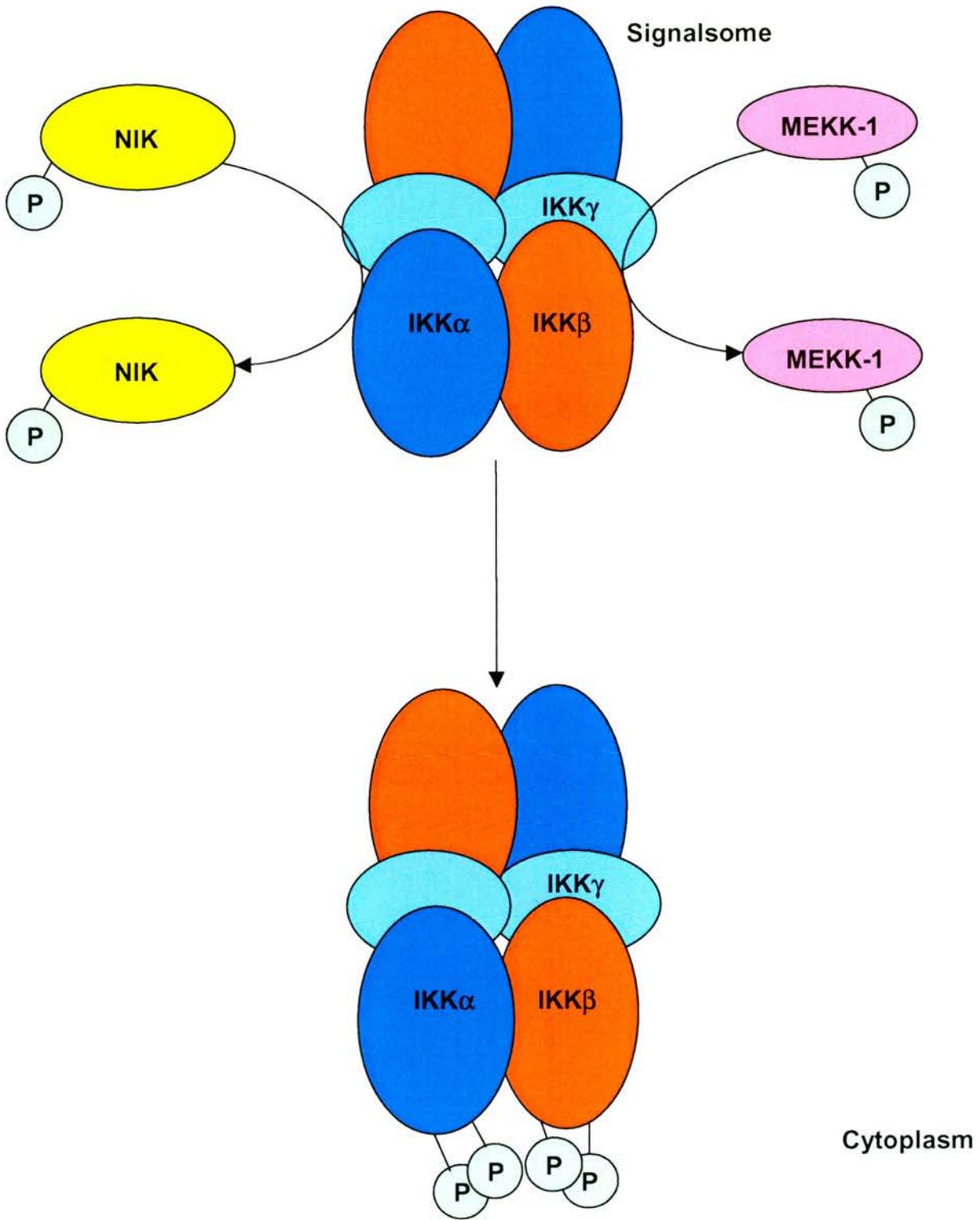
1.3.3.4 NIK, MEKK-1 and TPL-2

In NF- κ B signal cascade, all the interactions converge to the activation from the Mitogen-associated Protein kinase kinase kinase (MAPKKK/MAP3K/MEKK) family of enzymes: NF- κ B inducing kinase (NIK) is activated by its association to TRAF2 (Malinin et al., 1997). It then preferentially phosphorylates IKK α of the signalsome (Ling et al., 1998; Nakano et al., 1998). On the other hand, MEKK-1 is activated by its association with TRAF-6 and preferentially phosphorylates IKK β of the signalsome (Nakano et al., 1998; Nemoto et al., 1998; Yin et al., 1998b) (Figure 10). Finally, TPL-2, another member of MEKK family of proteins, is activated upon TNF α binding to TNFR. The activated kinase binds to p105. This causes the phosphorylation of p105 C-terminus (not by TPL-2 itself), which signals p105 proteolytic process which leads to the production of NF- κ B p50 (Belich et al., 1999; Lin et al., 1999). Therefore, p105 degradation is IKK independent.

1.3.3.5 Signalsome (IKK α /IKK β /IKK γ)

I κ B α , I κ B β , I κ B ϵ and Cactus can undergo polyubiquitination and proteolytic degradation once both of the conserved serine residues of their N-terminal regulatory region are phosphorylated (Brown et al., 1995; Chen et al., 1996; DiDonato et al., 1996; Régnier et al., 1997; Traenckner et al., 1995). The complex which is responsible for the phosphorylation of I κ Bs as a response to cell stimulation by TNF α , IL-1 and LPS, is a 900 kDa complex termed IKK complex or Signalsome (DiDonato et al., 1997; Mercurio et al., 1997). The signalsome consists of two catalytic subunits, IKK α (previously named as CHUK) and IKK β and a regulatory subunit termed IKK γ (also known as NEMO) (Mercurio et al., 1999; Rothwarf et al.,

Figure 10: Activation of IKK α and IKK β by activated NIK and MEKK-1 respectively. Activated NIK-1 interacts with IKK γ of the signalsome and preferably activates IKK α . In a similar manner, activated MEKK-1 interacts with the signalsome and preferably activates IKK β .



1998).

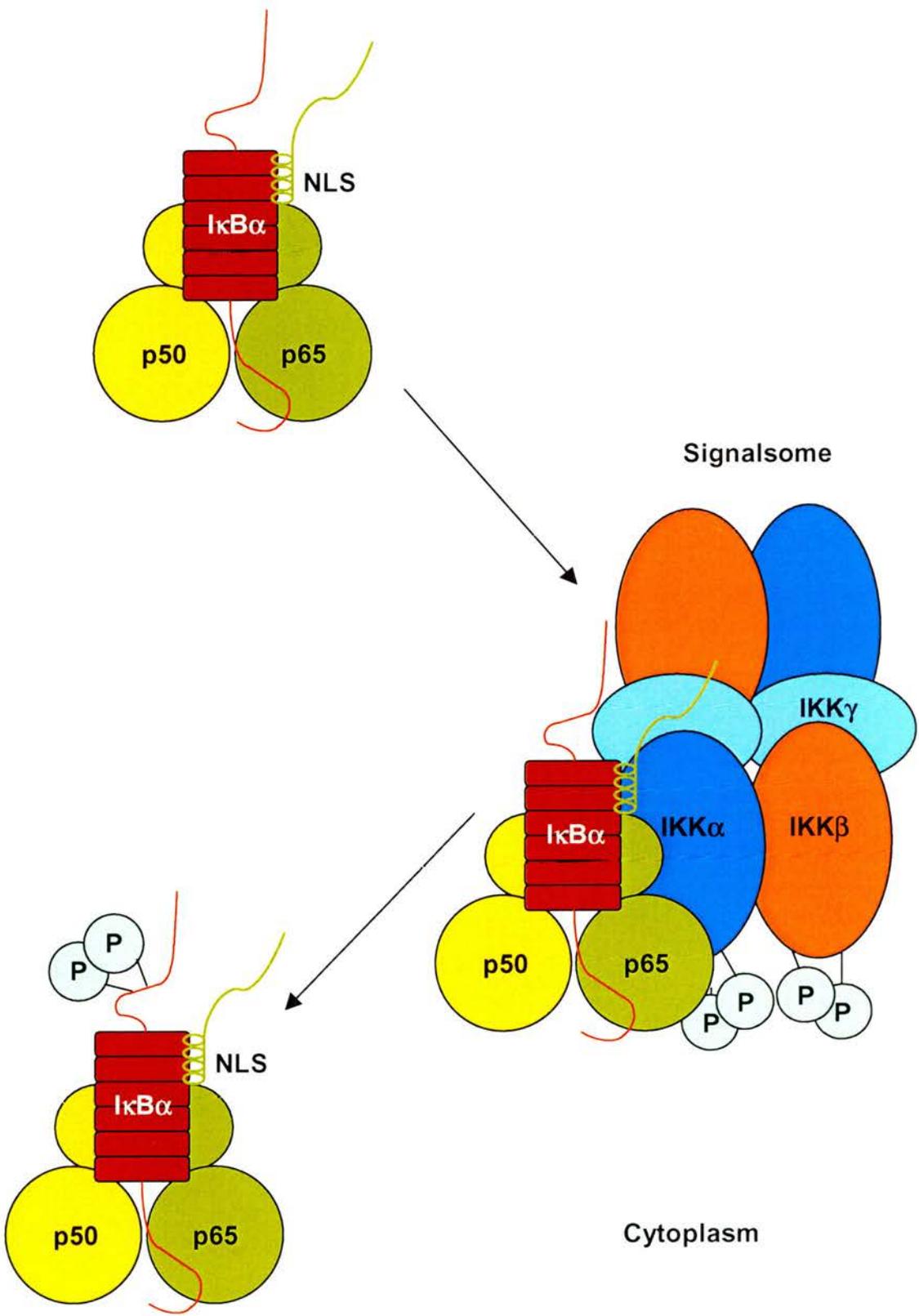
The molecular weight of IKK α and IKK β is similar (85 and 87 kDa respectively) (Woronicz et al., 1997; Zandi et al., 1997). They share 52% sequence identity and they have common structure: The kinase domain is N-terminally located and it is followed by a leucine zipper. The C-terminal domain has a helix-loop-helix motif. The leucine zipper domain is responsible for the dimerisation of IKK α and IKK β . Both homodimers and heterodimers are formed. Homodimerisation is necessary for kinase activity (Mercurio et al., 1999; Zandi et al., 1998; Zandi et al., 1997). While both (IKK α)₂ and (IKK β)₂ homodimers can catalyse the phosphorylation of I κ B α , kinetic analysis has proven that IKK β is more active (Li et al., 1998). IKK β plays more important role in this catalytic reaction in IKK α •IKK β heterodimers (Zandi et al., 1998) (Figure 11).

On the other hand, IKK γ is a 50 kDa protein containing coiled coil repeat motifs (helical regions) at its N-terminus, a leucine zipper and a zinc-finger domain at its C-terminus. IKK γ lacks any known catalytic domain (Rothwarf et al., 1998; Yamaoka et al., 1998). IKK γ can form homodimers or trimers. Fully active purified IKK complexes contain IKK α •IKK β heterodimers and several IKK γ molecules (Rothwarf et al., 1998).

Even if the substrate of the reaction is I κ B α S32 and S36, I κ B α is phosphorylated faster than its terminus alone (Mercurio et al., 1999; Zandi et al., 1998). The last reaction could be accelerated in the presence of a peptide which corresponded to the amino acids 279-303 of the C-terminus of I κ B α (Burke et al., 1998). Therefore both N- and C-terminal regions of I κ B α are necessary for its recognition by IKK. Furthermore, I κ B α •NF- κ B and I κ B β •NF- κ B complexes were more favourable substrates for IKK than I κ B α and I κ B β alone (Zandi et al., 1998). The lower turnover of free I κ B α is crucial for the termination of the NF- κ B activation which starts with the de novo synthesis of I κ B α while IKK is still active.

IKK γ interacts with IKK α and IKK β through its N-terminus (Mercurio et al., 1999; Rothwarf et al., 1998; Yamaoka et al., 1998). Its C-terminus, which has the leucine

Figure 11: Phosphorylation of I κ B α by IKKs of the signalsome: NF- κ B is cytoplasmically sequestered due to its interaction with I κ B α which masks its NLS. Activated signalsome can recognise the N-terminal signal responsive domain of I κ B α which is bound to NF- κ B. This leads to I κ B α phosphorylation in positions S32 and S36.



zipper and the zinc-finger domains, serves as an anchorage for IKK-kinases. This is why IKK γ plays a key role in activation of IKK complex and the consequent phosphorylation and degradation of I κ B and activation of NF- κ B upon TNF α , IL-1, LPS and dsRNA stimulation.

During IKK activation, phosphorylation of the serines of both IKK α and IKK β takes place. IKK γ is also phosphorylated but the nature of this phosphorylation has yet to be determined. Phosphorylation can be reversed by the protein phosphatase PP-2a (DiDonato et al., 1997). It is speculated that the IKK α and IKK β kinase domain and helix-loop-helix domain interact, in the unphosphorylated IKKs. Upon stimulation the 2 serines of the activation loop of one IKK are phosphorylated with the assistance of the helix-loop-helix domain (Figure 12). The serines of the other IKK which is bound to the phosphorylated IKK are consequently autophosphorylated. That causes the autophosphorylation of the serine rich C-terminal region. Accumulation of negative charges causes electrostatic repulsion between the helix-loop-helix and the phosphorylated kinase domain which abolishes the interaction between these two domains. The conformational change enables a phosphatase to dephosphorylate the activation loop and deactivate IKK. Therefore, this mechanism of autophosphorylation acts as a timing device that limits the duration of IKK activation. Even if IKK α and IKK β are highly homologous and are tightly bound in the signalsome, their roles are not identical: IKK β plays a critical role in NF- κ B activation by proinflammatory cytokines (Delhase et al., 1999; Mercurio and Manning, 1999; Mercurio et al., 1997) while IKK α plays an essential role in controlling the formation and differentiation of the epidermis (Seitz et al., 1998). As it was mentioned before, different classes of stimuli activate different IKK kinases (NIK or MEKK-1) and each of them preferably activates IKK α and IKK β , respectively. This provides a mechanism for differential activation of IKKs and eventually a mechanism which would lead to differential responses. Finally, another interesting observation is the striking degree of conservation among the mammals and the insects of the dual role of Rel/NF- κ B/Dorsal as molecules which mediate the innate immune

Figure 12: Identification of consensus sequences of phosphorylation sites of two kinases. Phosphoacceptor serines which are critical for kinase activation are boxed. (A) Homology of the IKK α , IKK β and MEK-1. All these proteins are phosphorylated by kinases which belong to the MAPKKK (MEKK) family of enzymes. (B) Homology of the N-terminal regulatory regions of “professional” I κ Bs and Cactus which contain the phosphorylation sites. S^P is phosphoserine, Ψ is hydrophobic amino acid, and X is any amino acid. Once both of the two conserved serines are phosphorylated by the IKKs of the signalsome, I κ Bs are recognised as substrates for ubiquitination.

A

IKKα	DLGYAKDVDQGS	SLCTS	SFVGT	LQYL	APE
IKKβ	DLGYAKELDQGS	SLCTS	SFVGT	LQYL	APE
MEK-1	DFGVSG-QLID	SMANS	SFVGT	RSYMS	PE

B

IκBα	26	LDDRHDS	GLDS	SMKDEE	YEQ
IκBβ	13	ADEWCDS	GLGSL	GPDA	AAAP
IκBϵ	151	EESQYDS	GIESL	RSLR	SLP
Cactus	68	PNETSDS	GFIS	GPQSS	QIF
Consensus		DSG ^P Ψ xS ^P			

system responses in adults, as well as morphogens which control developmental decisions in embryos.

1.3.3.6 Ubiquitination

Ubiquitin is a highly conserved 76 amino acid polypeptide. A multiple enzymatic process which leads to proteolytic degradation of proteins with short half-life which play a pivotal role in various regulatory mechanisms (cell cycle, immune response, biogenesis of subcellular organelles, cell differentiation, DNA repair, transcriptional control) depends on ubiquitin (Ciechanover, 1994; Hochstrasser, 1995; Jentsch and Schlenker, 1995).

Ubiquitin conjugation involves two or often three enzymatic reactions (Ciechanover et al., 1981; Hershko et al., 1983; Pickart and Rose, 1985). In the first step, a high energy thioester bond between ubiquitin and the ubiquitin-activating enzyme E1 is formed, assisted by ATP consumption. Then, ubiquitin is transferred to a conserved cysteine group of the ubiquitin-conjugating enzyme E2 which subsequently passes ubiquitin to a thiol group of the ubiquitin-ligating enzyme E3. E3 then attaches ubiquitin to the ϵ -amino group of a lysine residue of the target protein via an isopeptide bond. In some cases, ubiquitin attachment to the substrate can be performed by E2 alone, either with or without cooperation with E3. The ubiquitin moiety bound to the target protein offers its lysine groups as targets for subsequent ubiquitination. After several rounds of ubiquitination, the target proteins are multiubiquitinated. E2 and E3 determine the specificity of various ubiquitin pathways (Jentsch, 1992).

In the cascade of NF- κ B/Dorsal activation, the substrates of ubiquitin conjugation are the "professional" I κ Bs (I κ B α , I κ B β , I κ B ϵ and cactus) (Alkalay et al., 1995) containing the consensus sequence DS^PG Ψ XS^P, where Ψ is a hydrophobic amino acid and S^P is a phosphoserine (Yaron et al., 1997) (phosphorylation of both serine residues is essential for ubiquitination (Baldwin, 1996)). This sequence is located N-terminally to the ARD and is same for the ubiquitination of β catenins (Aberle et

al., 1997). S32/S36 phosphorylated I κ B α is the substrate for β -TrCP (β -transducin repeat-containing protein), a part of the SCF (Skp1p, Cullin1, F-box protein) ubiquitin ligase complex (Winston et al., 1999; Yaron et al., 1998). This leads to polyubiquitination of K21 and K22 (Chen et al., 1995; Scherer et al., 1995) (Figure 13).

1.3.3.7 SUMO-1 conjugation

A parallel but antagonistic process to I κ B α ubiquitination is SUMO-1 conjugation (Boddy et al., 1996; Kamitani et al., 1997; Mahajan et al., 1997; Matunis et al., 1996; Shen et al., 1996). SUMO-1 is a ubiquitin-like protein which can be conjugated to I κ B α with similar but not identical E1 and E2 conjugating enzymes (SAE1•SAE2 complex and Ubch9 respectively) without the requirement of an E3-like enzyme (Desterro et al., 1999; Desterro et al., 1997). As opposed to ubiquitination, SUMO-1 conjugation does not require S32 and S36 phosphorylation (which occurs upon stimulation). Another difference with ubiquitination in SUMO-1 conjugation is that only a single SUMO-1 molecule is conjugated in each K residue (K21 and K22, instead of the addition of multiple copies. That blocks ubiquitination and subsequent proteolytic degradation. Therefore, SUMO modification creates a pool of resistant to signal-induced degradation I κ B α (Desterro et al., 1998).

1.3.3.8 Proteasome/I κ B α degradation

The ubiquitinated I κ B α is degraded in the proteasome (Alkalay et al., 1995; Chen et al., 1995). The proteasome is an unusually large multisubunit proteolytic complex which consists of two 19S regulatory subunits termed PA700 bound to the ends of a central cylindrical 20S catalytic core (Coux et al., 1996). PA700 specifically recognises the polyubiquitinated chain. After binding to the regulatory subunit, the ubiquitinated substrate is unfolded and translocated into the inner core of the catalytic 20S where it undergoes proteolytic degradation, while via the actions of isopeptidases of the proteasome, free and reusable ubiquitin is released (Figure 14).

Figure 13: Ubiquitin conjugation of phosphorylated I κ B α by E2 and E3: The phosphorylation of S32 and S36 is the signal for the ubiquitin conjugating enzymes E2 and E3 to start forming ubiquitin chains in K21 and K22.

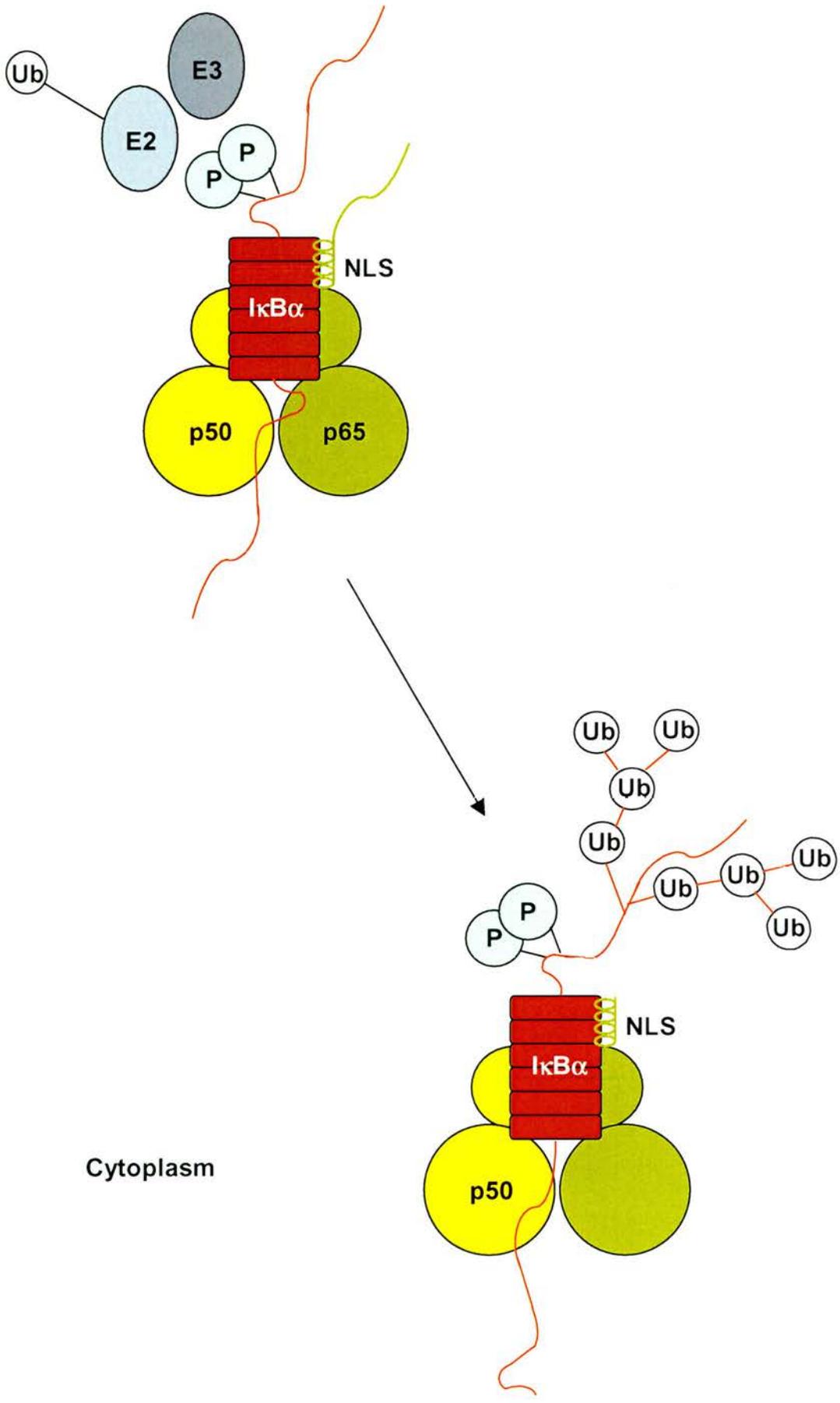
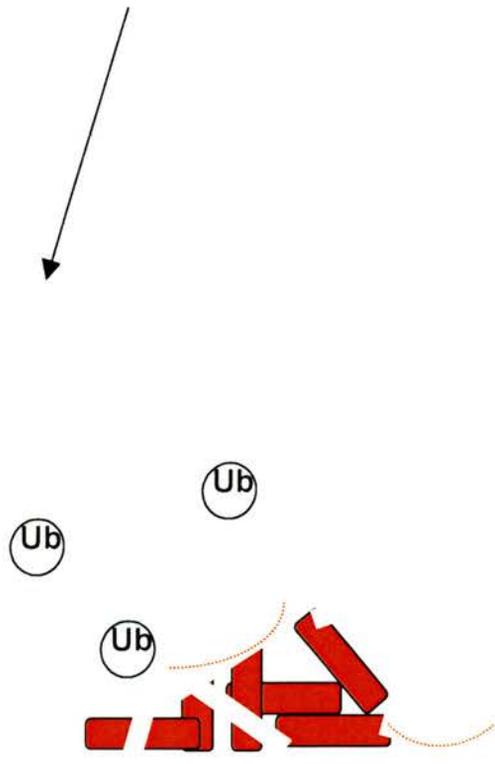
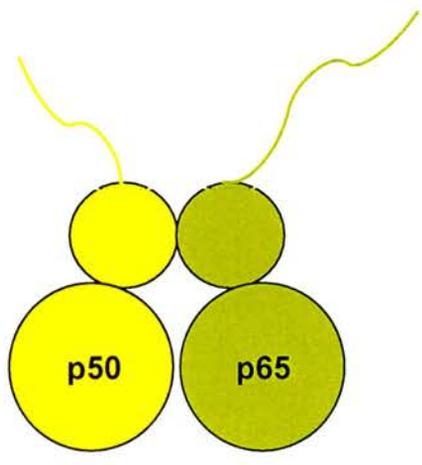
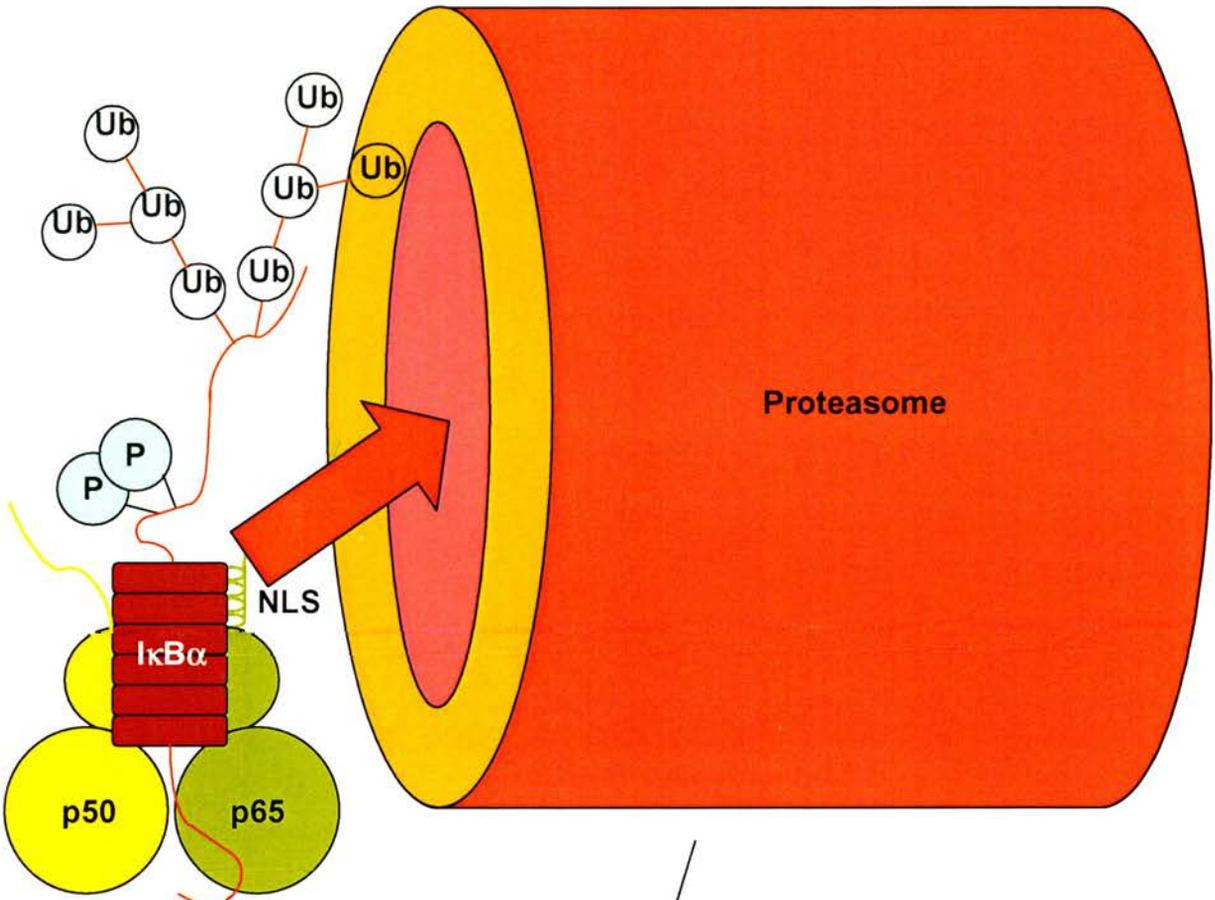


Figure 14: Proteolytic degradation of ubiquitinated I κ B α in the 26S proteasome: The ubiquitinated I κ B α , still attached to NF- κ B, is specifically recognised by the 19S particle of the 26S proteasome, and eventually I κ B α undergoes proteolytic degradation, which releases the ubiquitin molecules that can then be reused, and NF- κ B.



1.3.3.9 p105/p100 processing

There is only one known case where a protein undergoes partial proteasome mediated proteolytic degradation instead of complete destruction: The case of p105 and p100, the precursor molecules of NF- κ B p50 and p52 respectively. For many years it was hypothesised that p50 and p52 were produced either by alternative splicing of the p105 mRNA (in a similar manner with the way I κ B γ is produced from p105 mRNA), or that it was produced by the proteolytic cleavage of p105 by an unknown endopeptidase which would be activated in the signal cascade that follows proinflammatory stimulation. Today, we know that once the C-terminus of these two molecules is phosphorylated by TPL-2, it can also be polyubiquitinated by the action of the E2 enzyme UBCH5a or UBHC7 and a 320 kDa E3 ubiquitin ligase (Coux and Goldberg, 1998), therefore it can be proteolytically processed by the 26S proteasome. Nevertheless, the Gly-rich region (GRR) which locates between the NLS and the ankyrin repeat domain plays a protective role (Lin and Ghosh, 1996). An additional structural motif within p105 might make the molecule only partially susceptible to proteasome degradation (Orian et al., 1999). An explanation of how GRR prevents total destruction of p105 is if it serves as a physical barrier blocking the entry of p50 in the cavity, and thus preventing any consequent degradation (Orian et al., 1999).

I κ Bs as well as p105 and p100 contain a PEST (proline, glutamate, serine and threonine rich) region downstream ARD. PEST can modulate the speed of the partial degradation of p105: It can inhibit it unless it is phosphorylated (Coux and Goldberg, 1998). PEST region is constitutively phosphorylated by casein kinase II (CKII) therefore regulation results from signal sensitive phosphatase activity (MacKichan et al., 1996).

1.3.3.10 NF- κ B nuclear transport

The first step for the nuclear transport of most karyophilic proteins involves the recognition of their NLS by a cytoplasmic NLS receptor complex (reviewed in Görlich and Mattaj, 1996). The most important interaction in the NF- κ B•I κ B complex is the interaction between NF- κ B NLS and I κ B ARD. This masks the NLS, so NLS

cannot be recognised by the nucleopore transport machinery and thus NF- κ B is cytoplasmically sequestered. Therefore, I κ B proteolytic degradation which occurs upon stimulation leads to rapid nuclear NF- κ B translocation (Figure 15).

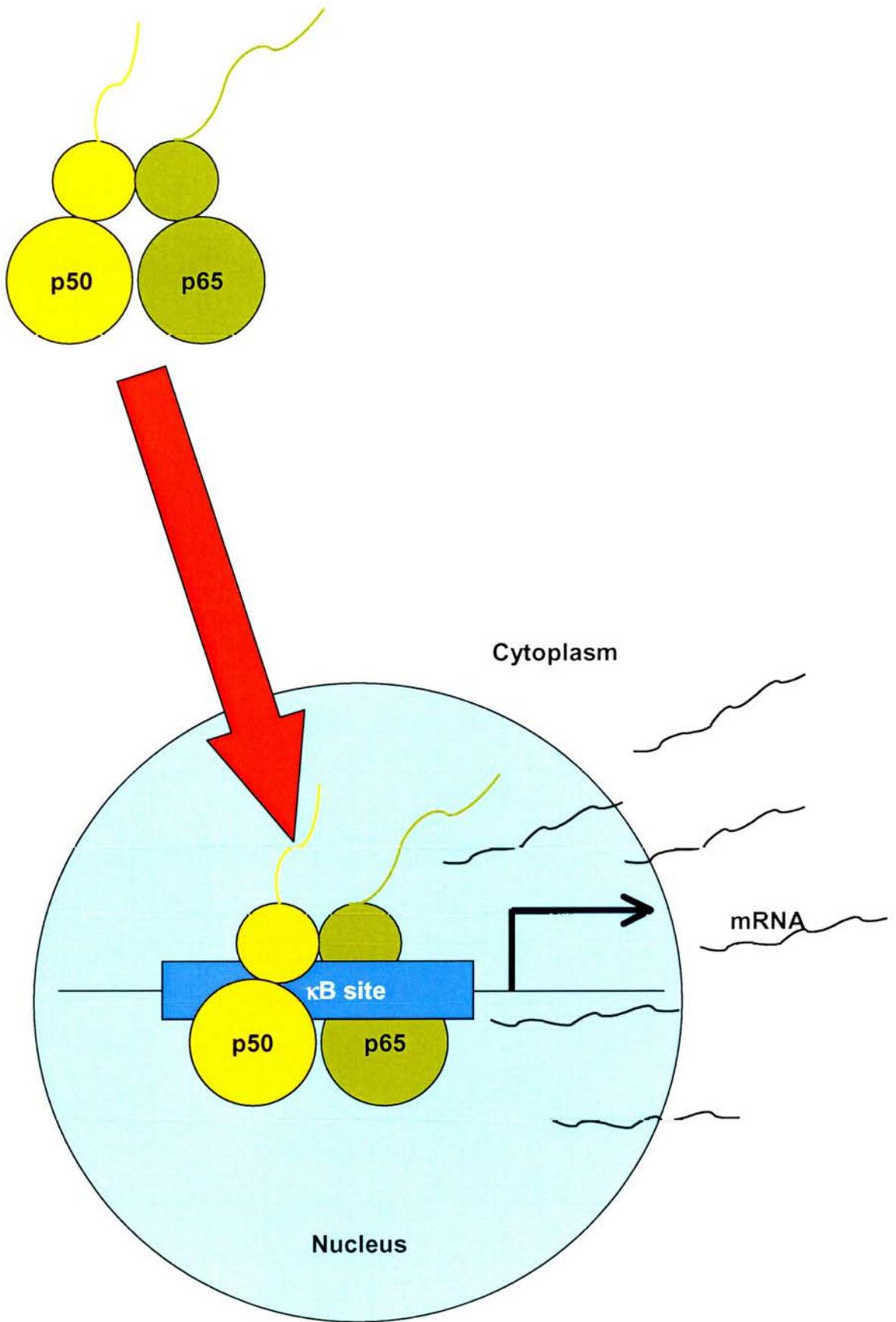
There are two main NLS motifs: The classical motif where there is only a single cluster of four or five basic amino acid residues and the bipartite motif where two clusters of basic amino acids are separated by an approximately 10 residue spacer. NF- κ B molecules have classical NLSs (Beg et al., 1992; Blank et al., 1991; Henkel et al., 1992; Zabel et al., 1993) while other transactivators have bipartite ones.

NF- κ B NLS receptor is a heterodimeric complex termed karyopherin- $\alpha\beta$. karyopherin- α binds to NLS motif in the cytoplasm while karyopherin- β 1 docks the entire complex at the cytoplasmic face of the nuclear pore. Once the complex is docked, it then translocates through the pore into the nucleus. This energy-dependent process involves the GTPase Ran/TC4 and the Ran interacting factor NTF2/p10 (reviewed in Görlich and Mattaj, 1996).

1.3.3.11 Induction of NF- κ B controlled genes

Once NF- κ B is in the nucleus, it specifically recognises its cognate DNA sequence in the promoter/enhancer elements of the genes it controls. NF- κ B activation is ubiquitous. That means that specificity is achieved through transcriptional synergy between NF- κ B and other transcription factors and cofactors and RNA pol II, leading to the formation of unique enhancer complexes. Therefore, the genes NF- κ B activates vary according to the cellular context (Maniatis et al., 1998; Perkins, 1997). In this process, selectivity and specificity is critical. There are many ways, this can be achieved: There are 5 different NF- κ B molecules, where two of them need an extra partial proteolytic step to be available. All these molecules form homo- and heterodimers, in almost all possible combinations. Therefore, the mechanisms which differentially activate NF- κ B molecules, contribute to selectivity. NF- κ B molecules have not exactly the same cognate sequence (see section 4.2.2), thus their differential sequence specificity enables them to target different genes. The homology between

Figure 15: NF- κ B nuclear translocation: Once NF- κ B is released from I κ B, its NLS is able to interact with the nuclear pore machinery, and it translocates to the nucleus. It then specifically recognises its own cognate DNA sequences and transactivates genes containing one or more κ B motifs in their regulatory sequences.



the transactivation domains of NF- κ B molecules is poor. That means that they form different complexes and therefore have non-overlapping roles, as it is also proven by the distinct phenotypes each NF- κ B gene knockout resulted in mice (reviewed in Gerondakis et al., 1999).

NF- κ B molecules interact with many factors and complexes, when bound on DNA. p65, which is the best characterised of all NF- κ B subunits, interacts with components of the TFIID complex and the general transcription factor TFIIB. By interaction with p300/CBP coactivator proteins, it also recruits acetyltransferase activity (Zhong et al., 1998), which is crucial for effective transcription (see section 1.2.2.2). That indirectly regulates p65-driven transactivation, since the activity and availability of both coactivators are regulated by a series of signalling (Hottiger et al., 1998). p65 directly interacts with other proteins which bind other DNA sequences, e.g. members of b-ZIP family of transcription factors (like c-Jun and CCAAT/enhancer-binding protein β (C/EBP β)), serum response factor and Sp1 (Perkins, 1997) etc. The enhancer complex forms a higher-order structure which is necessary for the assembly of the transcription machinery to the gene promoter. This higher-order nucleoprotein complex of the enhancer complex is called enhanceosome (Kim and Maniatis, 1997; Maniatis et al., 1998).

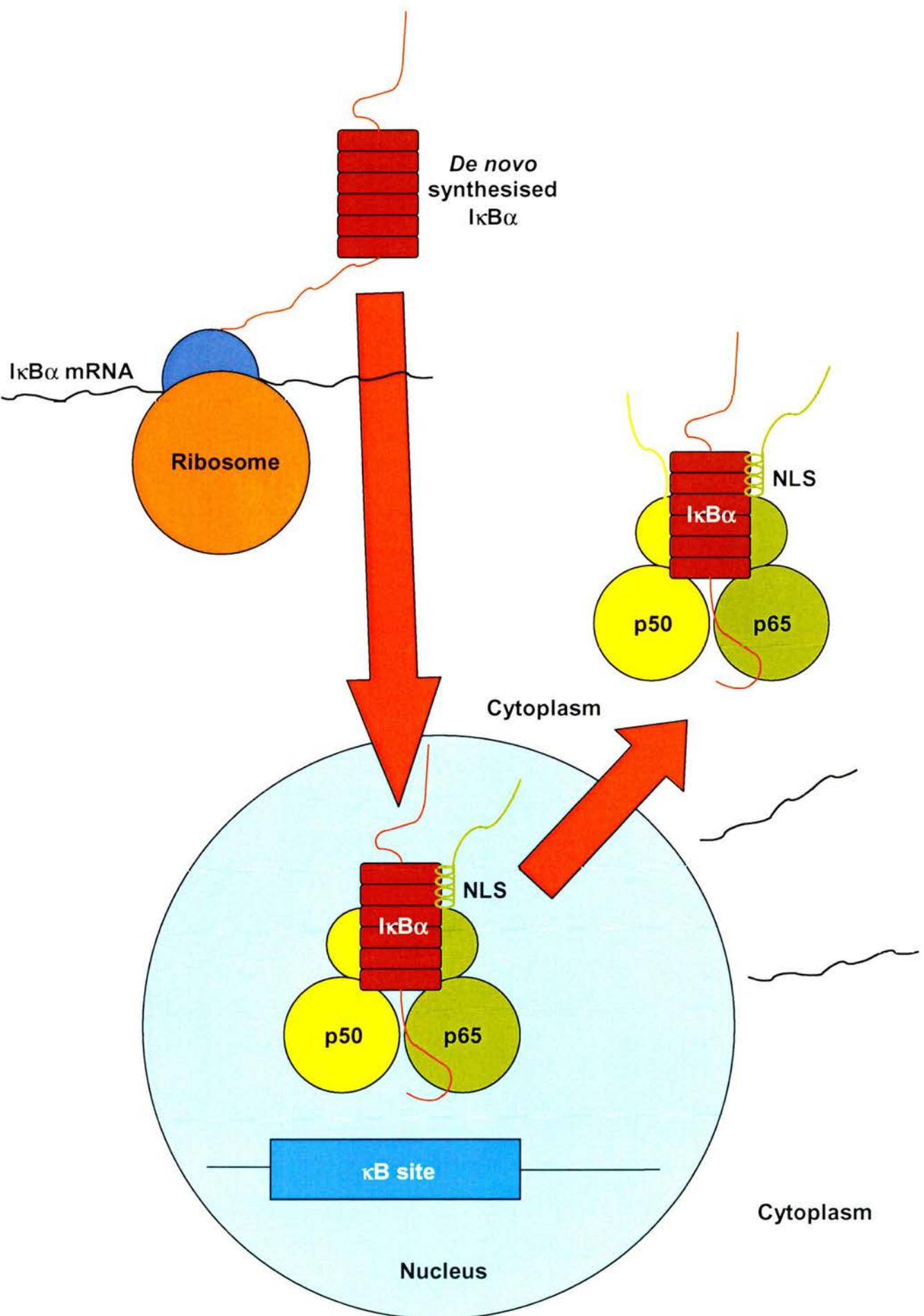
In many cases, NF- κ B activation and DNA binding, although necessary, is not by itself sufficient to lead to effective transcription, unless the full complement of promoter and enhancer-binding proteins is active. This is clear in the case of the interferon- β (IFN- β) regulatory sequence, where p50•p65 DNA binding alone is not sufficient for transcription. NF- κ B driven activation of this gene is only possible upon viral infection, and not upon any stimulation by other NF- κ B inducer. From that, it is apparent how NF- κ B can integrate to other signalling pathways.

1.3.3.12 NF- κ B•I κ B nuclear export

One of the genes that their transcription is NF- κ B dependent is NF- κ B inhibitor, I κ B α . Therefore, some minutes after the degradation of I κ B α , I κ B α mRNA is

translated in the cytoplasm. This *de novo* synthesised I κ B α translocates to the nucleus and associates with NF- κ B p50 and p65. This inhibits NF- κ B DNA activity and therefore NF- κ B dependent transcription. More interestingly I κ B α also transports NF- κ B back to the cytoplasm (Figure 16). This function of I κ B α is insured by a nuclear export sequence (NES) (Arenzana-Seisdedos et al., 1997; Johnson et al., 1999) located in the C-terminal domain of I κ B α and homologous to the previously described export signal found in HIV-1 Rev protein as well as in PKI (the inhibitor of the catalytic subunit of protein kinase A). Thus, inhibition of NF- κ B/DNA binding and the consecutive efficient nuclear export of the transcription factor of I κ B α could represent an important mechanism for the control of the expression of NF- κ B-dependent genes (Arenzana-Seisdedos et al., 1995; Arenzana-Seisdedos et al., 1997).

Figure 16: One of the genes that are activated by NF- κ B is the one that codes I κ B α . Translation of NF- κ B induced I κ B α . I κ B α translocates to the nucleus, it associates with DNA bound NF- κ B, and it dissociates the NF- κ B•DNA complex. NF- κ B•I κ B complex translocates to the nucleus. Therefore, NF- κ B driven transcription is inhibited.



1.4 Role of NF- κ B in diseases

1.4.1 Inflammation

NF- κ B activation is critical for many inflammatory diseases, like bronchial asthma and rheumatoid arthritis. This is mainly due to the fact that it is activated by cytokines and it also activates the expression of cytokines. NF- κ B activation is well regulated with various and complex mechanisms of feedback inhibition e.g. NF- κ B induces the expression of its very inhibitor I κ B α , but not I κ B β , which eventually inactivates NF- κ B by relocating it from the nucleus to the cytoplasm. NF- κ B also induces A20 which interacts with TRAF1/TRAF2 to inhibit NF- κ B activation (Song et al., 1996). Moreover, even if NF- κ B is activated by and activates cytokines which enable the spread of NF- κ B activation from one cell to another or even from one tissue to another (Pahl, 1999), the activation of NF- κ B is normally localised due to anti-inflammatory cytokine IL-10 which inhibits both IKK and NF- κ B DNA binding activity (Schottelius et al., 1999). Therefore, the cause of inflammatory diseases is the aberrant nuclear localisation of NF- κ B which leads to constitutive NF- κ B-driven transactivation of a series of genes. The difference between this situation and cancer is the fact that in inflammatory diseases, cells are deficient to regulate NF- κ B activation, while in cancer NF- κ B or I κ B molecules are mutated.

1.4.1.1 Septic Shock

Gram- bacteria produce a proinflammatory agonist, called Lipopolysaccharide (LPS). The presence of LPS in the blood is the main contributor to the 300,000-500,000 septic shocks recorded annually in US hospitals alone, with mortality rates from 20% to 40% (Wenzel, 1992). Monocytes and macrophages are LPS primary target cells (Wright et al., 1990). In the presence of LPS, NF- κ B is activated via I κ B α , I κ B β and I κ B ϵ degradation (see section 1.3.3.3.2). NF- κ B is a major intracellular mediator of the systemic inflammatory response syndrome known as sepsis and septic shock (Böhler et al., 1997). Activated NF- κ B stimulates transcription of genes encoding cytokines (TNF- α , IL1, IL-6, IL-8 and IL-12), procoagulants (tissue factor and

plasminogen activator inhibitor 1), signal transducers (inducible nitric oxide synthase and cyclooxygenase 2), cell adhesion molecules (endothelial selectin, intercellular cell adhesion molecule-1, and vascular cell adhesion molecule-1), and growth factors (granulocyte-colony stimulating factor). This massive production of proinflammatory cytokines, reactive oxygen- and nitrogen-intermediates, procoagulants and cell adhesion molecules by monocytes, macrophages and vascular endothelial contributes to the development of the septic shock, collapse of the circulatory system and to disseminated intravascular coagulation, leading to multiple organ failure and death (Bone, 1991).

1.4.2 Cancer

Various lines of evidence suggest for NF- κ B a regulatory role in apoptosis and cellular proliferation, which enables NF- κ B to act as a modulator of oncogenesis (reviewed in Foo and Nolan, 1999; reviewed in Mosialos, 1997).

The role of the Rel family of proteins and their inhibitors in oncogenesis was initially shown by the identification of forms of these genes in virally transformed cells or in chromosomal translocations found in leukaemia cells. The first evidence was the homology of v-Rel, the oncoprotein from the avian reticuloendotheliosis virus strain T (REV-T), with the Rel family of proteins like its cellular homologue c-Rel (Wilhelmsen et al., 1984), p50 (Ghosh et al., 1990; Kieran et al., 1990) and *Drosophila* morphogen Dorsal (Steward, 1987). v-Rel is a potent oncoprotein which transforms cells *in vivo* and *in vitro*. p52 was characterised as a potential oncogene when it was found in chromosomal translocations in lymphomas where its C-terminus was modified (Neri et al., 1991). Finally, Bcl-3 which was found in chromosomal translocations in chronic lymphocytic leukaemias (Ohno et al., 1990), was classified as a member of the I κ B family of proteins (Kerr et al., 1992), when the gene of I κ B α was cloned (Davis et al., 1991; Haskill et al., 1991). All these were the first indications that NF- κ B molecules and their inhibitors (I κ B molecules) were mediators of pro-mitogenic signals.

In the early stages of transformation, malignancy overcomes normal cellular growth

control, by rescuing from apoptosis and/or by providing constant proliferative stimuli. The role of NF- κ B in apoptosis became apparent with the observation that mouse p65 knockouts die by day 10 of their embryonic development, as a result of extensive liver apoptosis. Furthermore, embryonic fibroblasts from these mice were susceptible to TNF- α -mediated apoptosis, while wt or reconstituted cells were resistant (Beg et al., 1995). Similarly, ionising radiation or chemotherapy treatment activated NF- κ B which consequently conferred survival to cells (Wang et al., 1996). Oncogene overexpression which leads to oncogenesis, also induces a strong apoptotic response. Therefore, malignant cells need to activate mechanisms which can suppress the transformation-associated apoptosis. In many cases, this can be achieved through aberrant NF- κ B activation which can be induced by practically every oncogene, e.g. NF- κ B activates an anti-apoptotic signal to suppress oncogene H-Ras-associated (Mayo et al., 1997) and c-myc (Romashkova and Makarov, 1999) apoptosis. NF- κ B was shown to suppress the expression of the pro-apoptotic Caspase 8 gene, by inducing TNF receptor associated factor 1 and 2 (TRAF1 and TRAF2), inhibitor of apoptosis 1 and 2 (c-IAP1 and c-IAP2), A20, IEX-1 etc (Wang et al., 1998). Because apoptosis is the predominant mechanism exploited in chemotherapy and irradiation, resistance to programmed cell death, enables tumour cells to overcome these cancer therapies. NF- κ B, which is activated upon these therapies (e.g. DNA damaging chemotherapeutic agents), potently suppresses the apoptotic potential of these stimuli, leading to tumour chemoresistance. Therefore, NF- κ B inhibition is considered a new approach to adjuvant chemotherapy.

Activated NF- κ B also induces the expression of proteins which contribute to cell survival by sustaining positive growth signals, like IL2, IL6, IL8, GCSF (reviewed in Foo and Nolan, 1999) and cyclin D1 (Guttridge et al., 1999; Hinz et al., 1999) which leads to the hyperphosphorylation of the oncosuppressor protein Rb.

In the late events of malignancy, NF- κ B is definitely not the only survival factor, against apoptosis. It induces the cell adhesion molecule ICAM-1 which is known to be upregulated in 90% of the metastatic tumours (Kageshita et al., 1993) and Cox-2

and iNOS which promote angiogenesis. Therefore, by upregulating the expression of molecules which regulate cell adhesion and angiogenesis, NF- κ B is promoting metastasis.

1.4.2.1 Hodgkin's Disease

Hodgkin disease was the first recognised lymphoma as a distinct clinical lesion (Hodgkin, 1832). In that disease, clonal malignant Hodgkin/Reed-Sternberg (H/RS) cells are characteristically surrounded by a mixture of as many as 100 times more granulocytes, plasma cells and T cells. Molecular biology experimental approaches to the aetiology and pathogenesis of Hodgkin's disease, brought light on the cellular origins of H/RS cells through analysis of the antigen receptor genes of these cells (Emmerich et al., 1999). Moreover, it uncovered the role of NF- κ B signal cascade in the pathogenesis of the disease.

H/RS cells express surface markers that are normally expressed in distinct haematopoietic lineages. They can express T cell, B cell or myeloid markers (Drexler, 1993; Trumper et al., 1993). The clonal rearrangement of the V, D and J segments of the IgH chain locus (often resulting in non-functional Ig V regions), which can only occur in B cells (Jacob et al., 1991) indicated that these cells are descended from germinal centre B cell (or from cell at a later stage of differentiation). Analysis of rare patients with "composite lymphomas" (where Hodgkin disease coexists with non-Hodgkin's lymphoma), suggested that the initial transforming event in Hodgkin's disease takes place within the germinal centre B cell itself (Bräuninger et al., 1999; Marafioti et al., 1999).

In mature B cells p50•c-Rel is constitutively present in the nucleus while p50•p65 is inducible (Liou et al., 1994; Miyamoto et al., 1994). In H/RS cells both heterodimers are constitutively in the nucleus (Bargou et al., 1997; Bargou et al., 1996). The first molecular abnormality which was identified in H/RS cells was mutations in I κ B α (Cabannes et al., 1999; Emmerich et al., 1999; Jungnickel et al., 2000). Nevertheless, in many Hodgkin's cases, wild type I κ B α is expressed (Krappmann et al., 1999). In

such cases, even if the secretions of cytokines by H/RS could explain NF- κ B induction, the hypothesis of an abnormality in the IKK cascade should not be ignored: In many Hodgkin's disease cases, H/RS cells host EBV (Jarrett and MacKenzie, 1999) which expresses the latent membrane protein 1 (LMP-1). This oncoprotein is a constitutively aggregated pseudo-tumour factor receptor which activates NF- κ B via a pathway that included NIK, IKK α and IKK β (Sylla et al., 1998). Three TNF family receptor-ligand pairs can also activate NF- κ B: CD40 expressed on H/RS can associate with expressed on the surrounding T cells CD40 ligand (Carbone et al., 1995) and similarly CD30 expressed on H/RS can associate with expressed on the surrounding T cells or on H/RS themselves CD30 ligand (Gruss et al., 1996). Finally, TNF- α secreted by the H/RS can behave in an autocrine mode via TNF- α receptors (Messineo et al., 1998). The consequence of the constitutive nuclear p50•p65 in H/RS cells is the constitutive expression of many NF- κ B target genes, like I κ B α (Emmerich et al., 1999), intercellular adhesion molecule 1 (ICAM-1), GM-CSF, IL-6 and TNF- α . NF- κ B plays an important role in protecting various cells from apoptosis (Beg et al., 1995; Doi et al., 1999; Doi et al., 1997). This also applies to B cells (Wu et al., 1996b). H/SR cells contain non-functional Ig genes which suggests that they originate from germinal centre B cells. Such cells should be normally negatively selected (Küppers and Rajewsky, 1998), but it seems that aberrant NF- κ B activation protects them from apoptosis, in a possibly similar manner, that transient NF- κ B activation contributes to the normal positive selection of germinal centre B cells after their interaction with antigen-bearing follicular dendritic cells.

1.4.3 Viral infections

NF- κ B activation serves as one of the earliest cellular immune and inflammatory responses. This defensive cellular mechanism is effectively subverted by a series of viruses, like retroviruses, herpes viruses, adenoviruses etc to enhance their own gene expression and replication.

1.4.3.1 AIDS

HIV-1 infection is the aetiology of the progressive immune degeneration which leads to AIDS (Barre-Sinoussi et al., 1983; Gallo et al., 1984). HIV-1 is a retrovirus: Its RNA genome is reversibly transcribed to DNA (provirus) which is consequently integrated into the host genome. That means that during replication, the generation for its genomic RNA requires transcription of the proviral genome. Proviral transcription is regulated by a region termed HIV-1 LTR (Cullen, 1991; Greene, 1990). There are two tandem κ B motifs in the HIV-1 proviral enhancer (U3 region), between nucleotides -104 and -81, which enable NF- κ B to play an important regulatory role in proviral transcription. Another transcriptional factor which binds U3 is Sp1 (there are three tandem Sp1 binding sites downstream the two κ B motifs). It is reported that the interaction and synergistic cooperation of the two transcription factors (NF- κ B and Sp1) optimises HIV-1 LTR-driven transcription (Perkins et al., 1994; Perkins et al., 1993). Other transcription factors that are thought to have a positive effect on HIV-1 transcription, by binding to various regions of HIV-1 LTR are Myb, LEF-1, NF-AT and AP1, while factors like UBP-1/LBP-1, USF and COUP seems to have a negative effect (Roulston et al., 1995).

NF- κ B activation is not a compulsory requirement for viral replication in T lymphocytes. Nevertheless, NF- κ B is a potent HIV-1 transcriptional modulator, because it enables a positive transcriptional responsiveness towards a variety of stimuli, including cytokines, bacterial products, viral cofactors, oxidative stress etc. This modulation of HIV-1 expression has a strong impact on HIV-1 infection and AIDS progression (reviewed in Mosialos, 1997).

1.5 NF- κ B targeting drugs

It was shown that NF- κ B aberrant activation is responsible for a series of diseases, including inflammatory diseases, HIV-1 infection and cancer. The complex signal transduction of NF- κ B offers many potentials for effective intervention. Therefore, NF- κ B inhibitors could be designed and used in many diseases. Nevertheless, it should be taken into account the fact that NF- κ B is a physiological regulator of

defensive mechanisms and morphogenesis. Absolute inhibition of NF- κ B activity could cause strong side effects, even pharmacological immune deficiency. There are some diseases where NF- κ B activation is defective (e.g. auto-immune dysfunctions like systemic lupus erythematosus etc). Moreover, as it was demonstrated from the dysmorphic phenotype IKK α (-/-) mice (reviewed in Gerondakis et al., 1999), due to the unexpected critical role in keratinocyte differentiation of IKK α in morphogenesis, pharmacological NF- κ B inhibition could be extremely dangerous in embryogenesis, if it is given to pregnant women. The alternative is modulation, rather than inhibition e.g. design of compound that could block certain synergistic interactions of NF- κ B with other transcription factors, in order to block specific pathways, etc.

At the moment, there are no drugs which target specifically NF- κ B. Pharmaceutical companies are currently trying to develop such drugs. On the other hand, there are drugs which have been used for long time, and they target NF- κ B activation together with other processes.

1.5.1 Glucocorticoids

Glucocorticoids are steroid hormones secreted by the adrenal glands as a response to various stimuli. Due to their lipophilicity, they can diffuse freely across cell membranes. The glucocorticoid receptors (GR) are cytoplasmic proteins which reside in the cytoplasm bound to various protein factors including heat-shock protein 90 (hsp90) (Gustafsson et al., 1989; Sanchez et al., 1986). When the receptor binds the hormone, it undergoes a conformational change which enables it to dissociate from the protein factors it is bound. The released hormone-bound receptor translocates to the nucleus, where it forms homodimers which can recognise a consensus sequence termed glucocorticoid-responsive element (GRE). GR bound to DNA can interact with transcriptional machinery and alter the transcription rates of glucocorticoid-sensitive genes (Chandler et al., 1983; Giguere et al., 1986; Strahle et al., 1988). GR, though, can also play a transcriptionally repressive role (Drouin et al., 1989).

The use of glucocorticoids as potent anti-inflammatory and immunosuppressant drugs, is a routine practice in Medicine. The immunosuppressive action of

glucocorticoids is, partly, due to inhibition of NF- κ B-driven cytokine and adhesion molecule gene transcription (Caldenhoven et al., 1995; McKay and Cidlowski, 1998; Scheinman et al., 1995). NF- κ B and GR have a mutual antagonism: GR inhibits p65-driven transcription, while NF- κ B can inhibit glucocorticoid responsive transcription, in a dose-dependant manner. Hormone-bound GR have the ability to interact with p65-containing NF- κ B molecules and inhibit NF- κ B DNA binding. To do so, they require their DNA binding domain, but not their transactivation domain. This is how GR blocks TNF- α or IL-1 NF- κ B activation at the level both DNA binding and transcriptional activation.

1.5.2 Salicylates

Salicylates (aspirin-like drugs) are widely used non-steroidal anti-inflammatory drugs (NSAID). Their action was initially thought to be due to inhibition of cyclooxygenase (COX), also termed prostaglandin H₂ synthase (PGS) (Vane, 1971). COX exists in two isoforms, COX-1 (constitutive) and COX-2 (inducible upon proinflammatory stimulation). It first oxidises arachidonic acid to prostaglandin G₂, and it consequently peroxidises prostaglandin G₂, to prostaglandin H₂. The observation that although salicylate was much weaker inhibitor of COX than aspirin (because the latter acetylates COX), they were both equally effective against chronic inflammatory diseases and that higher doses were required for that than those for effective prostaglandin synthesis inhibition (Weissmann, 1991), were initially explained as a result of the fact that both drugs were relatively weak inhibitors of the inducible form COX-2 (Vane, 1994). Nevertheless, it was later discovered that salicylates could also reduce inflammation through inhibition of NF- κ B activation (Kopp and Ghosh, 1994). According to that report, it was shown that salicylates inhibit NF- κ B activation through interference to the pathway which leads to phosphorylation or degradation (or both) of I κ B. After the discovery of the IKKs, it was shown that the apparent blocking of NF- κ B activation by sodium salicylate and aspirin was due were selective inhibition of the enzymatic activity of IKK β , but not IKK α (Yin et al., 1998a). Aspirin was found to be a competitive inhibitor of ATP binding to IKK β , and that

although salicylates could not covalently bind to IKK β , they were irreversible or very slow reversible inhibitors. Since, IKK activation is the converging step of the signal cascade which leads to NF- κ B activation, and IKK activation is the last inducible event of this cascade (the downstream events of phosphorylated I κ B α ubiquitination and proteasome-mediated proteolytic ubiquitin-ligated I κ B α degradation are constitutive), the identification of IKK β as a salicylate target can help the design of more effective aspirin-like anti-inflammatory agents.

2 MATERIALS AND METHODS

2.1 Materials

TE

10 mM Tris-HCl, 1 mM EDTA (pH 7.6)

STE

0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 7.6)

5x TBE

0.45 M Tris-H₃BO₃, 10 mM EDTA (pH 8.3)

Luria broth

10 g/l bacto-tryptone (Difco), 5 g/l yeast extract (Difco), 10 mM NaCl (pH 7.5)

Bradford reagent

0.1 mg/ml Coomassie brilliant blue G250, 5% v/v methanol, 10% v/v H₃PO₄

Solution I

50 mM Glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 7.6)

Solution II / Buffer P2

0.2 N NaOH, 1% SDS

Solution III / Buffer P3

3 M KOAc (pH 5.5)

Buffer P1

0.1 mg/ml RNase A, 50 mM Tris-HCl, 10 mM EDTA (pH 8.0)

Buffer QBT

0.75 M NaCl, 50 mM MOPS, 15% ethanol, 0.15% Triton X-100 (pH 7.0)

Buffer QC

1 M NaCl, 50 mM MOPS, 15% ethanol (pH 7.0)

Buffer QF

1.25 M NaCl, 50 mM Tris-HCl, 15% ethanol (pH 8.5)

5x Sequenase Buffer

0.2 M Tris-HCl, 0.1 M MgCl₂, 0.25 M NaCl (pH 7.5)

5x Labelling Mix (dGTP)

7.5 μ M dGTP, 7.5 μ M dCTP, 7.5 μ M dTTP

ddG dGTP Termination Mix

80 μ M dGTP, 80 μ M dATP, 80 μ M dCTP, 80 μ M dTTP, 8 μ M ddGTP, 50 mM NaCl

ddA dGTP Termination Mix

80 μ M dGTP, 80 μ M dATP, 80 μ M dCTP, 80 μ M dTTP, 8 μ M ddATP, 50 mM NaCl

ddT dGTP Termination Mix

80 μ M dGTP, 80 μ M dATP, 80 μ M dCTP, 80 μ M dTTP, 8 μ M ddTTP, 50 mM NaCl

ddC dGTP Termination Mix

80 μ M dGTP, 80 μ M dATP, 80 μ M dCTP, 80 μ M dTTP, 8 μ M ddCTP, 50 mM NaCl

Stop Solution

95% Formamide, 20 mM EDTA, 0.05% Xylene Cyanol FF, 0.05% Bromophenol Blue

Enzyme Dilution Buffer

10 mM Tris-HCl, 5 mM DTT, 0.5 mg/ml BSA (pH 7.5)

Sequenase Version 2.0

13 units/ μ l T7 DNA Polymerase, 20 mM K_3PO_4 , 1 mM DTT, 0.1 mM EDTA, 50% Glycerol (pH 7.4)

PBS

0.137 M NaCl, 2.7 KCl, 4.3 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 (pH 7.3)

Bacterial Lysis buffer

0.5 M NaCl, 1 mM EDTA, 1 mM PMSF, 2 mM Benzamidine, 100 μ g/ml Lysozyme in PBS

Protein Purification Dilution buffer

0.5 M NaCl, 5 mM DTT, 1% Triton X-100 in PBS

Washing buffer

0.5 N NaCl in PBS

Elution buffer

0.5 M NaCl, 50 mM Tris-HCl, 10 mM Glutathione (pH 8.0)

2xHBS

50 mM HEPES, 0.28 M NaCl, 1.5 mM Na₂HPO₄ (pH 7.1)

Cell Lysis buffer

25 mM Tris-H₃PO₄, 8 mM MgCl₂, 1 mM DTT, 1% Triton X-100, 15% Glycerol (pH 8.0)

Luciferase buffer

25 mM Tris-H₃PO₄, 8 mM MgCl₂, 1 mM DTT, 1% Triton X-100, 15% Glycerol, 1 mM ATP, 0.25 mM Luciferin, 1% BSA (pH 8.0)

NHC/EDC

0.05 M N-hydroxysuccinimide, 0.2 M N-ethyl-N'-(dimethylaminopropyl) carbodiimide

Ethanolamine-HCl

1 M Ethanolamine hydrochloride (pH 8.5)

2.2 Methods

2.2.1 Cell lines and tissue culture procedures

293 cells (adenovirus transformed human foetal kidney cells) were grown in G-MEM (Gibco-BRL) supplemented with 10% (v/v) foetal calf serum (Sera-lab). Tissue cultures were grown at 37°C in 5% CO₂, and split in a 1 to 3 ratio on average every 3-4 days.

2.2.2 Bacterial strain

The genotype of the TOP10F' host strain (Invitrogen) which was used for bacterial transformations, is: F'_{tet^r} *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80Δ*lac*-ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara,leu*)7697 *galU galK λ⁻ rpsL endA1 nupG*.

2.2.3 Bacterial culture

Liquid bacterial cultures were grown in Luria broth supplemented with 100 µg/ml ampicillin (Sigma) when necessary. Solid media contained Luria broth supplemented with 1.5% agar (Difco) and ampicillin as above.

2.2.4 Preparation of competent cells

A 5 ml L-broth starter culture containing 100 µg/ml ampicillin was grown up overnight at 37°C. A 50 ml L-broth containing 100 µg/ml ampicillin was inoculated with 1 ml of the starter culture and incubated at 37°C. When the absorbance at 600 nm reached 0.5-0.6, the cells were harvested in bench centrifuge (3500 rpm, 3 minutes) and resuspended in total volume of 12.5 ml of 0.1 M. MgCl₂. The cells were harvested, resuspended in 12.5 ml of 0.1 M CaCl₂, and allowed to stand on ice for 20 minutes. They were then harvested and resuspended in 2.5 ml of 0.1 M. CaCl₂, 14% glycerol. They were aliquoted into sterile eppendorf tubes, put in liquid nitrogen, and stored at -70°C.

2.2.5 Transformation

Aliquots of competent cells were allowed to thaw in iced water. DNA (not more than 10 µl) was added into sterile eppendorf tubes on ice and 200 µl of cells were added to DNA, mixed gently and left on ice for 20 minutes. After a heat shock at 42°C for 2

minutes 1 ml L-broth was added and incubated at 37°C for 1 hour. A range of volumes were spread onto plates containing 100 µg/ml ampicillin. For every fresh batch of cells, the competency was tested by including a positive (DNA) and negative (no DNA) control.

2.2.6 Synthesis of Oligonucleotides

Synthetic oligonucleotides were prepared using solid-phase phosphoramidite chemistry in an Applied Biosystems ABI 394 DNA synthesiser. The 3' nucleoside that was covalently immobilised to a controlled pore glass (CPG) through a linker arm, was initially deprotected (detritylated) in order to provide a free 5'-hydroxyl group for the coupling with the next nucleotide. An excess of the next activated in 3'-phosphate position nucleotide was applied, followed by a capping step, necessary for the blocking any unreacted immobilised material. The formed dimer was then oxidised and the same cycle was repeated until the desired oligonucleotide was synthesised. The oligonucleotide was then cleaved from the solid-phase and was (optionally) purified by HPCL. In the case of the 3'-biotin labelled oligonucleotides, a biotin tagged CPG support was used in a manner identical to protected nucleoside support since it contained the DMT group. The synthesised DNA oligonucleotides were dissolved in water, ethanol precipitated, dried, and dissolved in water. To estimate the concentration of single stranded DNA oligonucleotides, the absorbance at 260 nm was measured using a spectrophotometer, and the concentration calculated based on the extinction coefficient of the oligonucleotide (Cantor et al., 1970; Fasman, 1975). Double stranded oligonucleotides were produced by addition of equimolar amounts of complementary single stranded oligonucleotides in TEN (100 mM NaCl, 10 mM Tris, pH 7.6, 1 mM EDTA, pH 8.0), boiling for 2 minutes and gradual cooling to room temperature (storage at 4°C or -20°C).

MHC-κB, HIV-L-κB, and IRE-κB double stranded oligonucleotides were used in DNA binding assays, 2xMHC-κB, and 2xHIV-L-κB double stranded oligonucleotides were cloned in a reporter gene vector, MHC-κB-Biotin, HIV-L-κB-Biotin, and NFIII-Biotin double stranded oligonucleotides were immobilised in SA5 sensor chips

for SPR, and pGL-2 was the primer for DNA sequencing of the recombinant plasmids (Table 4).

2.2.7 Expression and purification of NF- κ B molecules

Amino acids 35-381 of p105 (p50), 1-351 of p100 (p52) and 12-317 of p65 (Figure 17) were expressed in *E. coli* as part of a fusion protein with glutathione-S-transferase and purified by chromatography on glutathione agarose, thrombin cleavage and affinity chromatography on DNA-Sepharose as described previously (Matthews et al., 1992).

2.2.8 Expression and purification of I κ B molecules

Amino acids 1-317 of I κ B α , 509-807 of p105 (I κ B γ) and 118-358 of Bcl-3 (Figure 18) were expressed in *E. coli* as part of a fusion protein with glutathione-S-transferase. During their purification, 50 ml L-broth containing 100 μ l/ml ampicillin were inoculated (starter culture) with a single bacterial colony from a plate, incubated at 37°C overnight, and added to 1 l L-broth containing 100 μ l/ml ampicillin which was incubated at 25°C. When the OD at 600 nm was 0.4-0.8, the bacterial production of the I κ B molecules was induced by the addition of IPTG to 1 mM and incubation for 4 hours at 25°C. The bacterial cells were harvested, resuspended in bacterial lysis buffer, and stored on ice for 15 minutes followed by addition of DTT to 5 mM, N-lauryl sarcosine to 1.6%, MgSO₄ to 5 mM and DNase to 10 μ g/ml. The lysate was clarified by centrifugation at 4°C, for 30 minutes at 14000 rpm followed by addition of triton X-100 to 2% and EDTA to 8.5 mM. The mixture was recentrifuged at 4°C, for 10 minutes at 14000 rpm. The supernatant was diluted 1:5 in protein purification dilution buffer and was loaded in a 50 ml glutathione agarose column overnight. The column was washed with washing buffer and the GST fusion protein was eluted in 5 ml fractions with the addition of elution buffer in the column with flow rate 1 ml/min. The purified I κ B α was the entire molecule, whereas I κ B γ , and Bcl-3 protein which was used in our experiments was derived from the ankyrin repeat domain.

Figure 17: Homology of NF- κ B p50, p52 and p65 molecules used in the experiments. They all contain the RHR sequence.

```

p50 35 -----MALP-----TDGPYLQILEOPKORGFRFRYVCEGP 64
p52 1  MESCYNPGLDGIIIEYDDFKLNSSIIVEPKEPAPETADGPYLWIVEOPKORGFRFRYVCEGP 60
p65 12 -----EP-----ACASGPYVEIIEOPKORGFRFRYVCEGP 41

p50 65  SGGLPGASSEKNKKSYPQVKICNYVGPAAKVIIVQLVTNGKNILHAAISLVGKICEDGIC 123
p52 61  SGGLPGASSEKCRKTYPTVKICNYEGPAKIEVDLVTSDPRAHAAISLVGKICSELGIC 120
p65 42  SAGSIPGERSTDTTKTPTIKINGYTCGTVRISLVTKDLPRHHPHELVGKDCRDGFY 100

p50 124 TVTAGPKDMVVGFFNLGILVTKKKVFETLEARMTEACIRGYNPGLLVHFDLAYLQAEGG 183
p52 121 AVSVGPKDMTAQFNNLGVLVTKKNMMGTMIQKLRQRLRSRPG 165
p65 101 EAEIICPDRCI-SFENLGIQCVKKRDLEQAISSORIQTN----- 136

p50 184 GDRGLCDREKELIRQAALQOTKEMDLSVVRIMFTAFLDSTGSFTRRLEPVVSDAIYDSK 243
p52 165 ----LIEAEQRELEQEAKELEKKVMDLSIVRLRFSAFLRASDGSFSLKLPVTSPIIDSK 221
p65 137 -----NNPQVPIEEQRCDYDLNIVRLCFQVTVRDISG-RFLRLEPVLPPIFDNR 187

p50 244 APNASNLKIVRMDRTAGCVTGGEEIYLLCDKVQKDDIQIRFYEEEENGWVEFGDFSPIT 303
p52 222 SPQASNLKISRMDKTAGSVRGGDEVYIICDKVQKDDIEVRFYEDDENGVEAFGDFSPIT 279
p65 188 APNTAELKICRVNRNSGSCGGDEIFLLCDKVQKEDIENVETG--FG--VEARCSFSQA 242

p50 304 DVIRQFAIVFKTPKYKDINIIPASVVFQLRKSDLETSEEKIFLYYPEIKDKEEVQRKR 363
p52 280 DVIRQYAIVFRTPPYKMKIERPVTVFQLKRRKGGDVSDSKOFTYYPIVEDKKEEVQRKR 339
p65 243 DVIRQVAIVFRTPPYADISLQAPVRSVQLRRSDRELSEPMEFQYLPDIDDRRIIEKR 302

p50 364 QKLMNFSDSFGGGSGAG 381
p52 340 RKATPTFSDFEG----- 351
p65 303 KRTYETKKSIMKKS---- 317

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Figure 18: Homology of I κ B molecules I κ B α , I κ B γ and Bcl-3 used in the experiments. While full length I κ B α is purified for our experiments, only the ARDs of I κ B γ and Bcl-3 is purified.

IκBα	1	MFQAAERBQEWAMEGFRDGLKKERLLDDR-DSGLDSMKDEEYEQMVKELQEIIRLERQEVF	60
IκBγ	509	-----AKR-ANALFDYAVTGDVKMLLAVQR-----	532
Bcl-3	118	-----	118

Ankyrin Repeat 1

IκBα	61	RGSEFWKQQLTEDGDSFLLAIIIEEKALTMETVIROVKG-----DLAFLNQNNLOOTEL	116
IκBγ	533	-----ELTAVQDENGDVSLLAIIIEELSQLVRDLLEVTSGLISDDI-----INMRNDLYQOTEL	587
Bcl-3	118	----AMATRADEDGDTLLLAIVVQCNLPAVRLVNLFOQ--GGRE--LDIYNNLRQOTEL	169

Ankyrin Repeat 2

Ankyrin Repeat 3

IκBα	117	LAVITNQPEIIEALLGAGCDPELRDFRGNTELALACEQGCLASVGVLTQSCCTTPIISIL	176
IκBγ	588	LAVITKQEDVVEDLLRAGADLSLLDRIGNSVLLAAKEG-DKVLSTLLK-KKA-ALL--L	644
Bcl-3	170	LAVITTLPSVVRLLVITAGASPMALDR-GITAAALACE-RSFTCLRALLLSAAN-GTLD-L	227

Ankyrin Repeat 4

Ankyrin Repeat 5

IκBα	177	KATNYNG-TCLLASI-GYLGIVELLVSLGADVNAQENKNGRTALALAVDLCNEDLVSL	236
IκBγ	645	DINCDGLNATLHAMMSNSLQCLLLVAAGADVNAQENKSGRTALALAVE-DNISLAGGL	704
Bcl-3	228	EARNYDGLTALAVAVNTECQETVQLLERGADIDAVDIKSGRSLLAVENNSLSMVLL	287

Ankyrin Repeat 6

IκBα	237	LKCGADVNRVTYQGYSPYQLTWGRSTRILQQLGQLTLEN-----LQMLFESBEDES	288
IκBγ	705	LLECDAVDSITYDGTTPLLAAGRGSTRILAALLKAAGADFLVENFELYDLDDSWENAG	764
Bcl-3	288	LQGANVNAQMYSGSSALASASGRLLPLVRLVRSRGAD-----	326

Ankyrin Repeat 7

IκBα	289	YDTESEFTETFEDELFPYDDCVFGCORLTL-----	317
IκBγ	765	EDEGVVPGTTELDMATSWQVEDIILNGKPYEPEFTSDDLLAQQD	807
Bcl-3	327	SSLKNCNDTFLMVARSRRVIDLIRGKATRPA	358

Table 4: Various single and double stranded oligonucleotides used in the experiments.

MHC-κB	GCTGGGGATTCCCCATC CGACCCCTAAGGGGTAG
HIV-L-κB	GATCTAGGGACTTTCCGCG ATCCCTGAAAGGCGCCTAG
IRE-κB	GATCAAAGTGGGAAATTCCTCTG TTTACCCTTTAAGGAGACCTAG
2xMHC-κB	GATCTGGGGATTCCCCGCTGGGGATTCCCCAGG ACCCCTAAGGGGCGACCCCTAAGGGGTCCCTAG
2xHIV-κB	GATCAAGGGACTTTCCGCTGGGGACTTTCCAGG TTCCCTGAAAGGCGACCCCTGAAAGGTCCCTAG
MHC-κB-Biotin	GCTGGGGATTCCCCATCTT-Biotin CGACCCCTAAGGGGTAG
HIV-L-κB-Biotin	AAAGTGGGAAATTCCTCTGTT-Biotin TTTACCCTTTAAGGAGAC
NFIII-Biotin	GAGTTAATATGCAAATAAGTT-Biotin CTCAATTATACGTTTATTC
pGL-2	GACTATGGTTGCTGACTAAT

2.2.9 Determination of protein concentration

For the estimation of protein concentrations, a microscale variant of Bradford's dye binding method was used (Bradford, 1976). The protein samples were added to 1 ml of Bradford reagent, mixed and allowed to stand for 10 minutes before measurement of the absorbance at 595 nm. In each case absorbance measurements were performed against blanks containing an equal volume of the appropriate buffer in 1 ml Bradford reagent. These absorbance measurements were then correlated with a freshly-generated calibration curve (0-25 µg of bovine serum albumin in 1 ml Bradford reagent) to estimate the protein concentration of the unknown sample.

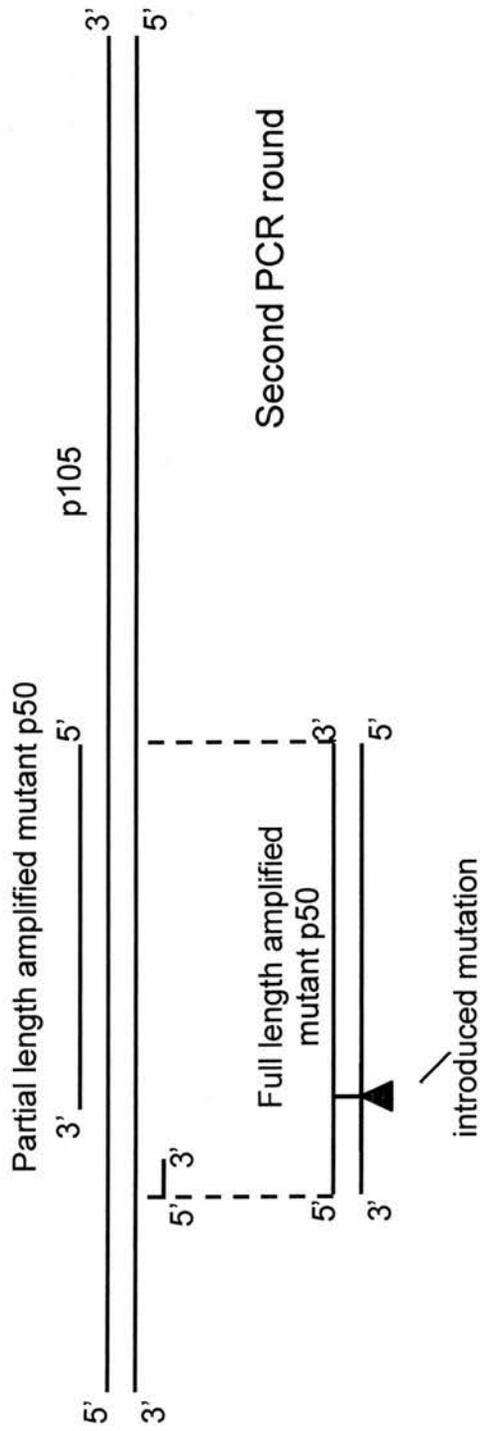
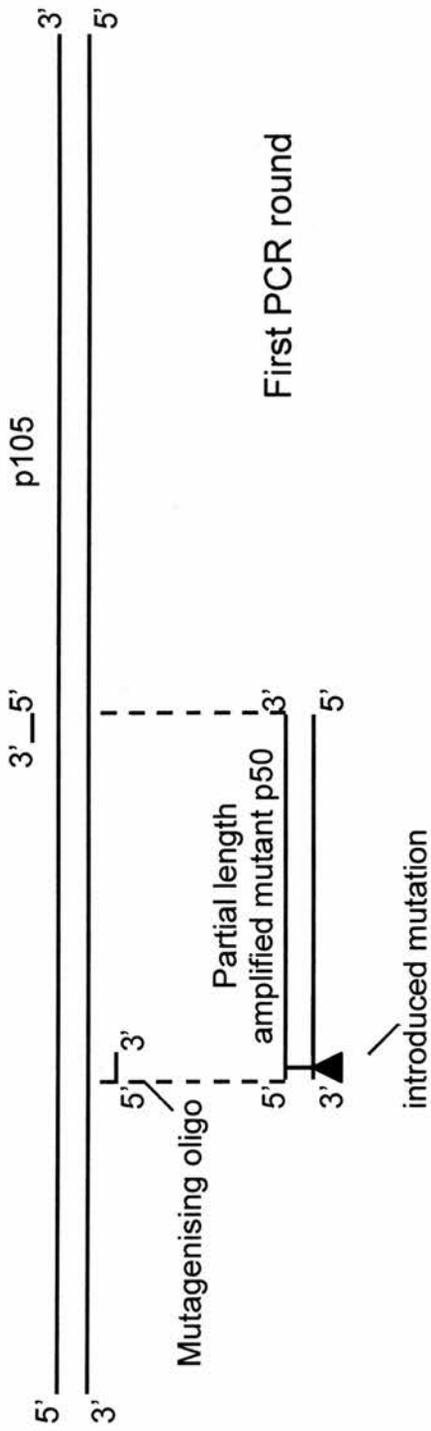
2.2.10 Preparation of the heterodimers of NF-κB proteins

Equal molar amounts of the two proteins (p50 and p65) were added, and incubated for 30 min at 37°C to form a heterodimer (p50•p65).

2.2.11 Site specific mutagenesis

The construction of an expression vector expressing p50, was previously performed (Matthews et al., 1992). For the construction of genes encoding mutated p50 molecules, a two step PCR mutagenesis technique was employed (Landt et al., 1990). In both reactions a plasmid encoding the p105 precursor of p50 (kindly provided by A. Israël) was used as a template. The purified product of a partial PCR amplification of p50 with a mutagenising internal primer and an external one (with the additional restriction enzyme cleavage site), was used, together with another external primer (with the different additional restriction enzyme cleavage site), as a primer for the amplification of full length mutant p50 genes (encoding amino acid 35 to amino acid 381) (Figure 19). The products of the second the PCR round were cleaved with BamHI and EcoRI and ligated into similarly cleaved pGEX-2T vectors (Smith and Johnson, 1988). All the plasmids were used for the transformation of E. coli to ampicillin resistance. The authenticity of the cloned products was checked by DNA sequencing (Sequenase 2.0, Amersham).

Figure 19: Two step PCR mutagenesis technique was employed (Landt et al., 1990). In the first PCR reaction a partial length DNA is amplified, using a downstream oligo and a mutagenising oligo, where the introduction of the mutation is to be made. In the second PCR round one of the two strands of the partial length amplified and mutated DNA fragment, serves as a primer. The other primer is an upstream oligo.



2.2.12 Analytical procedures

Gel electrophoresis, DNA affinity chromatography and SDS polyacrylamide gel electrophoresis were carried out as described previously (Matthews et al., 1993a; Matthews et al., 1992).

2.2.13 Partial proteolysis

To investigate whether the replacement of a positively charged hydrophilic amino acid residue (Lysine) by a non polar hydrophobic one (Alanine) could induce a major conformational change, purified wt p50 protein and p50 (K77A, K79A, K80A) were incubated with either proteinase K or chymotrypsin and the digestion products analysed by SDS PAGE and Coomassie staining as described previously (Matthews et al., 1995).

p50- Δ NLS was produced by proteolytic degradation by trypsin cleavage of previously purified p50. This fragment included the region between the amino acid 35 and 362 and it lacked the NLS sequence.

2.2.14 Radiolabelling of DNA oligonucleotides

To label a double stranded κ B motif oligonucleotide with [32 P], 5 pmols of single stranded κ B motif oligonucleotide were incubated in 10 μ l of 70 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 5 mM DTT buffer, 1 U/ μ l T4 Polynucleotide Kinase (New England Biolabs), and 0.66 μ M [γ - 32 P] ATP (Amersham, specific activity 3000 Ci/mmol) at 37°C for 45 minutes in a 1.5 ml screw-cap tube. 5 pmols of complementary strand κ B motif oligonucleotide were added. The reaction was then terminated by heating to 100°C for 2 minutes. The mixture was allowed to cool gradually to room temperature overnight.

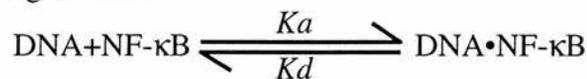
Radiolabelled, double stranded κ B motif oligonucleotides were separated from unincorporated [γ - 32 P] ATP and the single stranded oligonucleotides by size exclusion chromatography. A 5 ml column containing BIO-GEL P-6 Gel (BIO-RAD) in STE, was run, and the counts per second of 1 μ l from every fraction, were counted, using Cherenkov radiation, to identify fractions containing radioactive DNA.

2.2.15 Gel electrophoresis DNA binding assay

The DNA binding properties of the different purified p50 proteins (wild type and mutants) were determined on 6% polyacrylamide gels (Acrylamide: Bis-Acrylamide 55:1) in 0.5x TBE. Typically, the reaction (total volume 20 μ l) contained 0.16 M NaCl, 27.5 mM Tris-HCl (pH 7.6), 10 mM DTT, 1 μ g/ μ l BSA (Protease Free), 5% Glycerol, 0.5% Nonidet P40, NF- κ B protein, and 2 nM radiolabelled double stranded. After incubation for 30 minutes at room temperature, the samples were loaded in the gels to electrophoretically separate free DNA from the protein-bound DNA. The Gels were run for 1.5 hours at 150 V, placed on Whatman DE81 paper, dried, and exposed to a phosphorimager screen. Radioactive DNA was visualised using a FUJI phosphorimager. Quantitation of the radioactivity in each DNA species was determined using MacBas. Gel electrophoresis DNA binding assays were complicated by the symmetrical nature of the κ B motif oligonucleotides, as they had a propensity to form hairpin like molecules. Thus, only a proportion of the DNA was fully double stranded and competent for binding to NF- κ B.

2.2.16 Scatchard plot analysis

In an NF- κ B DNA binding reaction:



the direction of the reaction is controlled by dissociation constant Kd and the association constant, Ka where:

$$Ka = 1/Kd$$

In equilibrium:

$$Kd = \frac{[\text{DNA}][\text{NF-}\kappa\text{B}]}{[\text{DNA} \cdot \text{NF-}\kappa\text{B}]}$$

If $[F]$ is the concentration of protein-free DNA ($[F] = [\text{DNA}]$), $[B]$ the concentration of bound to protein DNA ($[B] = [\text{DNA} \cdot \text{NF-}\kappa\text{B}]$), $[P]$ is the concentration DNA-free protein ($[P] = [\text{NF-}\kappa\text{B}]$) and $[P_0]$ is the concentration of protein prior to the addition of DNA, then:

$$[P_0] = [P] + [B] \Leftrightarrow [P] = [P_0] - [B]$$

If [D] is the concentration of total DNA, then:

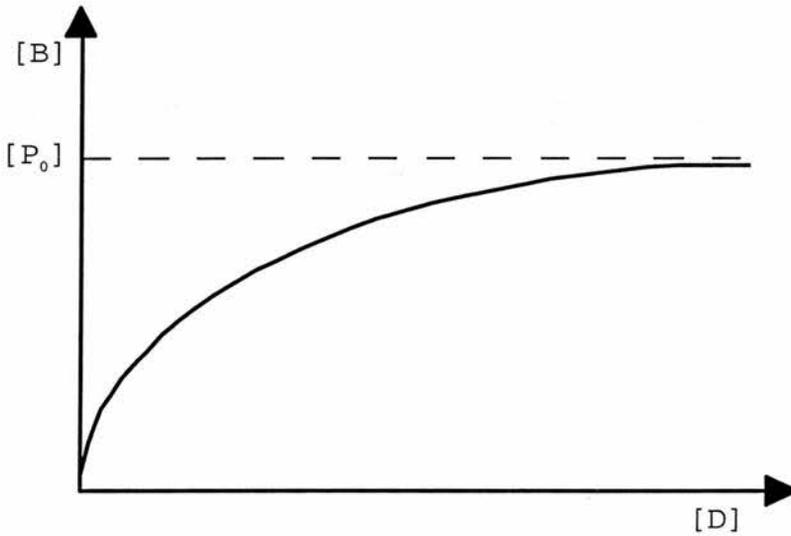
$$[D] = [F] + [B] \Leftrightarrow [F] = [D] - [B]$$

The previous equation is transformed to:

$$Kd = \frac{[F][P]}{[B]} \Leftrightarrow Kd = \frac{([D] - [B])([P_0] - [B])}{[B]} \Leftrightarrow$$

$$[B] = \frac{[D] + [P_0] + Kd - \sqrt{([D] + [P_0] + Kd)^2 - 4[D][P_0]}}{2}$$

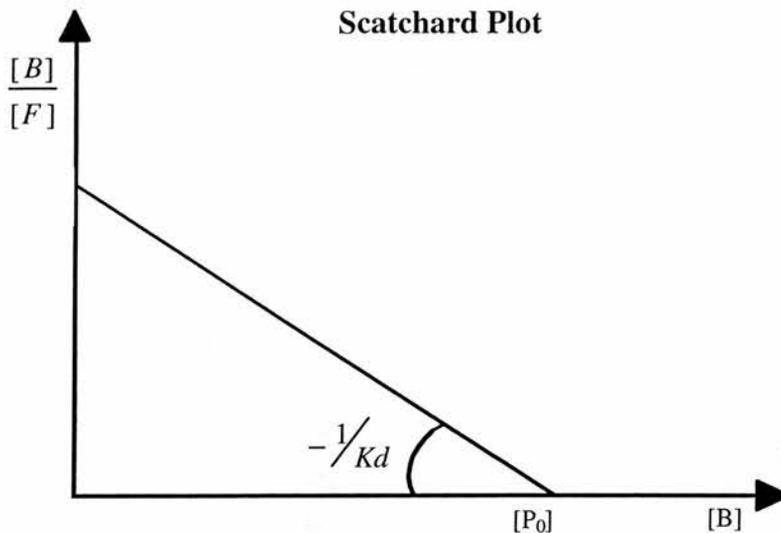
Thus, the plot of the concentration of total DNA against the concentration of bound to protein DNA, looks as follows:



In that curve data can not be fit easily and the calculation of Kd is not straight forward. On the other hand, in Scatchard analysis (Dahlquist, 1978):

$$Kd = \frac{[F][P]}{[B]} \Leftrightarrow Kd = \frac{[F]([P_0] - [B])}{[B]} \Leftrightarrow \frac{[B]}{[F]} = \left(-\frac{1}{Kd} \right) [B] + \frac{[P_0]}{Kd}$$

-1/Kd is the slope of a linear plot of the rate [B]/[F] (a value that can be easily produced by dividing the PSL of the band corresponding to the NF-κB•DNA complex by the PSL of the band corresponding to the NF-κB-free DNA) against the concentration of bound to protein DNA.



2.2.17 Surface plasmon resonance (SPR)

Surface plasmon resonance (**SPR**) detectors allow the direct visualisation of macromolecular interactions in real time, thus providing the data for the characterisation of the kinetics and thermodynamics (rate and equilibrium binding constants respectively) of these interactions (O'Shannessy et al., 1994).

During Total Internal Reflection (**TIR**), light which travels through an optically dense medium (e.g. glass) is totally reflected back to that medium at the interface with a less dense medium (e.g. buffer), provided the angle of incidence is greater than a critical angle required for the pair of optical media. Even if total light reflection occurs, a component of the incident light momentum (**evanescent wave**), penetrates the less dense medium a distance of an order of one wavelength. This wave can excite molecules which are in close proximity with the interface between the media. When the incident light is monochromatic and polarised (e.g. laser beam), and the interface is coated with a thin metal (e.g. golden) film with thickness of a fraction of the wavelength, the evanescent wave interacts with the free oscillating electrons (**plasmons**) of the metal layer. In Surface Plasmon Resonance, a portion of the incident light energy is lost to the metal surface, causing a measurable decrease of the intensity of the reflected light. SPR occurs in a precisely defined angle of the incident light, which depends on the refractive index of the medium which is close proximity

with the metal layer. Thus, changes of the refractive index of the buffer solution up to 300 nm from the metal coat, change the resonance angle. By continuous monitoring of the resonance angle, changes of the refractive index of the buffer in proximity with the metal film surface can be quantitated. Biosensors exploit this principle, since the change of angle of resonance (in Resonance Units or **RU**) is proportional to the surface concentration of molecules that can be bound close to the metal surface, during molecular binding reactions.

The SPR detector which was used was a BIAcore-X system, run by a PC, containing an SPR monitor and an integrated microfluidic cartridge which, together with an autosampler, regulate the delivery of sample plugs into a running buffer that continuously passes over a sensor surface in a flowcell.

In the DNA binding assays, SA Sensor chips were used (BIAcore) with streptavidin pre-immobilised on a dextran matrix. The removal of streptavidin, loosely bound to the chip, was performed by three applications of 0.07% SDS in water at 10 $\mu\text{l}/\text{min}$ flow rate, for 1 minute. Then, the surface was divided in two flow cells and double stranded biotinylated DNA was applied to the second flow cell such that the capture would be 72 RU. The theoretical binding capacity of the chip is represented by an R_{max} value of 500 RU. The dsDNAs (top strand shown) applied on the chip surfaces were MHC (5'-GCTGGGGATTCCCCATC-3') derived from H-2K κB motif, IRE (5'-AAAGTGGGAAATTCCTCTG-3') derived from the interferon responsive element κB motif and NFIII (5'-GAGTTAATATGCAAATAAG-3') derived from the octamer motif. One of the strands of each of these double stranded oligonucleotides had a 2 bases (TT) protruding 3' end which was biotinylated.

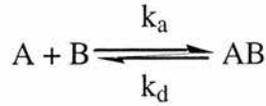
A 10 $\mu\text{l}/\text{min}$ continuous flow of running buffer 250 mM NaCl, 10 mM HEPES pH 7.4, 3 mM EDTA, 2 mM DTT and 0.05% (v/v) Tween-20 was applied. The proteins were diluted in the running buffer (concentration 100 nM) and were injected to both flow cells, for 2 minutes (association step). Running buffer was then applied for 10 minutes (dissociation step). The surface was regenerated by the application of 0.07% SDS in water, for 1 minute.

In the protein binding assays, CM5 Sensor Chips were used (BIAcore). Goat Anti-GST IgG antibody was immobilised on the dextran matrix, using amine coupling chemistry, as follows: A 5 μ l/min continuous flow of running buffer 250 mM NaCl, 10 mM HEPES pH 7.4, 3 mM EDTA, and 0.005% (v/v) Surfactant P20 (BIAcore) was applied. Freshly made NHS/EDC was applied for 7 minutes to activate carboxymethyl groups of the dextran matrix, followed by the injection of 30 μ g/ml Anti-GST up to 2000 RU. The application of Ethanolamine-HCl for 7 minutes deactivated the matrix and removed materials loosely bound to the chip. Then, the surface was divided in two flow cells and I κ B γ -GST was applied to the second flow cell such that the capture would be 350 RU. The theoretical binding capacity of the chip is represented by an R_{max} value of 500 RU, if each I κ B γ -GST bound a (p50)₂.

A 10 μ l/min continuous flow of running buffer 250 mM NaCl, 10 mM HEPES pH 7.4, 3 mM EDTA, and 0.05% (v/v) Tween-20 was applied. The proteins were diluted in 20 mM DTT in the running buffer (concentration 100 nM) until they were fully reduced and were injected to both flow cells, for 2 minutes (association step). As opposed to the DNA binding protocol, DTT was avoided in the running buffer since it would also reduce the S-S bonds which stabilise the IgG antibody. That would cause irreproducibility of the results. Running buffer was then applied for 10 minutes (dissociation step). The surface was regenerated by the application 10mM Glycine pH2., for 1 minute, which was removing both p50 and I κ B γ -GST.

The kinetic data were produced by subtracting the SPR signals generated by passing the protein solutions across a flow cell containing streptavidin without DNA, from those obtained from a flow cell containing streptavidin with biotinylated double stranded DNA, in DNA binding assays and across a flow cell containing anti-GST without I κ B γ -GST, from those obtained from a flow cell containing anti-GST with I κ B γ -GST, in the protein binding assays. These data were evaluated with BIAevaluation 3.0 software package (Bondeson et al., 1993; Parsons et al., 1995). The kinetics background of this analysis is as follows:

In every binding reaction where two molecules form a binary complex:



the association rate of the complex is:

$$\frac{d[AB]}{dt} = k_a[A][B]$$

and the dissociation rate is:

$$-\frac{d[AB]}{dt} = k_d[AB]$$

At equilibrium, the association and the dissociation rates are equal, thus:

$$k_a[A][B] = k_d[AB]$$

By rearranging that, the **equilibrium dissociation constant** is:

$$K_D = \frac{k_d}{k_a} = \frac{[A][B]}{[AB]}$$

and the **equilibrium association constant** is:

$$K_A = \frac{k_a}{k_d} = \frac{[AB]}{[A][B]}$$

In the *association phase*, the substance B (ligand) is immobilised on the surface and the substance A (analyte) is applied on the flow (and its concentration remains stable).

Under these conditions, the rate equation is:

$$\frac{d[AB]}{dt} = k_a[A][B] - k_d[AB]$$

The concentration of the unbound ligand [B] is the difference between the total amount of the immobilised ligand [B₀] and the amount of the complex [AB]:

$$[B] = [B_0] - [AB]$$

Thus:

$$\frac{d[AB]}{dt} = k_a[A]([B_0] - [AB]) - k_d[AB]$$

The last equation can be expressed in terms of SPR response (R), where R_{\max} is the response when all immobilised molecules are bound, C the concentration of the analyte:

$$\frac{dR}{dt} = k_a C(R_{\max} - R) - k_d R$$

The last equation can be rearranged to:

$$\frac{dR}{dt} = k_a C R_{\max} - (k_a C + k_d) R$$

After integration, the equation that describes the association curve, is as follows:

$$R = \frac{k_a C R_{\max}}{k_a C + k_d} \left(1 - e^{-(k_a C + k_d)t} \right)$$

In *dissociation phase*, the flow contains only the buffer solution, and the already formed complexes dissociate (we assume that once they dissociate, the analyte is removed with the flow and does not reassociates with the ligand). Then, the response rate is:

$$\frac{dR}{dt} = -k_d R$$

After integration, the equation that describes the dissociation curve, where R_0 is the response in time t_0 and R_t is the response at time t , is as follows:

$$R_t = R_0 \cdot e^{-k_d(t-t_0)}$$

The software which is based on curve fitting, initially estimates the k_d from the dissociation phase, and by subsequently substituting this value in the association equation, it estimates the k_a from the association phase.

2.2.18 Experimental protein extinction coefficient estimation

Protein concentration estimations (Bradford, 1976) were followed by measurement of absorbance of the proteins in native conditions, at 280 nm. From Beer-Lambert rule:

$$\varepsilon = \frac{A}{lc}$$

where ϵ is the extinction coefficient, A is the absorbance, l is the length of the cell, and c is the protein concentration (in mg/ml).

2.2.19 Sedimentation Equilibrium

The partial specific volume (\bar{v}) of proteins with known amino acid composition can be calculated by using Traube's rule:

$$\bar{v} = \frac{\sum n_i M_i \bar{v}_i}{\sum n_i M_i}$$

where n_i is the number of residues of each amino acid in a protein monomer, M_i is the molecular weight of the amino acid, and \bar{v}_i is its partial specific volume.

In the sedimentation equilibrium experiments, the rotor is run at a moderate speed which is high enough to allow the generation of a measurable distribution of the solute, but low enough, so the centrifugal force which tends to sediment the solute to the bottom of the cell, is counterbalanced by the diffusion force which tends to maintain the same concentration everywhere in the cell. In thermodynamic equilibrium, the solute distribution is described as follows (Williams et al., 1958):

$$M = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \times \frac{d(\ln c)}{dr^2}$$

where M is the molecular weight (in g/mol); R is the gas constant; T is the temperature in Kelvin; \bar{v} is the partial specific volume of the solute (in l/g); ρ is the density of the solvent; ω is the angular velocity; c is the concentration of the solute (in g/l) at a radial distance r from the axis of rotation. By integration:

$$c_r = c_{r_0} e^{\left[\frac{\omega^2}{2RT} M (1 - \bar{v}\rho) (r^2 - r_0^2) \right]}$$

where c_r is the concentration at radius r and c_{r_0} is the concentration at the reference radius r_0 . The exponential distribution of the protein, can also be expressed in terms of absorbance, since according to Beer-Lambert rule, the absorbance is proportional to concentration:

$$A_r = A_{r_0} e^{\left[\frac{\omega^2}{2RT} M (1-\bar{v}\rho)(r^2-r_0^2) \right]} + E$$

where A_r is the concentration at radius r ; A_{r_0} is the concentration at the reference radius r_0 and E is the optical baseline offset. So, curve fit of the data A_r versus r can find the least squares best estimate of M , provided that there is only one solute species. If, the solution is a heterogeneous mixture of i different solute species, then the analysis estimates the weight-average molecular mass M_w , which is defined as:

$$M_w = \frac{\sum M_i c_i}{\sum c_i}$$

In order to determine the stoichiometry and the strength of association of a self-associating system defined by up to three association constants (K_{a_2} , K_{a_3} , K_{a_4}) for oligomers composed of up to four monomers, we fit the curves to the following formula (Kim et al., 1977):

$$A_r = A_{r_0} e^{\left[\frac{\omega^2}{2RT} M (1-\bar{v}\rho)(r^2-r_0^2) \right]} + K_{a_2} A_{r_0}^2 e^{\left[\frac{\omega^2}{RT} M (1-\bar{v}\rho)(r^2-r_0^2) \right]} + K_{a_3} A_{r_0}^3 e^{\left[\frac{3\omega^2}{2RT} M (1-\bar{v}\rho)(r^2-r_0^2) \right]} + K_{a_4} A_{r_0}^4 e^{\left[\frac{2\omega^2}{RT} M (1-\bar{v}\rho)(r^2-r_0^2) \right]} + E$$

In a model-independent analysis, called M^* analysis, point average molecular weight M^* values are calculated (Creeth and Harding, 1982) and plotted against the radial displacement squared parameter ξ , which is defined as:

$$\xi = \frac{r^2 - a^2}{b^2 - a^2}$$

where a and b are the radial positions of the meniscus and cell bottom, respectively and r is the radius. Since:

$$M^*_{\xi=1} = M_{w,app}$$

by extrapolation of M^* to $\xi=1$ in the M^* versus ξ plot, a weight-averaged molecular mass can be estimated.

In 1 ml of each protein, fresh DTT was added to a final concentration of 20 mM, for ~2 h at room temperature. The fully reduced proteins were then dialysed against 500

ml of 350 mM NaCl, 10 mM NaH₂PO₄/Na₂HPO₄ pH 7.0, 1 mM EDTA for ~2 h, then dialysed overnight at 4°C in 500 ml of the same fresh buffer. The dialysates were used for buffer subtraction in the spectrophotometer and ultracentrifuge. All sedimentation experiments were performed in a Beckman (Palo Alto, CA) Optima XL-A analytical ultracentrifuge equipped with automatic absorbance optics in combination with a nine cell, An-60Ti rotor. The proteins were studied in native conditions, in equilibrium runs on the Beckman Optima XL-A analytical ultracentrifuge at 4°, obtaining absorbance data at 280 nm. In each run, absorbance values at low speed (3 krpm) were recorded for the samples so that the sample concentrations could be back calculated, then, the rotor speed was increased to a moderate speed to obtain equilibrium, and finally, the rotor was speeded up to high speed (47 krpm) to obtain a true optical baseline free from macromolecular species.

2.2.20 Sedimentation Velocity

Sedimentation velocity experiments are performed in higher speeds than Sedimentation equilibrium experiments, to cause rapid sedimentation of solute towards the cell bottom. While the solution is initially universal, soon the solute near the meniscus is depleted, while a sharp boundary between the depleted region and the uniform concentration of sedimenting solute is formed. These experiments can give us an idea of the form of the molecules in solution.

In sedimentation velocity experiments, the 3 forces act to each solute particle: The gravitational force (F_s), the buoyant force (F_b) and the frictional force (F_f).

The gravitational force (F_s) is the sedimenting force:

$$F_s = m\omega^2 r = \frac{M}{N}\omega^2 r$$

where m is the mass of the particle, ω is the angular velocity, r the distance of the particle from the axis of rotation, M the molar weight of the particle and N Avogadro's number.

The buoyant force (F_b), according to Archimedes's principle equals the weight of the displaced fluid:

$$F_b = -m_0 \omega^2 r$$

where m_0 is the mass of the fluid displaced by the particle:

$$m_0 = m \bar{v} \rho = \frac{M}{N} \bar{v} \rho$$

where \bar{v} is the partial specific volume of the particle (the inverse of its effective density) and ρ is the density of the solvent.

The frictional force (F_f) is the drag which acts to the particle while its moves through a viscous fluid:

$$F_f = -fu$$

where f is the friction coefficient (depends on the shape and size of the particle) and u the velocity of the particle.

For very small time periods, the forces which act on the particle come into balance:

$$F_s + F_b + F_f = 0$$

$$\frac{M}{N} \omega^2 r - \frac{M}{N} \bar{v} \rho \omega^2 r - fu = 0$$

$$\frac{M}{N} (1 - \bar{v} \rho) \omega^2 r - fu = 0$$

which gives:

$$\frac{M(1 - \bar{v} \rho)}{Nf} = \frac{u}{\omega^2 r} \equiv s$$

where s is the sedimentation coefficient and depends on the properties of the particle.

u can be experimentally measured as the rate of movement of the boundary:

$$s \equiv \frac{u}{\omega^2 r} = \frac{dr_{bnd} / dt}{\omega^2 r}$$

By solving this differential:

$$\ln(r_{bnd} / r_m) = s \omega^2 t$$

where r_m is the radial position of the meniscus. Thus, $s\omega^2$ can be estimated as the slope of a linear plot of $\ln r_{\text{bnd}}$ vs. time. Practically, two independent pieces of software (origin and svedberg) (Philo, 1997) were used in order to process the data. Since, s depends on the density and viscosity of solution, conventionally the observed s is consequently calculated in terms of a standard solvent (water) at 20°C, as follows:

$$s_{20,w} = s_{\text{obs}} \frac{\eta_{T,w} \eta_s (1 - \bar{v}\rho_{20,w})}{\eta_{20,w} \eta_w (1 - \bar{v}\rho_{T,s})}$$

where $s_{20,w}$ is the sedimentation coefficient in water at 20°C, s_{obs} is the measured sedimentation coefficient in buffer at the temperature of the experiment, $\eta_{T,w}$ is the viscosity of water at the temperature of the experiment, $\eta_{20,w}$ is the viscosity of water at 20°C, η_s and η_w the viscosity of buffer and water respectively at a common temperature, $\rho_{20,w}$ the density of water at 20°C, and $\rho_{T,s}$ the density of buffer at the temperature of the experiment. Finally, $s_{20,w}$ values at different concentrations are plotted against their concentrations, and the $s_{0/20,w}$ (the sedimentation coefficient in water at 20°C, in 0 concentration) is obtained by extrapolation to 0 concentration on the x axis.

All runs were performed on a Beckman Optima XL-A analytical ultracentrifuge. Absorbance values at 3 krpm were recorded for the samples so that the sample concentration could be back calculated. The rotor speed was increased and at least 200 scans were recorded at 30 min intervals. Finally, using the published atom coordinates of the molecules, sedimentation coefficients of these molecules were predicted using a bead model by computer (HYDRO), and the theoretically calculated values were compared with the once experimentally obtained, in order to suggest the solution form of the molecules.

2.2.21 Computational analyses

NCBI Entrez was used for search and transfer of DNA and protein sequences submitted in various databases. Genetics Computer Group software package GCG 8.0 (UNIX) and DNA Strider 1.0 (Macintosh) were used for computational analyses of

the sequence data. DNA and protein sequence alignments were performed with GCG and the outputs were presented with SeqVu 1.0.1 (Macintosh). The atomic coordinates of several crystal structures were obtained from the Protein Data Bank at Brookhaven National Laboratory. These data were manipulated and processed by Insight II (IRIX), O (IRIX) and RasMol 6 (Macintosh) molecular modelling software. Atomic distances were measured and figures were prepared with Insight II and O.

2.2.22 Molecular modelling

The atomic coordinate data of the human p50•DNA complex (Müller et al., 1995), were obtained from the Protein Data Bank run by Brookhaven National Laboratory (PDB ID code: 1SVC). The protein fragment contained the residues 2-366 of the mutated C62A p50 bound to a DNA 19-mer. These data were animated and processed by Insight II molecular modelling software in a Silicon Graphics computer. Atom distances were measured and figures were prepared with the same software.

2.2.23 Sequence alignment

DNA and protein alignments were performed with the GCG software package (Genetics Computer Group) for UNIX.

2.2.24 DNA cloning

Three recombinant plasmids were produced: pGL-2pv/2xHIV, pGL-2pv/2xMHC, and pcDNA I/Amp/p105. The plasmids were digested with the appropriate restriction enzymes, in the appropriate restriction buffers.

2.2.25 Colony hybridisation

Colonies from the transformed bacteria were transferred in new Agar plates. After an overnight incubation, the cells were blotted in circular 3MM Whatman filter paper. The filter was, then, put in 0.1 M HCl for 1 minute, in 0.5 M NaOH for 15 minutes, in 1 M Tris-HCl (pH 7.6) for 5 minutes, and in 0.1 M NaCl for 15 minutes. It was dried and placed on UV light box for 2 minutes. The filter was put in a hybridisation cylinder (Techne) and 5 ml of prehybridisation solution was mixed. After 2 hours of incubation at 50°C, the ssDNA radiolabelled probe was added. The cylinder was incubated overnight at 50°C. The paper was washed and put in a sealed plastic bag.

The plastic bag was placed in a cassette with a film for autoradiography. This showed the colonies which contained the dsDNA oligonucleotide insert.

2.2.26 DNA sequencing

DNA sequencing was used in order to verify the different plasmid used, and to check the orientation of the cloned inserts and the introduced sequence specific mutations.

Sequenase Version 2.0 DNA Sequencing Kit protocol was followed. 5 µg plasmid DNA was denatured with alkali, precipitated, and added to the annealing mixture which, also, contained equal molar amount of the primer and 2 µl 5x Sequenase buffer. The mixture was heated for 2 minutes at 65°C, cooled slowly to <35°C over 30 minutes, and then chilled on ice. 1 µl 0.1 M DTT, 2 µl 10-fold diluted dGTP Labelling Mix, 0.5 µl [³⁵S] dATP, and 9-fold diluted Sequenase polymerase version 2.0 were added to the annealed DNA mixture. 3.5 µl of this labelling reaction mixture were mixed with 2.5 µl of each dGTP termination mixture (G, A, T and C). This termination mixture was incubated at 42°C for 5 minutes. The reactions were stopped by adding 4 ml of stop solution. The samples were heated at 85°C for 2 minutes and chilled on ice. 2.5 µl of each sample were loaded in a DNA sequencing polyacrylamide gel containing 6% acrylamide: bis-acrylamide (19:1), 1x TBE, 7 M Urea, 0.1% TEMED, 0.025% APS, and 0.05% bromophenol blue.

The ABI automatic DNA Sequencer used the dRhodamine ABI PRISM BigDye Terminators Kit with the cycle sequencing enzyme AmpliTaq DNA Polymerase FS (a mutant form of Taq DNA polymerase). 0.5 µg of DNA template and 3.2 pmols of primer were added to a mix containing buffer, enzyme, dNTPs and fluorescently labelled dideoxy terminators in excess (because they are difficult to incorporate during extension), in a single tube, as each of the four dideoxies was labelled with a different colour fluorescent tag. The whole mixture went through 25 cycles of denaturation, annealing and extension in a thermal cycler (the use of Taq as the DNA polymerase allowed the reactions to be run at high temperatures to minimise secondary structure problems as well as non specific primer binding) and the completed reaction was ethanol precipitated, dried in a vacuum centrifuge for five

minutes and loaded in a gel. The energy transfer acceptors, dichlororhodamine dyes (ABI PRISM dRhodamines), were spectrally resolved and then software analysis of the four signals revealed the DNA sequence.

2.2.27 *Small scale preparation of plasmid DNA*

Mini preparations of plasmid DNA were obtained by the alkaline lysis method. A single bacterial colony was transferred into 2 ml of LB medium containing 100 µg/ml ampicillin. The culture was incubated overnight at 37°C with vigorous shaking. Cells were harvested by brief centrifugation in a microcentrifuge and the supernatant was discarded. The bacterial cell pellet was resuspended in 0.5 ml of STE and recentrifuged. The obtained pellet was resuspended in 100 µl of ice-cold Solution I by vigorous vortexing. 200 µl of freshly prepared Solution II were added, mixed by inversion and stored on ice. Then, 150 µl of ice-cold Solution III were added and mixed gently by vortexing for 10 seconds in order to disperse Solution III through the viscous bacterial lysate. The lysed bacterial suspensions were stored on ice for 5 minutes and then centrifuged at 13000 g for 5 minutes at 4°C in a microfuge. The supernatant was transferred and an equal volume of phenol: chloroform was added and mixed by vortexing. After centrifuging at 13000 g for 2 minutes at 4°C, the supernatant was transferred to a fresh tube. The double-stranded DNA was precipitated with the addition of 2 volumes of ethanol at room temperature. The mixture was allowed to stand for 2 minutes at room temperature and centrifuged at 13000 g for 6 minutes at 4°C. The supernatant was removed and the pellet of double-stranded DNA was rinsed with 1 ml of 70% ethanol at 4°C. The pellet of nucleic acid was dried in the air for 10 minutes and redissolved in 50 µl of TE (pH 8.0) containing DNase-free pancreatic RNase (20 mg/ml). The DNA was stored at -20°C.

2.2.28 *Large scale preparation of plasmid DNA*

Using QIAGEN-tip 500 DNA purification kit, up to 500 µg of plasmid DNA can be obtained from 100 ml of E. coli LB culture for high copy number plasmids. The cells were harvested with a centrifugation and resuspend in 10 ml of buffer P1. 10 ml of buffer P2 were added, mixed, and incubated at room temperature for 5 min. 10 ml of

chilled buffer P3 were added, mixed, and incubated on ice for 20 min. The lysate was centrifuged at 4°C for 30 min at >30000 g. The supernatant was removed promptly. A QIAGEN-tip 500 was equilibrated with 10 ml of buffer QBT. The supernatant was applied onto the QIAGEN-tip 500. The QIAGEN-tip 500 was washed with 2 x 30 ml of buffer QC. The DNA was eluted with 15 ml of buffer QF, and precipitated with 0.7 volumes of isopropanol. After a centrifugation at >15000 g at 4°C for 30 min, the DNA was washed with 15 ml of cold 70% ethanol, air dried for 5 min, and redissolved in 0.5 ml TE.

2.2.29 Transfection

5 µg plasmid DNA and 30 µl of 2M CaCl₂ were added and mixed. This solution was added dropwise to 240 µl of 2xHBS. The precipitate was allowed to form for 30 minutes at room temperature. The DNA precipitate was added dropwise to the Petri plates of the cells. After 4 hours incubation, the medium was replaced and the plates were incubated overnight.

2.2.30 Luciferase assay

The cells were washed twice with 4 ml PBS. 200 µl ice cold lysis buffer was added. After 5 minutes on ice, the cells were scraped and transferred. The protein concentration was determined and 100 µl of cell extract were added to a cuvette which was then placed in a luminometer. 100 µl of Luciferase buffer were injected and the light units were measured.

3 RESULTS

3.1 Interaction between NF- κ B proteins and Bcl-3

3.1.1 Expression and Purification of NF- κ B proteins and Bcl-3

To investigate the binding properties of various NF- κ B proteins on a series of κ B motifs, in the presence of Bcl-3, amino acids 35-381 of p50, 12-317 of p65 and 1-351 of p52 RHR were expressed in *E. coli* (Figure 17) as parts of fusion proteins with GST and purified by chromatography on glutathione agarose, thrombin cleavage and affinity chromatography on DNA-Sepharose. The amino acids 119-358 of Bcl-3 (Bcl-3 ARD) were also expressed in *E. coli* (Figure 18) as part a fused to GST protein and purified by chromatography on glutathione agarose. Attempts to consequently cleave with thrombin of fused Bcl-3 ARD/GST caused precipitation of Bcl-3 ARD, since Bcl-3 ARD was insoluble. Thus, in the experiments Bcl-3 ARD/GST was used. All proteins were analysed by SDS polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue, in order to determine the purity of each protein. All proteins were homogenous.

3.1.2 NF- κ B DNA binding activity in the presence of Bcl-3

To study how the interactions between NF- κ B proteins and Bcl-3 affect NF- κ B DNA binding activity, electrophoretic mobility shift assays were performed with NF- κ B (p50)₂, (p65)₂, (p52)₂ or p50•p65 bound to ³²P-labelled κ B motifs from MHC, HIV-L or IRE, in the presence or absence of Bcl-3 ARD, in a range of Bcl-3 ARD:NF- κ B molar ratio between 0 and 70, in triplicate. These assays showed that addition of increasing amounts of Bcl-3 ARD to (p50)₂ bound to the symmetrical MHC κ B motif, resulted in the dose-dependent appearance of a more slowly migrating DNA-protein complex that is consistent with the formation of a ternary complex (Figure 20). Quantitation of the bound DNA showed that addition of Bcl-3 ARD also increased the DNA binding affinity of (p50)₂ towards the MHC κ B motif in a dose-dependent manner (Figure 21). Addition of increasing amounts of Bcl-3 to (p50)₂ bound to the non symmetrical IRE and HIV-L κ B motifs, also resulted in the appearance of a more

Figure 20: Bcl-3 ARD forms a ternary complex when it interacts with $(p50)_2$ bound to symmetrical κB motifs (MHC- κB) but inhibits $(p50)_2$ DNA binding when $(p50)_2$ is bound to asymmetrical κB motifs (HIV-L and IRE κB).

Interaction of Bcl-3 with (p50)₂•DNA complex is sequence dependent

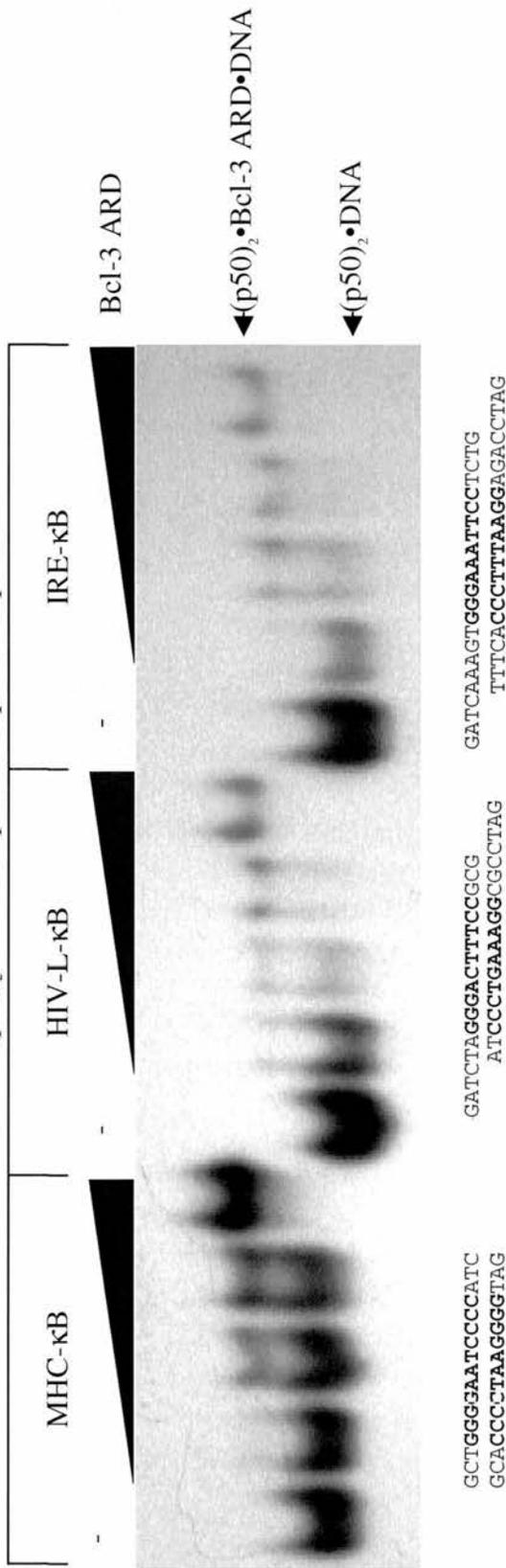
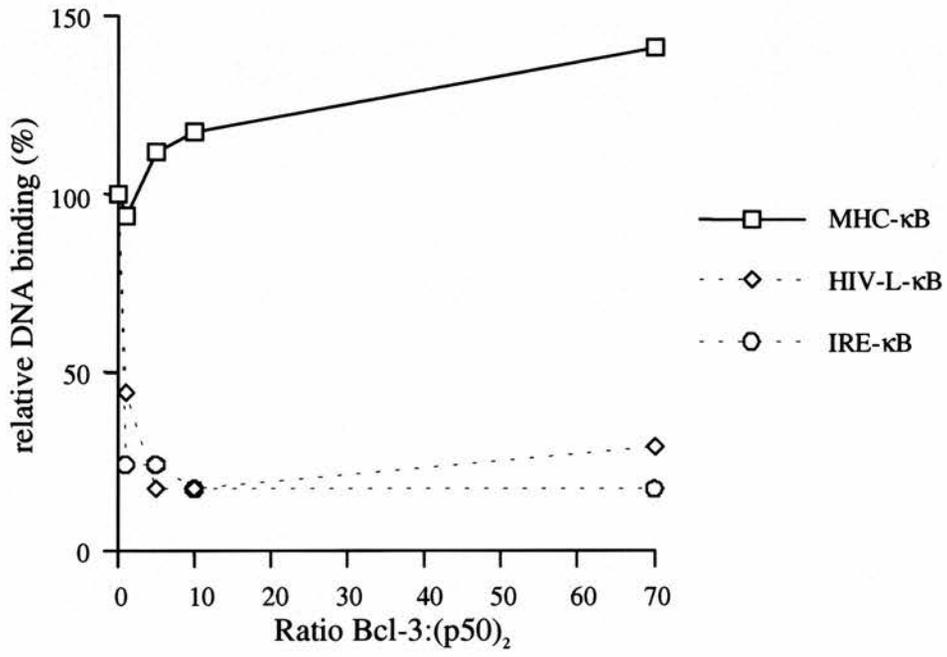


Figure 21: (p50)₂ DNA binding in the presence of Bcl-3 increases when the κB motif is symmetrical and decreases when the DNA target is not symmetrical.

The relative $(p50)_2$ DNA binding in the presence of Bcl-3 is sequence dependent



slowly migrating DNA-protein complex (Figure 20) but decreased the DNA binding affinity of (p50)₂ towards the IRE motif (Figure 21). On the other hand, addition of Bcl-3 ARD to NF-κB (p52)₂ bound to MHC and IRE κB motif, resulted in the appearance of a more slowly migrating DNA-protein complex irrespective of whether the DNA was symmetrical or non symmetrical (Figure 22). Addition of Bcl-3 ARD to (p65)₂ bound to MHC and IRE κB motifs, resulted in the decrease of the DNA binding affinity of (p65)₂ towards these κB motifs (Figure 22). Addition of Bcl-3 ARD to p50•65 bound to MHC and IRE motif had no effect in the DNA binding activity towards these κB motifs (Figure 22). Addition of Bcl-3 ARD to NF-κB molecules bound to HIV-L-κB motif, had very similar results to those of the addition of Bcl-3 ARD to NF-κB molecules bound to the non symmetrical IRE-κB motif (data not shown).

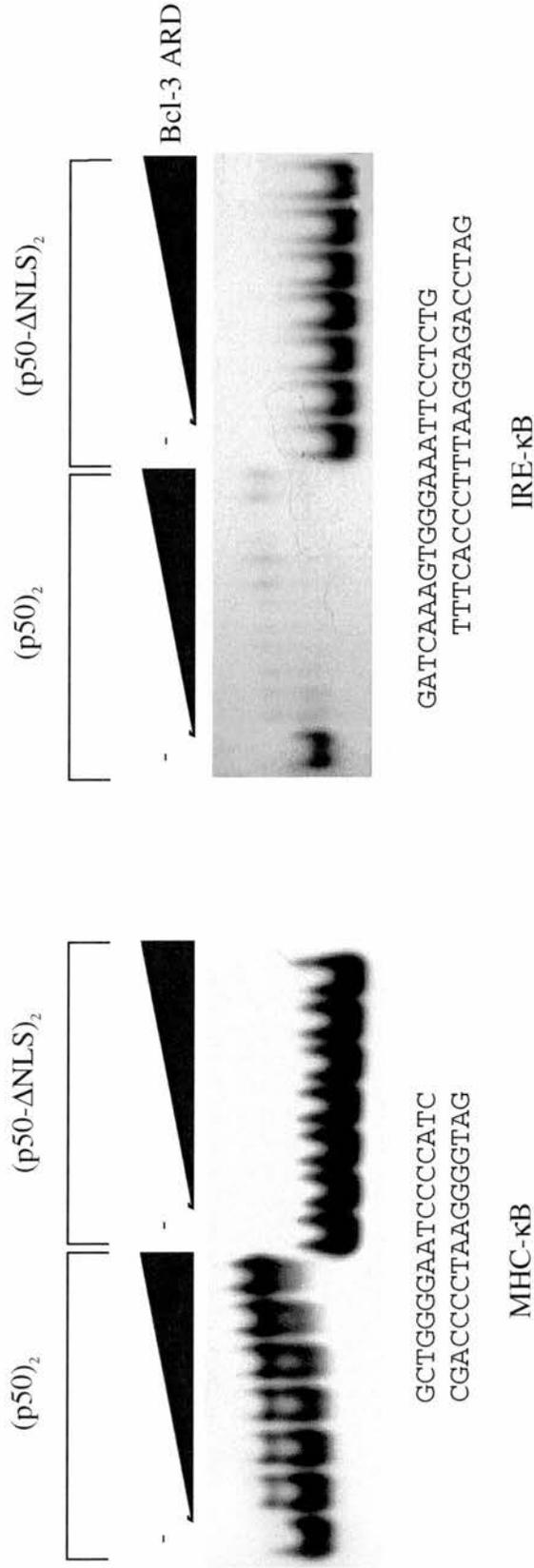
3.1.3 The NLS of p50 is required for interaction with Bcl-3

To define the role of the p50 NLS in the interaction of p50 with Bcl-3 ARD, this region was removed from p50 by trypsin cleavage. p50-ΔNLS contained amino acids 35-362 from the p105 precursor. (p50-ΔNLS)₂ bound to DNA with characteristics that were indistinguishable from (p50)₂. Untreated p50 or trypsinised p50 was incubated with Bcl-3 ARD and the DNA binding activity was determined in electrophoretic mobility shift assays. Addition of increasing amounts of Bcl-3 ARD to (p50-ΔNLS)₂ bound to MHC and IRE motifs had no effect in the DNA binding affinity of (p50-ΔNLS)₂ towards these κB motifs (Figure 23), whereas a ternary complex was found on MHC-κB and IRE-κB with the p50 containing the NLS. Bcl-3 ARD increased the DNA binding affinity of (p50)₂ towards the MHC-κB and it decreased the DNA binding affinity of (p50)₂ towards the IRE-κB motif. Addition of increasing amounts of Bcl-3 to (p50)₂ and (p50-ΔNLS)₂ bound to HIV-L-κB, had similar results to those of the addition of increasing amounts of Bcl-3 to (p50)₂ and (p50-ΔNLS)₂ bound to the non symmetrical IRE-κB motif (data not shown).

Figure 22: Bcl-3 ARD inhibits (p65)₂ DNA binding activity, it does not influence p50•p65 DNA binding activity and it forms a ternary complex when it interacts with (p52)₂. These interactions are non sequence dependent as opposed to the interaction of Bcl-3 with (p50)₂.

Figure 23: $(p50-\Delta NLS)_2$ is partially trypsin-digested $(p50)_2$ which lacks the nuclear localisation signal. Bcl-3 interacts with $(p50)_2 \cdot DNA$ but not with $(p50-\Delta NLS)_2 \cdot DNA$.

Interaction of Bcl-3 with (p50)₂•DNA requires the Nuclear Localisation Signal (NLS)



Role of the C-terminus of the loop L1 in stabilisation of p50 DNA binding

3.1.4 Mutant p50 protein production

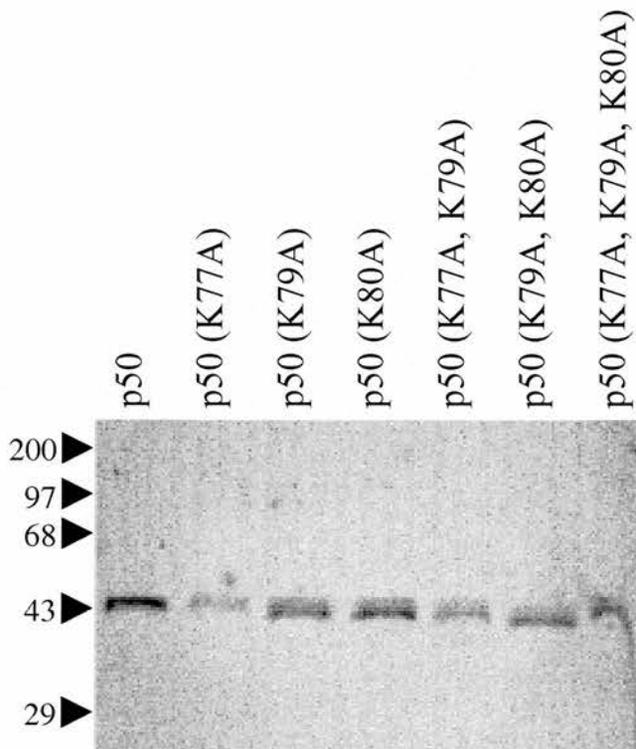
To determine the importance in DNA binding of each lysine residue of the p50 sequence 77-80 wt, single, double and triple mutants of p50, where lysines were replaced with alanines were purified (Figure 24A). The mutant proteins were: p50 (K77A), p50 (K79A), p50 (K80A), p50 (K77A, K79A), p50 (K79A, K80A) and p50 (K77A, K79A, K80A) (Michalopoulos and Hay, 1999) (Figure 24B). These mutations are located on the edge of the C-shaped, C-terminal part of the AB loop. The inserts of the expression vector plasmids were DNA sequenced, to confirm introduction of the mutations and check the integrity of the cDNA. Mutated proteins were isolated as described (see section 2.2.7) and the purity of each mutant protein determined by SDS PAGE followed by Coomassie Brilliant Blue staining (Figure 24A). Although some heterogeneity in the C-termini of the purified p50 proteins was apparent, it is outwith the rel-homology domains and does not influence the DNA binding and dimerisation properties of the proteins.

3.1.5 Chymotrypsin and proteinase K digestion of wt and triple mutant p50

The replacement of positively charged hydrophilic residues with non polar hydrophobic ones, could introduce a change of the overall structure of (p50)₂. Partial proteolysis experiments with Chymotrypsin or Proteinase K were performed with both wt p50 and p50 (K77A, K79A, K80A) (triple mutant). The sites where Chymotrypsin or Proteinase K cleave wt p50, were identified previously (Matthews et al., 1995). The two enzymes were selected because their cleavage specificities are such that K to A mutation in p50 would not alter substrate specificities and they could both cleave in the area of the C-terminus of AB loop (Chymotrypsin cleaves after N78 and Proteinase K after S74). The two enzymes could recognise many other cleavage sites in wt p50, that were protected in the inner core of the protein. Thus, if a small conformational change of the loop or a major change of the overall structure occurred, as a consequence of the mutations, then the sensitivity of some cleavage sites could be

Figure 24: Purification of wild type and mutant p50 proteins. **(A)** Coomassie blue stained 10% polyacrylamide gel showing purified p50 and mutant p50 proteins (100 ng). The lower band corresponds to a C-terminal degradation product. **(B)** List of mutants containing the names of the purified proteins and the sequences of their C-terminal part of the AB loop. The residues within the box are the residues of the turn of the C-shaped loop. The point mutations introduced are in bold letters.

A



B

p50	GASSEK N KKKSYPQV
p50 (K77A)	GASSE A NKKKSYPQV
p50 (K79A)	GASSEK N AKSYPQV
p50 (K80A)	GASSEK N K A SYPQV
p50 (K77A, K79A)	GASSE A N A KSYPQV
p50 (K79A, K80A)	GASSEK N A A SYPQV
p50 (K77A, K79A, K80A)	GASSE A N A A SYPQV

changed or completely abolished (especially the ones in close proximity to the mutations) or new cleavage sites could appear because of the exposure of protein sequences that were protected in the wt p50. The digestion patterns of both wt p50 and the triple mutant were almost identical (Michalopoulos and Hay, 1999) (Figure 25). Qualitatively, there was no change in the already characterised cleavages, and no new potential cleavage site was exposed to the surface, as no new protein fragments were detected. Quantitatively, the thermodynamics of the existing cleavages was not altered, as the appearance of the protein fragments occurred in the same molar ratios of protein substrate: enzyme. The dimeric state of wild type and mutant proteins was confirmed by polyacrylamide gel electrophoresis of the proteins under native conditions. Only the dimeric species were detected (data not shown). Those experiments indicate that no change of the overall structure of (p50)₂ (that could influence the DNA binding activity of these molecules), was introduced by the mutations of the lysine cluster in the AB loop.

3.1.6 DNA binding properties of mutants

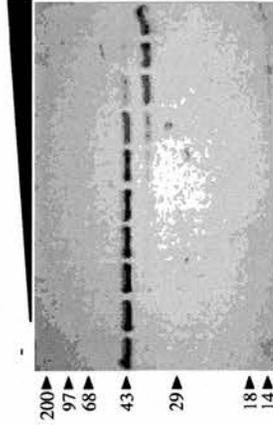
Gel electrophoresis DNA binding assays were performed with all proteins (wt and mutants). The data showed that the triple mutant was clearly more defective in binding to DNA than the other proteins (Figure 26). The single and double mutants were almost equally defective (more defective than the wt and less defective than the triple mutant) (Michalopoulos and Hay, 1999) (Figure 26). Unfortunately, the resolution of this method was insufficient to discriminate between the DNA binding activities of the single and double mutants. It was thus not possible to unambiguously identify the lysine residue responsible for the interaction with DNA. However, these experiments support the existence of the AB loop-DNA interaction and demonstrate its importance for stabilisation of the DNA-protein complex. Additional gel electrophoresis DNA binding assays carried out in the presence of a variety of specific and non-specific unlabelled competition DNAs (data not shown) indicated that while the triple mutant bound to DNA with reduced affinity, the specificity of the interaction was unchanged.

Figure 25: Proteolytic patterns of the wt and the triple mutant p50. (A) 5 μ g p50 and p50 (K77A, K79A, K80A) were digested with Chymotrypsin at substrate: protease ratios of 2560:1, 1280:1, 640:1, 320:1, 160:1, 80:1, 40:1, 20:1, 10:1 for 1 hour at room temperature. Digestion products were resolved in a 10% polyacrylamide gel containing SDS and stained with Coomassie blue. The masses of the molecular weight standards (M) are indicated in kDa. (B) 5 μ g p50 and p50 (K77A, K79A, K80A) were digested with Proteinase K at substrate: protease ratios of 20480:1, 10240:1, 5120:1, 2560:1, 1280:1, 640:1, 320:1, 160:1, 80:1 for 1 hour at room temperature. Digestion products were resolved in a 10% polyacrylamide gel containing SDS and stained with Coomassie blue. The masses of the molecular weight standards (M) are indicated in kDa.

Proteinase K



KNKK

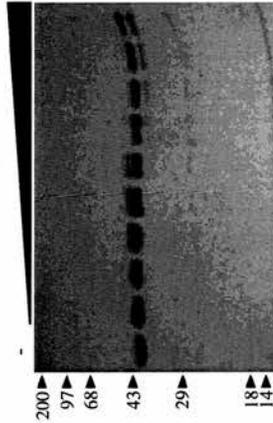


ANAA

Chymotrypsin

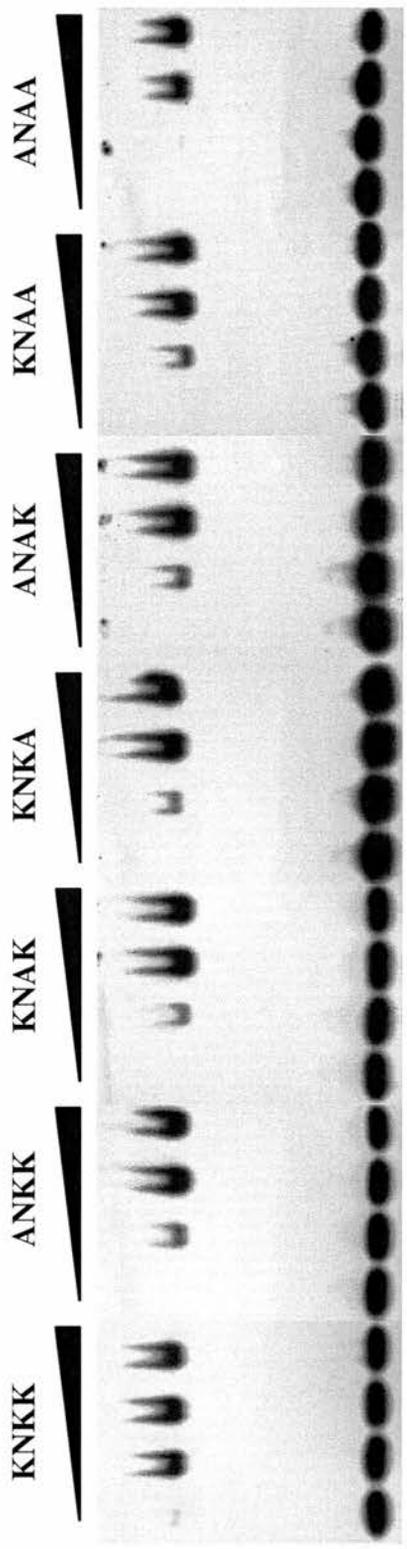


KNKK



ANAA

Figure 26: Gel electrophoresis DNA binding assays with all p50 proteins (wt and mutants) using a ^{32}P labelled double stranded 17-mer oligonucleotide containing the κB motif from the H-2K enhancer: 5'-GCTGGGGATTCCCCATC-3' (MHC). 1 fmol, 10 fmol, 100 fmol, and 1000 fmol of each protein were incubated for 30' at room temperature with 20 fmol of ^{32}P labelled double stranded MHC oligonucleotide. The samples were resolved in a 6% non denaturing polyacrylamide gel (55:1 acrylamide: bis-acrylamide). The upper band corresponds to the DNA-protein complex. The lower band corresponds to protein free DNA.



3.1.7 Discrimination between DNA binding activities of p50 mutants

To discriminate between the DNA binding activities of the p50 mutants SPR was employed. A biotinylated double stranded DNA containing an NF- κ B binding site was captured on the surface of a sensor chip by bound streptavidin. Wild type p50 and the various mutants were tested for DNA binding activity by passage over the sensor chip. DNA binding reactions were carried out under stringent conditions at 250 mM NaCl to eliminate non-specific interactions between the proteins and the sensor chip. Under these stringent conditions, wild type p50 bound efficiently to DNA containing a symmetrical κ B motif (MHC), but bound less well to DNA containing an asymmetrical κ B motif (HIV). Under these conditions binding to non-specific DNA sequence (NFIII) was negligible. Data were collected at a range of protein concentration between 2.5 and 1000 nM. At each protein concentration the analysis was carried out in triplicate. Although the data were highly reproducible it was not possible to derive association and dissociation rate constants for the interaction between p50 and DNA containing a κ B motif, as the data could not be fitted to the theoretical binding models of the evaluation software. Thus it appears that interaction between p50 and DNA immobilised on the sensor surface did not follow strictly the kinetics expected from a simple bimolecular reaction. This may be a consequence of conformational changes that accompany DNA binding, protein-protein interactions on the DNA (Dekker et al., 1998) or a "cage" effect at the surface of the sensor chip. Although the quantitative evaluation could not be completed, the experimental data could be used to resolve the differences in the DNA binding of the wild type and mutant proteins by direct comparison of the binding curves, at a given protein concentration. Consistent with the data in Figure 26 and Figure 27, wt p50 bound more tightly than any of its mutants (Figure 28). The protein least defective for DNA binding, of all p50 mutants was the single mutant p50 (K79A). The most defective of the 3 single mutants was p50 (K80A), while the affinity of p50 (K77A) was between the affinities of the other two single mutants. The double mutant p50 (K77A, K79A) was more defective in DNA binding than each of the two single mutants p50 (K77A)

Figure 27: DNA binding curves. Amount of bound to protein DNA over protein amount.

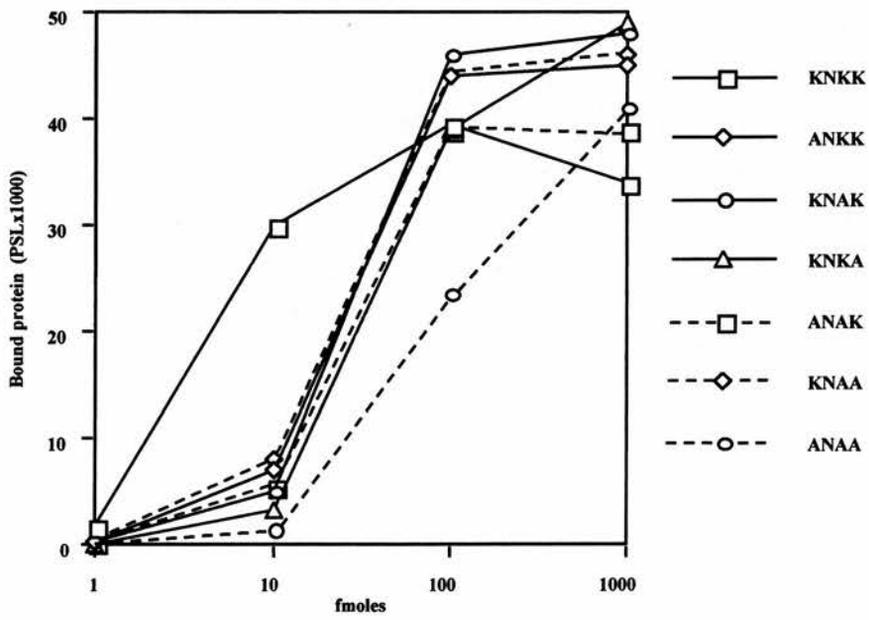
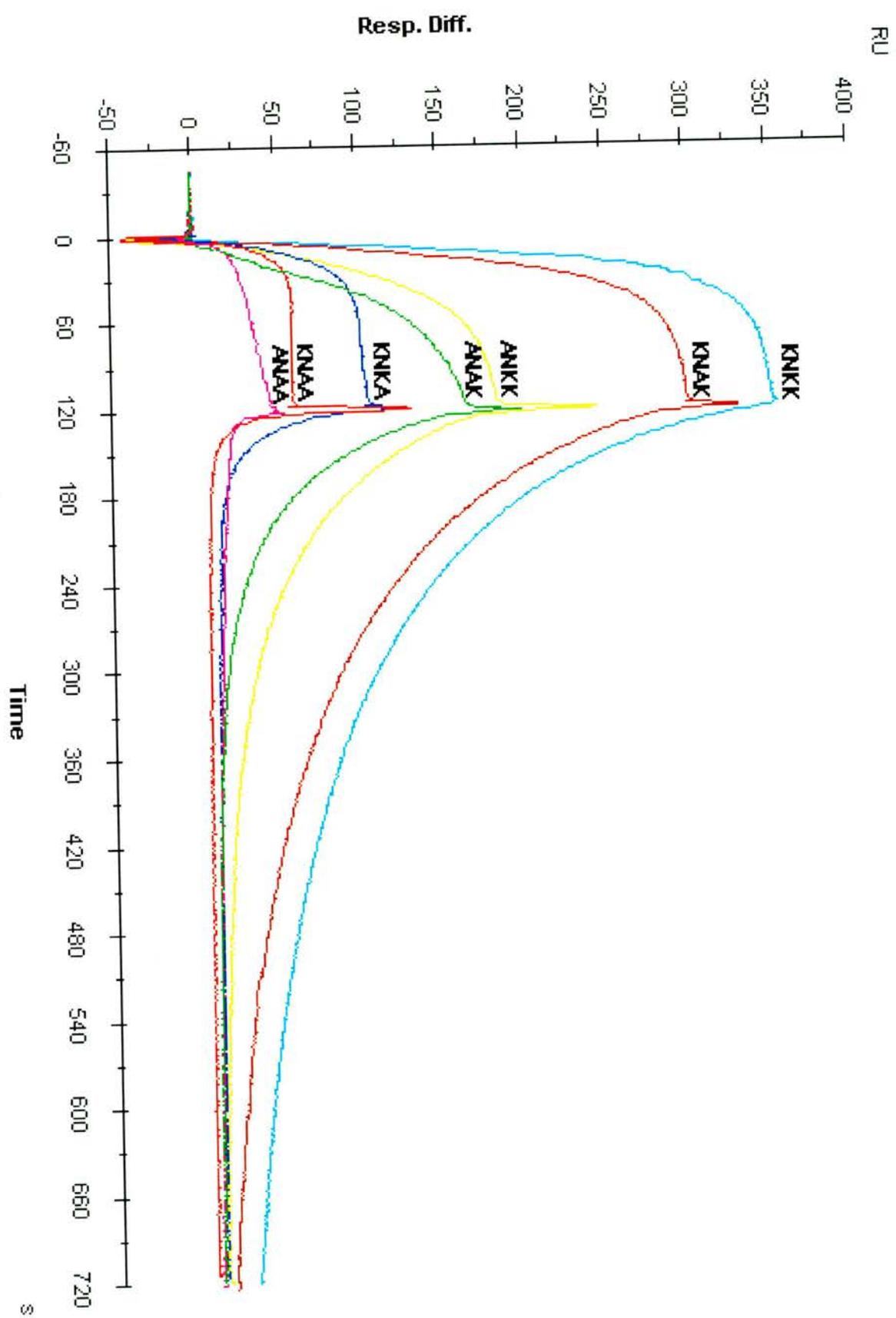
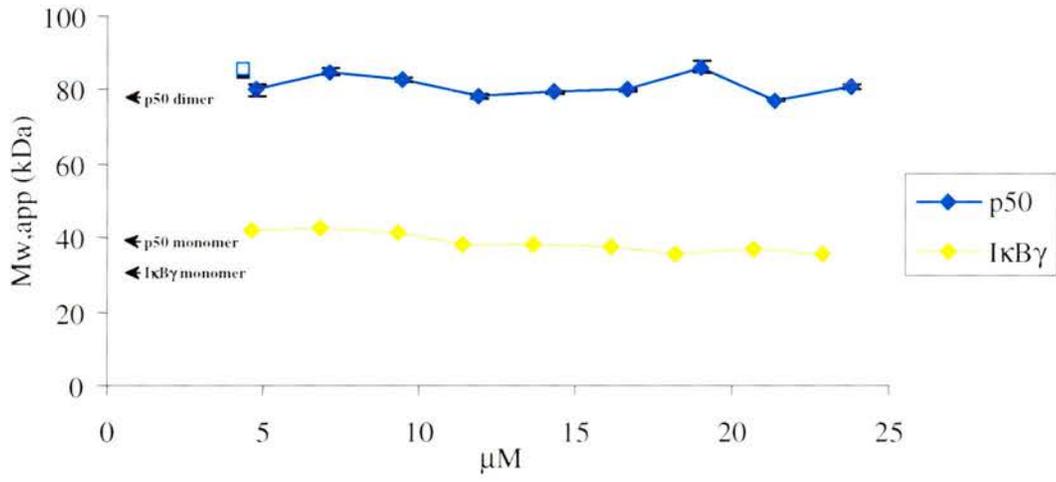


Figure 28: Typical sensogram (Surface Plasmon Resonance), showing the association and the dissociation of the p50 proteins (wt and mutants) to a double stranded DNA containing the H2-K κ B motif. In the association phase, 100 nM of each protein were injected for 2 minutes at 10 μ l/min flow rate. In the dissociation phase, buffer containing no protein was injected for 10 minutes at 10 μ l/min flow rate.



p50, IκBγ single ideal species fit



and p50 (K79A) but was less defective than the single mutant p50 (K80A). p50 (K79A, K80A) was more defective than p50 (K79A) and p50 (K80A) while the triple mutant p50 (K77A, K79A, K80A) was clearly the most defective in binding to DNA of all the mutant proteins (Michalopoulos and Hay, 1999) (Figure 28). These data provide experimental evidence for the suggested interaction between the C-terminus of the AB loop-DNA and demonstrate its importance for the stabilisation of the DNA-protein complex. K80 appears to play a critical role in this interaction with the phosphate backbone, although K77 may also contact DNA. Residue K79 does not seem to contribute to the DNA protein interaction.

3.2 p50•IκBγ complex stoichiometry and form

3.2.1 Absorbance vs. Wavelength scans

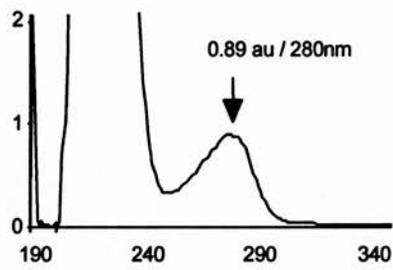
Purified p50 and IκBγ samples were fully reduced with DTT and consequently dialysed against a Phosphate/Saline buffer without DTT. Absorbance vs. wavelength scans of freshly dialysed p50 and IκBγ samples were performed on a Unicam spectrophotometer using dialysate for baseline absorbance correction (Figure 29). The buffer density was estimated by tables ($\rho=1.015\text{g/ml}$), the partial specific volumes of the proteins $\bar{v}_{p50} = 0.738 \text{ ml/g}$, $\bar{v}_{I\kappa B\gamma} = 0.741 \text{ ml/g}$, the monomer mass $M_{p50} = 38909 \text{ Da}$, $M_{I\kappa B\gamma} = 29399 \text{ Da}$ and the extinction coefficient of the proteins $\epsilon_{p50} = 19934 \text{ cm}^2/\text{mg}$ and $\epsilon_{I\kappa B\gamma} = 15908 \text{ cm}^2/\text{mg}$.

3.2.2 Sedimentation Equilibrium of p50, IκBγ and p50•IκBγ

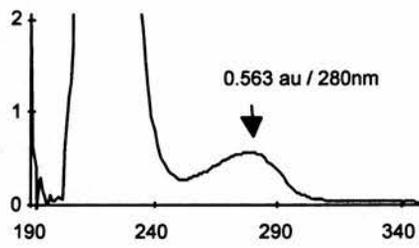
Nine concentrations of p50 (from 5 μM to 25 μM monomer concentration) were prepared by dilution with its dialysate, and equilibrium was obtained at 16 krpm. The distribution data obtained from the runs were fitted using the single ideal species fit model (Figure 30). From the curve fit plots obtained, apparent weight average molecular masses $M_{w,app,p50}$ for each p50 concentration were estimated and the quality of the curve fits was estimated by the residual plots. The estimated $M_{w,app,p50}$ values at different concentrations were between 77.3-86.1 kDa (Figure 32). This value shows that p50 is forming homodimers and is in accordance with all previous findings.

Nine concentrations of IκBγ (from 5 μM to 25 μM monomer concentration) were prepared by dilution with its dialysate, and equilibrium was obtained at 18 krpm. The distribution data obtained from the runs were fitted using the single ideal species fit model (Figure 31), the monomer/dimer model (Figure 33) and monomer/tetramer model (Figure 34). From the curve fit plots obtained, apparent weight average molecular masses $M_{w,app,I\kappa B\gamma}$ for each IκBγ concentration were estimated and the quality of the curve fits was estimated by the residual plots. Although the curve fitting was better using the monomer/dimer and monomer/tetramer fit models than using the

Figure 29: Absorbance vs. Wavelength scans of freshly dialysed samples of p50 and IκBγ.



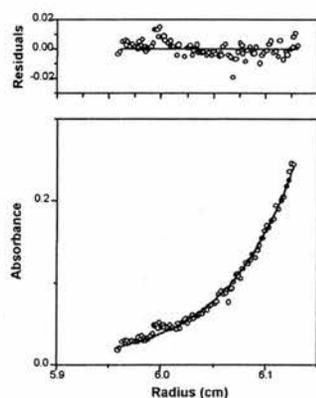
p50



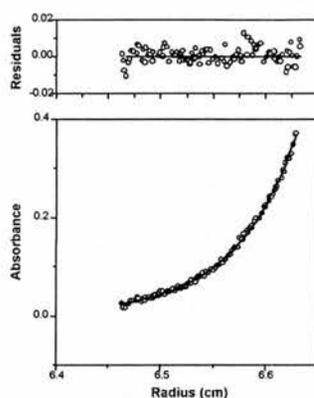
IκBγ

Figure 30: Distribution data from p50 at 16 krpm and the curve fits and the residual plots, using the single ideal species model.

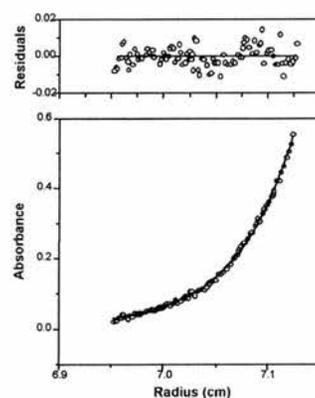
p50 at 16krmp (single ideal species model)



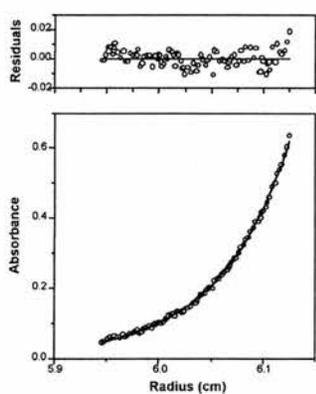
$M_{w,app}$ 80.1 (± 1.6) 4.8 μM



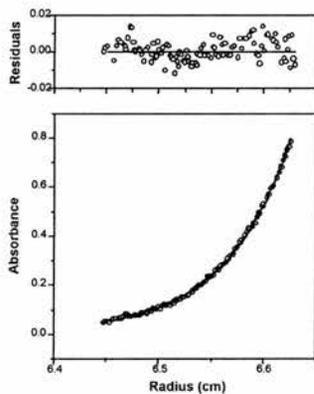
$M_{w,app}$ 84.9 (± 0.8) 7.1 μM



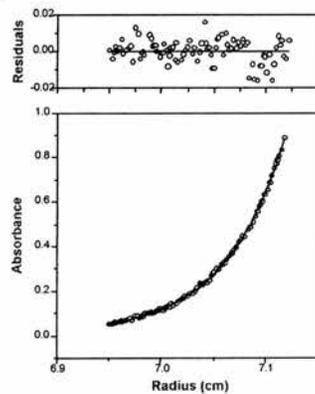
$M_{w,app}$ 82.8 (± 0.8) 9.5 μM



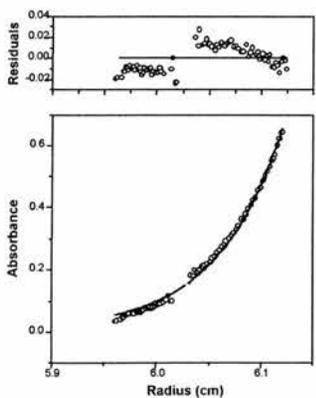
$M_{w,app}$ 78.1 (± 0.6) 11.9 μM



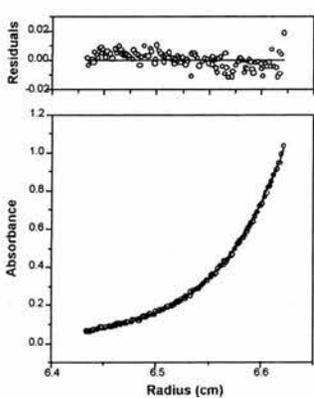
$M_{w,app}$ 79.3 (± 0.5) 14.3 μM



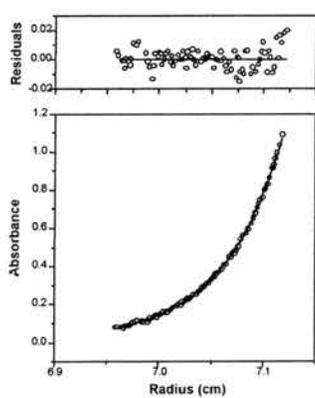
$M_{w,app}$ 80.0 (± 0.5) 16.7 μM



$M_{w,app}$ 86.1 (± 1.6) 19.0 μM



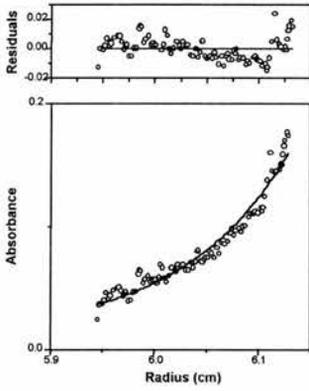
$M_{w,app}$ 77.3 (± 0.4) 21.4 μM



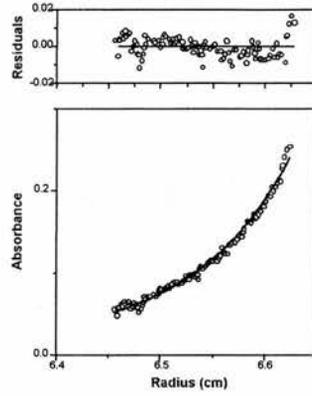
$M_{w,app}$ 81.0 (± 0.5) 23.8 μM

Figure 31: Distribution data from $I\kappa B\gamma$ at 18 krpm and the curve fits and the residual plots, using the single ideal species model.

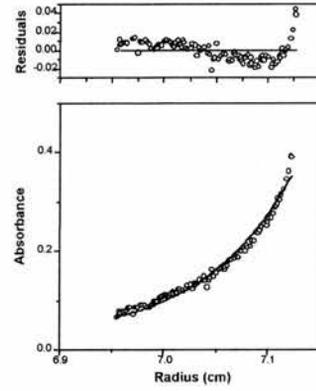
1kBy at 18krpm (single ideal species model)



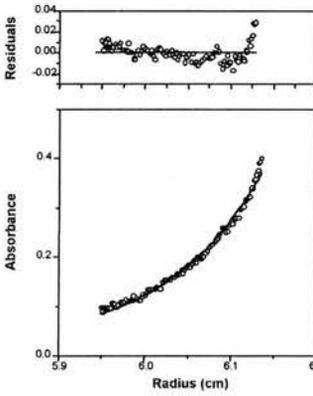
$M_{w,app}=41.8 (\pm 2.1) 4.6 \mu\text{M}$



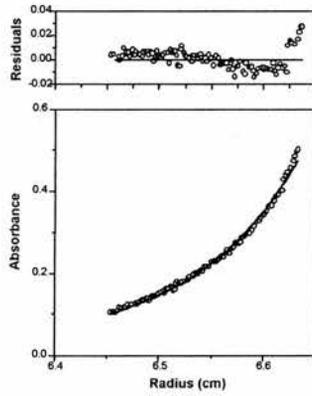
$M_{w,app}=42.5 (\pm 0.7) 6.8 \mu\text{M}$



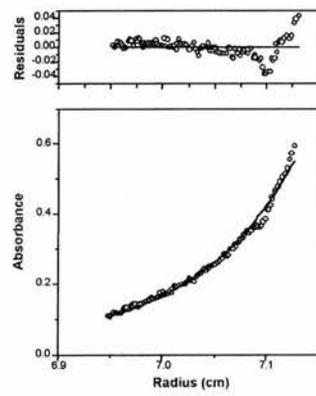
$M_{w,app}=41.5 (\pm 1.3) 9.3 \mu\text{M}$



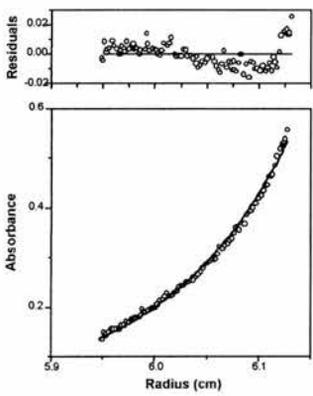
$M_{w,app}=37.9 (\pm 0.8) 11.4 \mu\text{M}$



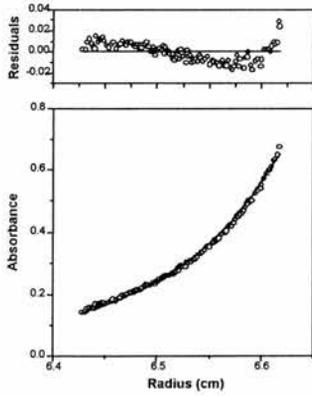
$M_{w,app}=38.3 (\pm 0.7) 13.6 \mu\text{M}$



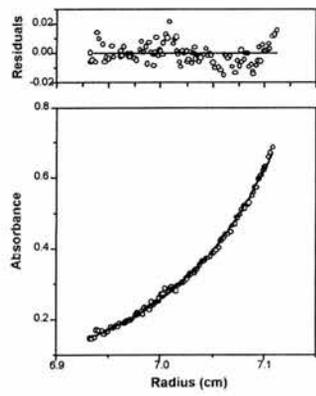
$M_{w,app}=37.3 (\pm 0.7) 16.1 \mu\text{M}$



$M_{w,app}=35.5 (\pm 0.4) 18.2 \mu\text{M}$



$M_{w,app}=36.7 (\pm 0.5) 20.7 \mu\text{M}$



$M_{w,app}=35.5 (\pm 0.3) 22.9 \mu\text{M}$

Figure 32: Estimation of the $M_{w,app}$ of p50 span at 16 krpm and $I_{kB\gamma}$ span at 18 krpm, using the single ideal species fit model.

p50, IκBγ single ideal species fit

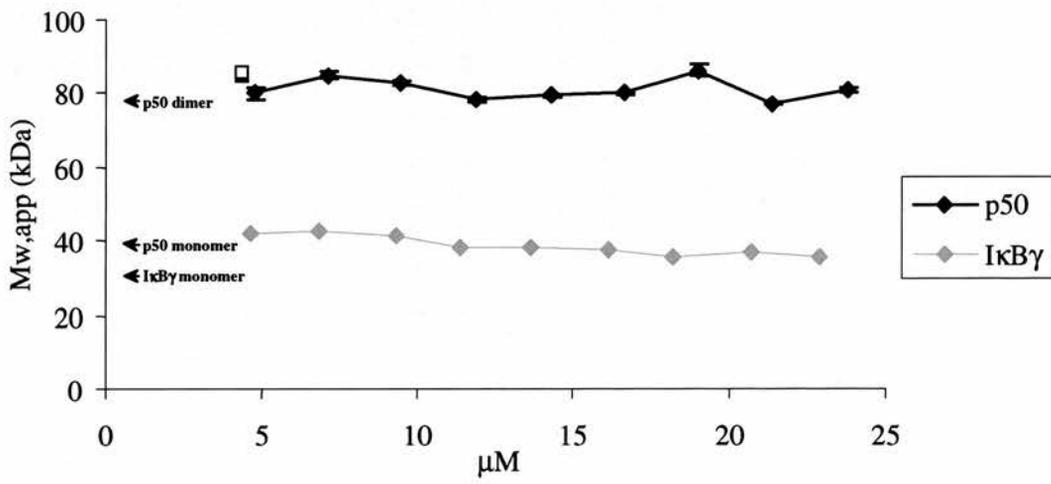
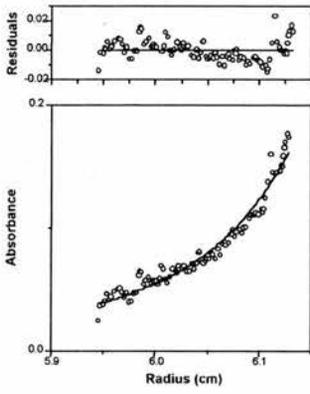
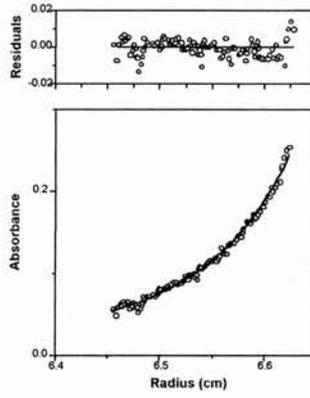


Figure 33: Distribution data from IκBγ at 18 krpm and the curve fits and the residual plots, using the monomer/dimer model.

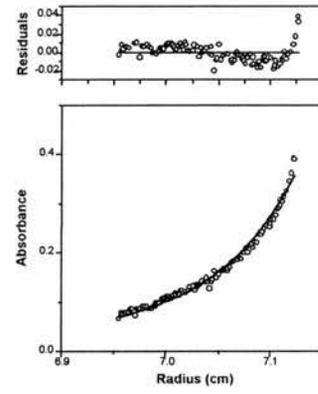
IκBγ at 18krpm (monomer/dimer model)



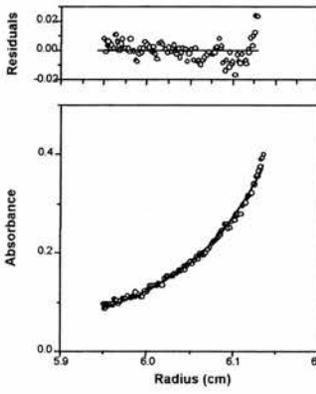
$Ka_2=17.4 (\pm 7.0) 4.6 \mu\text{M}$



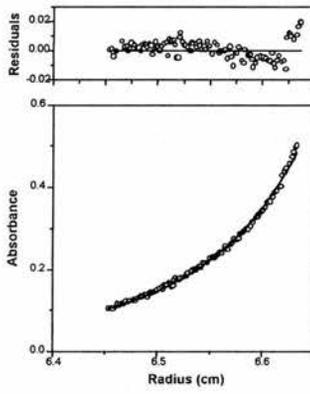
$Ka_2=12.9 (\pm 1.5) 6.8 \mu\text{M}$



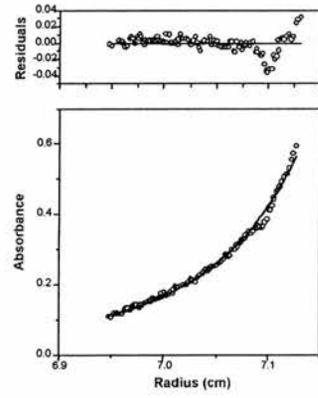
$Ka_2=7.3 (\pm 1.7) 9.3 \mu\text{M}$



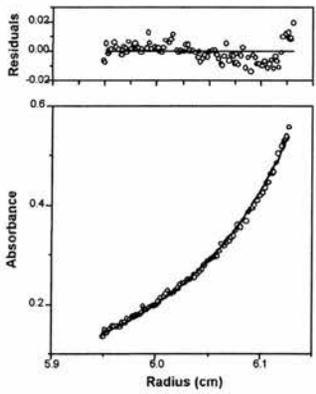
$Ka_2=3.1 (\pm 0.4) 11.4 \mu\text{M}$



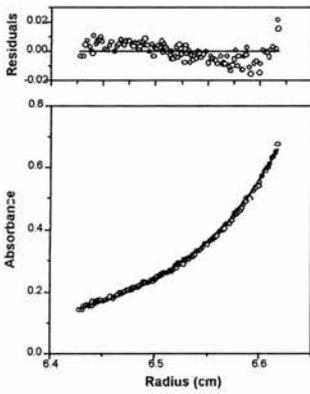
$Ka_2=2.8 (\pm 0.3) 13.6 \mu\text{M}$



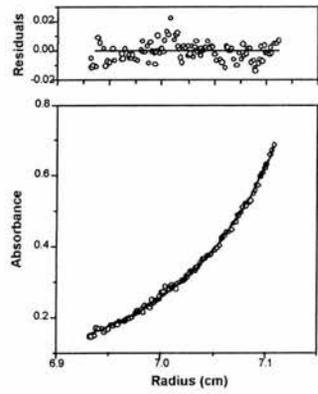
$Ka_2=2.0 (\pm 0.3) 16.1 \mu\text{M}$



$Ka_2=1.2 (\pm 0.1) 18.2 \mu\text{M}$



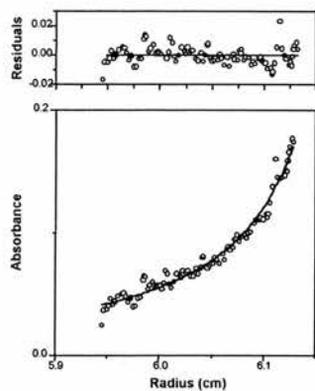
$Ka_2=1.3 (\pm 0.1) 20.7 \mu\text{M}$



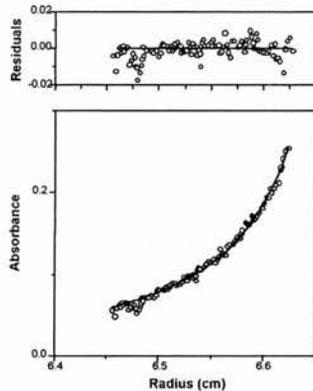
$Ka_2=1.0 (\pm 0.1) 22.9 \mu\text{M}$

Figure 34: Distribution data from IκBγ at 18 krpm and the curve fits and the residual plots, using the monomer/tetramer model.

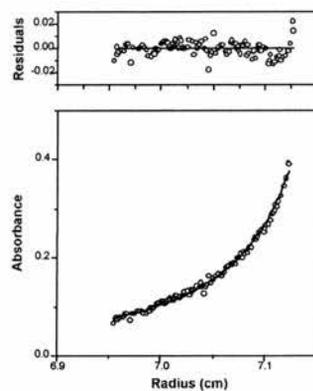
IκBγ at 18krpm (monomer/tetramer model)



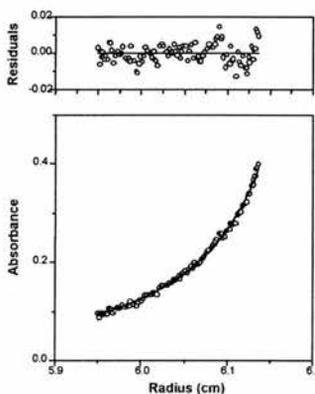
$Ka_4=569 (\pm 153) 4.6 \mu\text{M}$



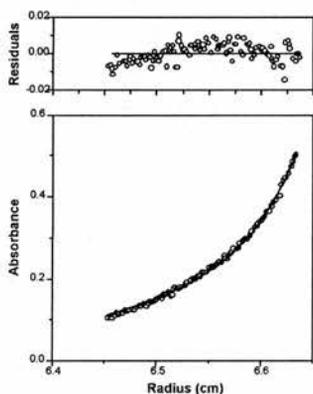
$Ka_4=157 (\pm 13) 6.8 \mu\text{M}$



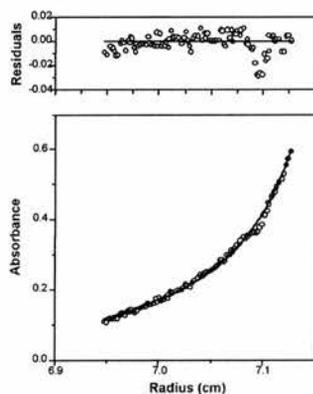
$Ka_4=45 (\pm 5) 9.3 \mu\text{M}$



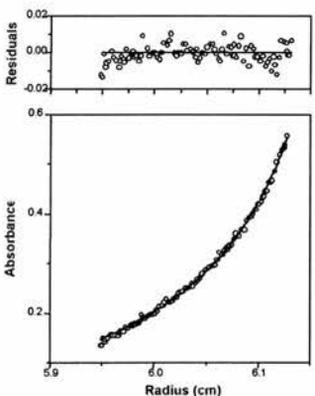
$Ka_4=18 (\pm 2) 11.4 \mu\text{M}$



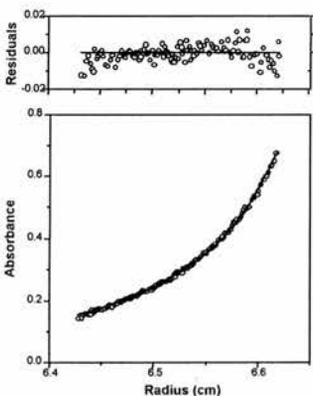
$Ka_4=10 (\pm 1) 13.6 \mu\text{M}$



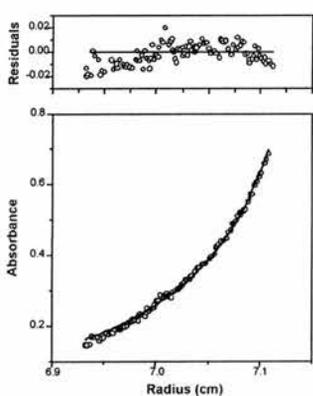
$Ka_4=6 (\pm 1) 16.1 \mu\text{M}$



$Ka_4=3 (\pm 0.2) 18.2 \mu\text{M}$



$Ka_4=2.5 (\pm 0.1) 20.7 \mu\text{M}$



$Ka_4=1.7 (\pm 0.2) 22.9 \mu\text{M}$

single ideal species model, the estimated $M_{w,app_{IkB\gamma}}$ values at different concentrations, using the monomer/dimer and monomer/tetramer fit models were not consistent (Figure 33 and Figure 34) while the estimated $M_{w,app_{IkB\gamma}}$ values at different concentrations, using the single ideal species model were between 35.5-42.5 kDa (Figure 32). The value is slightly higher than that of the monomer. The data suggest that $IkB\gamma$ in solution, is monomeric.

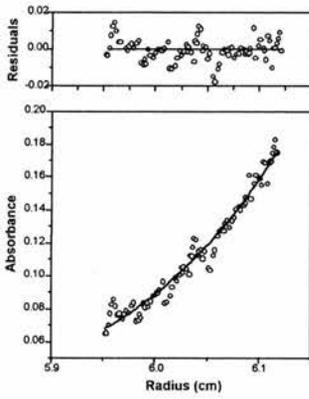
Nine samples with the p50 monomer concentration fixed at 5 μ M, and the $IkB\gamma$ monomer concentration varying from 1 μ M to 25 μ M were prepared by dilution with their dialysate, and equilibrium was obtained at 10 and 16 krpm. The distribution data obtained from the runs at 10 krpm and 16 krpm were fitted using the single ideal species fit model (Figure 35 and Figure 36, respectively). From the curve fit plots obtained from the two runs, apparent weight average molecular masses $M_{w,app_{p50 \cdot IkB\gamma}}$ for each p50: $IkB\gamma$ ratio were estimated and the quality of the curve fits was estimated by the residual plots. Using the single ideal species analysis, the highest estimated $M_{w,app_{p50 \cdot IkB\gamma}}$ value at 10 krpm was 114.4 kDa at p50: $IkB\gamma$ ratio=1.3 and at 16 krpm was 111.2 kDa at p50: $IkB\gamma$ ratio=1.7. Using the m^* analysis, the highest estimated $M_{w,app_{p50 \cdot IkB\gamma}}$ value at 10 krpm was 113.3 kDa at p50: $IkB\gamma$ ratio=1.3 and at 16 krpm was 111.9 kDa at p50: $IkB\gamma$ ratio=1.7 (Figure 37). Judging from the highest value of the $p50 \cdot IkB\gamma$, the p50: $IkB\gamma$ ratio is 2:1.

Analytical ultracentrifugation enables the characterisation of proteins in solution. It can operate in two modes to reveal data on solute molecular mass (sedimentation equilibrium experiments) and shape (sedimentation velocity experiments) in its near-native state in solution (Svedberg and Pederson, 1940). Thus, sedimentation equilibrium analysis was employed to directly measure the molecular weight M of the complex of unmodified p50 and $IkB\gamma$, as it exists in solution, and thus reveal the stoichiometry of the participating molecules in the complex.

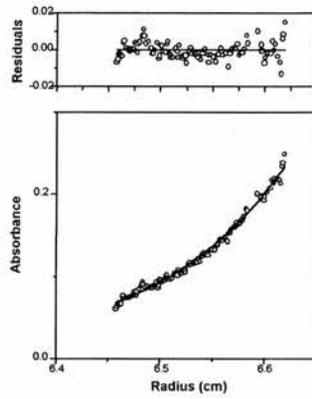
Analysis of the p50 data as a single, thermodynamically ideal species gave reasonable fits and values for $M_{w,app}$ corresponding to dimer. These data were in accordance with the crystallographic and NMR data.

Figure 35: Distribution data from p50/IκBγ at 10 krpm and the curve fits and the residual plots, using the single model.

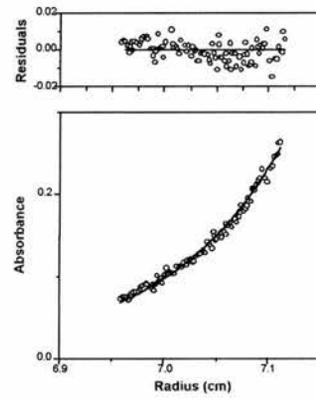
p50/IκBy at 10krpm (single ideal species model)



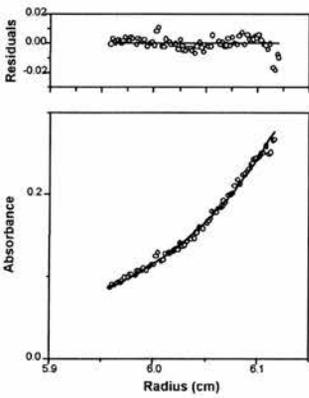
$M_{w,app} = 82.3 (2.9)$



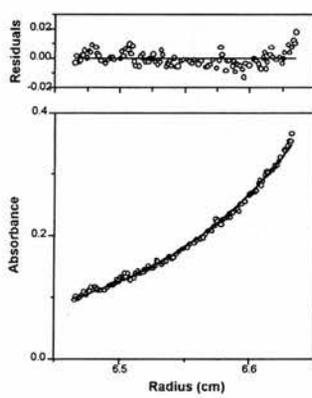
$M_{w,app} = 103.8 (1.6)$



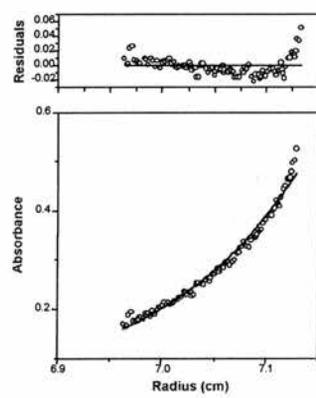
$M_{w,app} = 105.4 (1.7)$



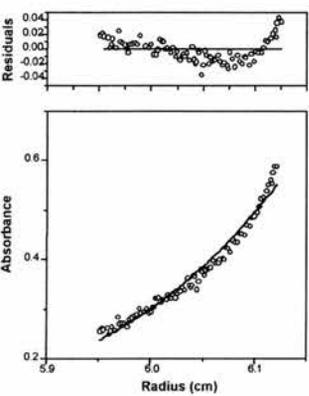
$M_{w,app} = 114.7 (1.7)$



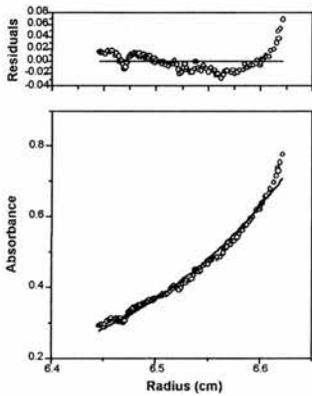
$M_{w,app} = 108.0 (1.7)$



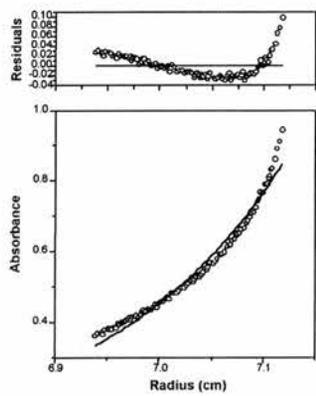
$M_{w,app} = 84.6 (2.0)$



$M_{w,app} = 71.8 (2.2)$



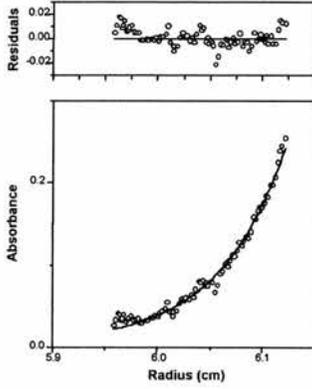
$M_{w,app} = 71.0 (1.7)$



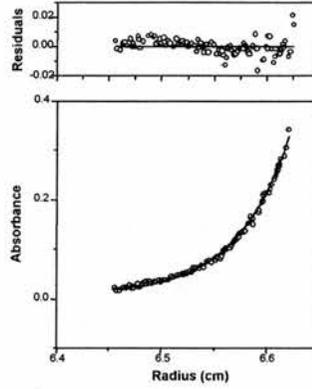
$M_{w,app} = 66.4 (2.1)$

Figure 36: Distribution data from p50/IκBγ at 16 krpm and the curve fits and the residual plots, using the single model.

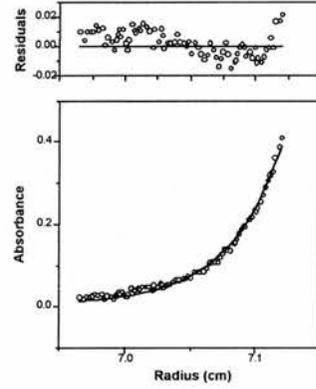
p50/IκBγ at 16krpm (single ideal species model)



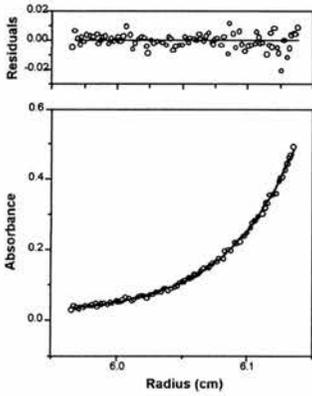
$M_{w,app} = 83.8 (2.6)$



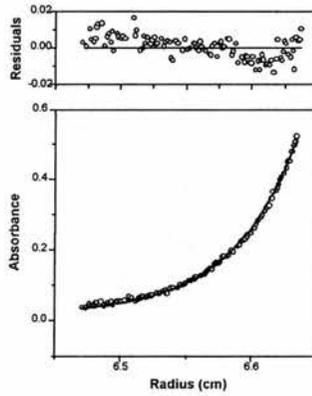
$M_{w,app} = 101.6 (1.5)$



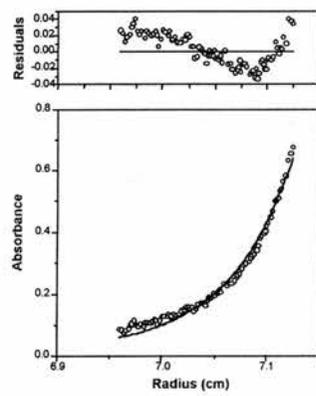
$M_{w,app} = 111.2 (2.7)$



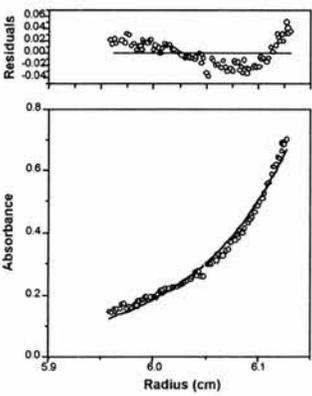
$M_{w,app} = 101.7 (0.9)$



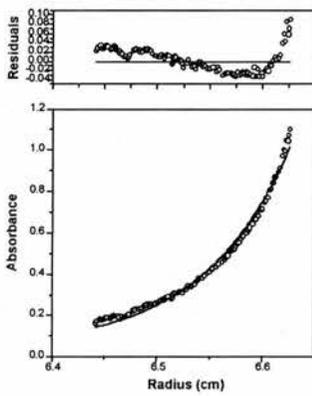
$M_{w,app} = 100.5 (1.0)$



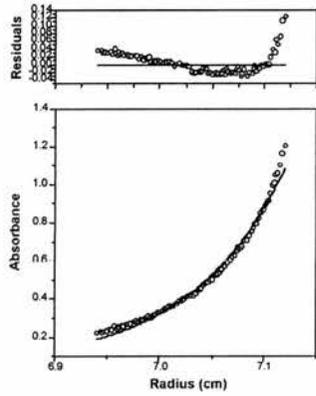
$M_{w,app} = 75.5 (2.0)$



$M_{w,app} = 57.2 (1.5)$



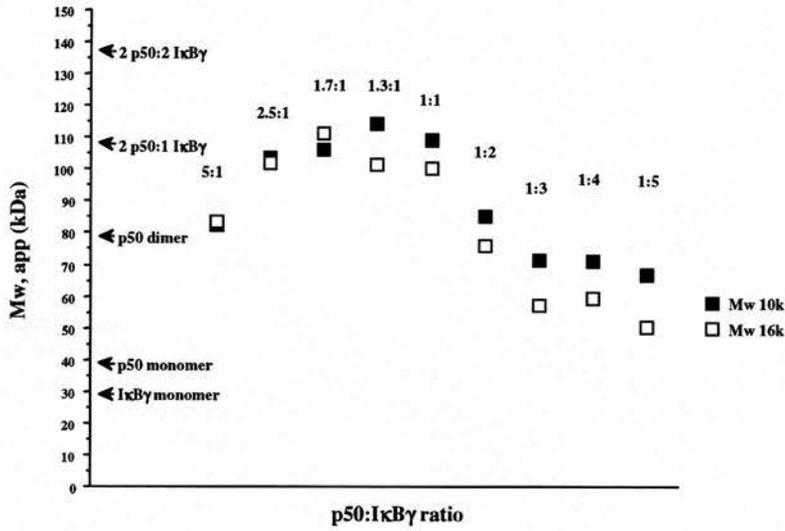
$M_{w,app} = 59.6 (1.4)$



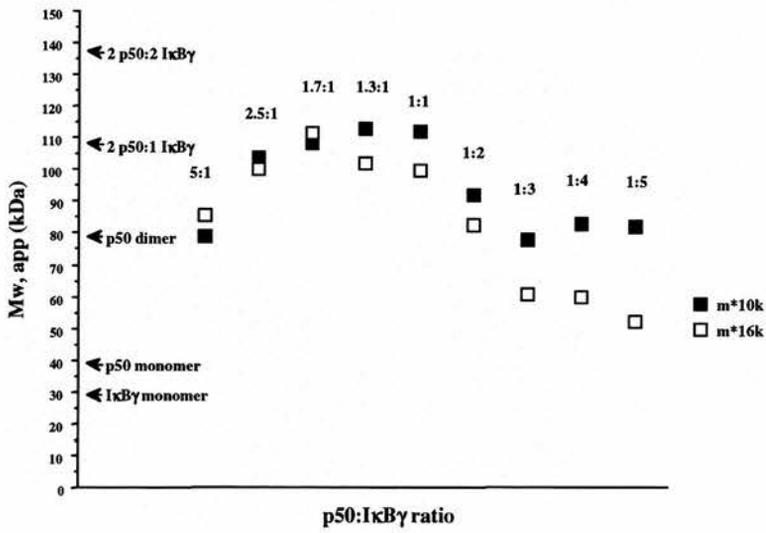
$M_{w,app} = 50.0 (1.0)$

Figure 37: Analysis of p50 / IkB γ mixtures span at 10 krpm and 16 krpm, using the single ideal species fit model and m* analysis.

Mixtures analysed using single ideal species fit



Mixtures analysed using m*



Fits to the raw sedimentation equilibrium IκBγ data with the model for a single, thermodynamically ideal species were poor. The tick shape of the residuals near the cell base was characteristic of the presence of higher mass species. Extending the model to include dimer does not satisfactorily account for this but the fits were improved with a monomer-tetramer model. The slight upside-down smile shape of the residuals for this fit was indicative of molecular elongation for the monomeric species. These data can be explained though in a far more convincing way: IκBγ in solution is monomeric. IκBγ was produced as a chimaeric GST fusion protein (Smith and Johnson, 1988), which was then cleaved with thrombin and purified by mixing with glutathione-agarose beads that should pull down all the cleaved GST and uncleaved IκBγ-GST molecules. It seems that during the purification process, a small portion of GST and IκBγ-GST did not bind to the beads and remained in the solution, contaminating the IκBγ sample. The molecular weight of monomer GST is 26968 Da which is very similar to the molecular weight IκBγ which is 29399 Da. GST forms stable homodimers, both in crystals and in solution (McTigue et al., 1995). Thus, the apparent "IκBγ dimers" and "IκBγ tetramers" are in fact GST and IκBγ-GST homodimers respectively. These two contaminants with molecular weight 53936 Da and 112734 Da respectively are responsible for both the tick shape of the residuals near the cell base and the overestimation of the apparent weight-average molecular mass of IκBγ (35.5-42.5 kDa instead of 29.4 kDa).

Fits of the raw data for the p50•IκBγ complex were poor, as would be expected for a system that contains at least 3 molecular species. However the so obtained are of sufficient trustworthiness to generate a titration curve for the experiment. From this treatment it is apparent that the weight-average molecular mass peaks at a p50:IκBγ ratio of 2:1. The hypothetical (IκBγ-GST)₂•(p50)₂ complex (which would consist of a (p50)₂ bound to an IκBγ-GST what would be also bound to another IκBγ-GST molecule through GST homodimerisation) with molecular weight 190552 kDa might be also present in the solution but because of its high molecular weight it would sediment rapidly in the cell bottom, without affecting the raw sedimentation

equilibrium data. For the same reason, even if IκBγ tends to aggregate during the purification process (p50 is very soluble and does not cause such problems during its purification), any IκBγ aggregates present in the solutions would readily sediment. That is why Analytical Ultracentrifugation is considered a "self-cleaning" method.

3.2.3 Sample preparation for Sedimentation Equilibrium analysis and Neutron Scattering

p50, IκBγ and 2:1 p50:IκBγ were dialysed in a Phosphate/Saline Buffer made up in D₂O. The buffer density was estimated ($\rho=1.119176$ g/ml) and the partial specific volumes of (p50)₂•IκBγ is $\bar{v}_{(p50)_2 \bullet I\kappa B\gamma}=0.739$ ml/g.

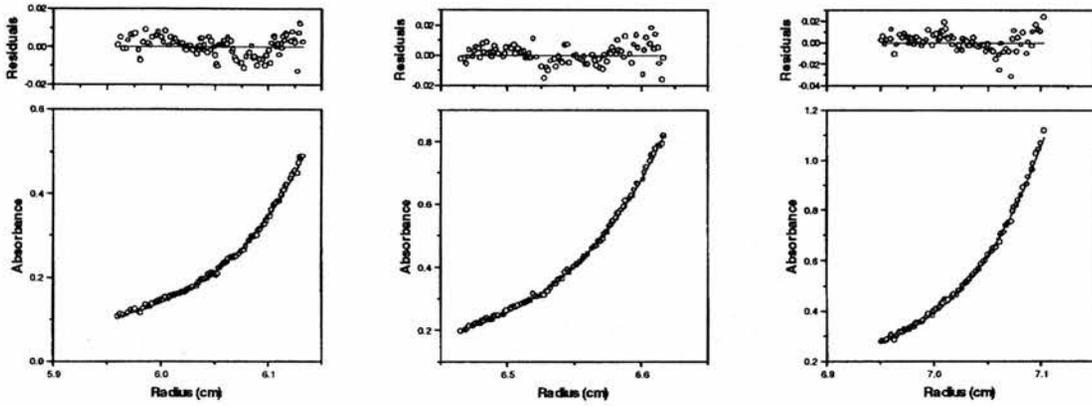
Three concentrations of p50 monomer (15, 31 and 44 μM) were prepared by dilution with its dialysate, and equilibrium was obtained at 16 krpm. The distribution data obtained from the runs were fitted using the single ideal species fit model (Figure 38). From the curve fit plots obtained, apparent weight average molecular masses $M_{w,app_{p50}}$ for each p50 concentration were estimated and the quality of the curve fits was estimated by the residual plots. The estimated $M_{w,app_{p50}}$ values at different concentrations were between 88.0-93.7 kDa (Figure 39). This value demonstrates that Deuterium containing p50 is dimeric in solution.

Three concentrations of IκBγ monomer (14, 27 and 38 μM) were prepared by dilution with its dialysate, and equilibrium was obtained at 16 krpm. The distribution data obtained from the runs were fitted using the single ideal species fit model (Figure 38). From the curve fit plots obtained, apparent weight average molecular masses $M_{w,app_{I\kappa B\gamma}}$ for each IκBγ concentration were estimated and the quality of the curve fits was estimated by the residual plots. The estimated $M_{w,app_{I\kappa B\gamma}}$ values at different concentrations were between 41.7-48.8 kDa (Figure 39). The molecular weight of Deuterium containing IκBγ is slightly higher than the theoretical one for the monomer.

Three concentrations of 2:1 p50:IκBγ (14, 20 and 38 μM) were prepared by dilution with its dialysate, and equilibrium was obtained at 16 krpm. The distribution data obtained from the runs were fitted using the single ideal species fit model (Figure

Figure 38: Distribution data from p50, IκBγ and 2:1 p50: IκBγ in D₂O at 16 krpm and the curve fits and the residual plots, using the single ideal species model.

p50 at 16krpm (single ideal species model)

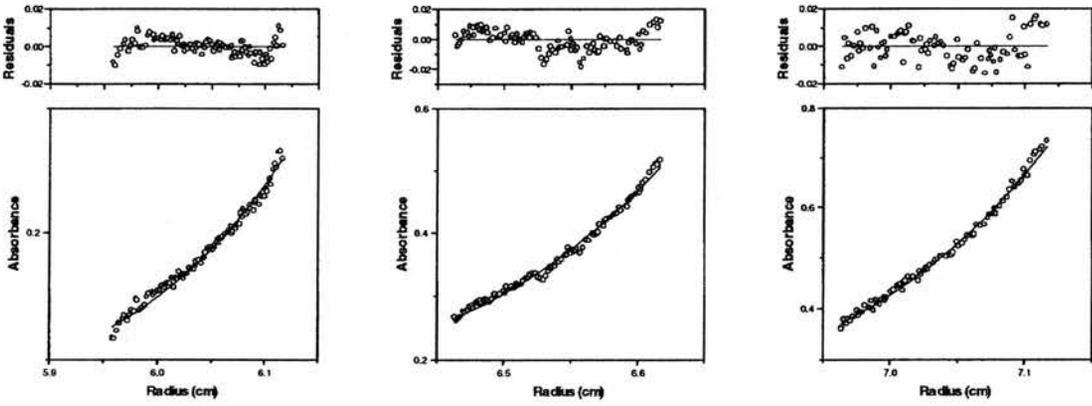


Mw,app=93.5 (\pm 1.1) 15 μ M

Mw,app=93.7 (\pm 0.8) 31 μ M

Mw,app=88.0 (\pm 0.7) 44 μ M

κ B γ at 16krpm (single ideal species model)

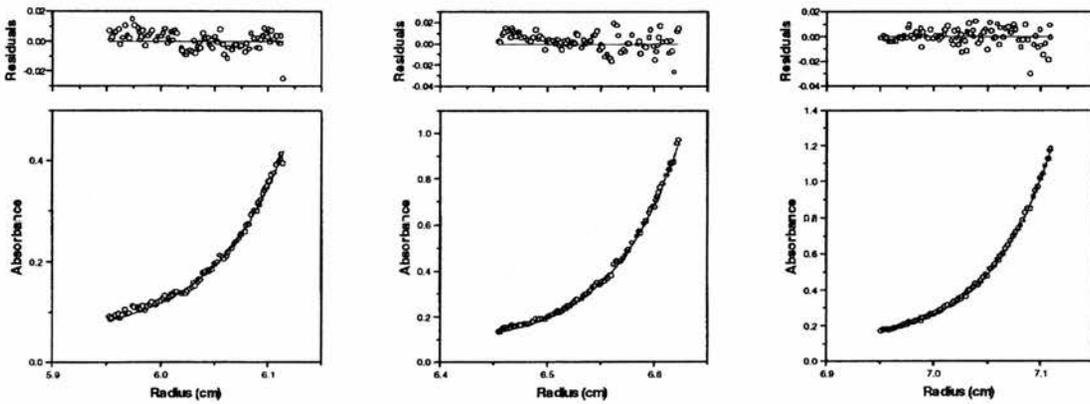


Mw,app=48.1 (\pm 0.9) 14 μ M

Mw,app=41.7 (\pm 0.7) 27 μ M

Mw,app=41.8 (\pm 0.7) 38 μ M

2:1 p50: κ B γ at 16krpm (single ideal species model)

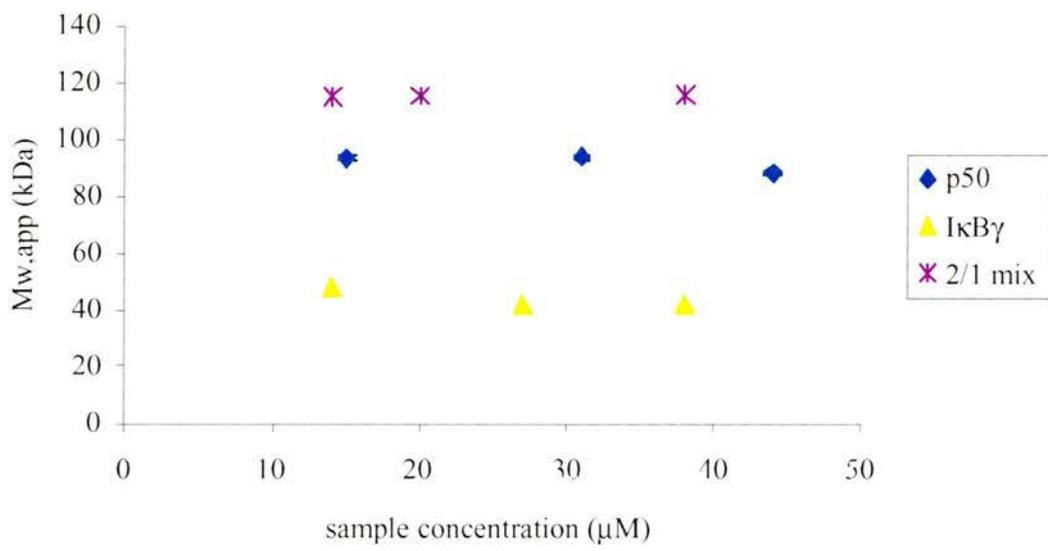


Mw,app=115.1 (\pm 1.5) 14 μ M

Mw,app=115.3 (\pm 0.9) 20 μ M

Mw,app=115.8 (\pm 0.5) 38 μ M

Figure 39: Estimation of the $M_{w,app}$ of p50, I κ B γ 2:1 p50:I κ B γ dialysed in D₂O and span at 16 krpm, using the single ideal species fit model.



38). From the curve fit plots obtained, apparent weight average molecular masses $M_{w,app(p50)_2 \cdot IkB\gamma}$ for each $IkB\gamma$ concentration were estimated and the quality of the curve fits was estimated by the residual plots. The estimated $M_{w,app(p50)_2 \cdot IkB\gamma}$ values at different concentrations were between 115.1-115.8 kDa (Figure 39), which is the molecular weight of a $p50 \cdot IkB\gamma$ complex with 2:1 $p50:IkB\gamma$ ratio.

3.2.4 Sedimentation Velocity

Three concentrations of $p50$ (35.5, 69.9, and 102.4 μM) were spun at 35 krpm at 4°C in a sedimentation velocity run. Sedimentation coefficients in buffer at 4°C for each concentration were obtained by data analysis using *Origin Velocity* and *Svedberg* data fitting programs. Sedimentation coefficients are normally calculated relative to water at 20°C i.e. $s_{20,w}$, therefore the values have to be corrected for temperature and experimental buffer. The viscosity of the water at 4°C $\eta_{T,w}=0.015670$ Poise, the viscosity of the water at 20°C $\eta_{20,w}=0.010020$ Poise, the viscosity of the buffer at 4°C $\eta_s=0.016265$ Poise, the viscosity of the water at 20°C $\eta_w=0.015670$ Poise, the density of the water at 20°C $\rho_{20,w}=0.99832$ g/ml and the density of the water at 4°C $\rho_{T,s}=1.119176$ g/ml. By plotting the corrected $s_{20,w}$ values, the sedimentation coefficient in water at 20°C to zero concentration $s_{0/20,w}$ was obtained by extrapolation to 0 concentration on the x axis. The measured $s_{0/20,w}=0.64$ S according to Origin Analysis and $s_{0/20,w}=3.86$ S according to Svedberg single species Analysis. The sedimentation coefficient predicted using crystallographic data with HYDRO using a bead model was for $p50$ monomer 3.40 S and for the dimer 4.96 S (Figure 40).

Similarly, three concentrations of $IkB\gamma$ (33.6, 66.1, and 102.9 μM) were spun at 45 krpm at 4°C in a sedimentation velocity run. The measured $s_{0/20,w}=1.86$ S according to Origin Analysis and $s_{0/20,w}=2.04$ S according to Svedberg single species Analysis. The predicted sedimentation coefficient for $IkB\gamma$ monomer which was constructed by the addition of one ankyrin repeat in the $IkB\alpha$ structure (Figure 41), was 3.39 S (Figure 40).

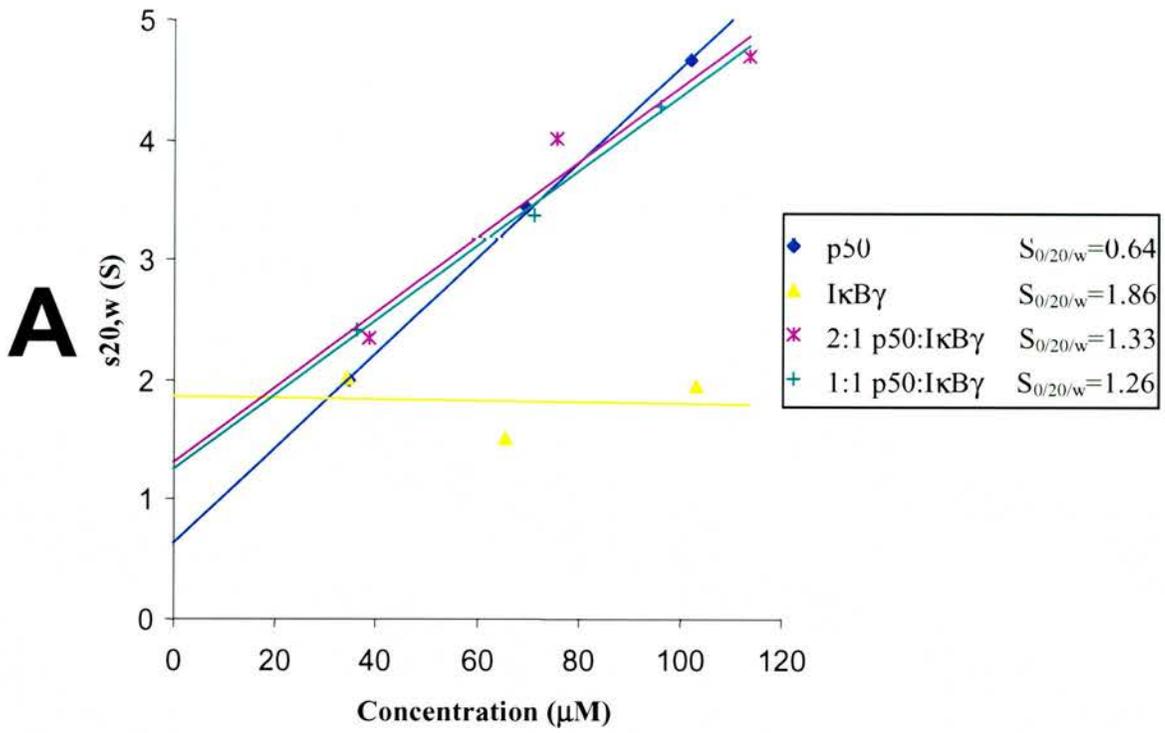
Three concentrations of 2:1 ratio $p50:IkB\gamma$ (34.7, 70.4, and 113.1 μM) were also spun at 35 krpm at 4°C in a sedimentation velocity run. The measured $s_{0/20,w}=1.33$ S

according to Origin Analysis and $s_{0/20,w}=4.71$ S according to Svedberg single species Analysis (Figure 40).

Finally, three concentrations of 1:1 ratio p50:IkB γ (25.8, 50.9, and 96.5 μ M) were span at 35 krpm at 4°C in a sedimentation velocity run. The measured $s_{0/20,w}=1.26$ S according to Origin Analysis and $s_{0/20,w}=4.63$ S according to Svedberg single species Analysis (Figure 40).

Figure 40: Estimation of s of p50, I κ B γ , 2:1 p50:I κ B γ and 1:1 p50:I κ B γ , by using *Origin* (A) and *Svedberg* (B) software for data analysis of sedimentation velocity runs, correcting observed s values and extrapolating them to zero concentration.

Estimates of s using Origin analysis



Estimates of s using Svedberg single species analysis

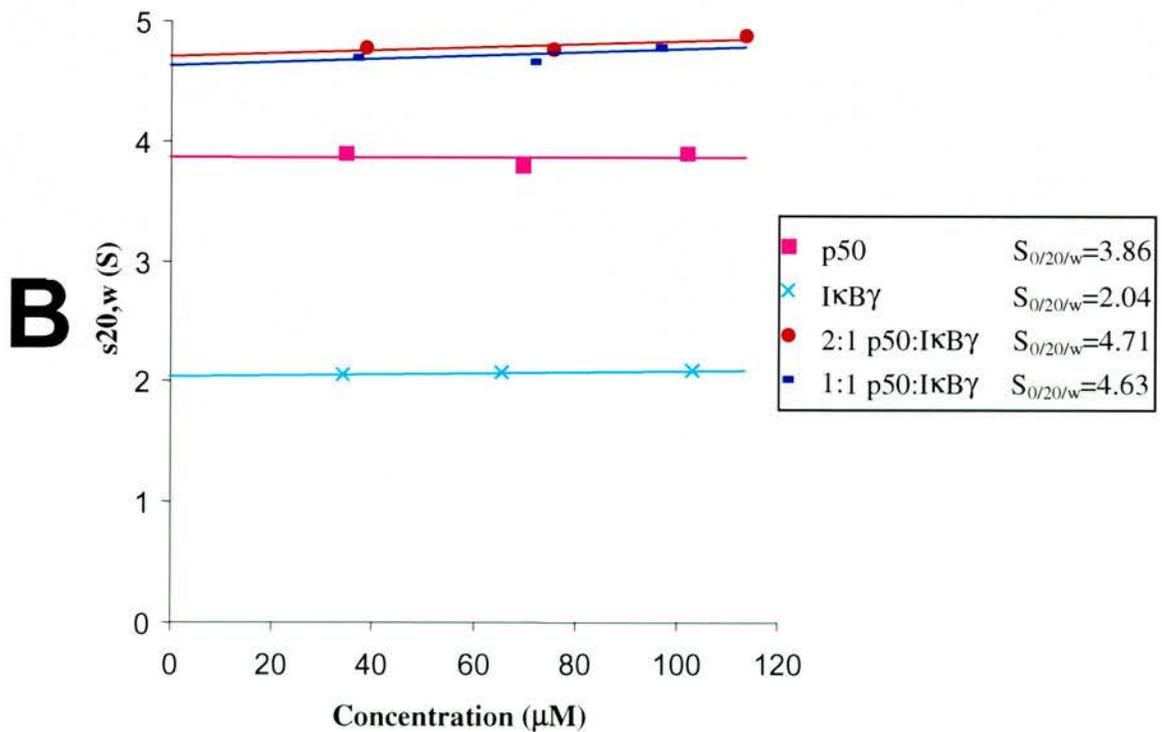
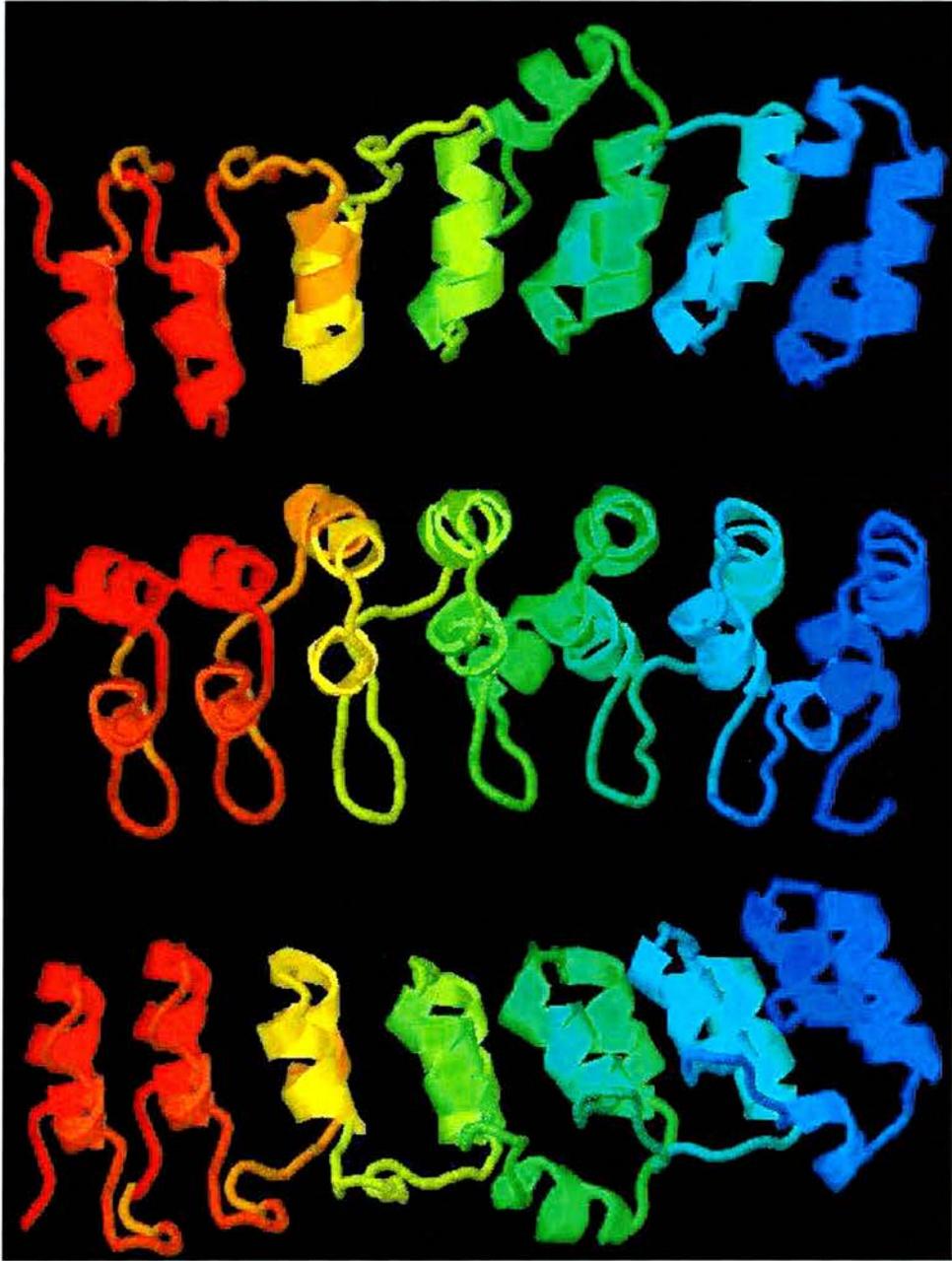


Figure 41: Predicted structure of I κ B γ ARD, using the known structure of I κ B α . This structure allows us to predict the theoretical S.

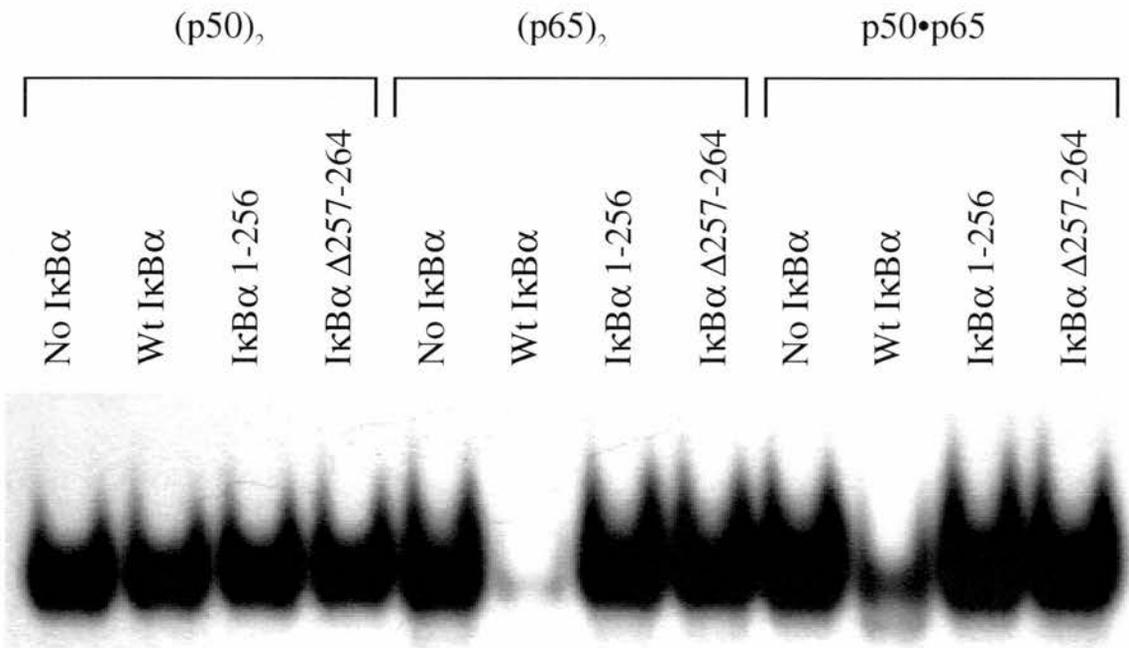


3.3 The C-terminus of I κ B α is required for interaction with NF- κ B proteins

To define the role of the C-terminus of I κ B α in the interaction of I κ B α with NF- κ B proteins, this region was totally or partially removed from I κ B α . I κ B α 1-256 contained amino acids 1-256 from the wild type I κ B α (deletion of the C-terminal 61 amino acids) and I κ B α Δ 257-264, where the amino acids 257-264 from the wild type I κ B α (deletion of a potential phosphorylation site by PKC) were replaced by the sequence PG. Wild type I κ B α or I κ B α mutants were incubated with NF- κ B proteins and the DNA binding activity was determined in electrophoretic mobility shift assays. Addition of wild type I κ B α to (p50)₂ bound to HIV-L- κ B motif had no effect in the DNA binding affinity of (p50)₂ towards this κ B motif. Addition of wild type I κ B α to (p65)₂ or p50•p65 bound to HIV-L- κ B motif, resulted in the decrease of the DNA binding affinity of (p65)₂ or p50•p65 towards this κ B motif. Addition of I κ B α 1-256 or I κ B α Δ 257-264 to (p50)₂, (p65)₂ or p50•p65 bound to HIV-L- κ B motif had no effect in the DNA binding affinity of these NF- κ B proteins towards the HIV-L NF- κ B motif (Figure 42).

Figure 42: I κ B α does not interact with p50•DNA complex. The wild type I κ B α molecule (wt I κ B α) can efficiently inhibit the DNA binding activity of (p65)₂ and p50•p65. The two modified forms of I κ B α , I κ B α 1-256 (deletion of the C-terminal 61 amino acids) and I κ B α Δ 257-264 (deletion of a potential phosphorylation site be PKC) cannot inhibit the DNA binding activity of (p65)₂ and p50•p65.

The C-terminus of IκBα is required for interaction with (p65)₂•DNA and p50•p65•DNA complexes



GATCTAGGGACTTTCCGCG
ATCCCTGAAAGGCGCCTAG
HIV-L-κB

3.4 Role of the C-terminus of the loop L1 in stabilisation of (p50)₂•IκBγ complex

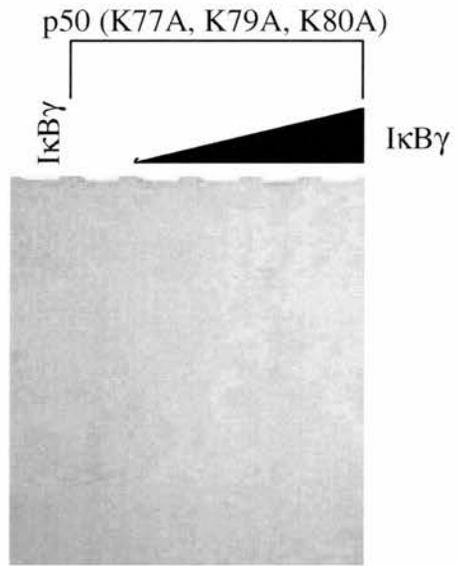
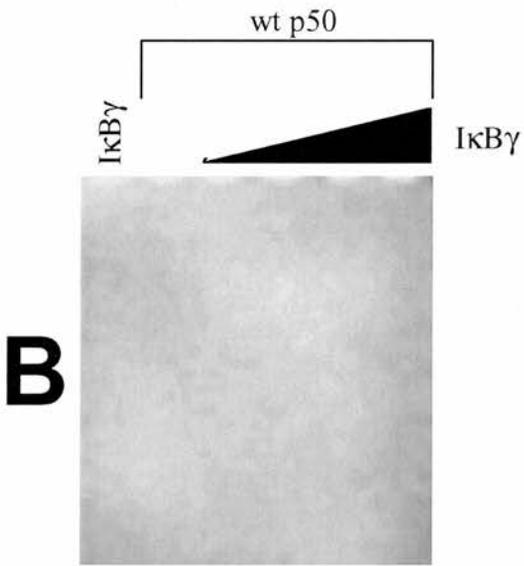
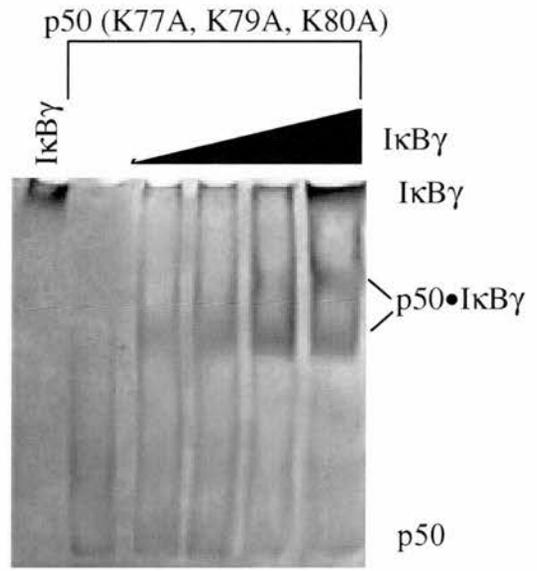
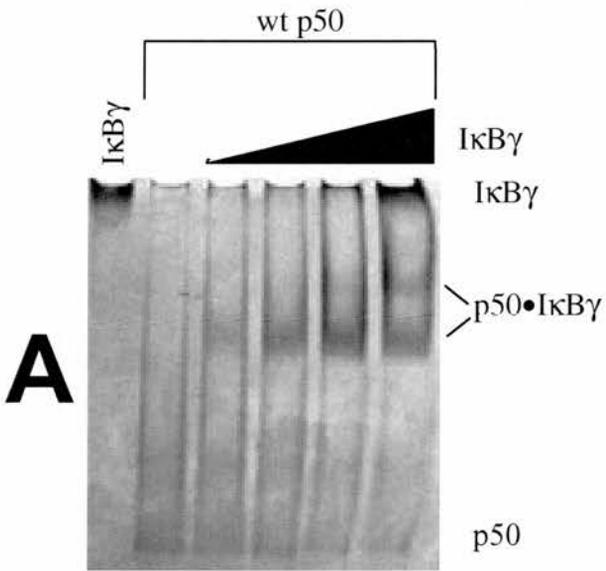
3.4.1 DNA binding properties of mutants

To determine the importance of each lysine residue of the C-terminal sequence of p50 (amino acids 77-80) the previously described p50 mutants (Figure 24), were employed. Gel electrophoresis IκBγ protein binding assays were performed with wt and the triple mutant. Unfortunately, the resolution of this method was insufficient to discriminate between the binding affinities of the wt and the triple mutant towards IκBγ (Figure 43). It was supposed that the this method would not show any differences in the affinity of the single and doubles mutants thus not possible to unambiguously identify the lysine residue(s) responsible for the interaction with IκBγ. This experiment could neither support the existence of the AB loop-IκBγ interaction (Bell et al., 1996) nor demonstrate its importance for stabilisation of the p50-IκBγ complex.

3.4.2 Discrimination between DNA binding activities of p50 mutants

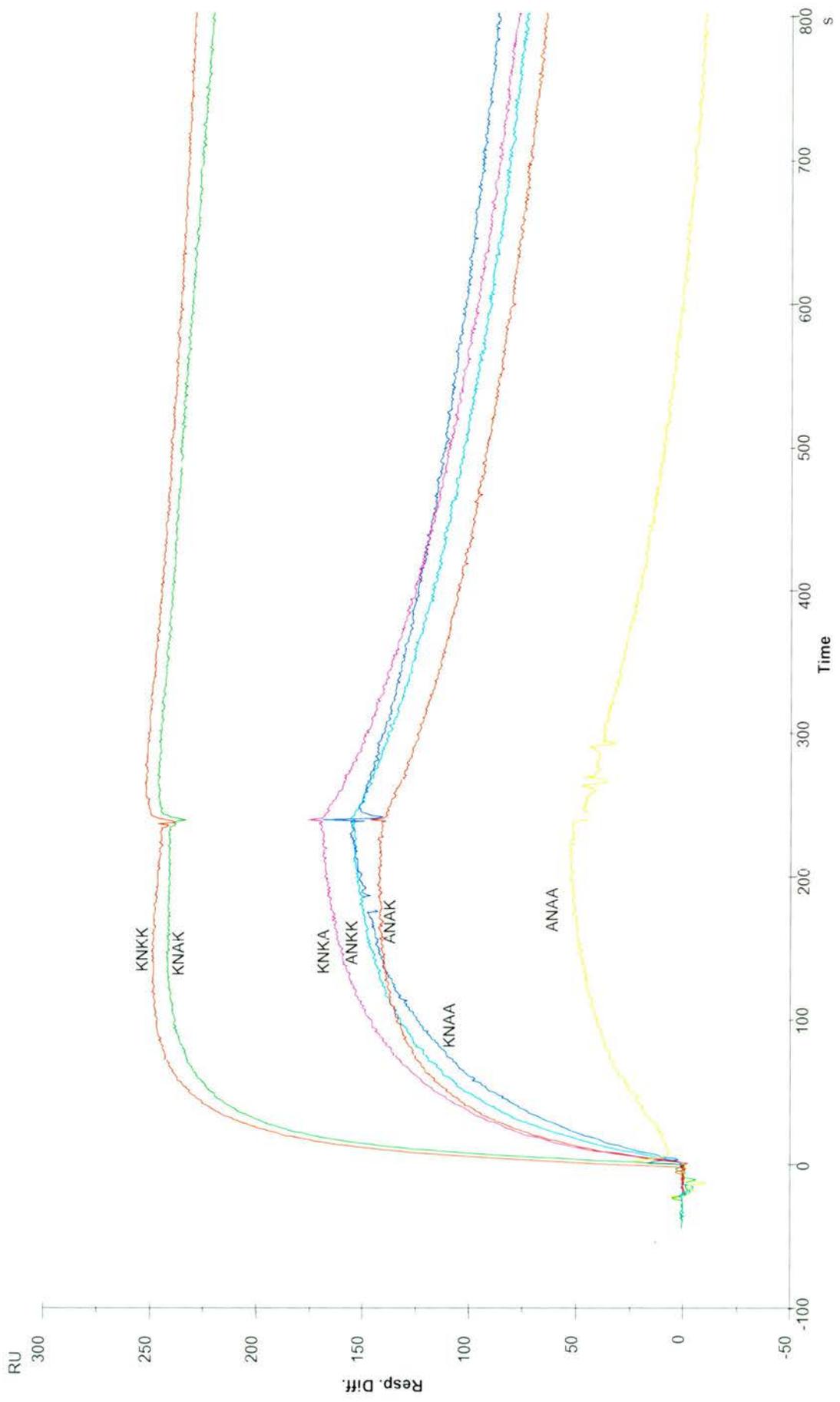
To discriminate between the DNA binding activities of the p50 mutants (Figure 24), SPR was employed. An anti-GST IgG antibody was covalently immobilised on the surface of a sensor chip and IκBγ-GST was applied on the surface until the response difference was 300 RU. Wild type p50 and the various mutants were tested for protein binding activity by passage over the sensor chip. Protein binding reactions were carried out under stringent conditions at 350 mM NaCl to eliminate non-specific interactions between p50 proteins and GST, IgG or the dextran surface of the sensor chip. Under these stringent conditions, wild type p50 bound efficiently to IκBγ-GST and p50 binding to GST immobilised by IgG was negligible. Data were collected at a range of protein concentration between 10 and 500 nM. At each protein concentration the analysis was carried out in triplicate. Although the data were reproducible it was not possible to derive association and dissociation rate constants for the interaction between p50 and IκBγ, as the data could not be fitted to the theoretical binding models of the evaluation software. The reason for that was the fact that the binding

Figure 43: Gel electrophoresis protein binding assays adding wt p50 or the triple mutant p50 (K77A, K79A, K80A) proteins to increasing amounts of IκBγ. To 1 μg wt p50 or the triple mutant p50 (K77A, K79A, K80A) increasing amounts of IκBγ (p50:IκBγ ratio 2:1, 1:1, 2:1 4:1) were incubated for 30' at room temperature. The samples were resolved in two 6% non denaturing polyacrylamide gel (55:1 acrylamide: bis-acrylamide) and the gel was stained with Coomassie Brilliant Blue. The upper band corresponds to free IκBγ the middle ones to p50•IκBγ complex and the lower band corresponds to unbound to IκBγ p50. A: Normal polarity B: Reverse polarity.



reaction did not follow strictly the kinetics expected from a simple bimolecular reaction, because the interaction between p50 and I κ B γ involves p50 conformational changes and interactions in more than one area (as it was revealed by structural analysis of p50•I κ B γ (Huxford et al., 1998; Jacobs and Harrison, 1998)). Although the quantitative evaluation could not be completed, the experimental data could be used to resolve the differences in the I κ B γ binding of the wild type and mutant proteins by direct comparison of the binding curves, at a given protein concentration. wt p50 bound more tightly than any of its mutants (Figure 44). The binding activity of the single mutant p50 (K79A) protein was almost the same with that of the wt. The most defective of the 3 single mutants was p50 (K77A), while the affinity of p50 (K80A) was slightly higher than the affinity of the p50 (K77A) mutant. The double mutant p50 (K79A, K80A) was more defective in I κ B γ binding than each of the two single mutants p50 (K79A) and p50 (K80A) but was almost as defective as the single mutant p50 (K77A). p50 (K77A, K79A) was more defective than p50 (K77A) and p50 (K80A) while the triple mutant p50 (K77A, K79A, K80A) was clearly the most defective in binding to I κ B γ of all the mutant proteins (Figure 44). These data provide experimental evidence for the suggested interaction between the C-terminus of the AB loop- I κ B γ and demonstrate its importance for the stabilisation of the I κ B γ -protein complex. K77, together with K80, appear to play a critical role in this interaction with I κ B γ . Residue K79 does not seem to contribute to the p50-I κ B γ protein interaction.

Figure 44: Typical sensogram (Surface Plasmon Resonance), showing the association and the dissociation of the p50 proteins (wt and mutants) to IκBγ. In the association phase, 100 nM of each protein were injected for 4 minutes at 10 μl/min flow rate. In the dissociation phase, buffer containing no protein was injected for 10 minutes at 10 μl/min flow rate.



4 DISCUSSION

4.1 Bcl-3-NF- κ B interactions

4.1.1 *Bcl-3 found in a chromosomal translocation*

Bcl-3 is a protooncogene. It was found on chromosome 19 adjacent to the breakpoint in the translocation t(14;19)(q32;q13.1) where a class switch region associated with the α constant region exons of the immunoglobulin heavy chain locus was fused head to head to the *bcl-3* gene, which occurred in some cases of B-cell lymphocytic leukaemia (Ohno et al., 1990). A more complex three way rearrangement t(7;19;14)(q21;q13;q32) was also reported (Michaux et al., 1996). This fusion to the Ig heavy chain locus resulted in a much stronger expression of *bcl-3* gene than in normal blood cells, while the coding region remained unmodified (Ohno et al., 1990). *Bcl-3* mRNA is widely expressed (Nolan et al., 1993), in a pattern similar to that observed with the p50 subunit of NF- κ B. The protein levels of Bcl-3 are not affected by ageing in heart, liver, kidney and brain of young, adult and old NMRI mice and Wistar rats (Helenius et al., 1996). Haemorrhage- induced Acute Respiratory Distress Syndrome (ARDS) appears to activate Bcl-3 expression (Moine et al., 1997). In normal peripheral mononuclear blood cells, *bcl-3* gene expression is induced upon T cell mitogenic stimulation, which suggests that the aberrant overexpression in the previously described translocations, mimics a growth-stimulating condition which can not be downregulated. This permanent proliferative stimulus is a step which can lead to neoplastic transformation. *Bcl-3* gene structural alteration is a rare abnormality in chronic lymphoproliferative disorders (Michaux et al., 1996) and was not found in paediatric Acute Lymphoblastic Leukaemia (ALL) (Liptay et al., 1997).

4.1.2 *I κ B-like properties of Bcl-3*

4.1.2.1 *Bcl-3 homology with the I κ B family of protein*

Bcl-3 contains 7 ankyrin repeats which suggested that Bcl-3 belongs to the I κ B family of proteins (Baeuerle and Baltimore, 1996; Baldwin, 1996; Franzoso et al., 1992; Wulczyn et al., 1992). *Bcl-3* gene contains 9 exons, spanning 11.5kb. In comparison

to other members of the I κ B family (*NFKB-2*, *cactus*, *MAD-3*), there is a remarkable conservation of the exon-intron boundaries in relation to the coding sequences, consistent with an origin from a common ancestral gene (McKeithan et al., 1994). Bcl-3 is more related to I κ B γ , as these proteins contain 7 seven ankyrin repeats, while I κ B α and I κ B β contain only six. Moreover, Bcl-3 and I κ B γ , but not I κ B α and I κ B β mainly target the p50 subunit of NF- κ B.

4.1.2.2 Interaction with NF- κ B molecules

Bcl-3 protein interacts *in vitro* with NF- κ B p50 (Bours et al., 1993; Franzoso et al., 1993; Franzoso et al., 1992; Hatada et al., 1992; Inoue et al., 1993; Wulczyn et al., 1992; Zhang et al., 1994) and p52 (Bours et al., 1993), which was also shown in our results. Bcl-3 protein does not interact *in vitro* with c-Rel (Inoue et al., 1993). Our data suggest that Bcl-3 interacts with p65, which is opposite to previous findings (Bours et al., 1993; Franzoso et al., 1992; Inoue et al., 1993).

Previous data which demonstrated that Bcl-3 ARD is sufficient for interaction with p50 (Wulczyn et al., 1992) and p52 (Bours et al., 1993), were also confirmed. We also displayed that this is in contrast with the fact that I κ B α ARD is necessary but not sufficient for interaction with p50•p65 heterodimers and (p65)₂ homodimers, because the C-terminus of MAD-3 is also required (Rodriguez et al., 1995) (Figure 42). Our findings, in accordance with the previously reported data, suggest that Bcl-3 ARD is necessary and sufficient for interaction with other homodimers (i.e. (p65)₂).

Our data showed that p50 NLS is required for association with I κ B proteins, as it was previously shown (Inoue et al., 1993).

4.1.2.3 DNA binding inhibition

In common with other members of the I κ B family of proteins Bcl-3 can inhibit the DNA binding of some members of the NF- κ B protein family. There are conflicting data on the DNA binding inhibition of the different NF- κ B molecules. The converging point is that Bcl-3, as opposed to I κ B α , preferentially interacts with (p50)₂ and (p52)₂ (Bours et al., 1993; Franzoso et al., 1992; Fujita et al., 1993; Hatada

et al., 1992; Inoue et al., 1993; Naumann et al., 1993; Nolan et al., 1993; Wulczyn et al., 1992). Nevertheless, our data suggest that Bcl-3 could alter DNA activity of other NF- κ B homodimers, like (p65)₂.

It was reported that Bcl-3 inhibits DNA binding activity of (p50)₂ (Franzoso et al., 1993; Inoue et al., 1993; Kerr et al., 1992; L veillard and Verma, 1993; Nolan et al., 1993). We found that Bcl-3 directed DNA binding inhibition is DNA sequence dependent. Bcl-3, which prefers to interact with homodimers, stabilises p50 binding to symmetrical DNA molecules, while it inhibits p50 binding to non symmetrical DNA sequences. Our data are not necessarily incompatible with these previous findings. They just reflect the fact that in all previous studies, non symmetrical DNA targets were selected.

According to previous reports, Bcl-3 inhibits DNA binding activity of (p52)₂ (Inoue et al., 1993; Kerr et al., 1992; L veillard and Verma, 1993; Nolan et al., 1993). Our data does not show any significant modification of p52 DNA binding activity in the presence of Bcl-3, irrespective of whether the DNA was symmetrical or non symmetrical.

We were unable to confirm previous data that Bcl-3 inhibits p52•p65 DNA binding activity (Kerr et al., 1992), since we were unable to form stable p52•p65 heterodimers, *in vitro*.

There is not universal agreement on whether Bcl-3 inhibits (p65)₂ DNA binding. Most reports suggest that it does not inhibit it (Inoue et al., 1993; L veillard and Verma, 1993; Nolan et al., 1993). Our findings though, are in accordance with the opposite view, which was also reported (Wulczyn et al., 1992). In fact (p65)₂ DNA binding inhibition by Bcl-3 was consistent in all DNA targets (symmetrical and non symmetrical).

Most observations suggest that Bcl-3 inhibits (c-Rel)₂ DNA binding activity (Inoue et al., 1993; Kerr et al., 1992; Nolan et al., 1993) but conflicting data have been reported (Wulczyn et al., 1992). We did not confirm any of the two findings, since we did not express c-Rel.

An early report suggests that Bcl-3 inhibits the DNA binding of p50•p65 (Kerr et al., 1992), while later studies suggest the opposite (Inoue et al., 1993; Nolan et al., 1993). Our data displayed that Bcl-3 did not cause inhibition of p50•p65 binding to either symmetrical or non symmetrical DNA targets.

Bcl-3 ankyrin repeat domain is necessary and sufficient for the destabilisation of the p50•DNA complexes (Franzoso et al., 1993; Wulczyn et al., 1992) *in vitro*. This was also demonstrated by our experiments.

Bcl-3 requires phosphorylation to efficiently inhibit (p50)₂ (Nolan et al., 1993).

4.1.2.4 Phosphorylation

Bcl-3 is constitutively phosphorylated (Bundy and McKeithan, 1997; Caamaño et al., 1996; Fujita et al., 1993; Nolan et al., 1993). Most of the phosphorylation of Bcl-3 occurs in the serine-proline rich C-terminal domain of Bcl-3 and is extensive and constitutive (Bundy and McKeithan, 1997), similarly to IκBα (Ernst et al., 1995) and IκBβ (Chu et al., 1996). Our protein that effectively induced p50 DNA binding inhibition in non symmetrical DNA targets, was not phosphorylated, because it was expressed in bacteria (since it was only the ARD and thus lacked the C-terminus and it would not be heavily phosphorylated in a prokaryotic expression system, anyway). By combining these lines of data, we can then speculate that phosphorylation of C-terminus might lead to exposure of Bcl-3 ARD, letting this domain efficiently interact with NF-κB molecules.

4.1.3 Atypical IκB properties of Bcl-3

4.1.3.1 Nuclear localisation

It was initially thought that Bcl-3, like p105, sequesters p50 in the cytoplasm and prohibits both nuclear translocation and DNA binding and that, unlike p105, does not sequester p65 in the cytoplasm (Naumann et al., 1993). It was eventually shown that Bcl-3 is a predominantly nuclear protein (Bours et al., 1993; Caamaño et al., 1996; Franzoso et al., 1993; Inoue et al., 1993; Nolan et al., 1993; Zhang et al., 1994) which promotes the nuclear localisation of the NF-κB molecules with which it interacts

(Heissmeyer et al., 1999; Watanabe et al., 1997). This is in contrast to other IκB proteins which block the NF-κB Nuclear Localisation Signal (NLS) and anchor NF-κB in the cytoplasm. In cells co-transfected with p50 lacking a functional NLS, Bcl-3 is relocated to the cytoplasm, showing that the two proteins interact in the cell (Nolan et al., 1993). The opposite effect (p50 lacking NLS was restricted in the cytoplasm in the absence of Bcl-3 and in the nucleus in the presence of Bcl-3) was also reported (Zhang et al., 1994). That means that the only requirement for efficient nuclear transport of the NF-κB•Bcl-3 complex is karyophilicity from either NF-κB or Bcl-3. Bcl-3 N-terminus is required for efficient nuclear localisation (Zhang et al., 1994). Interestingly, there is no report that this region is phosphorylated upon stimulation, ubiquitin or SUMO conjugated or proteolytically degraded through proteasome, as it happens to the other IκB proteins.

4.1.3.2 *Formation of a ternary complex containing Bcl-3 and the DNA bound form of (p50)₂ and (p52)₂*

One and/or two Bcl-3 molecules can also associate with (p50)₂ and (p52)₂ bound to DNA (Bours et al., 1993; Fujita et al., 1993), and form a ternary complex. This was also shown by our experiments. The ankyrin repeat domain of Bcl-3 is sufficient for the formation of a Bcl-3•p52•DNA ternary complex (Bours et al., 1993) *in vivo* and we demonstrated that the formation of Bcl-3 ARD•p50•DNA and Bcl-3 ARD•p52•DNA ternary complexes *in vitro*.

Our data indicate that p50 NLS sequence is crucial for the effective interaction of p50 with Bcl-3. Lack of p50 NLS stops Bcl-3 inhibiting DNA binding activity and also blocks the formation of the ternary complex. Previous experiments have shown that p50 and p65 NLS participates in interactions with IκBγ and IκBα, respectively (Beg et al., 1992; Henkel et al., 1992; Matthews et al., 1993b). Since the NLS is on the other side from p50 than its DNA binding area, and this interaction is necessary for modification of p50 DNA properties, it is apparent that NLS recognition by Bcl-3 ARD is necessary for anchoring of Bcl-3 to (p50)₂, rather than actively participating in the DNA binding itself (e.g. by directly competing DNA in the DNA binding

region of p50 or inducing allosteric rearrangements of the two proteins that would alter DNA binding activity). The fact that the supercomplex does not appear in the lack of p50 NLS indicates that the NLS is necessary for the stabilisation of the ternary complex.

Formation of this ternary complex depends on Bcl-3 phosphorylation and concentration (Franzoso et al., 1997), and shows direct transactivation potential. I κ B α can also be found in the nucleus (Arenzana-Seisdedos et al., 1995), but it does not associate with p65 to directly transactivate target promoters (Cressman and Taub, 1993). Bcl-3 can increase p50 DNA binding *in vivo*, by transition from p50•p105 to (p50)₂ which involves phosphorylation upon TNF α stimulation of 3 C-terminal serines of p105 by IKK α and IKK β which leads to degradation of p105 in the proteasome and release of p50 (Heissmeyer et al., 1999), but can also be done without proteolytic processing (Watanabe et al., 1997). Therefore, p50•p105 serves as a physiological reservoir to generate (p50)₂, through a mechanism of interchangeable interactions among NF- κ B and I κ B molecules.

The number of Bcl-3 molecules that participate in NF- κ B•DNA complexes is not known. A model of the formation of the Bcl-3•p52•DNA ternary complex, depending on the stoichiometry of the Bcl-3/p52 was proposed: Only 1:2 complexes can be bound to DNA, whereas 2:2 complexes are unable to bind DNA (Bundy and McKeithan, 1997).

4.1.3.3 *Bcl-3 dependent Transactivation*

Bcl-3 modulates transcription from κ B sites in a different way from I κ B α and I κ B γ (Inoue et al., 1993). There are reports indicating that Bcl-3 represses κ B-mediated transcription *in vivo* (Kerr et al., 1992), but most published work demonstrates that Bcl-3 enhances κ B-mediated transcription *in vivo* in a p50 and p52 dependent fashion (Franzoso et al., 1992). Cotransfection of Bcl-3 with p50 (Fujita et al., 1993; Pan and McEver, 1995), and p52 (Bours et al., 1993; Pan and McEver, 1995) enhanced NF- κ B dependent transcription.

Bcl-3 ankyrin repeat domain is flanked by a proline rich N-terminus and a serine-proline rich C-terminus, both of which cooperate in transcriptional activation (Bours et al., 1993). Bcl-3 interacts with the general transcription factors TFIIB, TBP and TFIIA (Na et al., 1998). The association of Bcl-3 to (p52)₂ bound to DNA at κB sites, functionally leads to a novel and potent form of transactivation through the κB motif: the tethering of Bcl-3 to DNA via the (p52)₂ allows Bcl-3 to transactivate directly, while (p52)₂ alone cannot (Bours et al., 1993). The amino acids 156-289 were identified as autonomous transactivation domains (Na et al., 1998).

Two-hybrid-system uncovered that Bcl-3 ARD itself can also interact with various co-activators like Tip60, Pirin, Bard1 and Jab1 (Dechend et al., 1999). Tip60 which displays histone acetylase activity (Yamamoto and Horikoshi, 1997), is a Tat-interacting protein (Claret et al., 1996), Pirin is an NFI co-activator (Wendler et al., 1997), Bard1 functionally interacts with the tumour suppressor Brca1 (Wu et al., 1996a) and Jab1 interacts with the transactivation domain of c-Jun (Claret et al., 1996). All four proteins are nuclear proteins which associate with gene regulators, but share no sequence homology. Pirin, Tip60 and Bard1 form ternary supercomplexes with Bcl-3•p50•DNA (whether one or two Bcl-3 molecules participate in these complexes has yet to be clarified), whereas Jab1 enhances the formation of Bcl-3•p50•DNA, without forming a complex with it. Furthermore, Bcl-3 interacts with the retinoid X receptor (RXR), and unlike IκBβ which also interacts with it, it coactivates the 9-*cis*-Retinoic Acid-dependent transactivation (Na et al., 1998). Thus, Bcl-3 interacting proteins (BIP) functionally relate NF-κB p50 and p52 to other transcription factor families. In this sense Bcl-3 is itself a co-factor in the establishment of such transactivation networks.

(p50)₂ has so strong affinity for select κB sites, that completely prevents binding of any other NF-κB molecules. These κB sites are candidates for regulation by Bcl-3, as this protein reverses this block (Franzoso et al., 1993). In this case Bcl-3 serves as an antirepressor.

4.1.4 Transgenic mice

Constitutive expression of Bcl-3 in the thymus, in transgenic mice, does not affect T-cell maturation and does not lead to rapid development of lymphoid tumours. p50 but not p52 DNA binding activity is enhanced in the presence of Bcl-3, and this occurs in a phosphorylation dependent manner (Caamaño et al., 1996).

Expression of I κ B and NF- κ B family members in the Bcl-3(-/-) mouse is unchanged, and B- and T- subsets are normal. Although Bcl-3(-/-) mice are able to generate greater than normal levels of antibodies in response to vaccination, they are unable to generate specific antibodies. This could be explained by the fact that the spleens of such mice lack germinal centres (Franzoso et al., 1997; Schwarz et al., 1997).

4.1.5 Bcl-3 dual mode of action

A model has been developed to describe the dynamic equilibrium and ternary complex formation between (p50)₂, its DNA binding site and Bcl-3. Accordingly, the formation of the supercomplex would mediate Bcl-3 induced DNA binding inhibition (Figure 45). This model could also be expanded to all NF- κ B molecules and their appropriate I κ B molecules, assuming that the ternary complex of NF- κ B•I κ B•DNA is quite unstable and short lived.

The previously described model suggests possible mechanisms for the action of Bcl-3, in transactivation of genes that contain NF- κ B motif sequences:

A direct model is the attachment of Bcl-3 to (p50)₂ (Bours et al., 1993; Fujita et al., 1993) or (p52)₂ (Bours et al., 1993), bound to DNA. The resulting complex transactivates through the Bcl-3 N- and C-terminal transactivation domains and Bcl-3 ARD possibly bound to nuclear co-regulators which link NF- κ B to other transcription factors, synergistically inducing transcription (Figure 46).

The indirect model is the inhibition of DNA-binding activity of (p50)₂ (Franzoso et al., 1993; Hatada et al., 1992; Nolan et al., 1993) which does not contain any transactivation domain. The removal of (p50)₂ from non-symmetrical κ B motifs, gives the opportunity to other molecules (p50•p65, (p65)₂, etc) that contain

Figure 45: The model for dynamic equilibrium and ternary complex formation between $(p50)_2$, its DNA binding site and Bcl-3.

Model for dynamic equilibrium

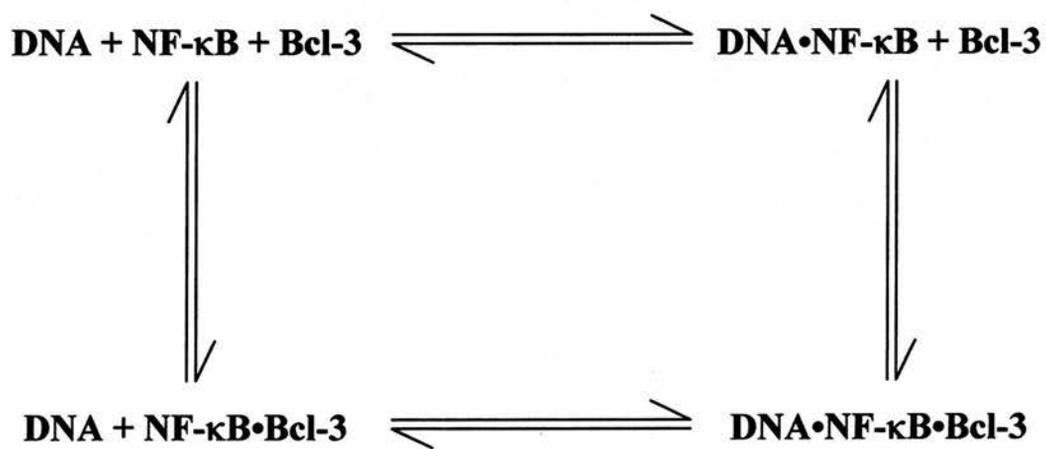
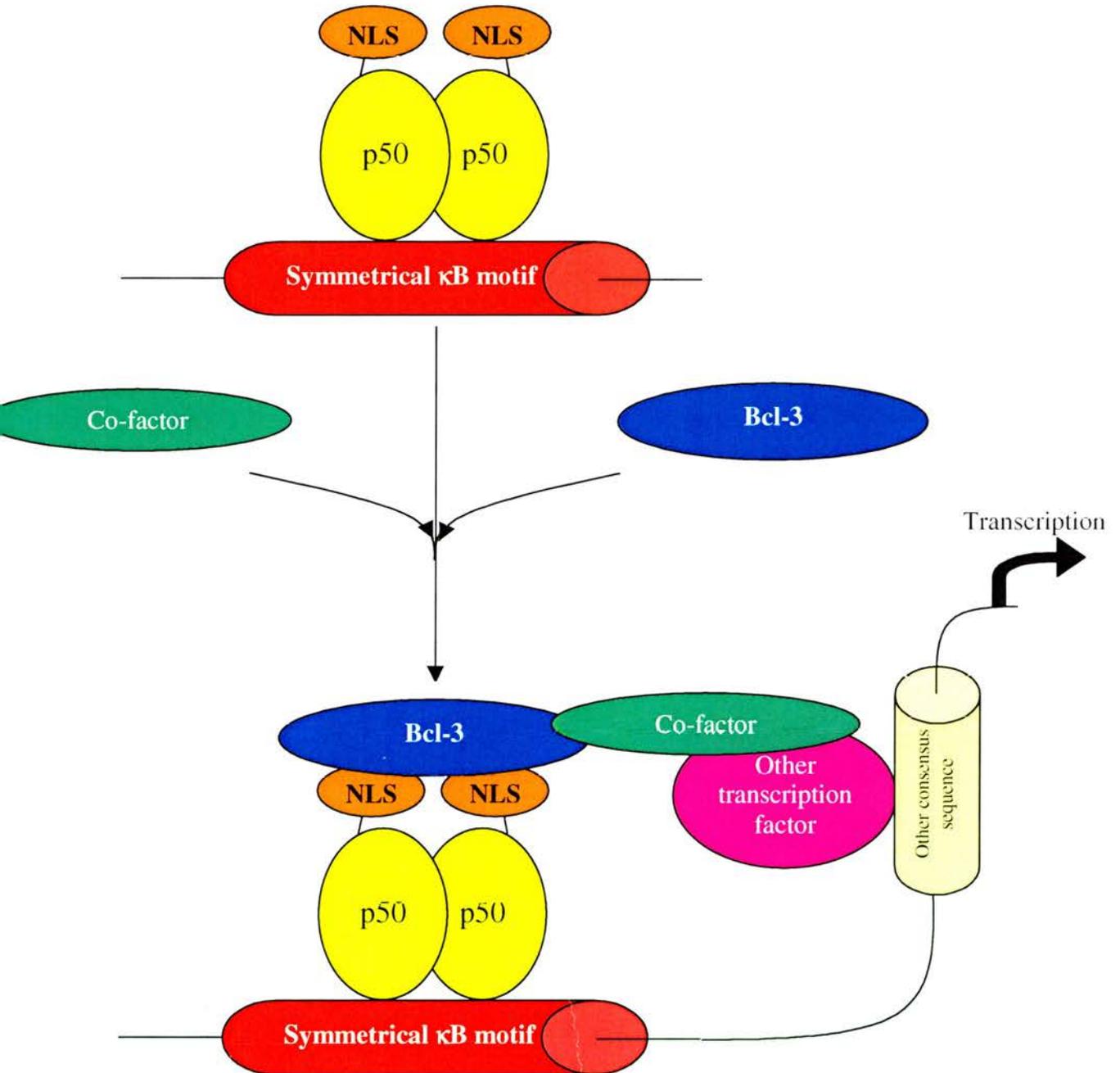


Figure 46: Attachment of Bcl-3 to (p50)₂ or (p52)₂, bound to symmetrical κB motifs, forms a ternary complex which transactivates through Bcl-3 binding proteins which also bind other transcription factors.

Direct transactivation through Bcl-3 binding co-factors

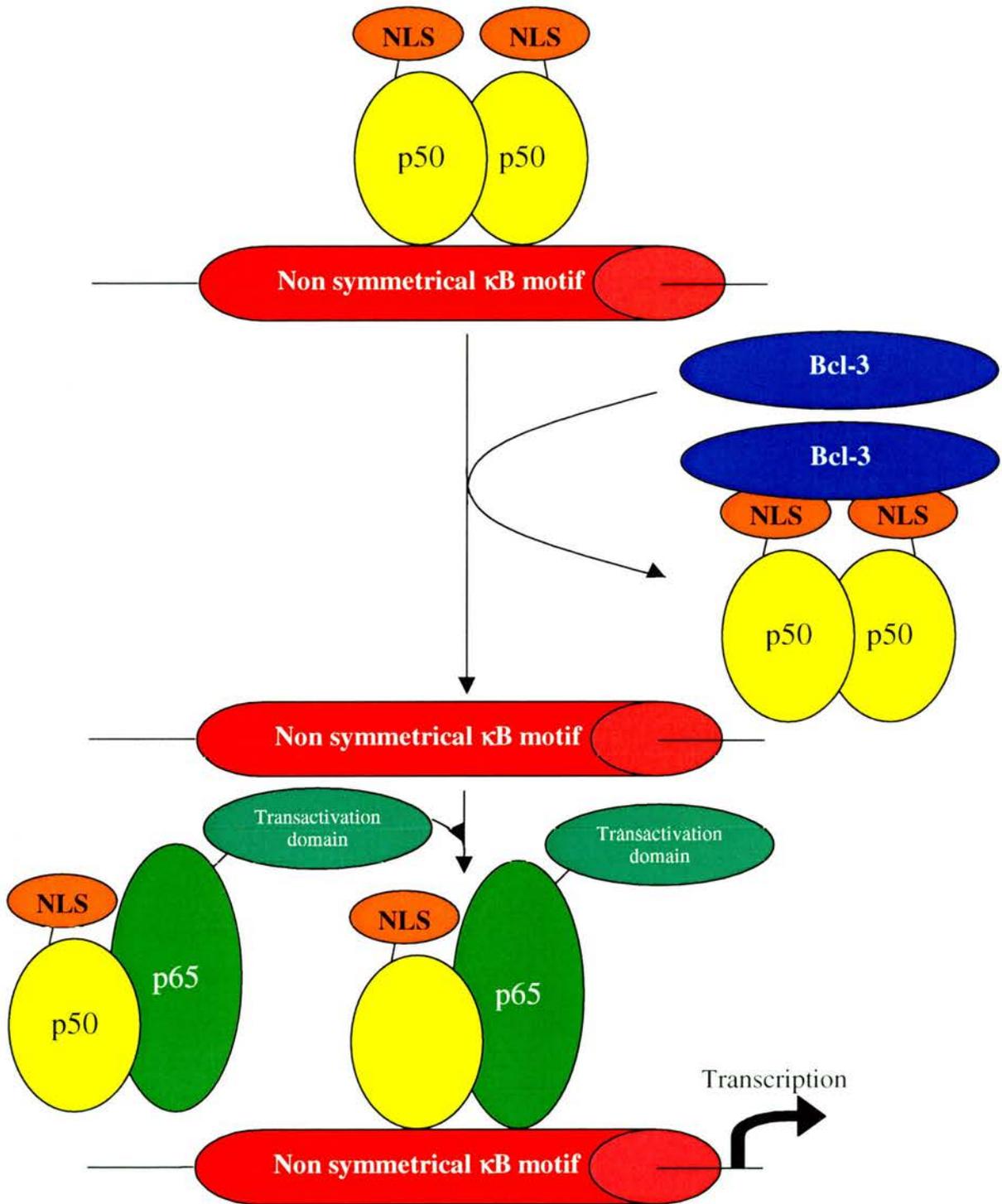


transactivation domains to bind to DNA and to transactivate (Franzoso et al., 1993; Franzoso et al., 1992) (Figure 47).

Our experiments show that the formation of Bcl-3•(p52)₂•DNA complexes is not dependent on the DNA sequence. Interestingly, when Bcl-3 interacts with (p50)₂, bound to DNA, it forms a ternary complex if the DNA sequence contains symmetrical NF-κB motifs and as a DNA binding inhibitor if the NF-κB motifs are not symmetrical. The sequence dependent formation of Bcl-3•(p50)₂•DNA complexes is another important way of differential transactivation of genes with different κB motifs, and might play an important role in the cases of leukaemia, caused by Bcl-3 overexpression.

Figure 47: Inhibition of DNA binding activity of (p50)₂ which does not contain any transactivation domain. The removal of (p50)₂ gives the opportunity to other molecules (e.g. p50•p65, (p65)₂, etc.) which contain transactivation domains, to bind to non symmetrical κB motifs and to transactivate.

Indirect transactivation



4.2 NF- κ B-DNA interactions

Protein footprinting by partial proteolysis (Hay and Nicholson, 1993; Matthews et al., 1995) and chemical modification (Bell et al., 1996) suggested that the lysine cluster which is located at positions 77 to 80 of NF- κ B p50 could contact DNA. Although the lysine cluster (K77, K79 and K80) was implicated the techniques employed did not have sufficient resolution to identify the lysine residue making the DNA contact (Bell et al., 1996). One of the objectives of this study was therefore to use site directed mutagenesis to investigate the role of each lysine in the sequence between 77 and 80. Our experiments demonstrated that conserved K80 interacts with the phosphate backbone of its DNA target molecule. These findings can be interpreted in the light of the discovery of the conserved structure of Rel/NF- κ B molecules (Chen et al., 1998c; Chen et al., 1998a; Cramer et al., 1997; Ghosh et al., 1995; Müller et al., 1995).

4.2.1 Overall NF- κ B•DNA structure

The members of the Rel family of proteins (p50, p52, p65, c-Rel, RelB) form most of the possible homo- and hetero-dimer combinations, both when they are free proteins or bound either to DNA or I κ Bs. All Rel family members contain a conserved region of approximately 300 amino acid with a pairwise identity of around 50%, termed Rel Homology Region (RHR) (Figure 7).

Crystal structure analysis revealed the structure of the RHR of NF- κ B (p50)₂ (Ghosh et al., 1995; Müller et al., 1995), (p52)₂ (Cramer et al., 1997), (p65)₂ (Chen et al., 1998c), and p50•p65 (Chen et al., 1998a) bound to κ B motifs. There were similarities and differences between the approaches and the results of these studies. All groups used NF- κ B proteins expressed in, and purified from, *Escherichia coli*. While some groups used human NF- κ B gene products, such as human p50 (Müller et al., 1995) and human p52 (Cramer et al., 1997), other groups used the murine NF- κ B homologues, such as mouse p50 (Ghosh et al., 1995), mouse p65 (Chen et al., 1998c) and mouse p50 and p65 (Chen et al., 1998a). The length of the purified NF- κ B molecules was not the same. The reason for omissions of certain parts (e.g.

N-terminus of NF- κ B prior to the N-terminus of RHR, the NLS in the end of the RHR C-terminus and the C-terminal transactivation domain of p65) was the fact that they were either out of the RHR and/or that they appeared unstructured in previous studies. In one case, the human p50 contained a C62A mutation (Müller et al., 1995). Previous site-specific mutagenesis experiments had shown that the sulphhydryl group of C62 is an important determinant of DNA recognition by the p50 subunit of NF- κ B (Matthews et al., 1993a), thus the introduced mutation abolished a very important interaction between p50 and its target DNA.

All studies showed that the overall structure of NF- κ B•DNA complexes is consistent, and has a butterfly shape in which protein domains resembling the wings, clamp a cylindrical body of DNA (Figure 48). Each NF- κ B subunit consists of two flexibly linked immunoglobulin-like domains (β -barrel), that are linked by a flexible 10 residue hinge. The 200-residue N-terminal region is based on an I-type Ig barrel with the addition of a partly α -helical subdomain of various length and low homology which is not part of the RHR consensus, termed Insert Region, while the 100-residue C-terminal domain is a C-type Ig barrel (Müller and Harrison, 1995) (Figure 49). While the C-terminal domain is only required for dimerisation (Huang et al., 1997), the entire RHR is necessary for DNA binding, in contrast to many other proteins where relatively small regions are needed for DNA binding. Another difference between NF- κ B and most DNA binding proteins, is that while the latter use either small α -helices or β -sheets to recognise their DNA targets, each NF- κ B subunit contacts DNA through 5 flexible loops which connect the β -sheets. Two of these loops are in the N-terminal domain (L1 and L2), two are in the C-terminal domain (L4 and L5) and the other one (L3) is the flexible linker connecting the two domains. These flexible loops combine to form an extensive protein-DNA interface, interacting with the functional groups of the major groove and the bordering sugar-phosphate backbone, making about 38 individual contacts with target DNA, leaving only the minor groove free (Figure 48B), so, minor groove group proteins, like high-mobility group protein I(Y) (HMG I(Y)), can still bind to DNA along with NF- κ B.

Figure 48: Views of the $(p50)_2 \bullet DNA$ complex. **(A)** Loop L1 penetrates deep into DNA (in orange), making site specific contacts. From this angle (the axis of DNA is perpendicularly related to the picture), DNA appears to be clamped all over by 10 loops and that the cysteine residues in the tip of the recognition loops (in green) are in close proximity. **(B)** In fact, by viewing the complex from the area in the insert region, it is clear that each p50 subunit only interacts with half of the DNA target (half site, in red/pink), leaving the minor groove accessible to other transcriptional factors which can anchor to the α -helices of the insert region, and that the two cysteine residues which can form S-S bond in oxidising conditions in the absence of DNA, are 20Å away.

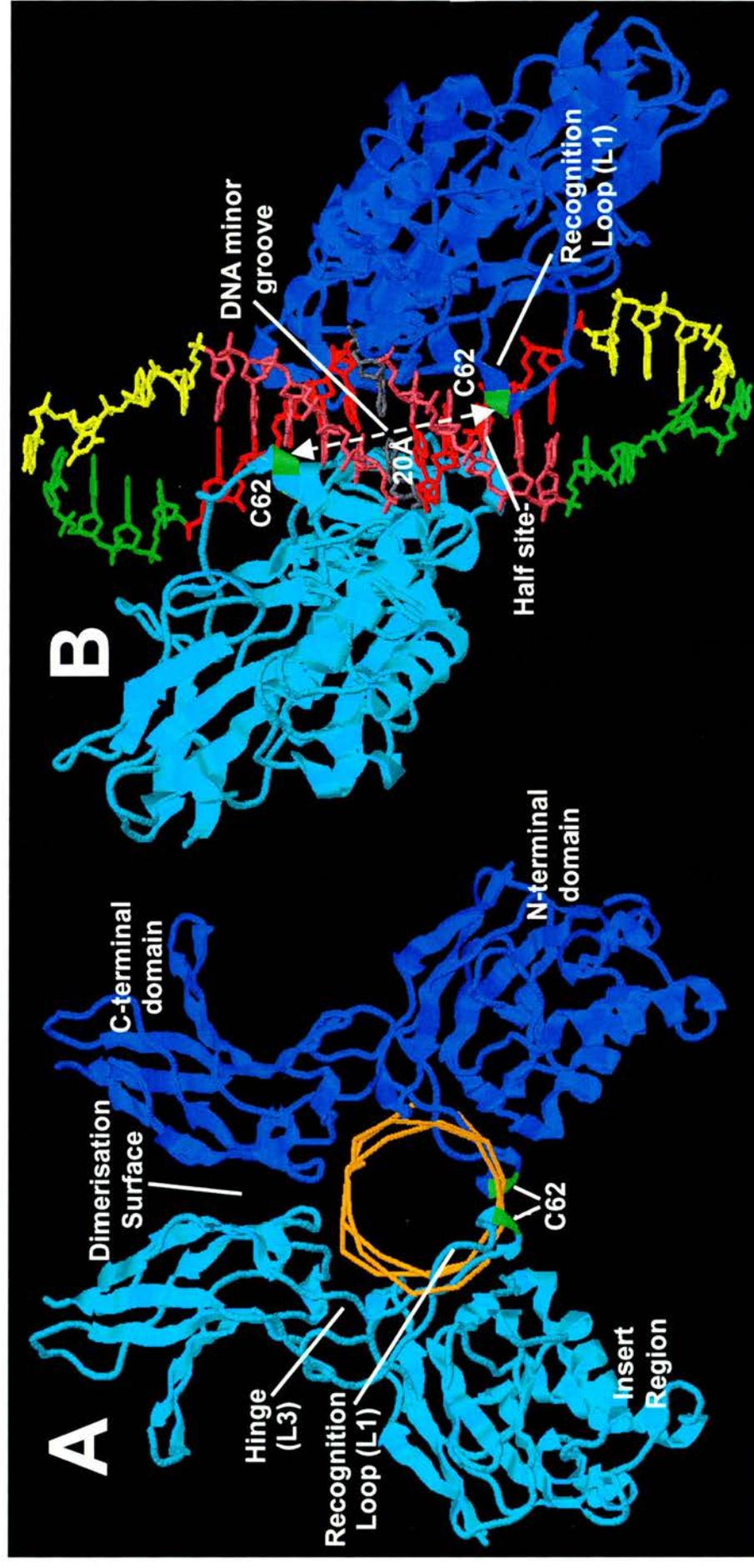


Figure 49: Views of the structure of a p50 subunit bound to DNA. **(A)** Each p50 subunit consists of two domains. The N-terminal domain also contains two α -helices in a non-conserved region (Insert Region). **(B)** Each domain comprises a β -barrel. β -sheets are connected through loops. Five of them (L1-L5) interact with DNA. L1 and L2 are in the N-terminus, L4 and L5 in the C-terminus and L3 is the hinge of the two domains. **(C)** Positions of K77 (in blue), K79 (in brown) and K80 in the c-terminus of the L1 loop.

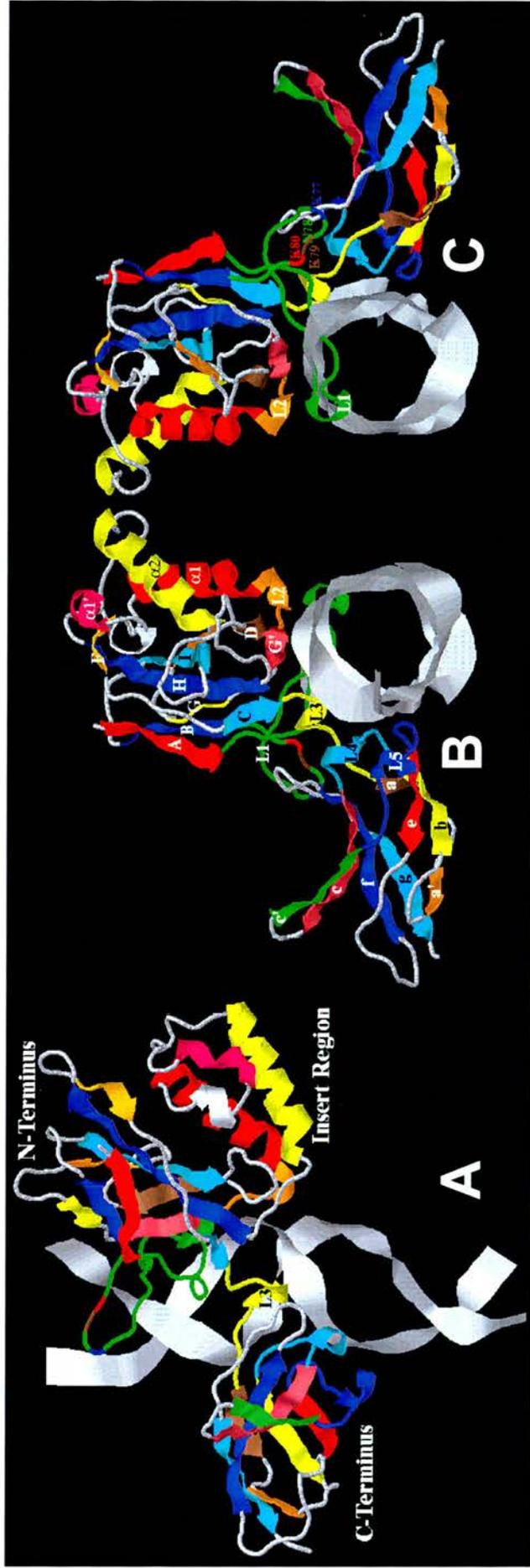


Figure 50: Mismatched bases cause a major distortion of the double helix of DNA of the p50•DNA complex. The distance between complementary bases is not more than 3 Å, while in this case the distance of the mismatched bases is more than 8 Å. Interestingly, K80 of the lysine cluster of the C-terminus of L1 loop, is in close proximity with the DNA backbone.

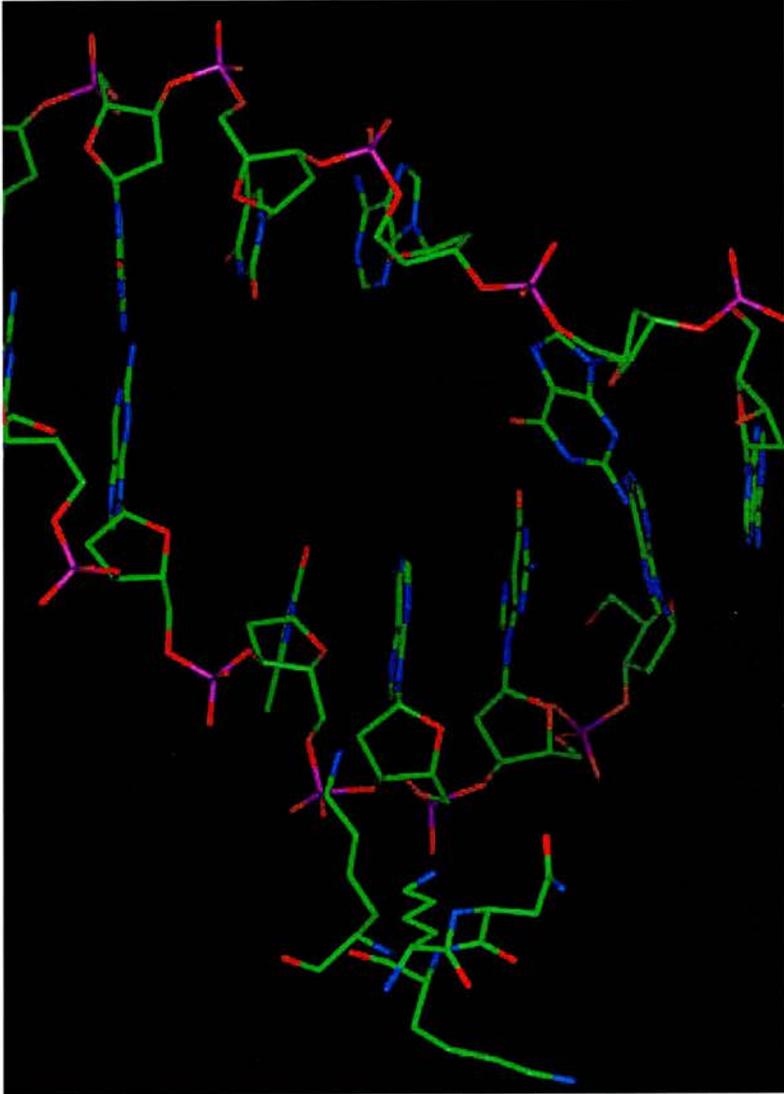


Table 5: Double stranded oligonucleotides used in the crystallographic studies of various NF- κ B molecules. Nucleotides in bold comprise κ B sites. Underlined nucleotides comprise κ B half sites while non-underlined nucleotides within κ B sites are central T:A dyads which separate the κ B subsites. Nucleotides in italics are mismatched and/or disordered.

p50 (Human)	5' AGAT <u>GGGGAATCCCC</u> TAGA 3'
	3' AGAT <u>CCCCTAAGGGG</u> TAGA 5'
p50 (Mouse)	5' <u>TGGGAATTCCC</u> 3'
	3' <u>CCCTTAAGGGT</u> 5'
p52	5' <u>TTGGGGATTCCCC</u> 3'
	3' <u>ACCCTAAGGGG</u> T 5'
p65	5' CGGCTG <u>GAAATTTCC</u> AGCCG 3'
	3' GCCGAC <u>CTTTAAAGG</u> TCGGC 5'
p50•p65	5' <u>TGGGGACTTTCC</u> 3'
	3' <u>CCCTGAAAGG</u> A 5'

The loops make base contacts with their target DNA bases, which determine NF- κ B specificity and sugar/phosphate contacts which contribute to the binding energy of the complex. All these contacts give the complex an affinity which is higher than the affinity of most eukaryotic transcription factors. This strategy for DNA recognition, in which an immunoglobulin fold (Bork et al., 1994) acts as a scaffold for the DNA contacting flexible loops, is also employed by p53 (Cho et al., 1994), STAT-1 (Chen et al., 1998b) and NFATC1 (Zhou et al., 1998), even although there is little recognisable sequence homology.

The crystallographic data show that all NF- κ B molecules interact with DNA over a complete turn. The most important NF- κ B—DNA interaction is between the N-terminus of the L1 loop that connects A and B β -sheets, and the bases of the major groove of target DNA. This part of the AB loop is termed the "recognition loop", because its interaction with DNA determines the sequence specificity of the different NF- κ B species. In the structural studies of the different NF- κ B•DNA complexes, the DNA targets formed slightly different κ B sites: The fully palindromic 10-mer oligonucleotide 5'-GGGAATTC^uCCC-3' for the murine p50, the 11-mer 5'-GGGGAATCCCC-3' (the underlined base was mismatched) comprising a site similar to MHC H-2 κ B except for an A:A mismatch at its very centre for the human p50 (according to binding studies, this mismatch has little effect on affinity, and analysis of crystals with p50 bound to target DNA with a central A:T base pair, prove that the mismatch mentioned above did not cause any significant distortion), the 11-mer 5'-GGGGATTCCC-3' comprising an MHC- κ B site for human p52, a 10-mer 5'-GGAAATTTCC-3' similar to IFN- γ - κ B for the murine p65 and the 10-mer 5'-GGGACTTTCC-3' comprising the Ig- κ B site for the p50•p65 (Table 5). In the case of the human p50, a 19-mer was used as a DNA target. The sequences that flanked the κ B site were mismatched (Table 5) and the double helix was heavily distorted (the distance of the mismatching bases is more than 8.5 Å, while the distance of opposite complementary bases in the canonical DNA double helix is not more than 3 Å) (Figure 50).

The DNA molecules of the complexes are slightly unwound and bent, but not severely distorted. It was initially thought that this was only induced by the interaction with the NF- κ B loops. Nevertheless, NMR analysis of a 16 bp dsDNA containing the HIV-1- κ B/Ig- κ B site and a mutated site that is not recognised by NF- κ B proteins, uncovered that the phosphate backbone of the sequences that flank a κ B motif can force this piece of DNA to adopt a dynamic conformation (intrinsic global curvature towards the major groove), which is very favourable for binding of the NF- κ B loops (the mutated duplex, in contrast, had a canonical conformation) (Tisné et al., 1999a; Tisné et al., 1998; Tisné et al., 1999b). The flexibility of both NF- κ B dimers and their cognate DNA enables NF- κ B to adopt multiple conformations in a promoter specific manner.

4.2.2 *Site specificity*

In all the crystal structures solved, each subunit contacts both DNA strands over a complete turn (10bp). The sequence specific contacts of each subunit, though, involve only half of the DNA target. Each subunit makes DNA specific contacts only with a half site, with residues from the loops L1 and L3, while the half site spacing is preferably one central A:T base pair (the A:T base pairing involves two hydrogen bonds, instead of a G:C which involves three, and that enables the widening of the DNA major groove which is essential for base specific contacts). The most important amino acids in L1 (recognition loop) seem to be the conserved residues R57, R59 and E63 (human p50 numbering). R57 contacts G³ and R59 G⁴ (numbering upstream starting from the first base adjacent to the central half site spacer base pair). The whole unit is stabilised by salt contacts of these two arginines with E63. This network recognises a 5'-G⁴G³-3' sequence in all Rel related proteins. p50 H67 and its p52 equivalent can recognise G⁵ while this residue in p65 is A43 (mouse p65 numbering). The lack of such interaction in p65, is responsible for the fact that while p50 and p52 optimally recognise five base pairs, p65 recognises only four. In MHC- κ B, p50 K244 of L3 contacts G² and A¹, while its homologous residue in p65, R187, anchored by E39, can only interact with the complementary T to A². That is why p50 prefers G²

whereas p65 prefers A². A conserved tyrosine (Y60 in p50, Y55 in p52 and Y36 in p65) makes van der Waals contacts with bases at positions 2' and 1'.

It seems that the interaction between the G³:C³, R33 and E39 is the most important p65 specificity determinant. That is why all sites that can be specifically recognised by p65 (and c-Rel) contain at least one 5'-G⁴G³A²A¹-3' cognate half site and a conserved G³ in the other half site (an A³:T³ base pair causes so unfavourable interactions that abolishes the crucial sequence specific interaction of the bases with the charged amino acids R33, R35, E39 of L1 and R187 of L3, leaving only the van der Waals interactions of Y36). In the absence of G³ on the other half site, the N-terminal domain is rotated by almost 20° from its normal conformation. Interestingly, it still maintains all its non sequence-specific sugar/phosphate backbone contacts with DNA. It was thus proposed that NF-κB dimers could bind to DNA targets which contain only one half site. This conformation could also explain how NF-κB dimers can bind DNA in a non sequence-specific way (binding of NF-κB molecules on *E. coli* bacterial chromosomal DNA is a step for their purification): It can be suggested that such binding takes place without any interactions of the DNA bases with the L1 loop, and is based on the DNA backbone contacts of the protein. The lack of any of these base-specific hydrogen bonds would reduce the affinity of NF-κB for heterologous DNA.

On the other hand, analysis of the structure, shows that p50 consensus half site is 5'-G⁵G⁴G³R²N¹-3'. Therefore, the higher affinity for p50 subunit to its cognate sequence, than that of p65 subunit for its own half site, can be explained due to the extra DNA base contacts made by p50, since p50 interacts with a 5 bp half site while p65 interacts with one which contains 4 bp. Furthermore, the difference on the sequence and the size of the half sites of the different NF-κB subunits explains why there is no unified κB consensus sequence for all Rel related proteins. (p50)₂ and (p52)₂ ideally recognise 11-mers, like MHC-κB (5'-GGGGATTCCCC-3'), (p65)₂ and (c-Rel)₂ 9-mers, like TF-κB (5'-GGAGTTTCC-3') and p50•p65 10-mers, like Ig-κB (5'-GGGACTTTCC-3'). So, it is not of any surprise why NF-κB p50•p65 was initially

discovered for its affinity to the intronic enhancer of the κ light chain gene and why the early studies were referring to the κ B consensus as a 10-mer.

4.2.3 Dimerisation domain

Around 1400 Å² of solvent accessible surface area is buried in the dimer interface of p50•p50, p52•p52, p65•p65 and p50•p65. Dimerisation does not depend of DNA binding. DNA-bound and unbound form of NF- κ B dimerisation domains do not show any noticeable change (Huang et al., 1997). Similarly, the overall structure of the dimerisation domain is not altered by I κ B binding (Huxford et al., 1998; Jacobs and Harrison, 1998). The dimer interface mainly consists of a hydrophobic core. Comparing the structural data of these dimerisation domains, we can understand that even if the overall structure of the C-terminal domain of p50, p52 and p65 is very similar (their backbones can be superimposed), the chemistry of the dimerisation of the interfaces of the 15 homo- and heterodimers, is different. It is also known, for instance, that the heterodimers p50•p65 and p52•p65 are thermodynamically preferred over p50•p50, p52•p52, and p65•p65 homodimers. Some asymmetries in the heterodimer interfaces may lead to conformational adjustments. For instance, the hydrogen bond between p50 D254 and p65 N200 appears only in p50•p65 heterodimer, because in p50•p50 and p65•p65 homodimers the interaction between homologous residues juxtaposes two like charges and thus it is not energetically favourable. This is supposed to be more intense in the case of RelB, where the two asparagine residues do not allow the formation of RelB homodimers, whereas a N→D RelB mutant can form homodimers, in a manner similar to p50 (Ryseck et al., 1995). On the other hand, based on sequence homology, it is proposed that c-Rel forms homodimers in the same manner with p65. Another nine contacts of side chains of uncharged polar residues that point from the β -sheets in towards the dimerisation interface, also contribute to the stronger affinity of p50•p65 heterodimers than that of p50 and p65 homodimers. All those are in accordance with our experiments, where mixture and incubation of equimolar amounts of p65 and p50 homodimers could form exclusively p50•p65 heterodimers.

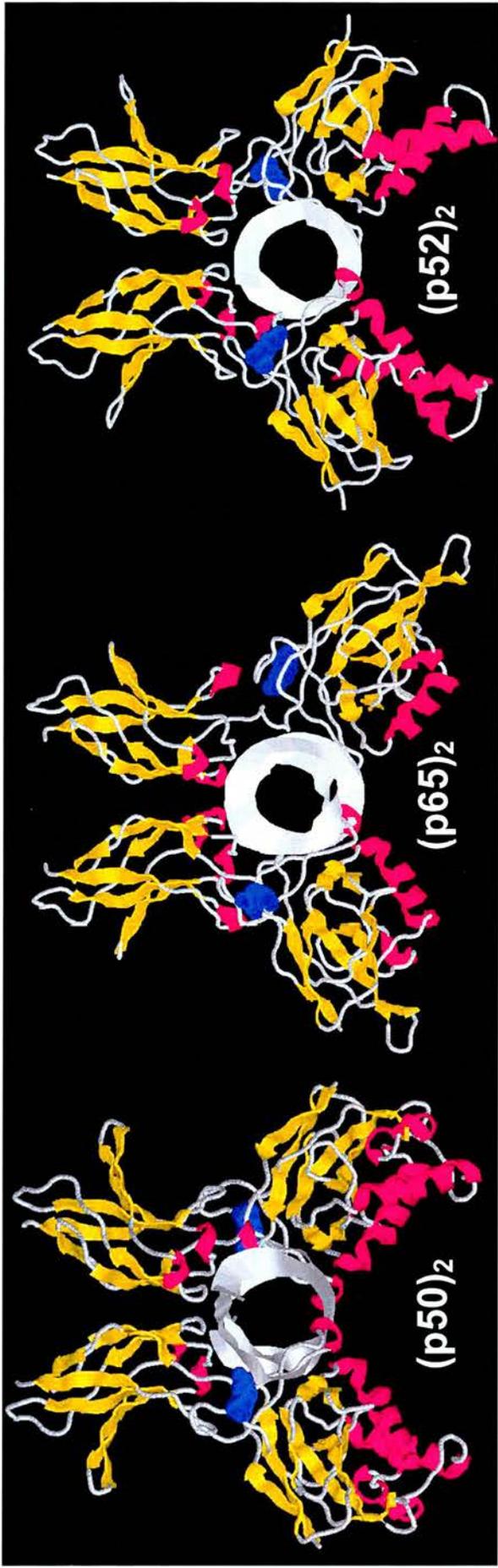
4.2.4 Insert Region

The only major difference between the NF- κ B p50, p52 and p65 subunit structures is in the "insert region" (Figure 48A and Figure 51). The insert region is located immediately after L2 loop of the N-terminal domain of the NF- κ B molecules. Its sequence homology is poor amongst different Rel family members and its length varies (66 residues in p50, 47 in p52, 34 in p65, and 36 in Dorsal), therefore it is not considered part of the RHR sequence. Moreover, while p50 and p52 insert region consists of two structurally conserved α -helices (α 1 and α 2), p65 insert region contains only one helical structure (α 1) (Figure 51). The α 1 N-terminus points towards the phosphate backbone of the minor groove side. The side chains of the α -helices is charged in order to attract other proteins. The insert region interacts with other DNA binding proteins. The differences of this region between all NF- κ B molecules, can lead to interactions with different transcription activators and/or coactivators, e.g. since the minor groove of DNA is not shielded by NF- κ B (Figure 48A), the insert region can be used for the interaction with the high mobility group protein I(Y) (HMGI(Y)) which contacts the minor groove at the AT-rich centre of the IFN β - κ B site (Thanos and Maniatis, 1992). Furthermore, NF- κ B molecules interact with basic region-leucine zipper (bZIP) families (Nolan, 1994; Stein et al., 1993a; Stein et al., 1993b). NF-ATc is a NF- κ B related monomeric transcription factor. NF-ATc homologous region to the NF- κ B insert region, is responsible for the interaction of NF-ATc with the bZIP transcription factor AP-1 in the interleukin-2 enhancer region (Wolfe et al., 1997).

4.2.5 Interaction with other transcription factors

Apart from the interactions through the insert region, NF- κ B molecules can interact with other transcription factors. It is generally accepted that dimers containing at least one Rel-related NF- κ B subunit (i.e. c-Rel, p65, RelB which contain a transactivation domain C-terminally of their NLS), can interact with the transcription machinery, while (p50)₂, (p52)₂ and p50•p52 which lack this region are considered repressors. The DNA sequence-induced changes of the protein structure can also modulate the

Figure 51: Crystal structure of three NF- κ B homodimers (p50)₂, (p65)₂ and (p52)₂ bound to DNA (the direction of the DNA axis is perpendicular to the picture surface). The structure of the RHR of all NF- κ B homo- and heterodimers is very similar, as far as the structure of the β -barrels is concerned. β -barrels from different NF- κ B molecules can be well superimposed. Interesting differences in the structures is the number of α -helices in the insert region (two in p50 and p52 and one in p65, shown in all structures as pink α -helices) and the distance of the conserved lysine (K80 in p50, shown in blue in all structures) of the C-terminal part of the L1 loop, which is in close proximity with the DNA phosphate backbone in p50 and p52 but 11Å away from it, in p65.



interaction with various transcription factors and thus cause activation differences (Menetski, 2000).

4.2.6 Redox regulation of NF- κ B binding

The knowledge of the structure of (p50)₂•DNA complex can explain the biochemical findings which suggested that the conserved among all Rel family members C62 would play a critical role in both the determination of p50 DNA sequence specificity and the redox regulation of p50 DNA binding (Matthews et al., 1993a; Matthews et al., 1992). When p50 is bound to DNA, the distance between the C62 residues of each subunit is 20 Å (Ghosh et al., 1995) (Figure 48B). C62 appears to be in the tip of the N-terminal part of L1 (Figure 48A) and contacts the sugar-phosphate backbone and other amino acids through van der Waals and hydrogen bonds (cysteine residues with such properties also interact with the phosphate backbone of DNA in Jun and Fos complexes). These cysteine residues though, in the absence of DNA and in oxidative conditions can form inter-strand disulphide bonds. This would lock the N-terminal "jaws", preventing the clamping of DNA and thus inhibiting DNA binding activity. Interestingly, NF- κ B is induced by oxidative signals, provided that the cell is able to retain reducing conditions in the intracellular environment (e.g. through the glutathione system).

The ability of the N-termini to move through the hinge, also proves the high degree of flexibility of the binding of the two N-terminal domains to the C-terminal dimerisation domains through the L3 loop. This flexibility is also demonstrated in the apparent difference of the angle of the axis of the N-terminus which is dictated by the sequence of the DNA target (and especially from the size of the half-site spacing).

4.2.7 The role of the C-terminus of the L1 loop in DNA binding

Previous biochemical experiments suggested an interaction between the C-terminus of the AB loop of (p50)₂ and DNA (Bell et al., 1996; Hay and Nicholson, 1993; Matthews et al., 1995). Although residues K77, K79 and K80 were thought to be involved, the resolution of the techniques employed were such that the contribution of individual residues to the interaction could not be determined. Site directed

mutagenesis of this region was therefore employed to remove functional groups (K to A changes) and thus identify lysine residues in contact with DNA. The various mutants were placed in a rank order of DNA binding affinity using Surface Plasmon Resonance (SPR). These experiments demonstrated that the residue involved in the DNA contact is predominantly K80, although K77 may also play a role (Michalopoulos and Hay, 1999). Partial proteolysis analysis of the wt p50 and the triple mutant showed that the defect of the binding is not due to major conformational changes induced by introduction of the K to A changes.

The first piece of evidence supporting the role of K80 in this interaction is its conservation among all of the NF- κ B/Rel/Dorsal molecules. The only change is in human c-Rel where there is an Arginine instead of a Lysine, suggesting that the interaction is due to the positive charge of the amino acid (the chicken c-Rel contains Lysine) (Michalopoulos and Hay, 1999) (Figure 52). The only other NF- κ B protein containing three positively charged amino acids in that loop is the p50-related protein p52.

The crystallographic data demonstrate how the lysine cluster on the C-terminus of L1 loop could interact with DNA, since there are another two lysine clusters in the tips of L2 and L4 loops. The DNA contacts mentioned in section 4.2.2, determine DNA sequence specificity of the different NF- κ B molecules, and play an important role in the stabilisation of the NF- κ B•DNA complexes. The other type of contacts which are significant for the affinity of Rel related proteins to DNA are the non sequence specific sugar-phosphate backbone contacts (2/3 of the phosphates of the κ B site are contacted by at least one hydrogen bond. Almost all these contacts are preserved among the Rel family members: p50 Y60, C62 in L1, K147, K148 in L2, Q309 in L5 and the homologous residues in p65 and p52 interact with the sugar/phosphate backbone through hydrogen bonds or salt bridges. Similarly, p50 R308 in L5 and its p65 homolog make contacts to the DNA backbone, while their homologs in p52 do not make any DNA interactions. p50 K275 and Q277 in L4 and their p52 homologs interact with DNA, while p65 L4 interacts with DNA backbone through K221 which

Figure 52: Conservation of K80 among NF- κ B/Rel/Dorsal proteins. Multiple sequence alignment of p50, p52, c-Rel (human and chicken), v-Rel, RelA, RelB, Dorsal and Dif. The arrow points to the lysine residue in NF- κ B/Rel/Dorsal proteins (K80 in p50).

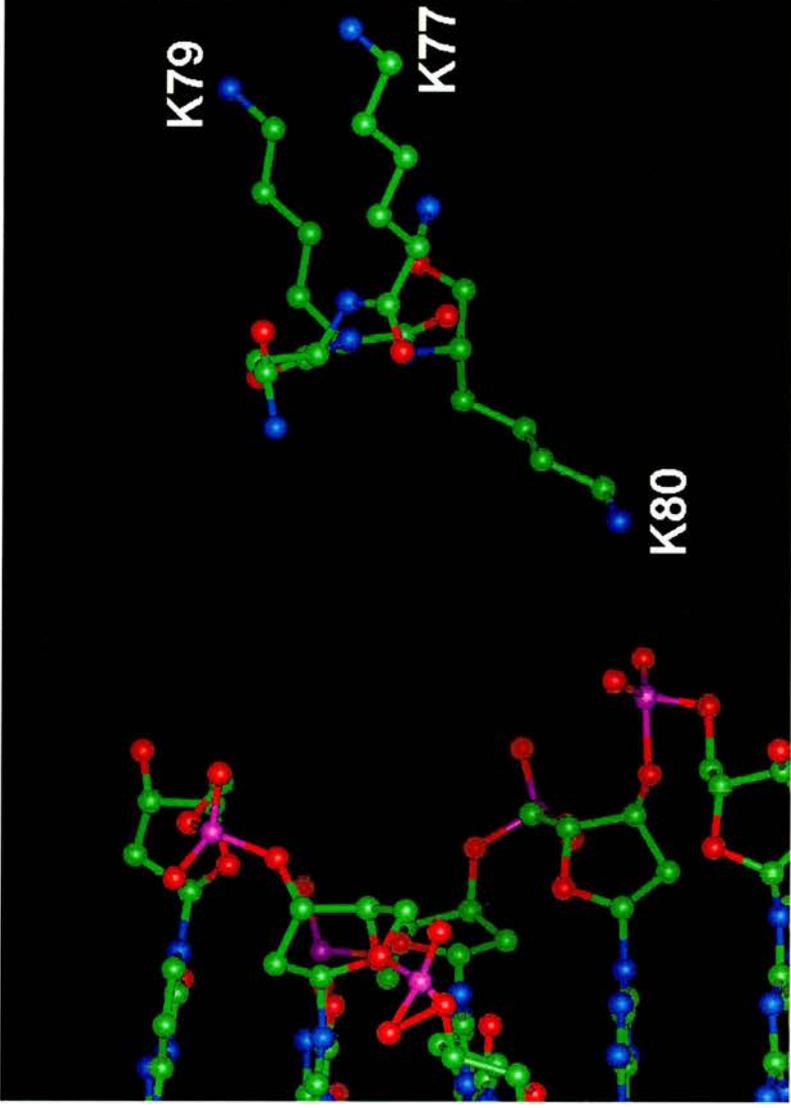
is homologous to p50 K278. p50 also contacts DNA through H144 in L2. This contact is not shown in the homologous His residue of p52, while the equivalent amino acid residue in p65 is conserved among Rel-related NF- κ B proteins C120 which does not make any interaction with DNA. All these data imply a role for the L1 loop lysine cluster in contacting the phosphate backbone of the target DNA. Furthermore, the observation that some NF- κ B molecules use the first and others the second of the two lysine residues of L4 to contact the DNA backbone, shows that neighbouring lysine residues can play similar roles one in the place of the other, thus demonstrating how K77 can partly substitute K80 in the K80A mutant, through adjustments.

The crystallographic data are also consistent with a DNA contact beyond the consensus sequence. However this interaction was not clearly resolved because the C-terminal part of the L1 loop is not highly ordered, and because of the small size of double stranded part of the oligonucleotides that were used to form the co-crystals (Chen et al., 1998a; Ghosh et al., 1995; Müller et al., 1995). More precisely, a double stranded 10-mer was used for the murine (p50)₂•DNA complex (Ghosh et al., 1995), a double stranded 11-mer for the p50•p65•DNA complex (Chen et al., 1998a) and a double stranded 11-mer for the human (p50)₂•DNA complex which was flanked by 4 mismatched base pair sequences (Table 5). Furthermore, it was shown that p50 affinity for a long oligonucleotide (16mer) was higher than of that for a shorter one (12mer), which corresponded to the oligonucleotide used in the crystallographic data (Bell et al., 1996). Analysis of the crystal structure of (p50)₂•DNA (Ghosh et al., 1995; Müller et al., 1995), indicated that although the two crystal structures were quite similar, they had a fundamentally different relationship to their DNA targets (Baltimore and Beg, 1995), as a consequence of the flexibility of the NRD contacts with DNA. This flexibility is due to the adjustability of the loops and the hinge (which links the two domains of each subunit) which allows recognition of different but related DNA sequences. One explanation for the apparent differences between the two structures is the slight differences of the DNA sequences of the double stranded parts of the two oligonucleotides: 5'-GGGAATTCCC-3' (Ghosh et al., 1995) and

5'-GGGGAATCCCC-3' (Müller and Harrison, 1995). Another reason is the slight difference of the length of the double stranded parts of the oligonucleotides: 10mer (Ghosh et al., 1995) and 11mer (Müller et al., 1995). This small difference of the size of the oligos played a dramatic role on the conformation of the AB (L1) loop in the two structures. While the 5' end of the double stranded 10mer is far from the C terminus of AB loop, the phosphate backbone beyond the duplex of the 19mer is in close proximity (2.87Å) with K80 of the loop (Figure 50). This interaction affects the conformation of the lysine cluster of the loop. In the complex of human p50 with the longer double stranded oligonucleotide, the lysines of the loop have different orientations than in that with the shorter one. In both structures the side chain of K79 points away from the target DNA molecule and K80 is ideally positioned to directly contact it. While the K77 side chain is positioned facing DNA in the complex with the 10mer, it has the opposite orientation in the complex with the 11 base pairs.

Even if the interaction of the L1 C-terminus was shown, in the case of the human (p50)₂•DNA complex, the significance of those data was not clear as the interaction occurred in a region of heavy DNA distortion, where DNA is not properly annealed (Figure 50). Using molecular modelling, the region of the oligo used in that crystallographic study (Müller et al., 1995) with these 4 distorted base pairs was replaced with a 4 base pair canonical B-form DNA. This clearly showed that the nitrogen atom of the positively charged K80 -NH₂ group is in close proximity (2.7Å) to the oxygen atom of the negatively charged phosphate backbone of DNA. While K80 was ideally positioned to contact the phosphate backbone, K77 and K79 were pointing away from the DNA (Michalopoulos and Hay, 1999) (Figure 53). In the other report (Ghosh et al., 1995), K77 is not ideally placed to make an interaction but, it could also make a phosphate back bone contact with minor adjustments. Therefore, molecular modelling suggested the interaction between K80 and DNA backbone, beyond the consensus sequence, since the positioning and the distance of K80 from the DNA backbone was ideal. The apparent differences in the positioning of K77 in

Figure 53: Structural representation of the interaction of the p50 sequence 77-80 (Lysine cluster of the C-terminal AB Loop) with the 4 base pair computer extended double stranded DNA. Orientation of the Lysine cluster: K80 is ideally positioned, because its ϵ -NH₂ group is 2.7Å from the DNA phosphate backbone. K77 and K79 point away from the DNA backbone.



the two studies also explained the important but not critical role of K77 in the interaction with DNA.

While the C-terminus of the p52 L1 loop has a very similar structure (Cramer et al., 1997) to its p50 homologue (the homologue to p50 K80 lysine residue of p52 points towards the 5' free phosphate, since the oligonucleotide used was short again), the same loop in p65 homodimers bound to a palindromic DNA 20-mer is at least 11Å from the DNA backbone (Chen et al., 1998c), although this may be a reflection of the complex and novel way that (p65)₂ binds to DNA. This loop could have other functions that are not related to DNA binding but may serve as a target for IκBα recognition, in the same way that the p50 homologous loop serves as a target for IκBγ recognition (Bell et al., 1996).

4.3 NF- κ B-I κ B interactions

After the discovery of the interaction of p50 K80 with the phosphate backbone of DNA, our objective was to clarify the role of the L1 C-terminus in the interaction between p50 and its inhibitor protein I κ B γ . Protein footprinting by chemical modification (Bell et al., 1996) suggested that the p50 L1 lysine cluster (K77, K79 and K80) could contact I κ B γ . Once again, although the C-terminus of L1 loop was implicated the techniques employed did not have sufficient resolution to identify the lysine residue(s) making the contact with I κ B γ (Bell et al., 1996). One of the objectives of this study was therefore to use site directed mutagenesis to investigate the role of each lysine in the sequence between 77 and 80. Our experiments demonstrated that K77, together with the conserved K80, interact with I κ B γ . We also determined the stoichiometry of the p50•I κ B γ complex and suggested how the complex might behave in solution. These findings can be interpreted in the light of the discovery of the structure of p50•p65•I κ B α complex (Huxford et al., 1998; Jacobs and Harrison, 1998) and by combining various biochemical data on different NF- κ B•I κ B complexes.

4.3.1 Ankyrin repeat domain structure

As mentioned elsewhere, members of the I κ B family of proteins contain a homologous region of 6 or 7 tandem ankyrin repeats (Ghosh et al., 1990; Kieran et al., 1990) (Figure 8). An ankyrin repeat is a 33 amino acids motif which is present in erythrocyte membrane-associated protein ankyrin, in Notch transmembrane protein of *Drosophila melanogaster* and in the proteins cdc10/SWI6 that control the yeast cell-cycle (Bennett, 1992). The periodicity of the structure of ankyrin was already known even before its sequence analysis, as electron microscopy of erythrocyte membranes uncovered that ankyrin was a long banded molecule (Margaritis et al., 1977). The size of the repetitive black and white bands corresponded to the size of an ankyrin repeat. Crystal structure analysis of the first ankyrin repeat containing proteins (p53-binding protein 2 (53BP2) (Gorina and Pavletich, 1996), the β subunit of GA-binding protein

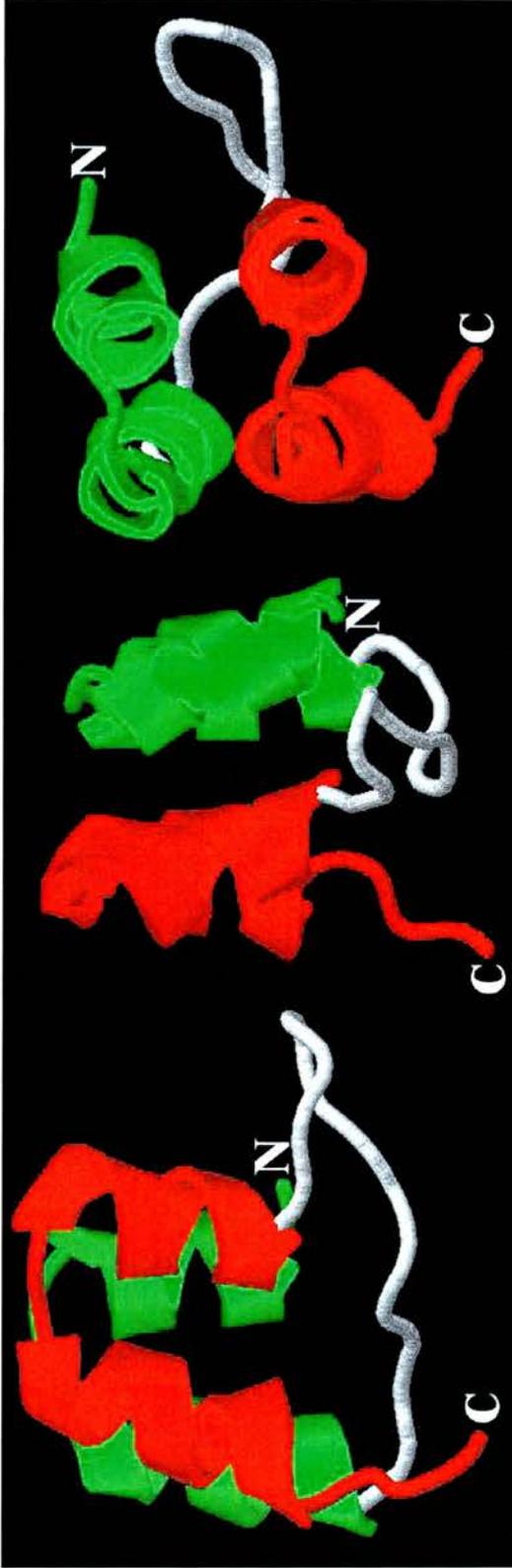
(GABP β) (Batchelor et al., 1998) and the CDK4/6 inhibitory protein p18^{INK4c} (Venkataramani et al., 1998)) revealed that its L-shaped structure consists of a β hairpin (an extended loop that contains a type I β turn in its tip) which is perpendicularly related to two α helices that form an antiparallel coiled-coil (Figure 54). The β hairpin is linked to the first helix with one amino acid. The packed repeats form a continuous antiparallel β sheet via their hairpins and helix bundles via their coiled-coils. The plane of the β sheet is perpendicular to the axes of the α helices. It is not likely that an ankyrin motif can fold stably in an isolated peptide sequence, as its folding also depends of the neighbouring repeats (the hydrophobic residues of the α helices form intra- and inter-repeat van der Waals interactions, while the conserved histidine residues contribute to the inter-repeat stabilisation through a network of hydrogen bonds). Other conserved amino acids are glycines (they facilitate the formation of sharp turns in the tips of β hairpins and the termination of α helices), threonines (they often initiate α helices), leucines and alanines (contribute to the stabilisation of the helix bundles). Although these amino acids are conserved among some repeat units of a variety of ankyrin repeat containing proteins, they are not uniformly conserved in each ankyrin repeat, which proves that the structures containing ankyrin repeat stacks tolerate small deviations from the consensus sequence.

The vertical spacing of the helices in close proximity to the β hairpins is smaller than that of the helices that are away from the β hairpins, causing a curvature of the ankyrin repeat stack. All the crystallographic data show that ankyrin repeats mediate protein interactions through the β hairpin protruding tips, although the helix bundles may also offer possibilities for various macromolecular interactions.

4.3.2 *NF- κ B/I κ B α complex structure*

The revelation of the novel L-shaped structure of the individual ankyrin repeat, together with sequence alignment analysis, prompted the prediction of the structure of I κ B molecules, so that even before the crystallographic data of I κ B α , experiments of swapping ankyrin repeats between the I κ Bs were performed and demonstrated that the

Figure 54: An ankyrin repeat is an L-shaped structure which consists of a β hairpin loop which is perpendicularly related to two α helices that form an antiparallel coiled-coil.



first ankyrin repeat of I κ B α confers strong inhibitory properties on I κ B β and I κ B ϵ (Simeonidis et al., 1999). Data like that should be treated though with extreme caution, as swapping of these highly packed modules might abolish highly important inter-repeat interactions (the recombination technique is far more reliable when distinct domains are swapped). The regions of NF- κ B molecules that were required for interaction with I κ Bs were already known before crystal structure analysis of I κ B α /NF- κ B: The NLS peptide of various NF- κ B molecules (Beg et al., 1992; Inoue et al., 1993; Malek et al., 1998; Matthews et al., 1993b), the same surface of NF- κ B p50 that is occupied, according to crystal structure data, by specific DNA (Bell et al., 1996) and the C-Terminus of AB loop (Bell et al., 1996; Malek et al., 1998) whose interaction with the DNA backbone our results demonstrated (Michalopoulos and Hay, 1999) (see section 4.2.7).

Two independent crystallographic studies of the complex between "classical" NF- κ B heterodimer and I κ B α (p50•p65•I κ B α) (Huxford et al., 1998; Jacobs and Harrison, 1998) (Figure 55) revealed mechanisms of the interaction between I κ B α •NF- κ B. Since obtaining crystals of the complexes was a complicated aim, the two laboratories selected the participating protein constructs, based on results of various proteolysis (Jaffray et al., 1995) and protein binding studies (Malek et al., 1998). The lengths of I κ B α , p50, and p65 constructs the two groups selected were similar but not identical. More precisely, using the numbering of the human homologue (in order to have direct comparisons):

Protein	(Jacobs and Harrison, 1998)	(Huxford et al., 1998)
p65	20-320 (human)	19-304 (murine)
p50	248-354 (human)	247-365 (murine)
I κ B α	70-282 (human)	67-302 (human)

The NF- κ B constructs had almost identical N-termini, in both studies. The C-termini of the murine constructs were exactly the NF- κ B NLS sequences. On the contrary, human p65 construct extended another 16 amino acids in the C-terminus of NLS, while in human p50 construct NLS was omitted. In the study of the murine

Figure 55: Different views of the crystal structure of p65•p50•IκBα. p65 RHR (in blue), p50 C-terminal (dimerisation) domain (in light green) and IκBα ARD (in green) were co-crystallised. The first two ankyrin repeats of IκBα interact with p65 NLS.



NF- κ B•human I κ B α , the I κ B α construct was 4 amino acid N-terminally, and 20 amino acid C-terminally longer, than the I κ B α molecule used in the study of the entirely human complex. So, it was rather the difference in size than the difference of the origin of the molecules, that explained the apparent major differences on the intermolecular interactions reported in the two studies: While totally 87 residues were reported to participate in protein-protein interactions, only 32 of them were common in both studies.

The combination of the structural data of the two studies revealed the nature of NF- κ B-I κ B interactions: The stoichiometry of the complex is 1 I κ B α :1 NF- κ B dimer and their chains run in an antiparallel fashion, so that the N-terminus of I κ B α is in the side of the C-terminal transactivation domain of p65 and the C-terminal PEST sequence of I κ B α interacts with the N-terminal p65 domain. This topology is consistent with the hypothesis of the autoinhibition of p105 and p100, where the part of p105 or p100 which comprises a p50 and p52 respectively, is bound to an NF- κ B subunit and the part of p105 or p100 which comprises I κ B γ or I κ B δ respectively, is folded backwards (probably in the glycine rich region) and masks the NLSs sequestering the complex in the cytoplasm. The binding interface of NF- κ B-I κ B α is extensive, but not continuous. It consists of three predominately polar patches: Ankyrin repeats 1 and 2 interact with p65 NLS and the sequence which is C-terminal to it. The β -loops of the ankyrin repeats 4-6 interact with the dimerisation domain of p50 and the C-termini of the inner helices of the ankyrin repeats 5 and 6 interact predominantly with p65, giving the complex its asymmetry. Ankyrin repeat six and the serpentine-structured C-terminal PEST-like domain of I κ B α make contacts with the N-terminus of p65. Since most of the NF- κ B residues which contact I κ B α are conserved, it was assumed that subtle differences in molecular contacts are responsible for the I κ B/NF- κ B specificity.

4.3.3 Interaction with NLS

Biochemical data had already uncovered the role of NLS in the NF- κ B-I κ B interactions, long before the discovery of the crystal structure of an NF- κ B•I κ B complex. NF- κ B NLS is required for interactions with I κ B molecules. Both I κ B α and

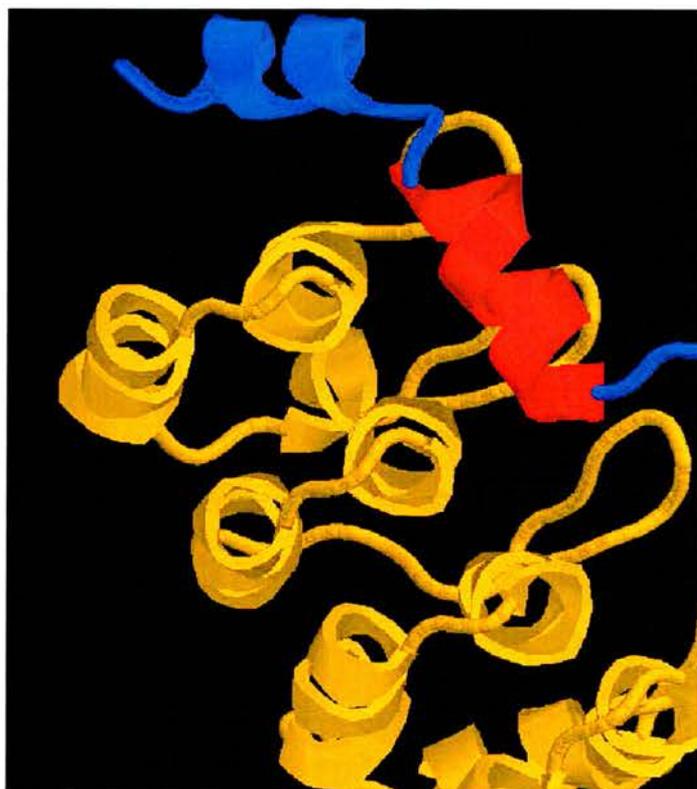
I κ B γ interact with p50 NLS (Matthews et al., 1993a). Although p50 and p65 NLS are both involved in direct interactions with I κ B α , p65 NLS•I κ B α interaction is more important (Malek et al., 1998). What was unclear before the solution of the crystal structure of the NF- κ B•I κ B complexes was the mechanism that was employed for the masking. It was eventually discovered that p65 NLS forms an α -helix when it interacts with and is masked by, the ankyrin repeats 1 and 2 (Jacobs and Harrison, 1998). Another sequence located C-terminally to the NLS which also forms an α -helix, capping ankyrin repeat 1, is necessary for the stabilisation of the helical form of NLS (Figure 56A). Interestingly, a sequence very similar to the NF- κ B NLS (from SV40) appears as an extended chain when bound to karyopherin- α (Figure 56B) and NF- κ B NLS which does not interact with DNA, is not structured in NF- κ B•DNA complexes. Therefore, it is accepted that NLS formation depends on the molecular context. On the other hand, the NLS of p50 appeared unstructured in both structural studies. Since a dimer needs at least one NLS for nuclear entry, and therefore I κ B α must mask both NLSs (Latimer et al., 1998), the crystallographic data failed to explain how p50 NLS is masked and therefore NF- κ B remains sequestered in the cytoplasm. What is proposed is that either steric hindrance by ankyrin repeat 1 and 2 and p65 NLS is sufficient for blocking p50 NLS to interact with import factors, or the N-terminal sequence of I κ B α folds back and masks p50 NLS (Latimer et al., 1998). Interestingly, our data on p50-Bcl-3 interactions and previous data on p50-I κ B γ interactions (Matthews et al., 1993a) showed that p50 NLS is necessary for DNA binding inhibition. This interaction occurs far from the site of DNA binding, therefore, it is necessary for stable anchoring of the I κ B molecule to NF- κ B, a step which is apparently a precondition for effective DNA inhibition.

4.3.4 Interaction with the NF- κ B dimerisation domain

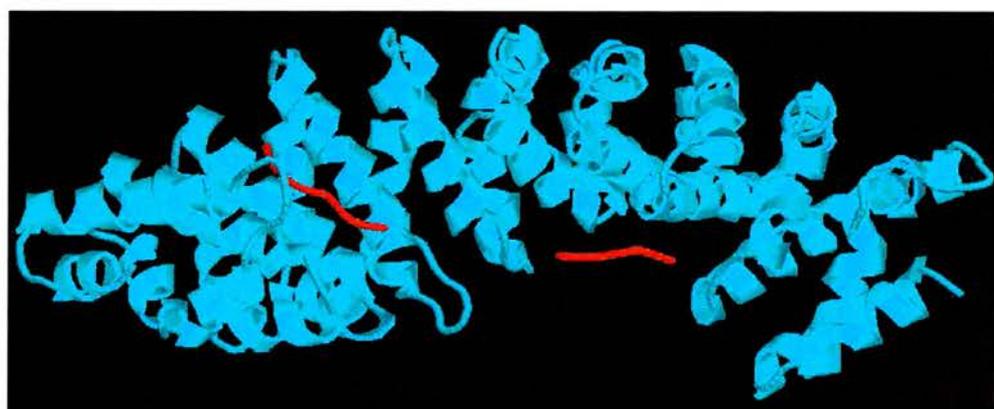
Although the overall architecture of the dimerisation domains of the p50•p65 complex is the same when NF- κ B interacts with DNA or I κ B α , there are some interesting alterations. E.g. p50 R305 and its homologous p65 R246 make non-specific DNA backbone contacts in the presence of DNA. When I κ B α is bound to p50•p65, p50

Figure 56: NLS conformation depends on its protein context. **(A)** p65 NLS (in red) forms an α -helix when it interacts with the I κ B α (in orange). **(B)** SV40 NLS appears as a linear fragment when it interacts with karyopherin- α . NF- κ B NLS is not structured when it does not interact with any other protein (e.g. when NF- κ B is bound to DNA).

A



B

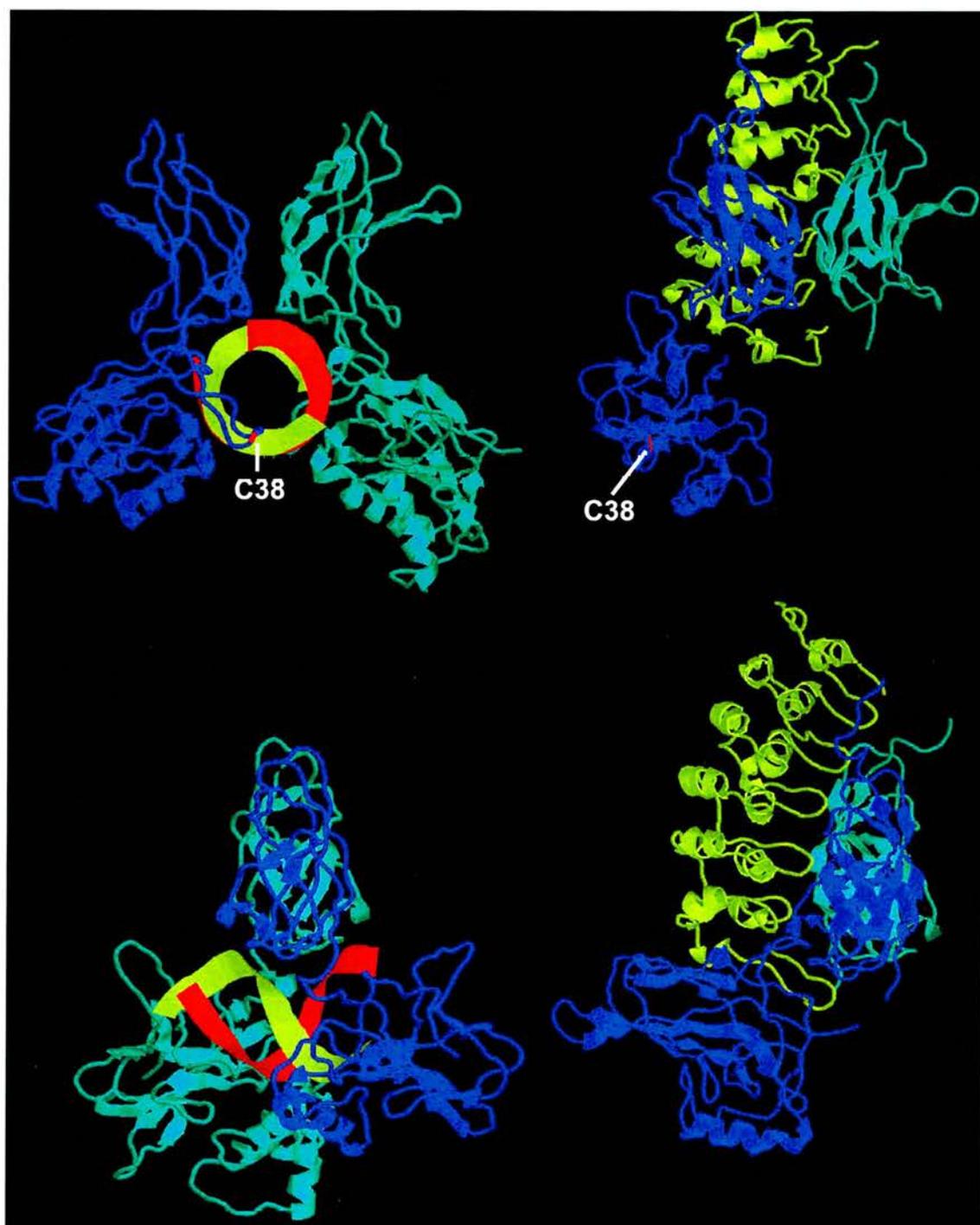


R305 and p65 R246 make salt bridges with p65 D217 and its homologous p50 D271 respectively. An opening of the dimer interface is also apparent. Finally, the interaction between p50 D254 and p65 N200 which only occurs in heterodimers (see section 4.2.3), is abolished upon I κ B α binding. I κ B α is aligned closer to p50, thus allowing more interactions between the ARD and the p50 subunit, although interactions like the salt bridges p65 R246-I κ B α E282 and p65 D243-I κ B α R246 contribute significantly to the I κ B α -p65 dimerisation domain interactions.

4.3.5 Domain movement and DNA binding inhibition

Comparison the structure of p50•p65•DNA and p50•p65•I κ B α complexes shows that p65 N-terminal domain orientation in relation to the homodimerisation p50 and p65 domains is almost opposite: In the NF- κ B•DNA complex conserved p65 C38 (homologous to p50 C62) which locates on the tip of the "recognition loop" points inside, towards the cleft of DNA. In the NF- κ B•I κ B α complex C38 points in the opposite direction (Figure 57). Apart from the almost 180° rotation, there is a translation of 40Å. This movement allows this domain to loosely associate with the ankyrin repeat 6. As it was previously mentioned, the NF- κ B•I κ B α complexes of both studies did not include the p50 N-terminal domain. Whether this apparent rotation/translation is due to the specific interactions between the serpentine PEST-like I κ B α C-terminus and the ankyrin repeat 6 with the p65 N-terminus or due to simply favourable crystal packing, has yet to be clarified. Nevertheless, such allosteric mechanism could disrupt NF- κ B DNA binding. Our data are consistent with this hypothesis. Our sedimentation velocity findings showed that (p50)₂, I κ B γ and (p50)₂•I κ B γ in solution are more extended than in the crystal structure. As far as I κ B γ alone is concerned, we can assume that a proportion of it is elongated/misfolded because free I κ B is unstable and is stabilised by forming complex with NF- κ B (Li et al., 1998). The "extended" form of (p50)₂ in solution explains the "paradox" that while full length p50 RHR NF- κ B alone failed to form crystals, a heterodimer of p50 and p65 dimerisation domains alone (Huang et al., 1997) and various NF- κ B•DNA complexes (Chen et al., 1998; Chen et al., 1998; Cramer et al., 1997; Ghosh et al.,

Figure 57: Comparison of the structure of the NF- κ B•DNA (left) and NF- κ B•I κ B α (right). p65 is shown in blue, p50 in light green, the two DNA strands are shown in yellow and orange, and I κ B α conserved C38 (homologous to C62 in p50) is showed in red, as a “marker”. In both pairs of pictures the dimerisation domains of NF- κ B have the same direction. It is apparent that p65 N-terminal domain points almost to opposite directions in the two conformations.



1995; Müller et al., 1995) could produce crystallographic data. That happened because the N-terminal domains can freely rotate and translate, due to the flexible hinge (L3 loop) that links them to their C-terminal dimerisation domains, unless they interact with other molecules which stabilise them (e.g. DNA). The 20 Å translation of p50 C62 during oxidative conditions also shows the flexibility given by L3 hinge (see section 4.2.6). In the same way, the (p50)₂•IκBγ appeared more extended because the 2 p50:1 IκBγ stoichiometry in which our data suggested that the molecules participate in the complex (in accordance with the crystallographic data for the NF-κB•IκBα complex) makes the complex NF-κB•IκBγ asymmetrical and IκBγ which is one ankyrin repeat longer and therefore can play roles similar to those of C-terminus, can only stabilise one of the two p50 N-termini, leaving the other N-terminus free to rotate. That explains why both the studies of the NF-κB•IκBα used p50 constructs that were lacking the N-terminus whose rotation was not stabilised by any interaction. If we accept that p50 N-terminus could not be co-crystallised due to lack of any interaction that would stabilise it, then we can assume that it would not make any contacts with the p65 N-terminus, and therefore the 180° rotated domain is not an artefact. Another suggested way of DNA binding inhibition, which is not necessarily mutually exclusive with the one previously proposed, is the competition between the C-terminal ankyrin repeats and/or the C-terminal domain of IκBs for the same binding sites on NF-κB with DNA. Indeed, protein footprinting using chemical modification and partial proteolysis showed that IκBγ and DNA interact with the K77-K80 of the C-terminus of p50 L1, K147-K149 of L2, K244 and K252 of L3, and K275 and K279 of L4, while IκBα does not. Many of the lysine residues that were contacted by DNA were also contacted by IκBγ, but not IκBα (Bell et al., 1996). Furthermore, this hypothesis is also based on the negative charge of IκB PEST-like C-termini, which form electrostatic bonds with the positively charged NF-κBs and on the finding that IκBβ and Bcl-3 C-termini are able to effectively inhibit DNA binding after phosphorylation (Bundy and McKeithan, 1997; Tran et al., 1997), which increases the negative charge of that region. Our finding of the “non classical” IκB property of Bcl-

3 ARD to form stable complexes with p50 and p52 bound to DNA, together with a similar finding on the formation of ternary complexes between I κ B β and NF- κ B bound to DNA (Tran et al., 1997), suggests that during I κ B-driven NF- κ B DNA binding inhibition, a ternary I κ B•NF- κ B•DNA complex is formed. Our data showed that the stability of such a ternary complex containing Bcl-3 ARD was DNA sequence dependent (the more symmetrical the DNA site, the more stable the ternary complex). Since it is known that p50 and p52 have stronger affinities for symmetrical κ B motifs, it seems that Bcl-3 ANK (or maybe full length Bcl-3 with unphosphorylated PEST-like sequence) is able to bind to the NLS and the dimerisation region but unable to compete with DNA for binding to specific NF- κ B residues, especially in the N-terminus, thus forming a ternary complex. The ability of Bcl-3 to compete with DNA and the affinity of an NF- κ B subunit for its half site depends on how its N-terminus and its flexible loops are related to DNA.

On the other hand, I κ B γ ARD is able to effectively inhibit p50 DNA binding activity. The ability of the C-terminus of I κ B γ ARD to compete with DNA for the same binding areas of p50 (Bell et al., 1996) and/or to consequently rotate p50 N-terminus to an unfavourable angle for DNA binding, destabilises any ternary complex. We demonstrated that K77 and K80 of the C-terminus of p50 L1 loop interact with I κ B γ , confirming previous biochemical findings (Bell et al., 1996). In the case of the conserved K80, we have already shown that it forms a very important bond with the DNA phosphate backbone, therefore competition between I κ B γ and DNA for that residue, would result in loosening of this bond. Furthermore, our data show that I κ B γ makes another interaction of equal importance with K77. K77 can also, under certain circumstances, recognise the DNA backbone. Since K80 is conserved in all NF- κ B/Rel/Dorsal, but its p65 homologue K46 was 11Å from the phosphate backbone it was hypothesised that this lysine residue could be a specific target for I κ B α . The crystallographic data of NF- κ B•I κ B α did not confirm the interaction between p65 K46 and the C-terminus of I κ B α , either because there is no such specific interaction, or because the C-terminus of the study was very short.

Our data demonstrated that the deletion of the amino acids 257-264 could efficiently block NF- κ B DNA binding inhibition. At the time when this experiment was conducted, we considered this area of significant importance, as it was supposed that it belonged to the C-terminal serpentine domain and could be a recognition signal for phosphorylation by PKA. We, now, know that phosphorylation is due to constitutive casein kinase II (CKII) (MacKichan et al., 1996) and that this sequence is N-terminally adjacent to the I κ B α nuclear export signal (NES) which extends in the region 265-277 of I κ B α and is responsible for the fast export of I κ B α out of the nucleus (Arenzana-Seisdedos et al., 1997). In the light of the crystal structure, it was apparent that the deletion was not in the C-terminal domain but in the linker sequence of the two antiparallel α -helices of the imperfect ankyrin repeat 6 (Figure 58) and that this region was necessary for the interaction with the dimerisation domain of p50, p65 and probably the p65 N-terminus. Therefore, removal of such sequence would result in the abolition of this interaction and/or the destruction of the ankyrin repeat 6. Combining our data with those of the crystal structures, it can be suggested that the interaction between the area 257-264 of the I κ B α with NF- κ B is critical for effective DNA binding of p65 containing NF- κ B molecules because it stabilises the NF- κ B•I κ B α and/or stabilises the 180° rotation of the p65 N-terminus which is unfavourable for DNA recognition. If the latter is correct (and not a crystal packing artefact), then we can assume that our data suggest that the I κ B α region 257-264 contributes to the specificity of I κ B α for p65.

Figure 58: Location in I κ B α structure and sequence of the deletions in the mutants used in the experiments: The part that is common in all constructs (wt and mutants) is shown in green. The peptide which is removed in I κ B α Δ 257-264 is shown in red and the C-terminus of I κ B α which is clipped in I κ B α 1-256 is shown in red and purple. The C-terminal domain of p65 is shown in blue. The peptide missing in I κ B α Δ 257-264 mutant is the linking sequence between the inner and outer anti-parallel α -helix of the sixth ankyrin repeat which interacts with p65 c'd loop. I κ B α 1-256 even lacks the entire outer α -helix together with the PEST sequence.



Ank VI: 243 **VNRRVTYQGYSFYQLTWGRPSTR** **IQQLGQLTLE** 275

5 FUTURE DEVELOPMENTS

5.1 Bcl-3 as a transactivator

The model of the direct and indirect transactivation through the interaction of Bcl-3 with p50 bound to symmetrical and asymmetrical κ B sites has to be tested by a series of transfection experiments. More precisely, cotransfection in cell lines with genotype RelA+/+ and RelA-/- of a plasmid with a reporter gene under the transcriptional control of various symmetrical and asymmetrical sites, a plasmid that constitutively expresses p50 (or this plasmid without p50 insert) and a plasmid which that constitutively expresses Bcl-3 (or this plasmid without Bcl-3 insert), would prove if the *in vitro* findings are correct. This experiment was tried under many different conditions but it failed, mainly because the commercially available reporter plasmid had distant sites that could be recognised by NF- κ B, as it was shown, once the precise mechanisms of NF- κ B DNA half-site recognition were uncovered by crystal structure analysis and the idea of the “NF- κ B consensus sequence” was abolished.

A more ambitious project would be the co-crystallisation of a p50•Bcl-3 ARD•DNA complex, provided that an efficient method for the production of notoriously insoluble Bcl-3 is found. Moreover, even if complexes between p50, Bcl-3 and asymmetrical κ B motifs are more unstable than those containing symmetrical κ B motifs, crystallisation of both ternary complexes would reveal the exact mechanisms that allow Bcl-3 to form ternary complexes with p50 bound to symmetrical sites and dissociate p50 bound to asymmetrical sites.

5.2 (p50)₂•I κ B γ in solution

Since we demonstrated that replacement of hydrogen with deuterium in p50 and I κ B γ does not influence the properties of these molecules and their complex, intend to find more on the structure on (p50)₂•I κ B γ in solution, using Neutron Scattering. This method will reveal more information on the native state of the entire complex.

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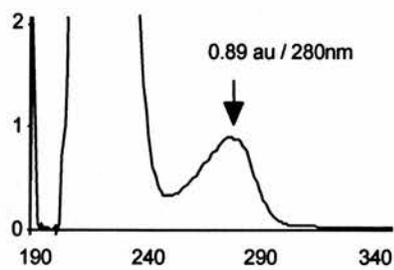
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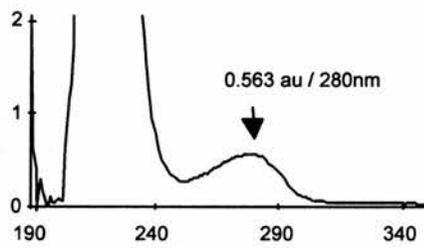
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Figure 29: Absorbance vs. Wavelength scans of freshly dialysed samples of p50 and IκBγ.



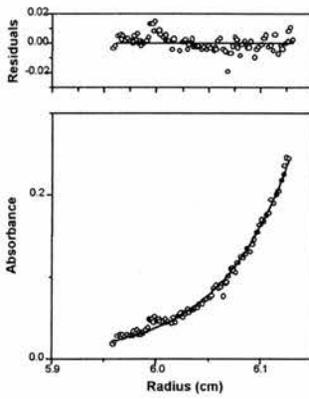
p50



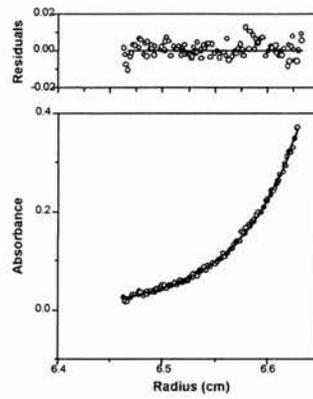
IκBγ

Figure 30: Distribution data from p50 at 16 krpm and the curve fits and the residual plots, using the single ideal species model.

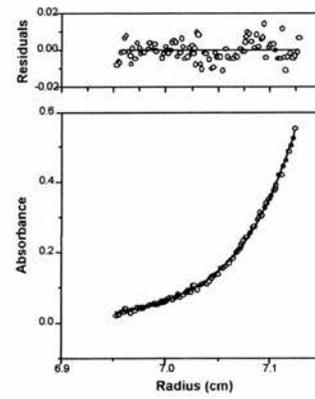
p50 at 16krmp (single ideal species model)



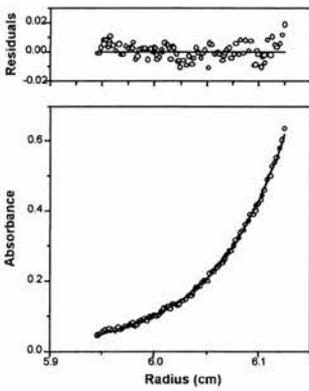
$M_{w,app}$ 80.1 (± 1.6) 4.8 μM



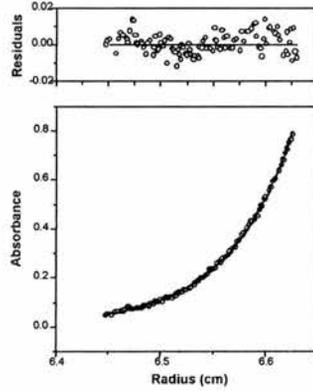
$M_{w,app}$ 84.9 (± 0.8) 7.1 μM



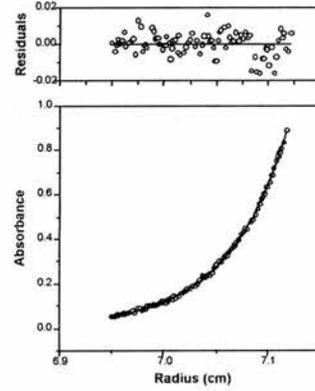
$M_{w,app}$ 82.8 (± 0.8) 9.5 μM



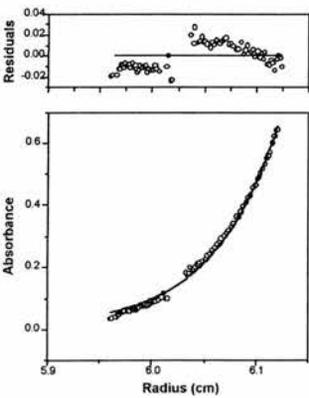
$M_{w,app}$ 78.1 (± 0.6) 11.9 μM



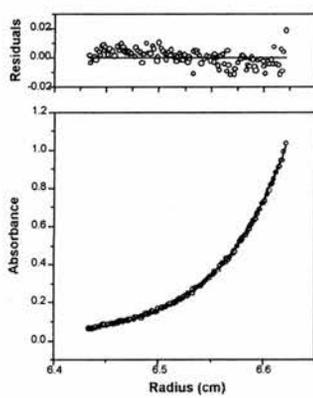
$M_{w,app}$ 79.3 (± 0.5) 14.3 μM



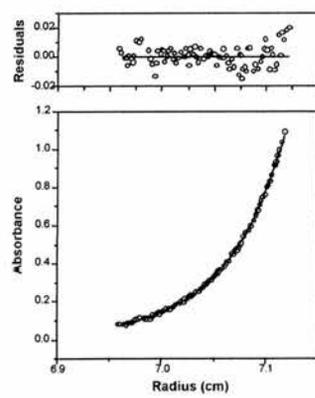
$M_{w,app}$ 80.0 (± 0.5) 16.7 μM



$M_{w,app}$ 86.1 (± 1.6) 19.0 μM



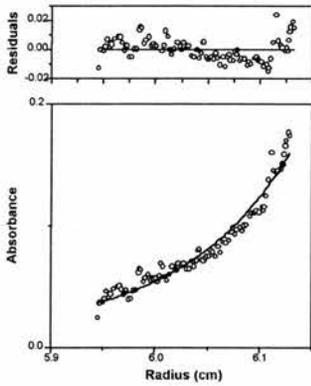
$M_{w,app}$ 77.3 (± 0.4) 21.4 μM



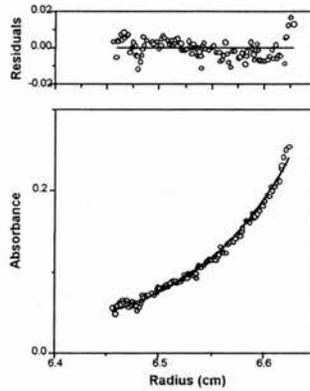
$M_{w,app}$ 81.0 (± 0.5) 23.8 μM

Figure 31: Distribution data from IκBγ at 18 krpm and the curve fits and the residual plots, using the single ideal species model.

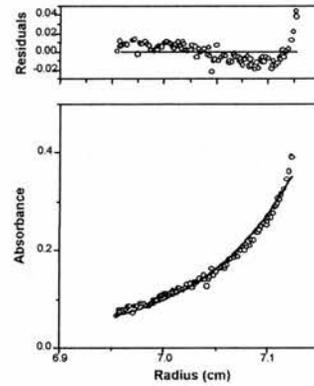
IκBy at 18krpm (single ideal species model)



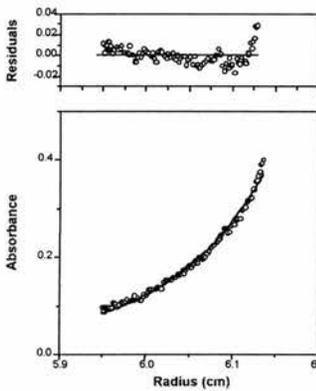
$M_{w,app}=41.8 (\pm 2.1) 4.6 \mu\text{M}$



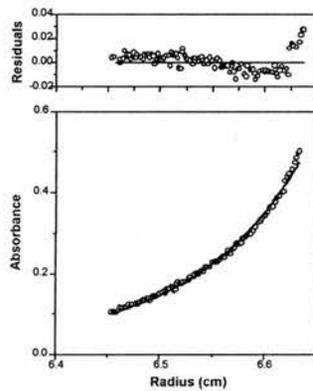
$M_{w,app}=42.5 (\pm 0.7) 6.8 \mu\text{M}$



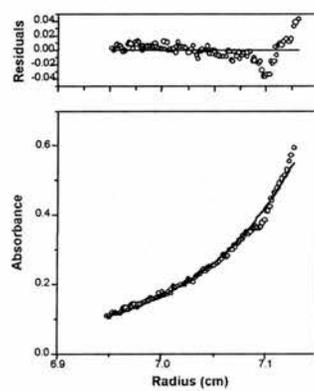
$M_{w,app}=41.5 (\pm 1.3) 9.3 \mu\text{M}$



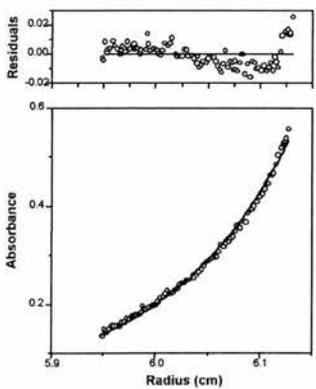
$M_{w,app}=37.9 (\pm 0.8) 11.4 \mu\text{M}$



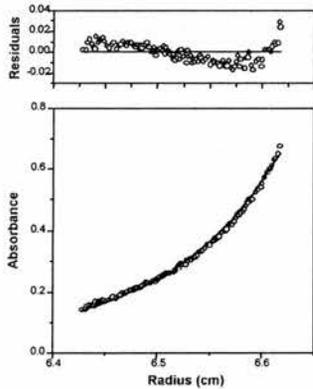
$M_{w,app}=38.3 (\pm 0.7) 13.6 \mu\text{M}$



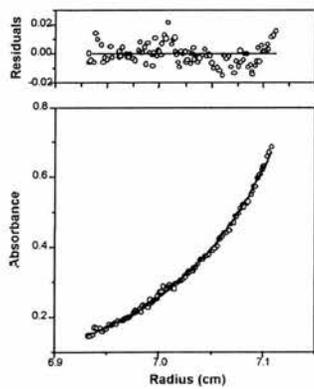
$M_{w,app}=37.3 (\pm 0.7) 16.1 \mu\text{M}$



$M_{w,app}=35.5 (\pm 0.4) 18.2 \mu\text{M}$



$M_{w,app}=36.7 (\pm 0.5) 20.7 \mu\text{M}$

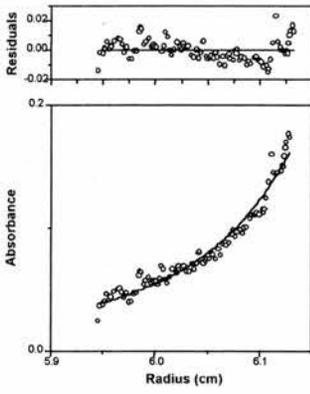


$M_{w,app}=35.5 (\pm 0.3) 22.9 \mu\text{M}$

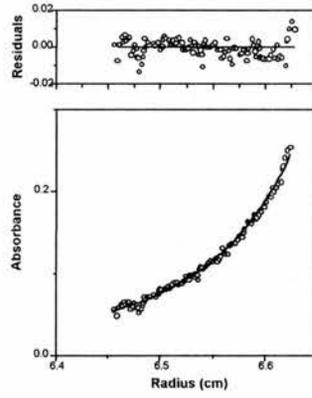
Figure 32: Estimation of the $M_{w,app}$ of p50 span at 16 krpm and $I_{kB\gamma}$ span at 18 krpm, using the single ideal species fit model.

Figure 33: Distribution data from IκBγ at 18 krpm and the curve fits and the residual plots, using the monomer/dimer model.

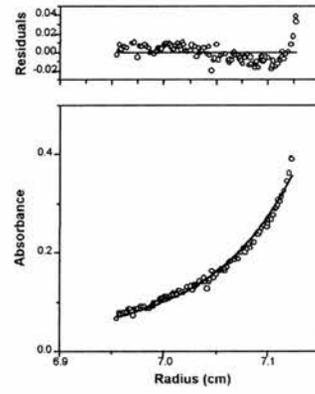
IκBγ at 18krpm (monomer/dimer model)



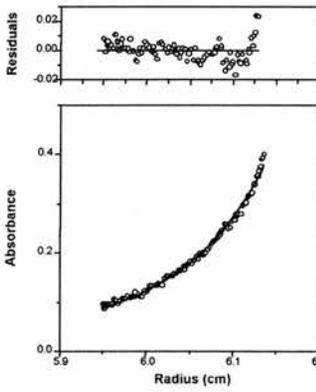
$Ka_2=17.4 (\pm 7.0) 4.6 \mu\text{M}$



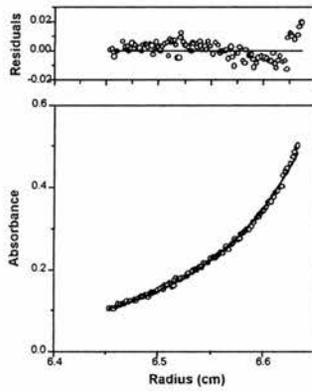
$Ka_2=12.9 (\pm 1.5) 6.8 \mu\text{M}$



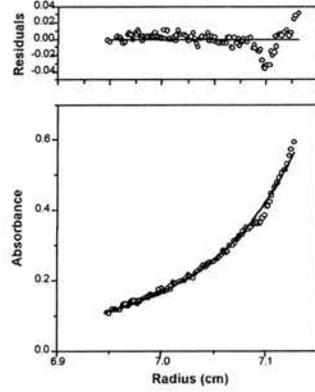
$Ka_2=7.3 (\pm 1.7) 9.3 \mu\text{M}$



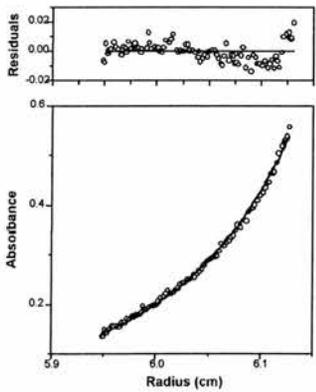
$Ka_2=3.1 (\pm 0.4) 11.4 \mu\text{M}$



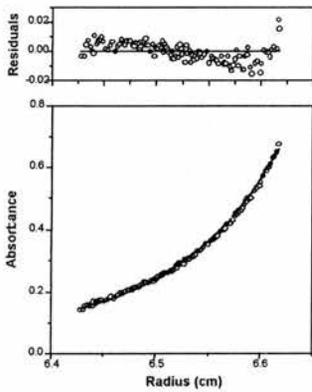
$Ka_2=2.8 (\pm 0.3) 13.6 \mu\text{M}$



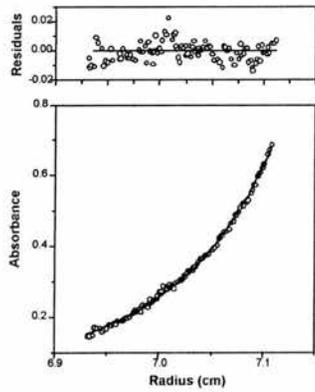
$Ka_2=2.0 (\pm 0.3) 16.1 \mu\text{M}$



$Ka_2=1.2 (\pm 0.1) 18.2 \mu\text{M}$



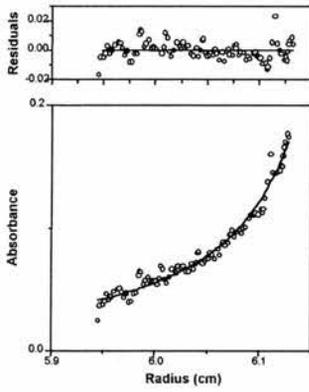
$Ka_2=1.3 (\pm 0.1) 20.7 \mu\text{M}$



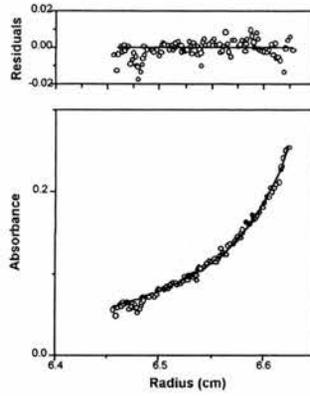
$Ka_2=1.0 (\pm 0.1) 22.9 \mu\text{M}$

Figure 34: Distribution data from IκBγ at 18 krpm and the curve fits and the residual plots, using the monomer/tetramer model.

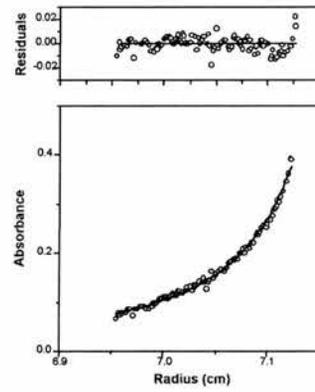
IκBγ at 18krpm (monomer/tetramer model)



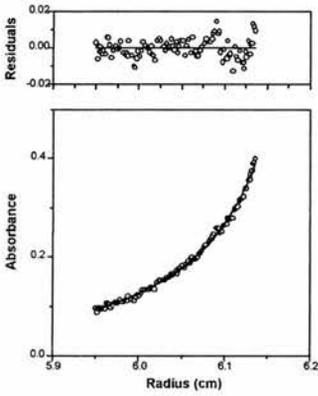
$Ka_4=569 (\pm 153) 4.6 \mu\text{M}$



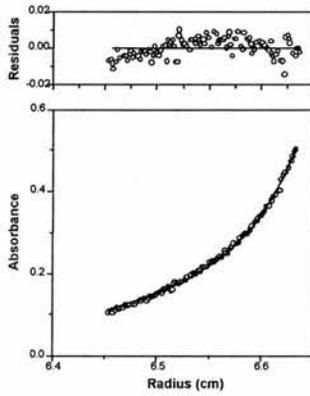
$Ka_4=157 (\pm 13) 6.8 \mu\text{M}$



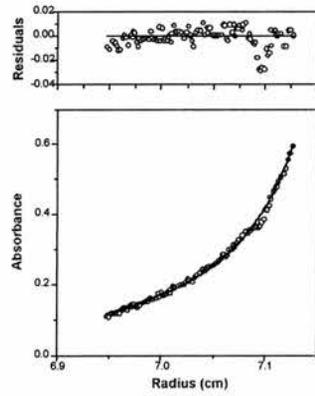
$Ka_4=45 (\pm 5) 9.3 \mu\text{M}$



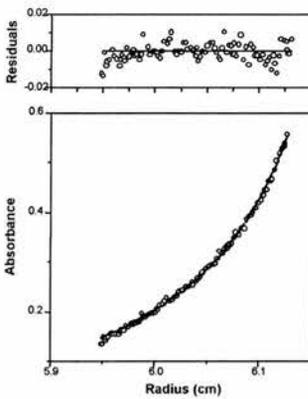
$Ka_4=18 (\pm 2) 11.4 \mu\text{M}$



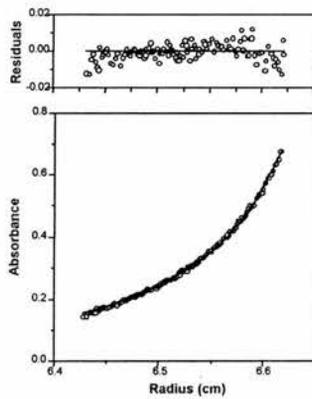
$Ka_4=10 (\pm 1) 13.6 \mu\text{M}$



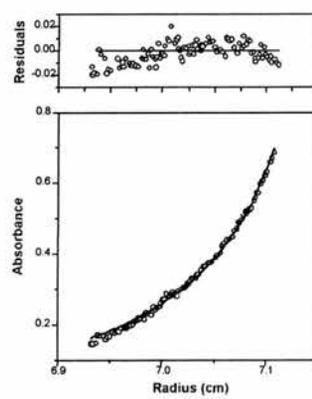
$Ka_4=6 (\pm 1) 16.1 \mu\text{M}$



$Ka_4=3 (\pm 0.2) 18.2 \mu\text{M}$



$Ka_4=2.5 (\pm 0.1) 20.7 \mu\text{M}$



$Ka_4=1.7 (\pm 0.2) 22.9 \mu\text{M}$

single ideal species model, the estimated $M_{w,appI\kappa B\gamma}$ values at different concentrations, using the monomer/dimer and monomer/tetramer fit models were not consistent (Figure 33 and Figure 34) while the estimated $M_{w,appI\kappa B\gamma}$ values at different concentrations, using the single ideal species model were between 35.5-42.5 kDa (Figure 32). The value is slightly higher than that of the monomer. The data suggest that $I\kappa B\gamma$ in solution, is monomeric.

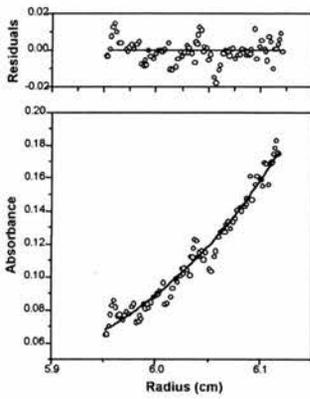
Nine samples with the p50 monomer concentration fixed at 5 μM , and the $I\kappa B\gamma$ monomer concentration varying from 1 μM to 25 μM were prepared by dilution with their dialysate, and equilibrium was obtained at 10 and 16 krpm. The distribution data obtained from the runs at 10 krpm and 16 krpm were fitted using the single ideal species fit model (Figure 35 and Figure 36, respectively). From the curve fit plots obtained from the two runs, apparent weight average molecular masses $M_{w,appp50\cdot I\kappa B\gamma}$ for each p50: $I\kappa B\gamma$ ratio were estimated and the quality of the curve fits was estimated by the residual plots. Using the single ideal species analysis, the highest estimated $M_{w,appp50\cdot I\kappa B\gamma}$ value at 10 krpm was 114.4 kDa at p50: $I\kappa B\gamma$ ratio=1.3 and at 16 krpm was 111.2 kDa at p50: $I\kappa B\gamma$ ratio=1.7. Using the m^* analysis, the highest estimated $M_{w,appp50\cdot I\kappa B\gamma}$ value at 10 krpm was 113.3 kDa at p50: $I\kappa B\gamma$ ratio=1.3 and at 16 krpm was 111.9 kDa at p50: $I\kappa B\gamma$ ratio=1.7 (Figure 37). Judging from the highest value of the $p50\cdot I\kappa B\gamma$, the p50: $I\kappa B\gamma$ ratio is 2:1.

Analytical ultracentrifugation enables the characterisation of proteins in solution. It can operate in two modes to reveal data on solute molecular mass (sedimentation equilibrium experiments) and shape (sedimentation velocity experiments) in its near-native state in solution (Svedberg and Pederson, 1940). Thus, sedimentation equilibrium analysis was employed to directly measure the molecular weight M of the complex of unmodified p50 and $I\kappa B\gamma$, as it exists in solution, and thus reveal the stoichiometry of the participating molecules in the complex.

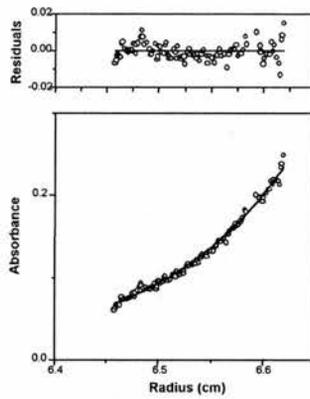
Analysis of the p50 data as a single, thermodynamically ideal species gave reasonable fits and values for $M_{w,app}$ corresponding to dimer. These data were in accordance with the crystallographic and NMR data.

Figure 35: Distribution data from p50/κBγ at 10 krpm and the curve fits and the residual plots, using the single model.

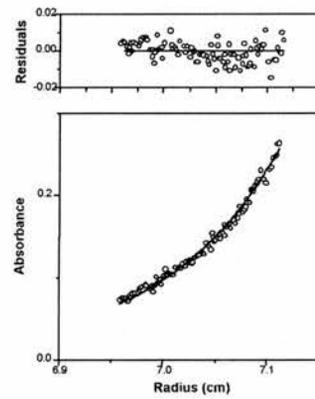
p50/IκBγ at 10krpm (single ideal species model)



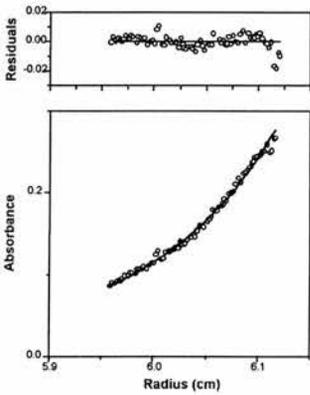
$M_{w,app} = 82.3 (2.9)$



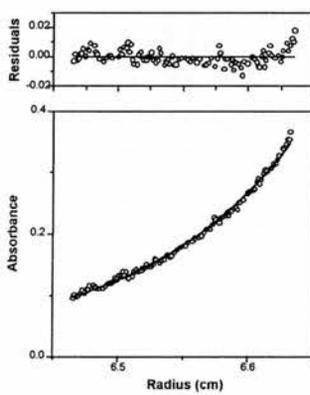
$M_{w,app} = 103.8 (1.6)$



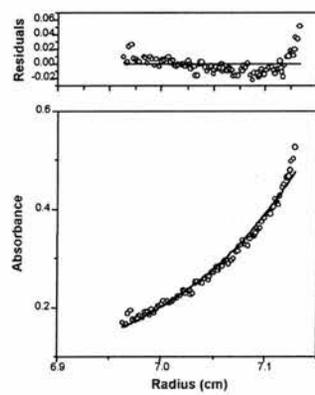
$M_{w,app} = 105.4 (1.7)$



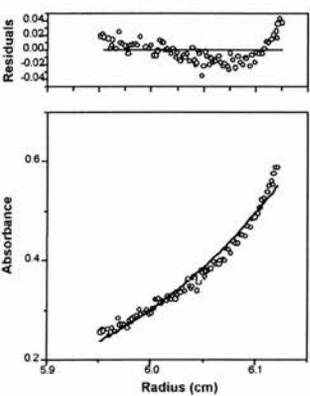
$M_{w,app} = 114.7 (1.7)$



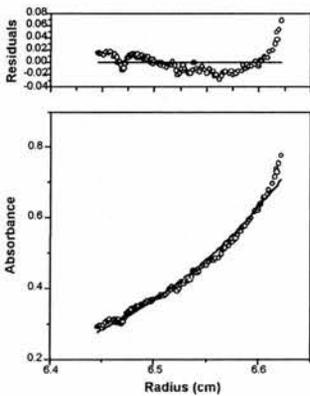
$M_{w,app} = 108.0 (1.7)$



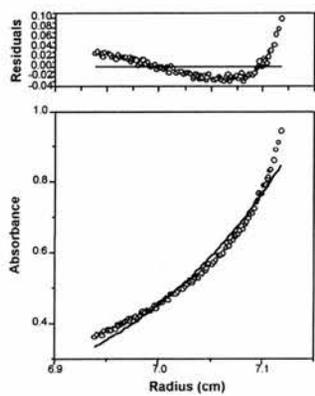
$M_{w,app} = 84.6 (2.0)$



$M_{w,app} = 71.8 (2.2)$



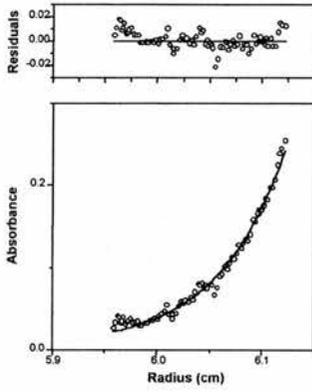
$M_{w,app} = 71.0 (1.7)$



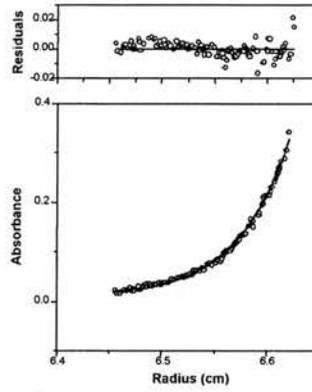
$M_{w,app} = 66.4 (2.1)$

Figure 36: Distribution data from p50/IκBγ at 16 krpm and the curve fits and the residual plots, using the single model.

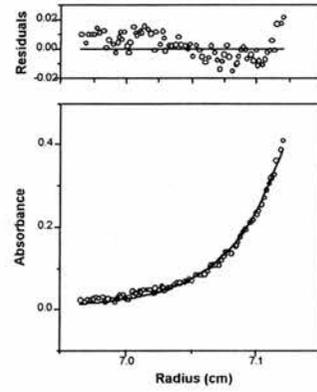
p50/IκBγ at 16krpm (single ideal species model)



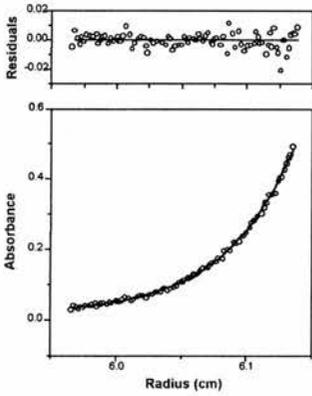
$M_{w,app} = 83.8 (2.6)$



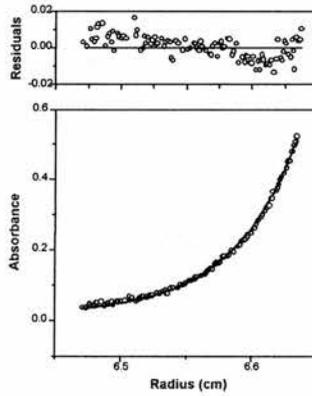
$M_{w,app} = 101.6 (1.5)$



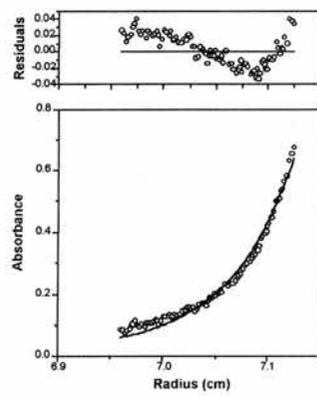
$M_{w,app} = 111.2 (2.7)$



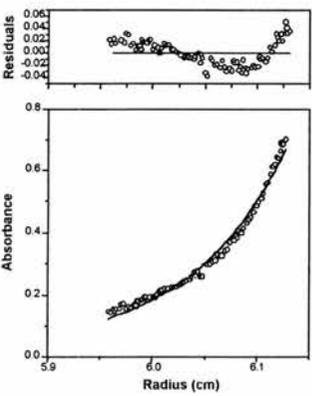
$M_{w,app} = 101.7 (0.9)$



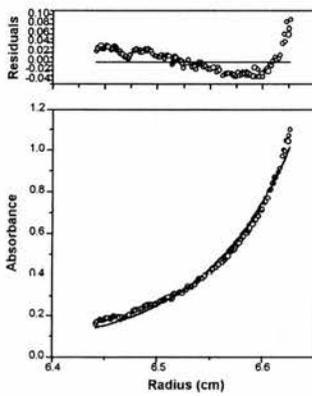
$M_{w,app} = 100.5 (1.0)$



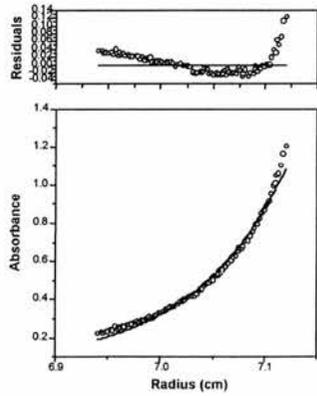
$M_{w,app} = 75.5 (2.0)$



$M_{w,app} = 57.2 (1.5)$



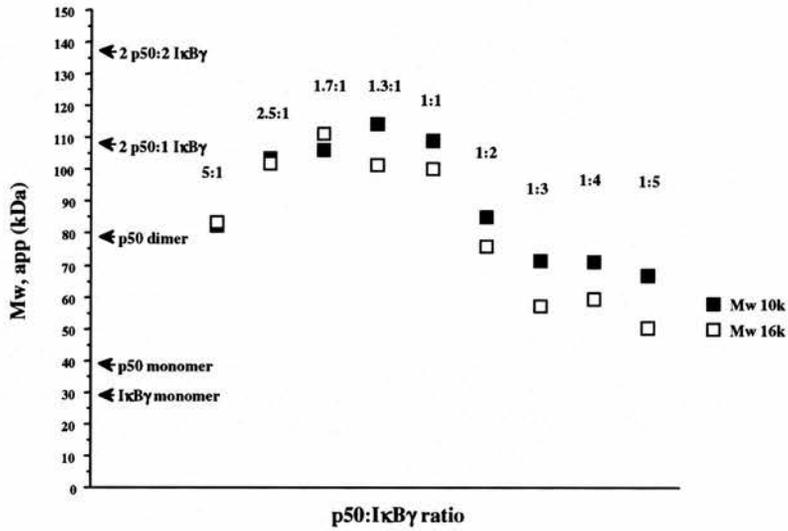
$M_{w,app} = 59.6 (1.4)$



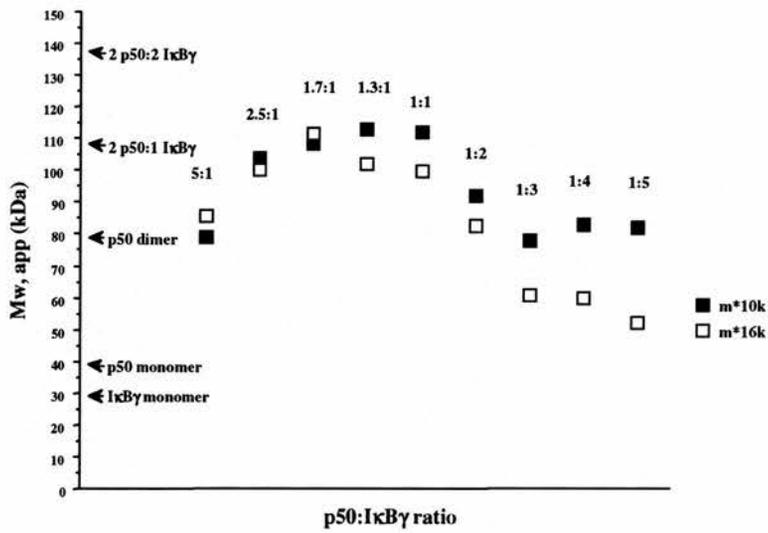
$M_{w,app} = 50.0 (1.0)$

Figure 37: Analysis of p50 / I κ B γ mixtures span at 10 krpm and 16 krpm, using the single ideal species fit model and m* analysis.

Mixtures analysed using single ideal species fit



Mixtures analysed using m*



Fits to the raw sedimentation equilibrium IκBγ data with the model for a single, thermodynamically ideal species were poor. The tick shape of the residuals near the cell base was characteristic of the presence of higher mass species. Extending the model to include dimer does not satisfactorily account for this but the fits were improved with a monomer-tetramer model. The slight upside-down smile shape of the residuals for this fit was indicative of molecular elongation for the monomeric species. These data can be explained though in a far more convincing way: IκBγ in solution is monomeric. IκBγ was produced as a chimaeric GST fusion protein (Smith and Johnson, 1988), which was then cleaved with thrombin and purified by mixing with glutathione-agarose beads that should pull down all the cleaved GST and uncleaved IκBγ-GST molecules. It seems that during the purification process, a small portion of GST and IκBγ-GST did not bind to the beads and remained in the solution, contaminating the IκBγ sample. The molecular weight of monomer GST is 26968 Da which is very similar to the molecular weight IκBγ which is 29399 Da. GST forms stable homodimers, both in crystals and in solution (McTigue et al., 1995). Thus, the apparent "IκBγ dimers" and "IκBγ tetramers" are in fact GST and IκBγ-GST homodimers respectively. These two contaminants with molecular weight 53936 Da and 112734 Da respectively are responsible for both the tick shape of the residuals near the cell base and the overestimation of the apparent weight-average molecular mass of IκBγ (35.5-42.5 kDa instead of 29.4 kDa).

Fits of the raw data for the p50•IκBγ complex were poor, as would be expected for a system that contains at least 3 molecular species. However the so obtained are of sufficient trustworthiness to generate a titration curve for the experiment. From this treatment it is apparent that the weight-average molecular mass peaks at a p50:IκBγ ratio of 2:1. The hypothetical (IκBγ-GST)₂•(p50)₂ complex (which would consist of a (p50)₂ bound to an IκBγ-GST what would be also bound to another IκBγ-GST molecule through GST homodimerisation) with molecular weight 190552 kDa might be also present in the solution but because of its high molecular weight it would sediment rapidly in the cell bottom, without affecting the raw sedimentation

equilibrium data. For the same reason, even if IκBγ tends to aggregate during the purification process (p50 is very soluble and does not cause such problems during its purification), any IκBγ aggregates present in the solutions would readily sediment. That is why Analytical Ultracentrifugation is considered a "self-cleaning" method.

3.2.3 Sample preparation for Sedimentation Equilibrium analysis and Neutron Scattering

p50, IκBγ and 2:1 p50:IκBγ were dialysed in a Phosphate/Saline Buffer made up in D₂O. The buffer density was estimated ($\rho=1.119176$ g/ml) and the partial specific volumes of (p50)₂•IκBγ is $\bar{v}_{(p50)_2 \bullet I\kappa B\gamma} = 0.739$ ml/g.

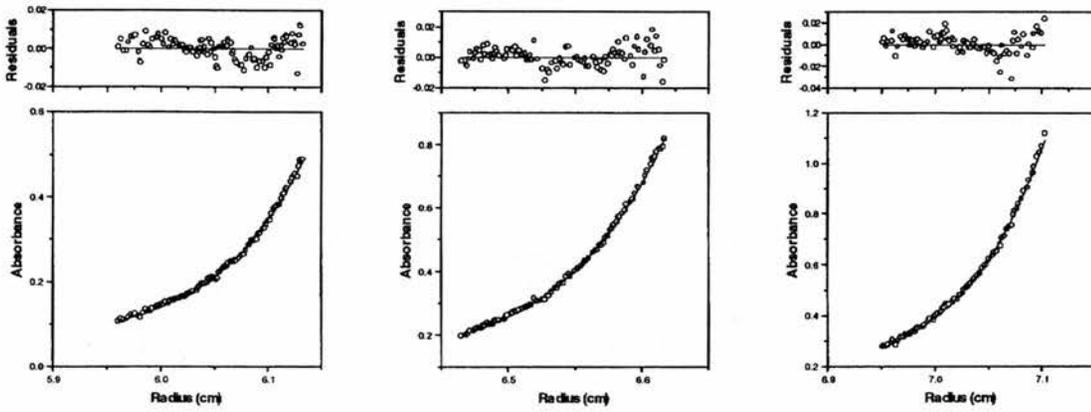
Three concentrations of p50 monomer (15, 31 and 44 μM) were prepared by dilution with its dialysate, and equilibrium was obtained at 16 krpm. The distribution data obtained from the runs were fitted using the single ideal species fit model (Figure 38). From the curve fit plots obtained, apparent weight average molecular masses $M_{w,app_{p50}}$ for each p50 concentration were estimated and the quality of the curve fits was estimated by the residual plots. The estimated $M_{w,app_{p50}}$ values at different concentrations were between 88.0-93.7 kDa (Figure 39). This value demonstrates that Deuterium containing p50 is dimeric in solution.

Three concentrations of IκBγ monomer (14, 27 and 38 μM) were prepared by dilution with its dialysate, and equilibrium was obtained at 16 krpm. The distribution data obtained from the runs were fitted using the single ideal species fit model (Figure 38). From the curve fit plots obtained, apparent weight average molecular masses $M_{w,app_{I\kappa B\gamma}}$ for each IκBγ concentration were estimated and the quality of the curve fits was estimated by the residual plots. The estimated $M_{w,app_{I\kappa B\gamma}}$ values at different concentrations were between 41.7-48.8 kDa (Figure 39). The molecular weight of Deuterium containing IκBγ is slightly higher than the theoretical one for the monomer.

Three concentrations of 2:1 p50:IκBγ (14, 20 and 38 μM) were prepared by dilution with its dialysate, and equilibrium was obtained at 16 krpm. The distribution data obtained from the runs were fitted using the single ideal species fit model (Figure

Figure 38: Distribution data from p50, IκBγ and 2:1 p50: IκBγ in D₂O at 16 krpm and the curve fits and the residual plots, using the single ideal species model.

p50 at 16krpm (single ideal species model)

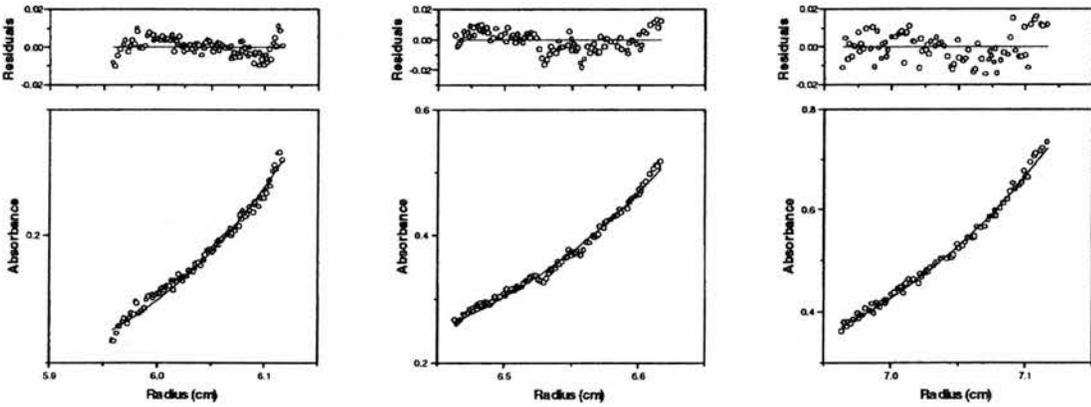


Mw,app=93.5 (± 1.1) 15 μ M

Mw,app=93.7 (± 0.8) 31 μ M

Mw,app=88.0 (± 0.7) 44 μ M

IkBy at 16krpm (single ideal species model)

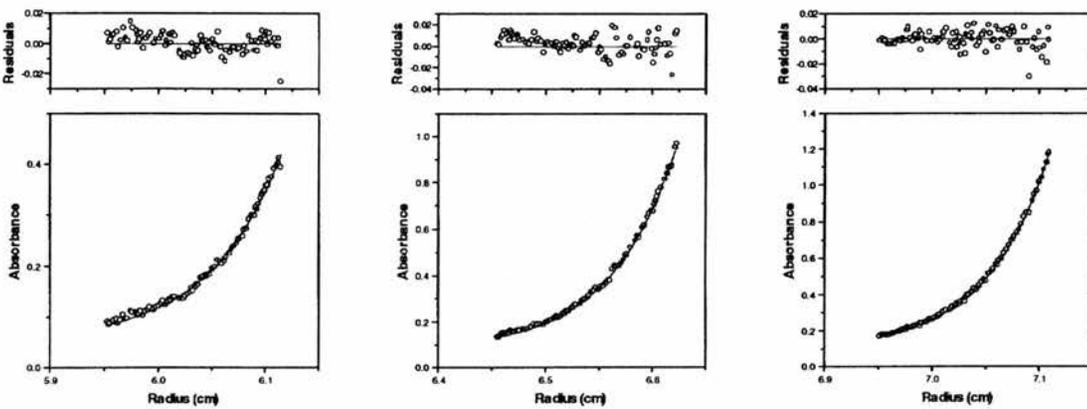


Mw,app=48.1 (± 0.9) 14 μ M

Mw,app=41.7 (± 0.7) 27 μ M

Mw,app=41.8 (± 0.7) 38 μ M

2:1 p50:IkBy at 16krpm (single ideal species model)

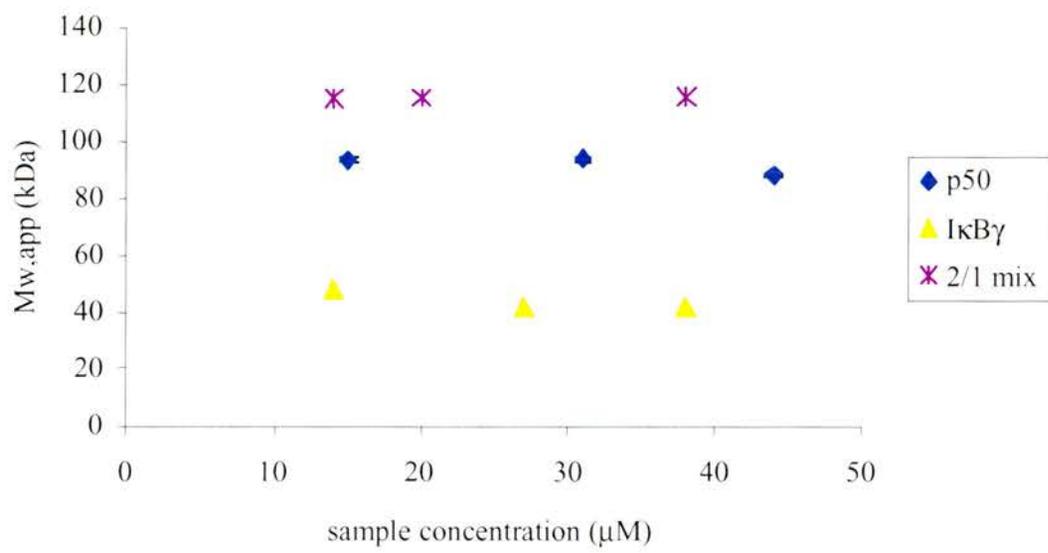


Mw,app=115.1 (± 1.5) 14 μ M

Mw,app=115.3 (± 0.9) 20 μ M

Mw,app=115.8 (± 0.5) 38 μ M

Figure 39: Estimation of the $M_{w,app}$ of p50, I κ B γ 2:1 p50:I κ B γ dialysed in D₂O and span at 16 krpm, using the single ideal species fit model.



38). From the curve fit plots obtained, apparent weight average molecular masses $M_{w,app(p50)_2 \cdot IkB\gamma}$ for each $IkB\gamma$ concentration were estimated and the quality of the curve fits was estimated by the residual plots. The estimated $M_{w,app(p50)_2 \cdot IkB\gamma}$ values at different concentrations were between 115.1-115.8 kDa (Figure 39), which is the molecular weight of a $p50 \cdot IkB\gamma$ complex with 2:1 $p50:IkB\gamma$ ratio.

3.2.4 Sedimentation Velocity

Three concentrations of p50 (35.5, 69.9, and 102.4 μ M) were spun at 35 krpm at 4°C in a sedimentation velocity run. Sedimentation coefficients in buffer at 4°C for each concentration were obtained by data analysis using *Origin Velocity* and *Svedberg* data fitting programs. Sedimentation coefficients are normally calculated relative to water at 20°C i.e. $s_{20,w}$, therefore the values have to be corrected for temperature and experimental buffer. The viscosity of the water at 4°C $\eta_{T,w}=0.015670$ Poise, the viscosity of the water at 20°C $\eta_{20,w}=0.010020$ Poise, the viscosity of the buffer at 4°C $\eta_s=0.016265$ Poise, the viscosity of the water at 20°C $\eta_w=0.015670$ Poise, the density of the water at 20°C $\rho_{20,w}=0.99832$ g/ml and the density of the water at 4°C $\rho_{T,s}=1.119176$ g/ml. By plotting the corrected $s_{20,w}$ values, the sedimentation coefficient in water at 20°C to zero concentration $s_{0/20,w}$ was obtained by extrapolation to 0 concentration on the x axis. The measured $s_{0/20,w}=0.64$ S according to Origin Analysis and $s_{0/20,w}=3.86$ S according to Svedberg single species Analysis. The sedimentation coefficient predicted using crystallographic data with HYDRO using a bead model was for p50 monomer 3.40 S and for the dimer 4.96 S (Figure 40).

Similarly, three concentrations of $IkB\gamma$ (33.6, 66.1, and 102.9 μ M) were span at 45 krpm at 4°C in a sedimentation velocity run. The measured $s_{0/20,w}=1.86$ S according to Origin Analysis and $s_{0/20,w}=2.04$ S according to Svedberg single species Analysis. The predicted sedimentation coefficient for $IkB\gamma$ monomer which was constructed by the addition of one ankyrin repeat in the $IkB\alpha$ structure (Figure 41), was 3.39 S (Figure 40).

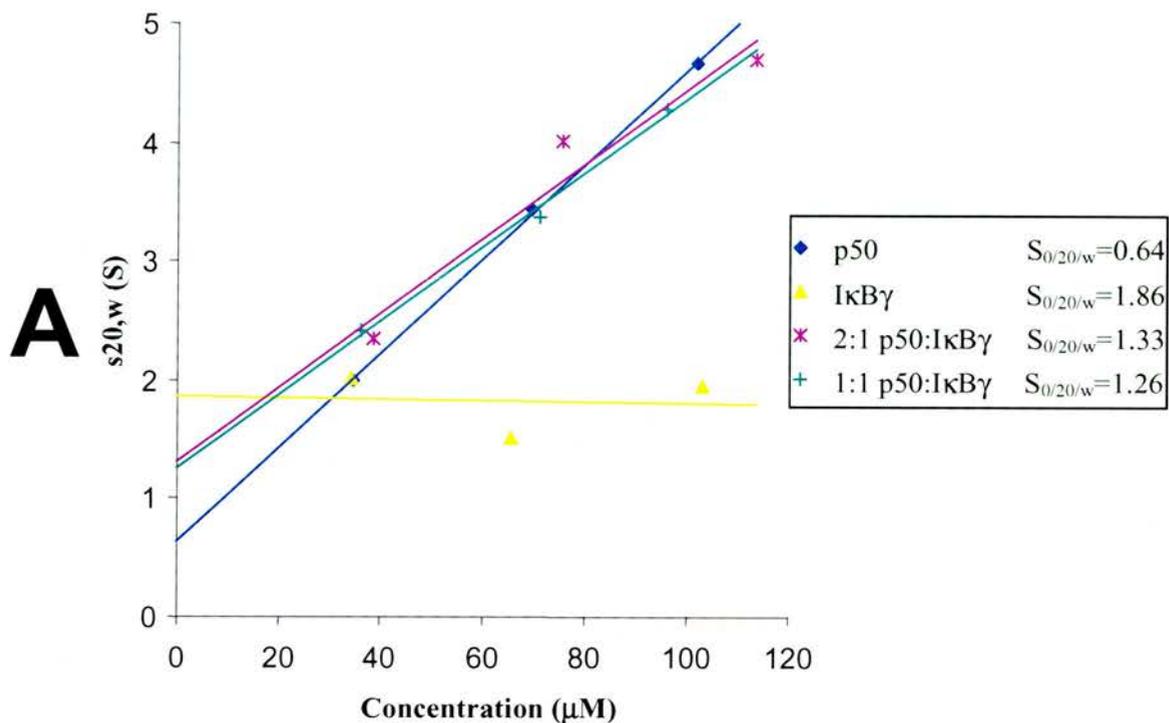
Three concentrations of 2:1 ratio $p50:IkB\gamma$ (34.7, 70.4, and 113.1 μ M) were also span at 35 krpm at 4°C in a sedimentation velocity run. The measured $s_{0/20,w}=1.33$ S

according to Origin Analysis and $s_{0/20,w}=4.71$ S according to Svedberg single species Analysis (Figure 40).

Finally, three concentrations of 1:1 ratio p50:IkB γ (25.8, 50.9, and 96.5 μ M) were span at 35 krpm at 4°C in a sedimentation velocity run. The measured $s_{0/20,w}=1.26$ S according to Origin Analysis and $s_{0/20,w}=4.63$ S according to Svedberg single species Analysis (Figure 40).

Figure 40: Estimation of s of p50, I κ B γ , 2:1 p50:I κ B γ and 1:1 p50:I κ B γ , by using *Origin* (A) and *Svedberg* (B) software for data analysis of sedimentation velocity runs, correcting observed s values and extrapolating them to zero concentration.

Estimates of s using Origin analysis



Estimates of s using Svedberg single species analysis

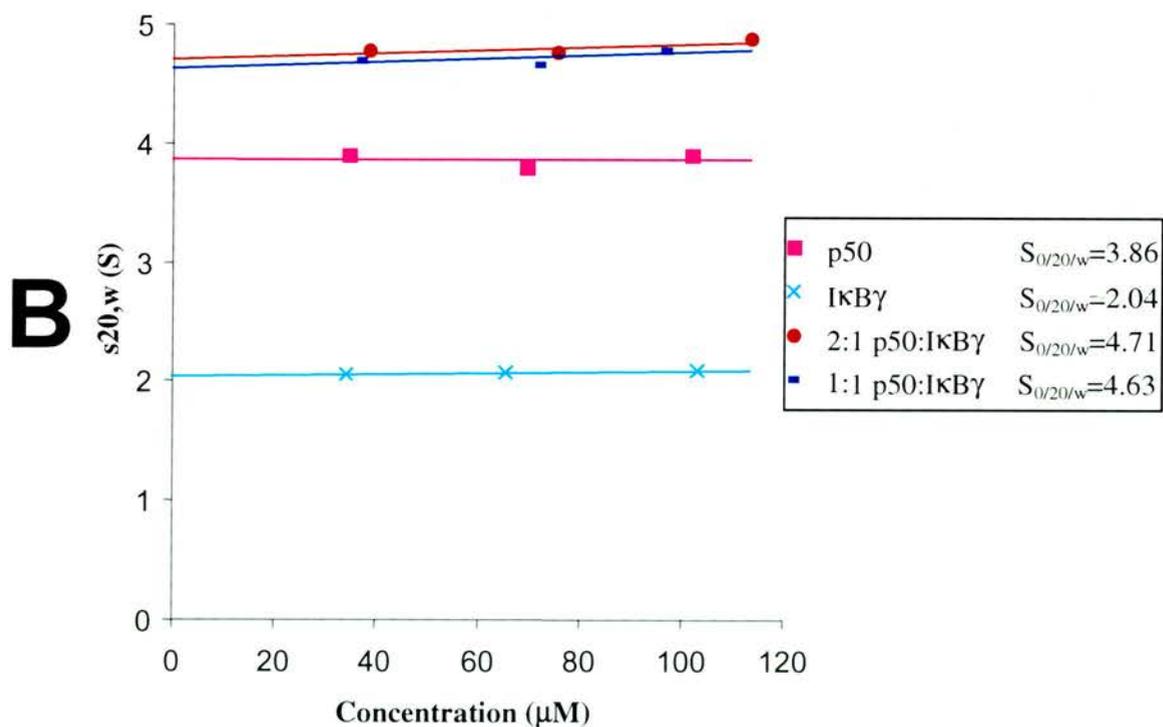
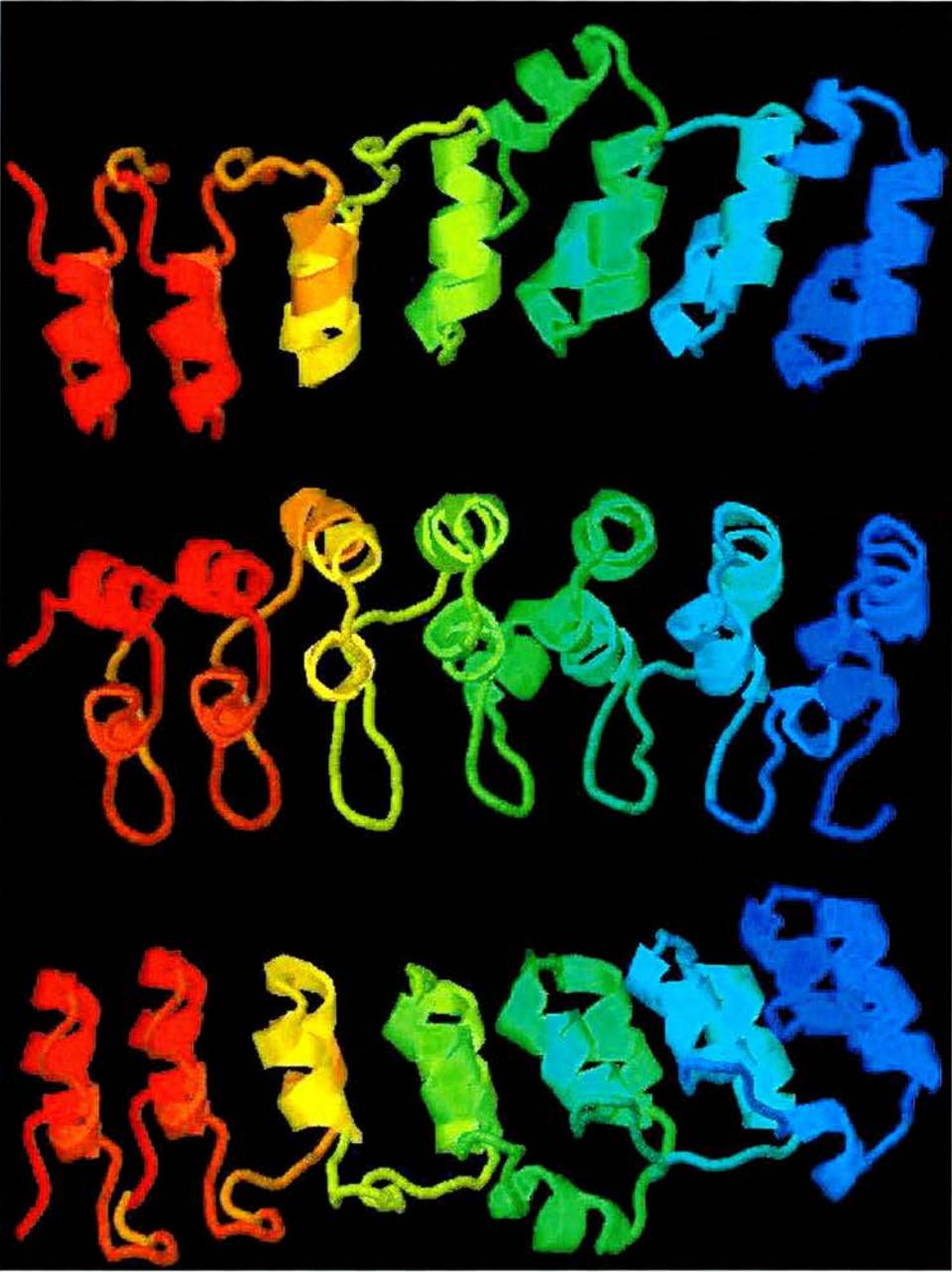


Figure 41: Predicted structure of I κ B γ ARD, using the known structure of I κ B α . This structure allows us to predict the theoretical S.

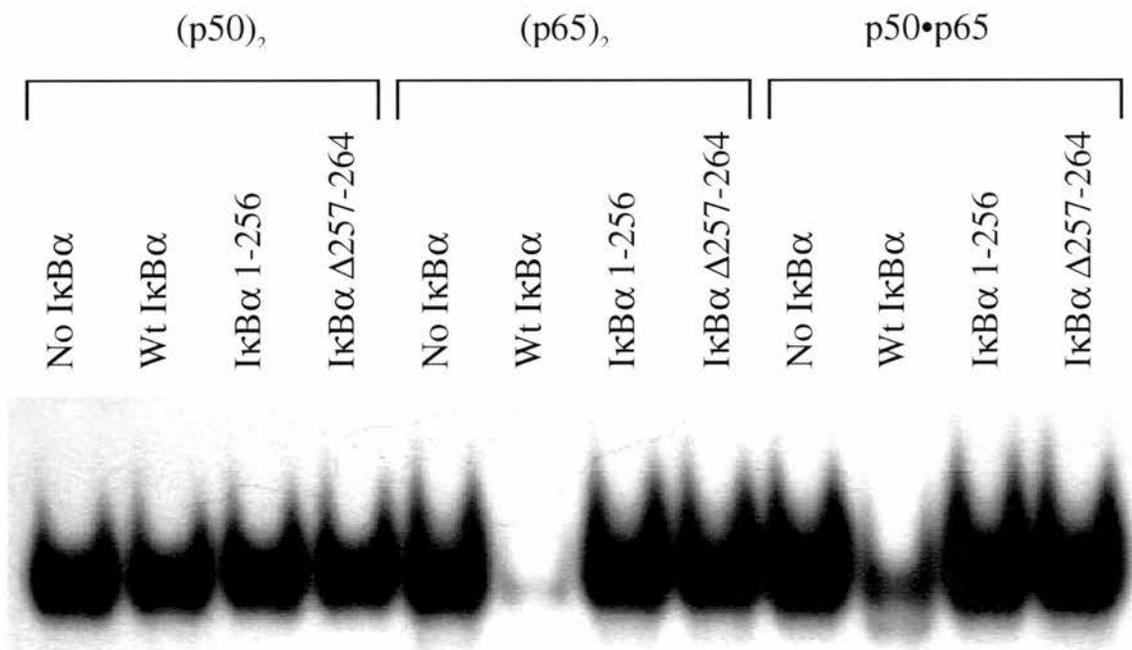


3.3 The C-terminus of I κ B α is required for interaction with NF- κ B proteins

To define the role of the C-terminus of I κ B α in the interaction of I κ B α with NF- κ B proteins, this region was totally or partially removed from I κ B α . I κ B α 1-256 contained amino acids 1-256 from the wild type I κ B α (deletion of the C-terminal 61 amino acids) and I κ B α Δ 257-264, where the amino acids 257-264 from the wild type I κ B α (deletion of a potential phosphorylation site by PKC) were replaced by the sequence PG. Wild type I κ B α or I κ B α mutants were incubated with NF- κ B proteins and the DNA binding activity was determined in electrophoretic mobility shift assays. Addition of wild type I κ B α to (p50)₂ bound to HIV-L- κ B motif had no effect in the DNA binding affinity of (p50)₂ towards this κ B motif. Addition of wild type I κ B α to (p65)₂ or p50•p65 bound to HIV-L- κ B motif, resulted in the decrease of the DNA binding affinity of (p65)₂ or p50•p65 towards this κ B motif. Addition of I κ B α 1-256 or I κ B α Δ 257-264 to (p50)₂, (p65)₂ or p50•p65 bound to HIV-L- κ B motif had no effect in the DNA binding affinity of these NF- κ B proteins towards the HIV-L NF- κ B motif (Figure 42).

Figure 42: I κ B α does not interact with p50•DNA complex. The wild type I κ B α molecule (wt I κ B α) can efficiently inhibit the DNA binding activity of (p65)₂ and p50•p65. The two modified forms of I κ B α , I κ B α 1-256 (deletion of the C-terminal 61 amino acids) and I κ B α Δ 257-264 (deletion of a potential phosphorylation site be PKC) cannot inhibit the DNA binding activity of (p65)₂ and p50•p65.

The C-terminus of IκBα is required for interaction with (p65)₂•DNA and p50•p65•DNA complexes



GATCTAGGGACTTTCCGCG
ATCCCTGAAAGGCGCCTAG
HIV-L-κB

3.4 Role of the C-terminus of the loop L1 in stabilisation of (p50)₂•IκBγ complex

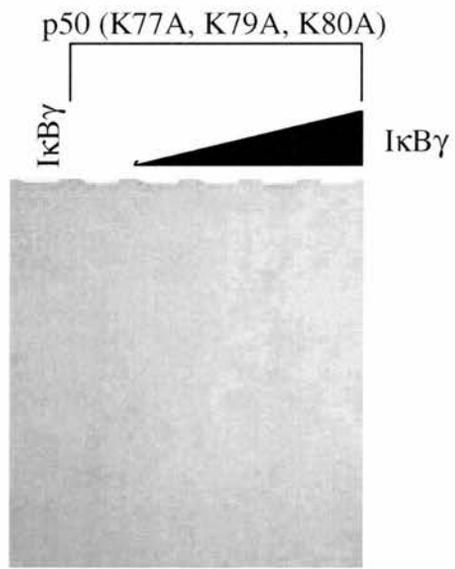
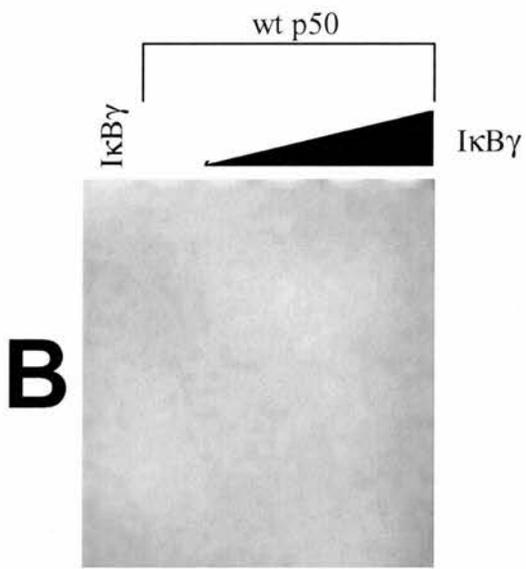
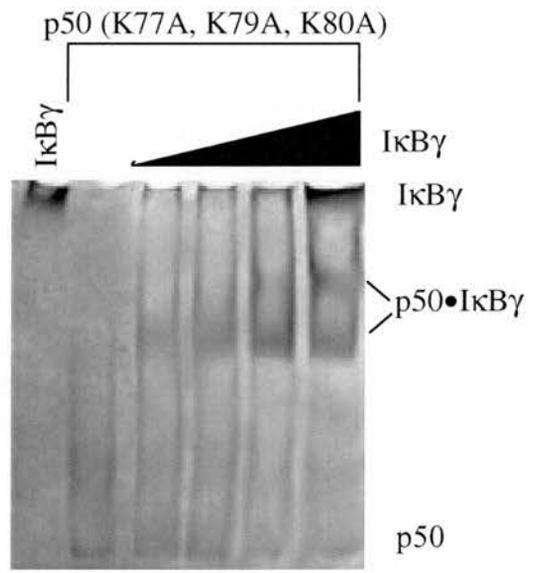
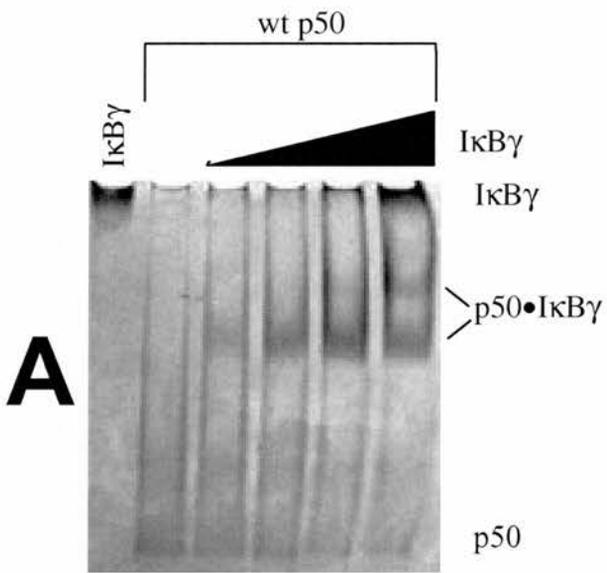
3.4.1 DNA binding properties of mutants

To determine the importance of each lysine residue of the C-terminal sequence of p50 (amino acids 77-80) the previously described p50 mutants (Figure 24), were employed. Gel electrophoresis IκBγ protein binding assays were performed with wt and the triple mutant. Unfortunately, the resolution of this method was insufficient to discriminate between the binding affinities of the wt and the triple mutant towards IκBγ (Figure 43). It was supposed that the this method would not show any differences in the affinity of the single and doubles mutants thus not possible to unambiguously identify the lysine residue(s) responsible for the interaction with IκBγ. This experiment could neither support the existence of the AB loop-IκBγ interaction (Bell et al., 1996) nor demonstrate its importance for stabilisation of the p50-IκBγ complex.

3.4.2 Discrimination between DNA binding activities of p50 mutants

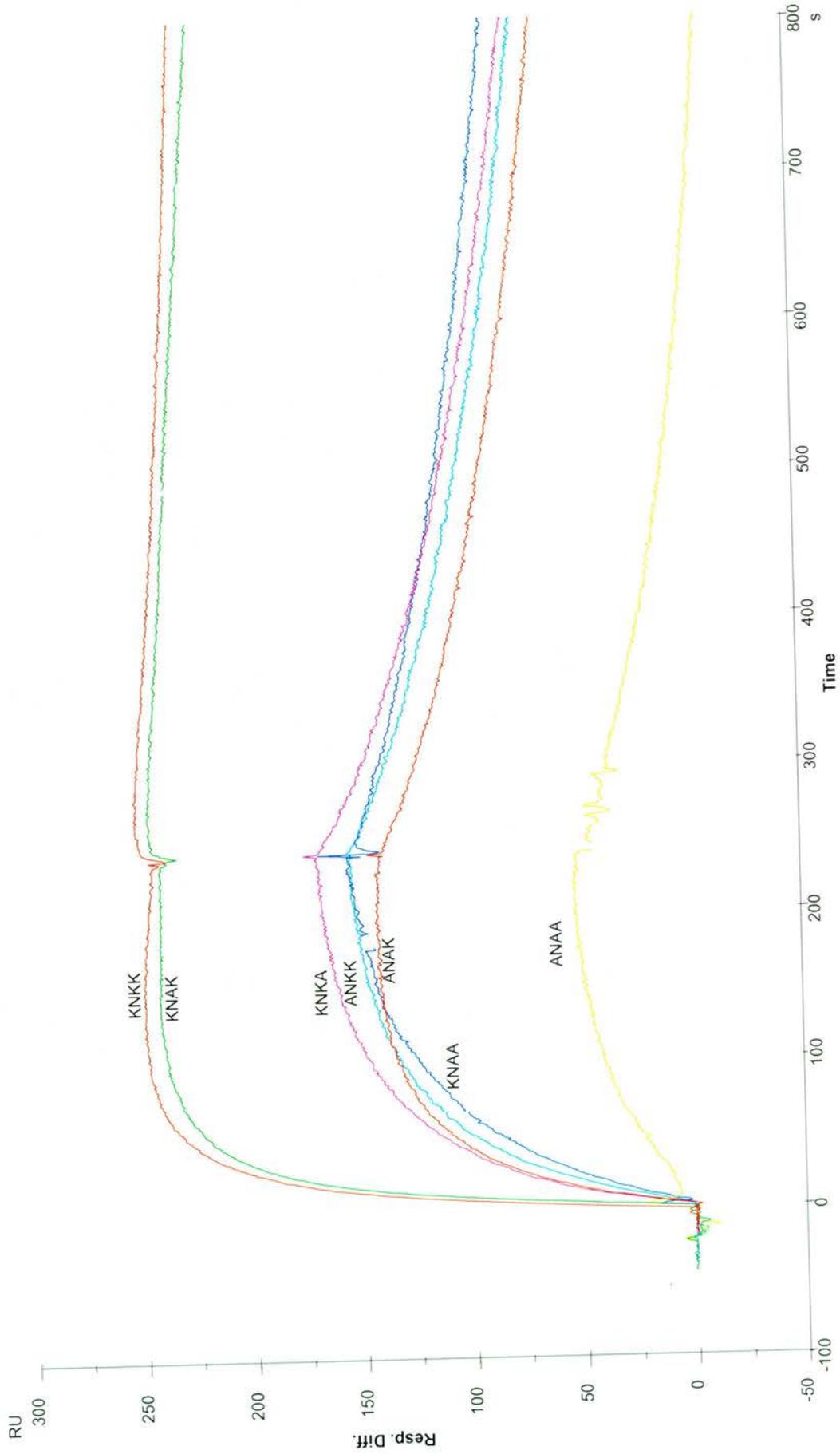
To discriminate between the DNA binding activities of the p50 mutants (Figure 24), SPR was employed. An anti-GST IgG antibody was covalently immobilised on the surface of a sensor chip and IκBγ-GST was applied on the surface until the response difference was 300 RU. Wild type p50 and the various mutants were tested for protein binding activity by passage over the sensor chip. Protein binding reactions were carried out under stringent conditions at 350 mM NaCl to eliminate non-specific interactions between p50 proteins and GST, IgG or the dextran surface of the sensor chip. Under these stringent conditions, wild type p50 bound efficiently to IκBγ-GST and p50 binding to GST immobilised by IgG was negligible. Data were collected at a range of protein concentration between 10 and 500 nM. At each protein concentration the analysis was carried out in triplicate. Although the data were reproducible it was not possible to derive association and dissociation rate constants for the interaction between p50 and IκBγ, as the data could not be fitted to the theoretical binding models of the evaluation software. The reason for that was the fact that the binding

Figure 43: Gel electrophoresis protein binding assays adding wt p50 or the triple mutant p50 (K77A, K79A, K80A) proteins to increasing amounts of IκBγ. To 1 μg wt p50 or the triple mutant p50 (K77A, K79A, K80A) increasing amounts of IκBγ (p50:IκBγ ratio 2:1, 1:1, 2:1 4:1) were incubated for 30' at room temperature. The samples were resolved in two 6% non denaturing polyacrylamide gel (55:1 acrylamide: bis-acrylamide) and the gel was stained with Coomassie Brilliant Blue. The upper band corresponds to free IκBγ the middle ones to p50•IκBγ complex and the lower band corresponds to unbound to IκBγ p50. A: Normal polarity B: Reverse polarity.



reaction did not follow strictly the kinetics expected from a simple bimolecular reaction, because the interaction between p50 and I κ B γ involves p50 conformational changes and interactions in more than one area (as it was revealed by structural analysis of p50•I κ B γ (Huxford et al., 1998; Jacobs and Harrison, 1998)). Although the quantitative evaluation could not be completed, the experimental data could be used to resolve the differences in the I κ B γ binding of the wild type and mutant proteins by direct comparison of the binding curves, at a given protein concentration. wt p50 bound more tightly than any of its mutants (Figure 44). The binding activity of the single mutant p50 (K79A) protein was almost the same with that of the wt. The most defective of the 3 single mutants was p50 (K77A), while the affinity of p50 (K80A) was slightly higher than the affinity of the p50 (K77A) mutant. The double mutant p50 (K79A, K80A) was more defective in I κ B γ binding than each of the two single mutants p50 (K79A) and p50 (K80A) but was almost as defective as the single mutant p50 (K77A). p50 (K77A, K79A) was more defective than p50 (K77A) and p50 (K80A) while the triple mutant p50 (K77A, K79A, K80A) was clearly the most defective in binding to I κ B γ of all the mutant proteins (Figure 44). These data provide experimental evidence for the suggested interaction between the C-terminus of the AB loop- I κ B γ and demonstrate its importance for the stabilisation of the I κ B γ -protein complex. K77, together with K80, appear to play a critical role in this interaction with I κ B γ . Residue K79 does not seem to contribute to the p50-I κ B γ protein interaction.

Figure 44: Typical sensogram (Surface Plasmon Resonance), showing the association and the dissociation of the p50 proteins (wt and mutants) to I κ B γ . In the association phase, 100 nM of each protein were injected for 4 minutes at 10 μ l/min flow rate. In the dissociation phase, buffer containing no protein was injected for 10 minutes at 10 μ l/min flow rate.



4 DISCUSSION

4.1 Bcl-3-NF- κ B interactions

4.1.1 *Bcl-3 found in a chromosomal translocation*

Bcl-3 is a protooncogene. It was found on chromosome 19 adjacent to the breakpoint in the translocation t(14;19)(q32;q13.1) where a class switch region associated with the α constant region exons of the immunoglobulin heavy chain locus was fused head to head to the *bcl-3* gene, which occurred in some cases of B-cell lymphocytic leukaemia (Ohno et al., 1990). A more complex three way rearrangement t(7;19;14)(q21;q13;q32) was also reported (Michaux et al., 1996). This fusion to the Ig heavy chain locus resulted in a much stronger expression of *bcl-3* gene than in normal blood cells, while the coding region remained unmodified (Ohno et al., 1990). *Bcl-3* mRNA is widely expressed (Nolan et al., 1993), in a pattern similar to that observed with the p50 subunit of NF- κ B. The protein levels of Bcl-3 are not affected by ageing in heart, liver, kidney and brain of young, adult and old NMRI mice and Wistar rats (Helenius et al., 1996). Haemorrhage- induced Acute Respiratory Distress Syndrome (ARDS) appears to activate Bcl-3 expression (Moine et al., 1997). In normal peripheral mononuclear blood cells, *bcl-3* gene expression is induced upon T cell mitogenic stimulation, which suggests that the aberrant overexpression in the previously described translocations, mimics a growth-stimulating condition which can not be downregulated. This permanent proliferative stimulus is a step which can lead to neoplastic transformation. *Bcl-3* gene structural alteration is a rare abnormality in chronic lymphoproliferative disorders (Michaux et al., 1996) and was not found in paediatric Acute Lymphoblastic Leukaemia (ALL) (Liptay et al., 1997).

4.1.2 *I κ B-like properties of Bcl-3*

4.1.2.1 *Bcl-3 homology with the I κ B family of protein*

Bcl-3 contains 7 ankyrin repeats which suggested that Bcl-3 belongs to the I κ B family of proteins (Baeuerle and Baltimore, 1996; Baldwin, 1996; Franzoso et al., 1992; Wulczyn et al., 1992). *Bcl-3* gene contains 9 exons, spanning 11.5kb. In comparison

to other members of the I κ B family (*NFKB-2*, *cactus*, *MAD-3*), there is a remarkable conservation of the exon-intron boundaries in relation to the coding sequences, consistent with an origin from a common ancestral gene (McKeithan et al., 1994). Bcl-3 is more related to I κ B γ , as these proteins contain 7 seven ankyrin repeats, while I κ B α and I κ B β contain only six. Moreover, Bcl-3 and I κ B γ , but not I κ B α and I κ B β mainly target the p50 subunit of NF- κ B.

4.1.2.2 Interaction with NF- κ B molecules

Bcl-3 protein interacts *in vitro* with NF- κ B p50 (Bours et al., 1993; Franzoso et al., 1993; Franzoso et al., 1992; Hatada et al., 1992; Inoue et al., 1993; Wulczyn et al., 1992; Zhang et al., 1994) and p52 (Bours et al., 1993), which was also shown in our results. Bcl-3 protein does not interact *in vitro* with c-Rel (Inoue et al., 1993). Our data suggest that Bcl-3 interacts with p65, which is opposite to previous findings (Bours et al., 1993; Franzoso et al., 1992; Inoue et al., 1993).

Previous data which demonstrated that Bcl-3 ARD is sufficient for interaction with p50 (Wulczyn et al., 1992) and p52 (Bours et al., 1993), were also confirmed. We also displayed that this is in contrast with the fact that I κ B α ARD is necessary but not sufficient for interaction with p50•p65 heterodimers and (p65)₂ homodimers, because the C-terminus of MAD-3 is also required (Rodriguez et al., 1995) (Figure 42). Our findings, in accordance with the previously reported data, suggest that Bcl-3 ARD is necessary and sufficient for interaction with other homodimers (i.e. (p65)₂).

Our data showed that p50 NLS is required for association with I κ B proteins, as it was previously shown (Inoue et al., 1993).

4.1.2.3 DNA binding inhibition

In common with other members of the I κ B family of proteins Bcl-3 can inhibit the DNA binding of some members of the NF- κ B protein family. There are conflicting data on the DNA binding inhibition of the different NF- κ B molecules. The converging point is that Bcl-3, as opposed to I κ B α , preferentially interacts with (p50)₂ and (p52)₂ (Bours et al., 1993; Franzoso et al., 1992; Fujita et al., 1993; Hatada

et al., 1992; Inoue et al., 1993; Naumann et al., 1993; Nolan et al., 1993; Wulczyn et al., 1992). Nevertheless, our data suggest that Bcl-3 could alter DNA activity of other NF- κ B homodimers, like (p65)₂.

It was reported that Bcl-3 inhibits DNA binding activity of (p50)₂ (Franzoso et al., 1993; Inoue et al., 1993; Kerr et al., 1992; L veillard and Verma, 1993; Nolan et al., 1993). We found that Bcl-3 directed DNA binding inhibition is DNA sequence dependent. Bcl-3, which prefers to interact with homodimers, stabilises p50 binding to symmetrical DNA molecules, while it inhibits p50 binding to non symmetrical DNA sequences. Our data are not necessarily incompatible with these previous findings. They just reflect the fact that in all previous studies, non symmetrical DNA targets were selected.

According to previous reports, Bcl-3 inhibits DNA binding activity of (p52)₂ (Inoue et al., 1993; Kerr et al., 1992; L veillard and Verma, 1993; Nolan et al., 1993). Our data does not show any significant modification of p52 DNA binding activity in the presence of Bcl-3, irrespective of whether the DNA was symmetrical or non symmetrical.

We were unable to confirm previous data that Bcl-3 inhibits p52•p65 DNA binding activity (Kerr et al., 1992), since we were unable to form stable p52•p65 heterodimers, *in vitro*.

There is not universal agreement on whether Bcl-3 inhibits (p65)₂ DNA binding. Most reports suggest that it does not inhibit it (Inoue et al., 1993; L veillard and Verma, 1993; Nolan et al., 1993). Our findings though, are in accordance with the opposite view, which was also reported (Wulczyn et al., 1992). In fact (p65)₂ DNA binding inhibition by Bcl-3 was consistent in all DNA targets (symmetrical and non symmetrical).

Most observations suggest that Bcl-3 inhibits (c-Rel)₂ DNA binding activity (Inoue et al., 1993; Kerr et al., 1992; Nolan et al., 1993) but conflicting data have been reported (Wulczyn et al., 1992). We did not confirm any of the two findings, since we did not express c-Rel.

An early report suggests that Bcl-3 inhibits the DNA binding of p50•p65 (Kerr et al., 1992), while later studies suggest the opposite (Inoue et al., 1993; Nolan et al., 1993). Our data displayed that Bcl-3 did not cause inhibition of p50•p65 binding to either symmetrical or non symmetrical DNA targets.

Bcl-3 ankyrin repeat domain is necessary and sufficient for the destabilisation of the p50•DNA complexes (Franzoso et al., 1993; Wulczyn et al., 1992) *in vitro*. This was also demonstrated by our experiments.

Bcl-3 requires phosphorylation to efficiently inhibit (p50)₂ (Nolan et al., 1993).

4.1.2.4 Phosphorylation

Bcl-3 is constitutively phosphorylated (Bundy and McKeithan, 1997; Caamaño et al., 1996; Fujita et al., 1993; Nolan et al., 1993). Most of the phosphorylation of Bcl-3 occurs in the serine-proline rich C-terminal domain of Bcl-3 and is extensive and constitutive (Bundy and McKeithan, 1997), similarly to IκBα (Ernst et al., 1995) and IκBβ (Chu et al., 1996). Our protein that effectively induced p50 DNA binding inhibition in non symmetrical DNA targets, was not phosphorylated, because it was expressed in bacteria (since it was only the ARD and thus lacked the C-terminus and it would not be heavily phosphorylated in a prokaryotic expression system, anyway). By combining these lines of data, we can then speculate that phosphorylation of C-terminus might lead to exposure of Bcl-3 ARD, letting this domain efficiently interact with NF-κB molecules.

4.1.3 Atypical IκB properties of Bcl-3

4.1.3.1 Nuclear localisation

It was initially thought that Bcl-3, like p105, sequesters p50 in the cytoplasm and prohibits both nuclear translocation and DNA binding and that, unlike p105, does not sequester p65 in the cytoplasm (Naumann et al., 1993). It was eventually shown that Bcl-3 is a predominantly nuclear protein (Bours et al., 1993; Caamaño et al., 1996; Franzoso et al., 1993; Inoue et al., 1993; Nolan et al., 1993; Zhang et al., 1994) which promotes the nuclear localisation of the NF-κB molecules with which it interacts

(Heissmeyer et al., 1999; Watanabe et al., 1997). This is in contrast to other IκB proteins which block the NF-κB Nuclear Localisation Signal (NLS) and anchor NF-κB in the cytoplasm. In cells co-transfected with p50 lacking a functional NLS, Bcl-3 is relocated to the cytoplasm, showing that the two proteins interact in the cell (Nolan et al., 1993). The opposite effect (p50 lacking NLS was restricted in the cytoplasm in the absence of Bcl-3 and in the nucleus in the presence of Bcl-3) was also reported (Zhang et al., 1994). That means that the only requirement for efficient nuclear transport of the NF-κB•Bcl-3 complex is karyophilicity from either NF-κB or Bcl-3. Bcl-3 N-terminus is required for efficient nuclear localisation (Zhang et al., 1994). Interestingly, there is no report that this region is phosphorylated upon stimulation, ubiquitin or SUMO conjugated or proteolytically degraded through proteasome, as it happens to the other IκB proteins.

4.1.3.2 *Formation of a ternary complex containing Bcl-3 and the DNA bound form of (p50)₂ and (p52)₂*

One and/or two Bcl-3 molecules can also associate with (p50)₂ and (p52)₂ bound to DNA (Bours et al., 1993; Fujita et al., 1993), and form a ternary complex. This was also shown by our experiments. The ankyrin repeat domain of Bcl-3 is sufficient for the formation of a Bcl-3•p52•DNA ternary complex (Bours et al., 1993) *in vivo* and we demonstrated that the formation of Bcl-3 ARD•p50•DNA and Bcl-3 ARD•p52•DNA ternary complexes *in vitro*.

Our data indicate that p50 NLS sequence is crucial for the effective interaction of p50 with Bcl-3. Lack of p50 NLS stops Bcl-3 inhibiting DNA binding activity and also blocks the formation of the ternary complex. Previous experiments have shown that p50 and p65 NLS participates in interactions with IκBγ and IκBα, respectively (Beg et al., 1992; Henkel et al., 1992; Matthews et al., 1993b). Since the NLS is on the other side from p50 than its DNA binding area, and this interaction is necessary for modification of p50 DNA properties, it is apparent that NLS recognition by Bcl-3 ARD is necessary for anchoring of Bcl-3 to (p50)₂, rather than actively participating in the DNA binding itself (e.g. by directly competing DNA in the DNA binding

region of p50 or inducing allosteric rearrangements of the two proteins that would alter DNA binding activity). The fact that the supercomplex does not appear in the lack of p50 NLS indicates that the NLS is necessary for the stabilisation of the ternary complex.

Formation of this ternary complex depends on Bcl-3 phosphorylation and concentration (Franzoso et al., 1997), and shows direct transactivation potential. I κ B α can also be found in the nucleus (Arenzana-Seisdedos et al., 1995), but it does not associate with p65 to directly transactivate target promoters (Cressman and Taub, 1993). Bcl-3 can increase p50 DNA binding *in vivo*, by transition from p50•p105 to (p50)₂ which involves phosphorylation upon TNF α stimulation of 3 C-terminal serines of p105 by IKK α and IKK β which leads to degradation of p105 in the proteasome and release of p50 (Heissmeyer et al., 1999), but can also be done without proteolytic processing (Watanabe et al., 1997). Therefore, p50•p105 serves as a physiological reservoir to generate (p50)₂, through a mechanism of interchangeable interactions among NF- κ B and I κ B molecules.

The number of Bcl-3 molecules that participate in NF- κ B•DNA complexes is not known. A model of the formation of the Bcl-3•p52•DNA ternary complex, depending on the stoichiometry of the Bcl-3/p52 was proposed: Only 1:2 complexes can be bound to DNA, whereas 2:2 complexes are unable to bind DNA (Bundy and McKeithan, 1997).

4.1.3.3 *Bcl-3 dependent Transactivation*

Bcl-3 modulates transcription from κ B sites in a different way from I κ B α and I κ B γ (Inoue et al., 1993). There are reports indicating that Bcl-3 represses κ B-mediated transcription *in vivo* (Kerr et al., 1992), but most published work demonstrates that Bcl-3 enhances κ B-mediated transcription *in vivo* in a p50 and p52 dependent fashion (Franzoso et al., 1992). Cotransfection of Bcl-3 with p50 (Fujita et al., 1993; Pan and McEver, 1995), and p52 (Bours et al., 1993; Pan and McEver, 1995) enhanced NF- κ B dependent transcription.

Bcl-3 ankyrin repeat domain is flanked by a proline rich N-terminus and a serine-proline rich C-terminus, both of which cooperate in transcriptional activation (Bours et al., 1993). Bcl-3 interacts with the general transcription factors TFIIB, TBP and TFIIA (Na et al., 1998). The association of Bcl-3 to (p52)₂ bound to DNA at κB sites, functionally leads to a novel and potent form of transactivation through the κB motif: the tethering of Bcl-3 to DNA via the (p52)₂ allows Bcl-3 to transactivate directly, while (p52)₂ alone cannot (Bours et al., 1993). The amino acids 156-289 were identified as autonomous transactivation domains (Na et al., 1998).

Two-hybrid-system uncovered that Bcl-3 ARD itself can also interact with various co-activators like Tip60, Pirin, Bard1 and Jab1 (Dechend et al., 1999). Tip60 which displays histone acetylase activity (Yamamoto and Horikoshi, 1997), is a Tat-interacting protein (Claret et al., 1996), Pirin is an NFI co-activator (Wendler et al., 1997), Bard1 functionally interacts with the tumour suppressor Brca1 (Wu et al., 1996a) and Jab1 interacts with the transactivation domain of c-Jun (Claret et al., 1996). All four proteins are nuclear proteins which associate with gene regulators, but share no sequence homology. Pirin, Tip60 and Bard1 form ternary supercomplexes with Bcl-3•p50•DNA (whether one or two Bcl-3 molecules participate in these complexes has yet to be clarified), whereas Jab1 enhances the formation of Bcl-3•p50•DNA, without forming a complex with it. Furthermore, Bcl-3 interacts with the retinoid X receptor (RXR), and unlike IκBβ which also interacts with it, it coactivates the 9-*cis*-Retinoic Acid-dependent transactivation (Na et al., 1998). Thus, Bcl-3 interacting proteins (BIP) functionally relate NF-κB p50 and p52 to other transcription factor families. In this sense Bcl-3 is itself a co-factor in the establishment of such transactivation networks.

(p50)₂ has so strong affinity for select κB sites, that completely prevents binding of any other NF-κB molecules. These κB sites are candidates for regulation by Bcl-3, as this protein reverses this block (Franzoso et al., 1993). In this case Bcl-3 serves as an antirepressor.

4.1.4 Transgenic mice

Constitutive expression of Bcl-3 in the thymus, in transgenic mice, does not affect T-cell maturation and does not lead to rapid development of lymphoid tumours. p50 but not p52 DNA binding activity is enhanced in the presence of Bcl-3, and this occurs in a phosphorylation dependent manner (Caamaño et al., 1996).

Expression of I κ B and NF- κ B family members in the Bcl-3(-/-) mouse is unchanged, and B- and T- subsets are normal. Although Bcl-3(-/-) mice are able to generate greater than normal levels of antibodies in response to vaccination, they are unable to generate specific antibodies. This could be explained by the fact that the spleens of such mice lack germinal centres (Franzoso et al., 1997; Schwarz et al., 1997).

4.1.5 Bcl-3 dual mode of action

A model has been developed to describe the dynamic equilibrium and ternary complex formation between (p50)₂, its DNA binding site and Bcl-3. Accordingly, the formation of the supercomplex would mediate Bcl-3 induced DNA binding inhibition (Figure 45). This model could also be expanded to all NF- κ B molecules and their appropriate I κ B molecules, assuming that the ternary complex of NF- κ B•I κ B•DNA is quite unstable and short lived.

The previously described model suggests possible mechanisms for the action of Bcl-3, in transactivation of genes that contain NF- κ B motif sequences:

A direct model is the attachment of Bcl-3 to (p50)₂ (Bours et al., 1993; Fujita et al., 1993) or (p52)₂ (Bours et al., 1993), bound to DNA. The resulting complex transactivates through the Bcl-3 N- and C-terminal transactivation domains and Bcl-3 ARD possibly bound to nuclear co-regulators which link NF- κ B to other transcription factors, synergistically inducing transcription (Figure 46).

The indirect model is the inhibition of DNA-binding activity of (p50)₂ (Franzoso et al., 1993; Hatada et al., 1992; Nolan et al., 1993) which does not contain any transactivation domain. The removal of (p50)₂ from non-symmetrical κ B motifs, gives the opportunity to other molecules (p50•p65, (p65)₂, etc) that contain

Figure 45: The model for dynamic equilibrium and ternary complex formation between $(p50)_2$, its DNA binding site and Bcl-3.

Model for dynamic equilibrium

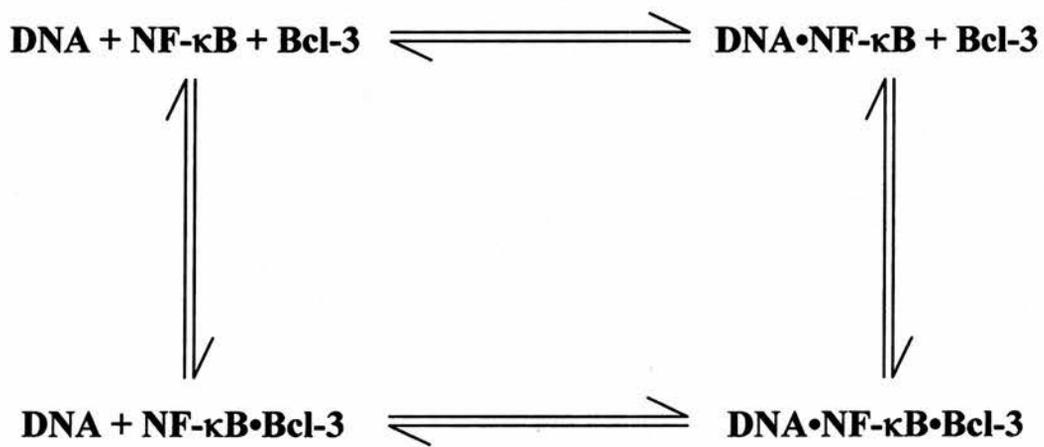
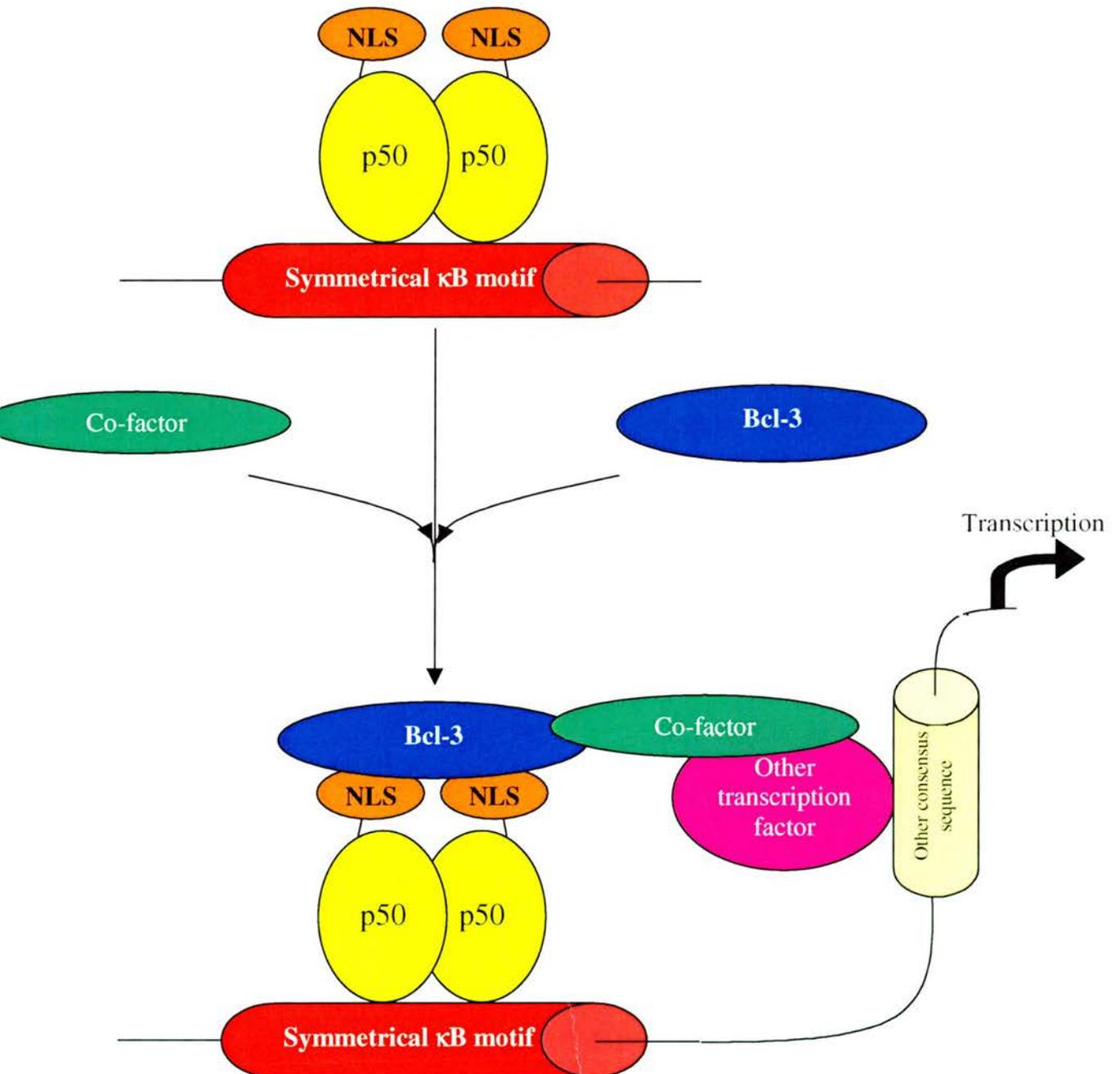


Figure 46: Attachment of Bcl-3 to (p50)₂ or (p52)₂, bound to symmetrical κB motifs, forms a ternary complex which transactivates through Bcl-3 binding proteins which also bind other transcription factors.

Direct transactivation through Bcl-3 binding co-factors

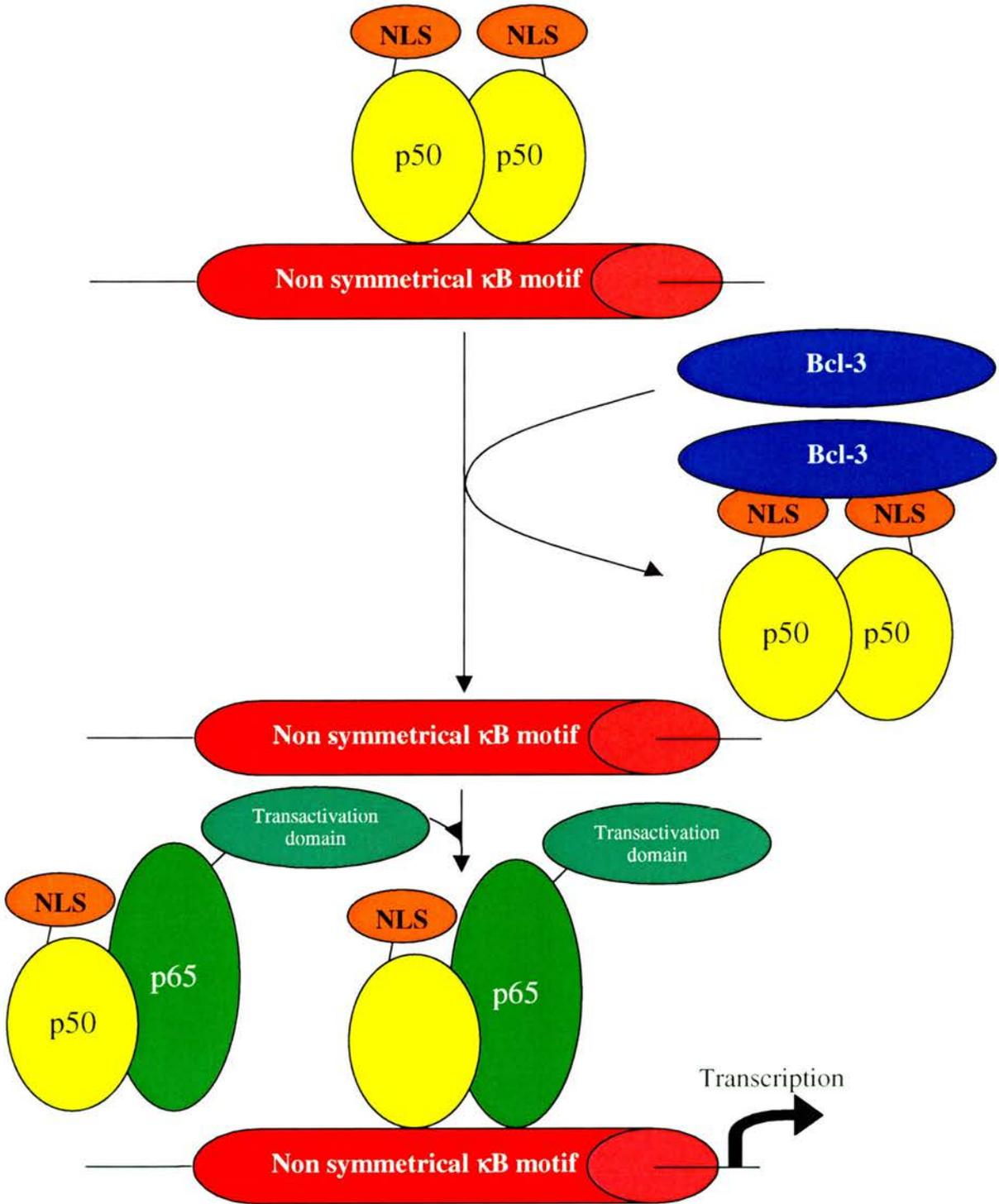


transactivation domains to bind to DNA and to transactivate (Franzoso et al., 1993; Franzoso et al., 1992) (Figure 47).

Our experiments show that the formation of Bcl-3•(p52)₂•DNA complexes is not dependent on the DNA sequence. Interestingly, when Bcl-3 interacts with (p50)₂, bound to DNA, it forms a ternary complex if the DNA sequence contains symmetrical NF-κB motifs and as a DNA binding inhibitor if the NF-κB motifs are not symmetrical. The sequence dependent formation of Bcl-3•(p50)₂•DNA complexes is another important way of differential transactivation of genes with different κB motifs, and might play an important role in the cases of leukaemia, caused by Bcl-3 overexpression.

Figure 47: Inhibition of DNA binding activity of $(p50)_2$ which does not contain any transactivation domain. The removal of $(p50)_2$ gives the opportunity to other molecules (e.g. $p50 \cdot p65$, $(p65)_2$, etc.) which contain transactivation domains, to bind to non symmetrical κB motifs and to transactivate.

Indirect transactivation



4.2 NF- κ B-DNA interactions

Protein footprinting by partial proteolysis (Hay and Nicholson, 1993; Matthews et al., 1995) and chemical modification (Bell et al., 1996) suggested that the lysine cluster which is located at positions 77 to 80 of NF- κ B p50 could contact DNA. Although the lysine cluster (K77, K79 and K80) was implicated the techniques employed did not have sufficient resolution to identify the lysine residue making the DNA contact (Bell et al., 1996). One of the objectives of this study was therefore to use site directed mutagenesis to investigate the role of each lysine in the sequence between 77 and 80. Our experiments demonstrated that conserved K80 interacts with the phosphate backbone of its DNA target molecule. These findings can be interpreted in the light of the discovery of the conserved structure of Rel/NF- κ B molecules (Chen et al., 1998c; Chen et al., 1998a; Cramer et al., 1997; Ghosh et al., 1995; Müller et al., 1995).

4.2.1 Overall NF- κ B•DNA structure

The members of the Rel family of proteins (p50, p52, p65, c-Rel, RelB) form most of the possible homo- and hetero-dimer combinations, both when they are free proteins or bound either to DNA or I κ Bs. All Rel family members contain a conserved region of approximately 300 amino acid with a pairwise identity of around 50%, termed Rel Homology Region (RHR) (Figure 7).

Crystal structure analysis revealed the structure of the RHR of NF- κ B (p50)₂ (Ghosh et al., 1995; Müller et al., 1995), (p52)₂ (Cramer et al., 1997), (p65)₂ (Chen et al., 1998c), and p50•p65 (Chen et al., 1998a) bound to κ B motifs. There were similarities and differences between the approaches and the results of these studies. All groups used NF- κ B proteins expressed in, and purified from, *Escherichia coli*. While some groups used human NF- κ B gene products, such as human p50 (Müller et al., 1995) and human p52 (Cramer et al., 1997), other groups used the murine NF- κ B homologues, such as mouse p50 (Ghosh et al., 1995), mouse p65 (Chen et al., 1998c) and mouse p50 and p65 (Chen et al., 1998a). The length of the purified NF- κ B molecules was not the same. The reason for omissions of certain parts (e.g.

N-terminus of NF- κ B prior to the N-terminus of RHR, the NLS in the end of the RHR C-terminus and the C-terminal transactivation domain of p65) was the fact that they were either out of the RHR and/or that they appeared unstructured in previous studies. In one case, the human p50 contained a C62A mutation (Müller et al., 1995). Previous site-specific mutagenesis experiments had shown that the sulphhydryl group of C62 is an important determinant of DNA recognition by the p50 subunit of NF- κ B (Matthews et al., 1993a), thus the introduced mutation abolished a very important interaction between p50 and its target DNA.

All studies showed that the overall structure of NF- κ B•DNA complexes is consistent, and has a butterfly shape in which protein domains resembling the wings, clamp a cylindrical body of DNA (Figure 48). Each NF- κ B subunit consists of two flexibly linked immunoglobulin-like domains (β -barrel), that are linked by a flexible 10 residue hinge. The 200-residue N-terminal region is based on an I-type Ig barrel with the addition of a partly α -helical subdomain of various length and low homology which is not part of the RHR consensus, termed Insert Region, while the 100-residue C-terminal domain is a C-type Ig barrel (Müller and Harrison, 1995) (Figure 49). While the C-terminal domain is only required for dimerisation (Huang et al., 1997), the entire RHR is necessary for DNA binding, in contrast to many other proteins where relatively small regions are needed for DNA binding. Another difference between NF- κ B and most DNA binding proteins, is that while the latter use either small α -helices or β -sheets to recognise their DNA targets, each NF- κ B subunit contacts DNA through 5 flexible loops which connect the β -sheets. Two of these loops are in the N-terminal domain (L1 and L2), two are in the C-terminal domain (L4 and L5) and the other one (L3) is the flexible linker connecting the two domains. These flexible loops combine to form an extensive protein-DNA interface, interacting with the functional groups of the major groove and the bordering sugar-phosphate backbone, making about 38 individual contacts with target DNA, leaving only the minor groove free (Figure 48B), so, minor groove group proteins, like high-mobility group protein I(Y) (HMG I(Y)), can still bind to DNA along with NF- κ B.

Figure 48: Views of the (p50)₂•DNA complex. **(A)** Loop L1 penetrates deep into DNA (in orange), making site specific contacts. From this angle (the axis of DNA is perpendicularly related to the picture), DNA appears to be clamped all over by 10 loops and that the cysteine residues in the tip of the recognition loops (in green) are in close proximity. **(B)** In fact, by viewing the complex from the area in the insert region, it is clear that each p50 subunit only interacts with half of the DNA target (half site, in red/pink), leaving the minor groove accessible to other transcriptional factors which can anchor to the α -helices of the insert region, and that the two cysteine residues which can form S-S bond in oxidising conditions in the absence of DNA, are 20Å away.

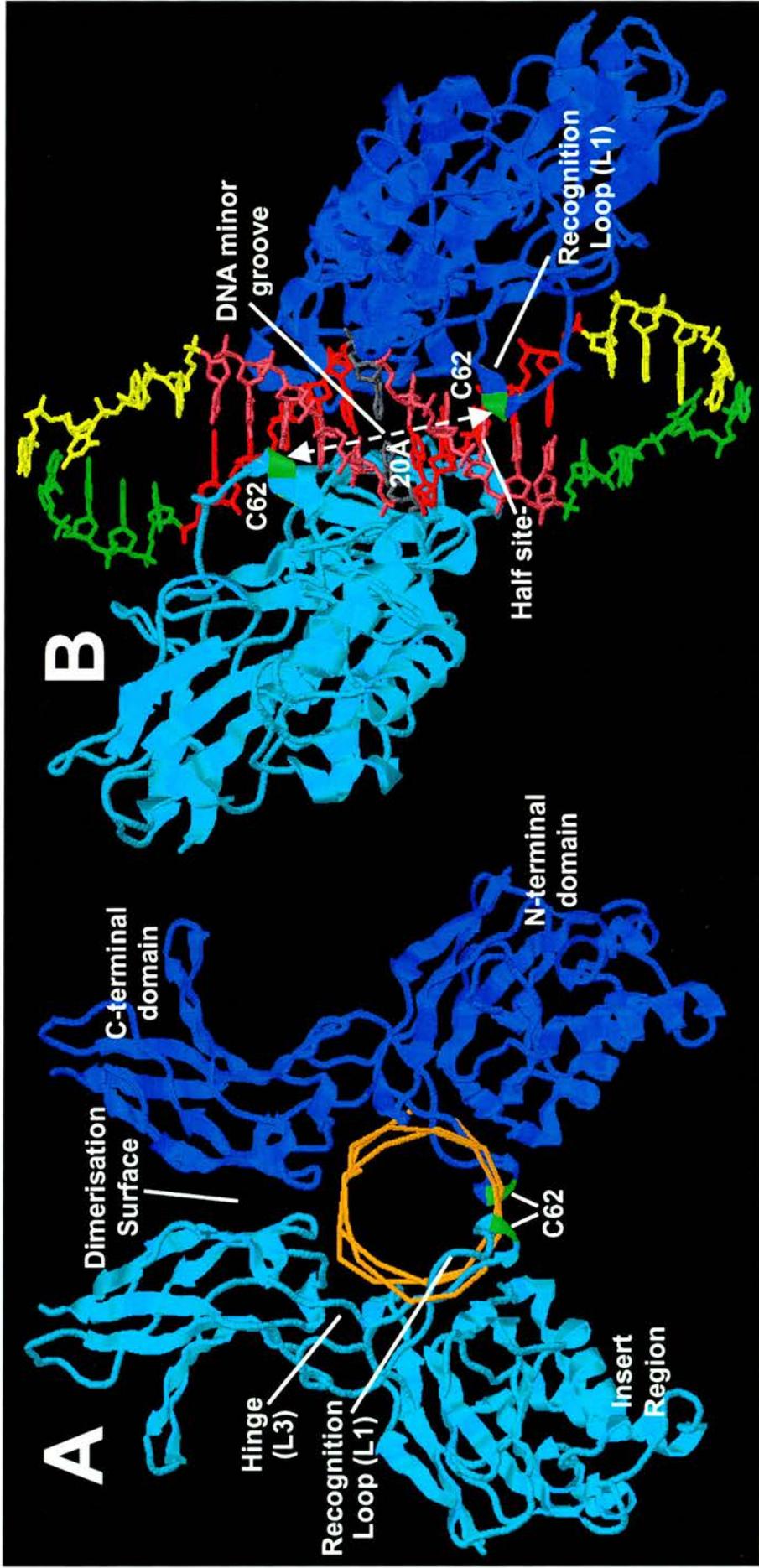


Figure 49: Views of the structure of a p50 subunit bound to DNA. **(A)** Each p50 subunit consists of two domains. The N-terminal domain also contains two α -helices in a non-conserved region (Insert Region). **(B)** Each domain comprises a β -barrel. β -sheets are connected through loops. Five of them (L1-L5) interact with DNA. L1 and L2 are in the N-terminus, L4 and L5 in the C-terminus and L3 is the hinge of the two domains. **(C)** Positions of K77 (in blue), K79 (in brown) and K80 in the c-terminus of the L1 loop.

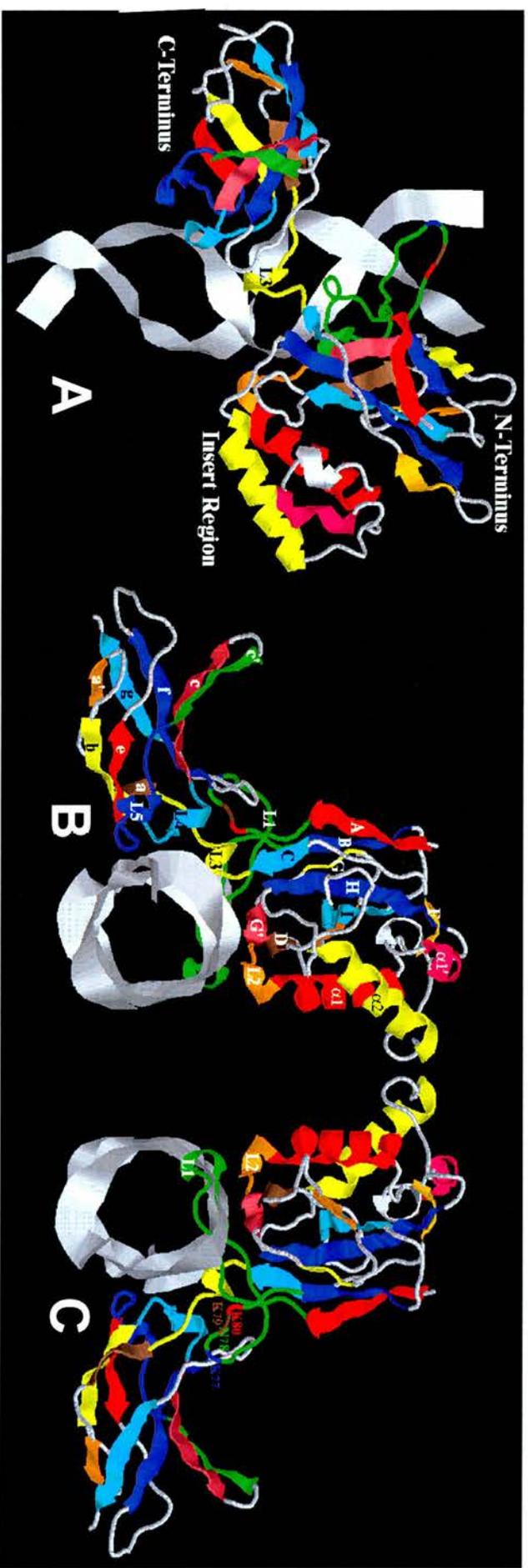


Figure 50: Mismatched bases cause a major distortion of the double helix of DNA of the p50•DNA complex. The distance between complementary bases is not more than 3 Å, while in this case the distance of the mismatched bases is more than 8 Å. Interestingly, K80 of the lysine cluster of the C-terminus of L1 loop, is in close proximity with the DNA backbone.

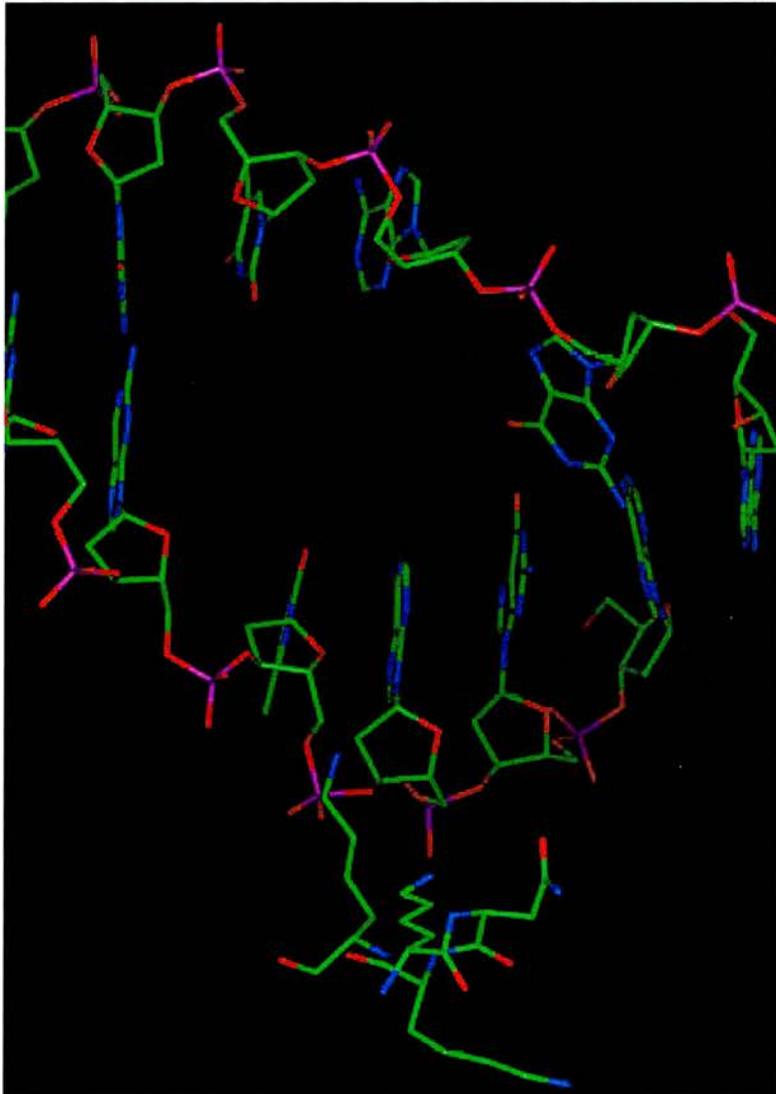


Table 5: Double stranded oligonucleotides used in the crystallographic studies of various NF- κ B molecules. Nucleotides in bold comprise κ B sites. Underlined nucleotides comprise κ B half sites while non-underlined nucleotides within κ B sites are central T:A dyads which separate the κ B subsites. Nucleotides in italics are mismatched and/or disordered.

p50 (Human)	5' AGAT <u>GGGGAATCCCC</u> TAGA 3'
	3' AGAT <u>CCCCTAAGGGG</u> TAGA 5'
p50 (Mouse)	5' <u>TGGGAATTCCC</u> 3'
	3' <u>CCCTTAAGGGT</u> 5'
p52	5' <u>TTGGGGATTCCCC</u> 3'
	3' <u>ACCCCTAAGGGT</u> 5'
p65	5' CGGCTG <u>GAAATTTCC</u> AGCCG 3'
	3' GCCGAC <u>CTTTAAAGG</u> TCGGC 5'
p50•p65	5' TG <u>GGGACTTTCC</u> 3'
	3' <u>CCCTGAAAGG</u> A 5'

The loops make base contacts with their target DNA bases, which determine NF- κ B specificity and sugar/phosphate contacts which contribute to the binding energy of the complex. All these contacts give the complex an affinity which is higher than the affinity of most eukaryotic transcription factors. This strategy for DNA recognition, in which an immunoglobulin fold (Bork et al., 1994) acts as a scaffold for the DNA contacting flexible loops, is also employed by p53 (Cho et al., 1994), STAT-1 (Chen et al., 1998b) and NFATC1 (Zhou et al., 1998), even although there is little recognisable sequence homology.

The crystallographic data show that all NF- κ B molecules interact with DNA over a complete turn. The most important NF- κ B—DNA interaction is between the N-terminus of the L1 loop that connects A and B β -sheets, and the bases of the major groove of target DNA. This part of the AB loop is termed the "recognition loop", because its interaction with DNA determines the sequence specificity of the different NF- κ B species. In the structural studies of the different NF- κ B•DNA complexes, the DNA targets formed slightly different κ B sites: The fully palindromic 10-mer oligonucleotide 5'-GGGAATTCCC-3' for the murine p50, the 11-mer 5'-GGGGAATTCCCC-3' (the underlined base was mismatched) comprising a site similar to MHC H-2 κ B except for an A:A mismatch at its very centre for the human p50 (according to binding studies, this mismatch has little effect on affinity, and analysis of crystals with p50 bound to target DNA with a central A:T base pair, prove that the mismatch mentioned above did not cause any significant distortion), the 11-mer 5'-GGGGATTCCC-3' comprising an MHC- κ B site for human p52, a 10-mer 5'-GGAAATTTCC-3' similar to IFN- γ - κ B for the murine p65 and the 10-mer 5'-GGGACTTTCC-3' comprising the Ig- κ B site for the p50•p65 (Table 5). In the case of the human p50, a 19-mer was used as a DNA target. The sequences that flanked the κ B site were mismatched (Table 5) and the double helix was heavily distorted (the distance of the mismatching bases is more than 8.5 Å, while the distance of opposite complementary bases in the canonical DNA double helix is not more than 3 Å) (Figure 50).

The DNA molecules of the complexes are slightly unwound and bent, but not severely distorted. It was initially thought that this was only induced by the interaction with the NF- κ B loops. Nevertheless, NMR analysis of a 16 bp dsDNA containing the HIV-1- κ B/Ig- κ B site and a mutated site that is not recognised by NF- κ B proteins, uncovered that the phosphate backbone of the sequences that flank a κ B motif can force this piece of DNA to adopt a dynamic conformation (intrinsic global curvature towards the major groove), which is very favourable for binding of the NF- κ B loops (the mutated duplex, in contrast, had a canonical conformation) (Tisné et al., 1999a; Tisné et al., 1998; Tisné et al., 1999b). The flexibility of both NF- κ B dimers and their cognate DNA enables NF- κ B to adopt multiple conformations in a promoter specific manner.

4.2.2 Site specificity

In all the crystal structures solved, each subunit contacts both DNA strands over a complete turn (10bp). The sequence specific contacts of each subunit, though, involve only half of the DNA target. Each subunit makes DNA specific contacts only with a half site, with residues from the loops L1 and L3, while the half site spacing is preferably one central A:T base pair (the A:T base pairing involves two hydrogen bonds, instead of a G:C which involves three, and that enables the widening of the DNA major groove which is essential for base specific contacts). The most important amino acids in L1 (recognition loop) seem to be the conserved residues R57, R59 and E63 (human p50 numbering). R57 contacts G³ and R59 G⁴ (numbering upstream starting from the first base adjacent to the central half site spacer base pair). The whole unit is stabilised by salt contacts of these two arginines with E63. This network recognises a 5'-G⁴G³-3' sequence in all Rel related proteins. p50 H67 and its p52 equivalent can recognise G⁵ while this residue in p65 is A43 (mouse p65 numbering). The lack of such interaction in p65, is responsible for the fact that while p50 and p52 optimally recognise five base pairs, p65 recognises only four. In MHC- κ B, p50 K244 of L3 contacts G² and A¹, while its homologous residue in p65, R187, anchored by E39, can only interact with the complementary T to A². That is why p50 prefers G²

whereas p65 prefers A². A conserved tyrosine (Y60 in p50, Y55 in p52 and Y36 in p65) makes van der Waals contacts with bases at positions 2' and 1'.

It seems that the interaction between the G³:C³, R33 and E39 is the most important p65 specificity determinant. That is why all sites that can be specifically recognised by p65 (and c-Rel) contain at least one 5'-G⁴G³A²A¹-3' cognate half site and a conserved G³ in the other half site (an A³:T³ base pair causes so unfavourable interactions that abolishes the crucial sequence specific interaction of the bases with the charged amino acids R33, R35, E39 of L1 and R187 of L3, leaving only the van der Waals interactions of Y36). In the absence of G³ on the other half site, the N-terminal domain is rotated by almost 20° from its normal conformation. Interestingly, it still maintains all its non sequence-specific sugar/phosphate backbone contacts with DNA. It was thus proposed that NF-κB dimers could bind to DNA targets which contain only one half site. This conformation could also explain how NF-κB dimers can bind DNA in a non sequence-specific way (binding of NF-κB molecules on *E. coli* bacterial chromosomal DNA is a step for their purification): It can be suggested that such binding takes place without any interactions of the DNA bases with the L1 loop, and is based on the DNA backbone contacts of the protein. The lack of any of these base-specific hydrogen bonds would reduce the affinity of NF-κB for heterologous DNA.

On the other hand, analysis of the structure, shows that p50 consensus half site is 5'-G⁵G⁴G³R²N¹-3'. Therefore, the higher affinity for p50 subunit to its cognate sequence, than that of p65 subunit for its own half site, can be explained due to the extra DNA base contacts made by p50, since p50 interacts with a 5 bp half site while p65 interacts with one which contains 4 bp. Furthermore, the difference on the sequence and the size of the half sites of the different NF-κB subunits explains why there is no unified κB consensus sequence for all Rel related proteins. (p50)₂ and (p52)₂ ideally recognise 11-mers, like MHC-κB (5'-GGGGATTCCCC-3'), (p65)₂ and (c-Rel)₂ 9-mers, like TF-κB (5'-GGAGTTTCC-3') and p50•p65 10-mers, like Ig-κB (5'-GGGACTTTCC-3'). So, it is not of any surprise why NF-κB p50•p65 was initially

discovered for its affinity to the intronic enhancer of the κ light chain gene and why the early studies were referring to the κ B consensus as a 10-mer.

4.2.3 *Dimerisation domain*

Around 1400 Å² of solvent accessible surface area is buried in the dimer interface of p50•p50, p52•p52, p65•p65 and p50•p65. Dimerisation does not depend of DNA binding. DNA-bound and unbound form of NF- κ B dimerisation domains do not show any noticeable change (Huang et al., 1997). Similarly, the overall structure of the dimerisation domain is not altered by I κ B binding (Huxford et al., 1998; Jacobs and Harrison, 1998). The dimer interface mainly consists of a hydrophobic core. Comparing the structural data of these dimerisation domains, we can understand that even if the overall structure of the C-terminal domain of p50, p52 and p65 is very similar (their backbones can be superimposed), the chemistry of the dimerisation of the interfaces of the 15 homo- and heterodimers, is different. It is also known, for instance, that the heterodimers p50•p65 and p52•p65 are thermodynamically preferred over p50•p50, p52•p52, and p65•p65 homodimers. Some asymmetries in the heterodimer interfaces may lead to conformational adjustments. For instance, the hydrogen bond between p50 D254 and p65 N200 appears only in p50•p65 heterodimer, because in p50•p50 and p65•p65 homodimers the interaction between homologous residues juxtaposes two like charges and thus it is not energetically favourable. This is supposed to be more intense in the case of RelB, where the two asparagine residues do not allow the formation of RelB homodimers, whereas a N→D RelB mutant can form homodimers, in a manner similar to p50 (Ryseck et al., 1995). On the other hand, based on sequence homology, it is proposed that c-Rel forms homodimers in the same manner with p65. Another nine contacts of side chains of uncharged polar residues that point from the β -sheets in towards the dimerisation interface, also contribute to the stronger affinity of p50•p65 heterodimers than that of p50 and p65 homodimers. All those are in accordance with our experiments, where mixture and incubation of equimolar amounts of p65 and p50 homodimers could form exclusively p50•p65 heterodimers.

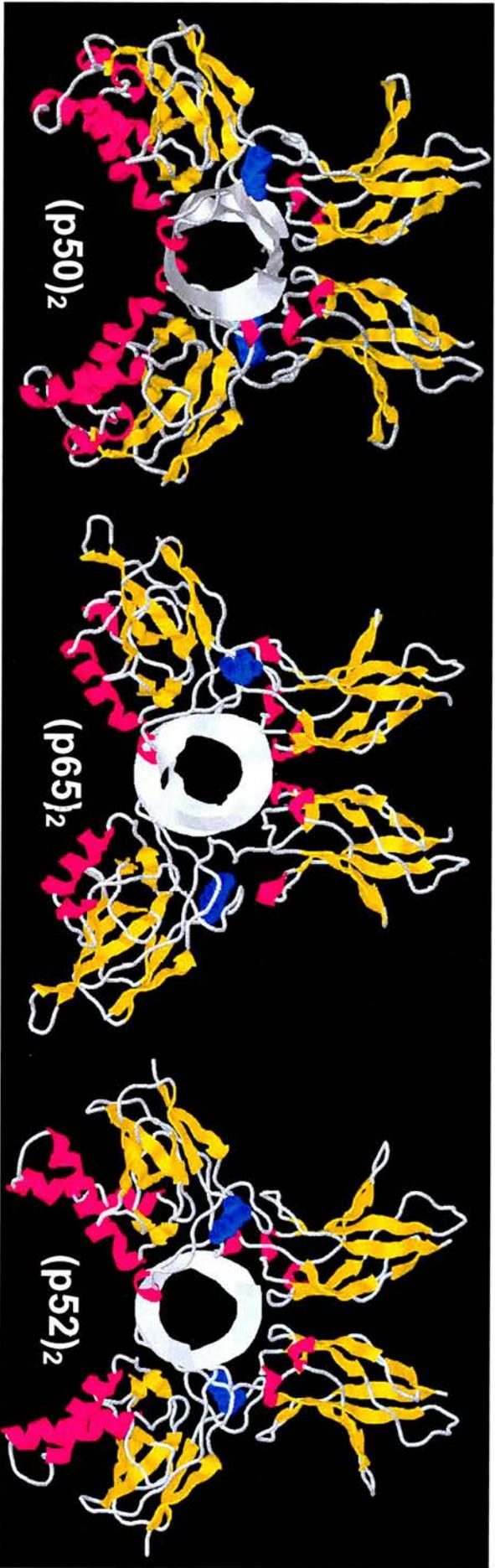
4.2.4 Insert Region

The only major difference between the NF- κ B p50, p52 and p65 subunit structures is in the "insert region" (Figure 48A and Figure 51). The insert region is located immediately after L2 loop of the N-terminal domain of the NF- κ B molecules. Its sequence homology is poor amongst different Rel family members and its length varies (66 residues in p50, 47 in p52, 34 in p65, and 36 in Dorsal), therefore it is not considered part of the RHR sequence. Moreover, while p50 and p52 insert region consists of two structurally conserved α -helices (α 1 and α 2), p65 insert region contains only one helical structure (α 1) (Figure 51). The α 1 N-terminus points towards the phosphate backbone of the minor groove side. The side chains of the α -helices is charged in order to attract other proteins. The insert region interacts with other DNA binding proteins. The differences of this region between all NF- κ B molecules, can lead to interactions with different transcription activators and/or coactivators, e.g. since the minor groove of DNA is not shielded by NF- κ B (Figure 48A), the insert region can be used for the interaction with the high mobility group protein I(Y) (HMGI(Y)) which contacts the minor groove at the AT-rich centre of the IFN β - κ B site (Thanos and Maniatis, 1992). Furthermore, NF- κ B molecules interact with basic region-leucine zipper (bZIP) families (Nolan, 1994; Stein et al., 1993a; Stein et al., 1993b). NF-ATc is a NF- κ B related monomeric transcription factor. NF-ATc homologous region to the NF- κ B insert region, is responsible for the interaction of NF-ATc with the bZIP transcription factor AP-1 in the interleukin-2 enhancer region (Wolfe et al., 1997).

4.2.5 Interaction with other transcription factors

Apart from the interactions through the insert region, NF- κ B molecules can interact with other transcription factors. It is generally accepted that dimers containing at least one Rel-related NF- κ B subunit (i.e. c-Rel, p65, RelB which contain a transactivation domain C-terminally of their NLS), can interact with the transcription machinery, while (p50)₂, (p52)₂ and p50•p52 which lack this region are considered repressors. The DNA sequence-induced changes of the protein structure can also modulate the

Figure 51: Crystal structure of three NF- κ B homodimers (p50)₂, (p65)₂ and (p52)₂ bound to DNA (the direction of the DNA axis is perpendicular to the picture surface). The structure of the RHR of all NF- κ B homo- and heterodimers is very similar, as far as the structure of the β -barrels is concerned. β -barrels from different NF- κ B molecules can be well superimposed. Interesting differences in the structures is the number of α -helices in the insert region (two in p50 and p52 and one in p65, shown in all structures as pink α -helices) and the distance of the conserved lysine (K80 in p50, shown in blue in all structures) of the C-terminal part of the L1 loop, which is in close proximity with the DNA phosphate backbone in p50 and p52 but 11 Å away from it, in p65.



interaction with various transcription factors and thus cause activation differences (Menetski, 2000).

4.2.6 Redox regulation of NF- κ B binding

The knowledge of the structure of (p50)₂•DNA complex can explain the biochemical findings which suggested that the conserved among all Rel family members C62 would play a critical role in both the determination of p50 DNA sequence specificity and the redox regulation of p50 DNA binding (Matthews et al., 1993a; Matthews et al., 1992). When p50 is bound to DNA, the distance between the C62 residues of each subunit is 20 Å (Ghosh et al., 1995) (Figure 48B). C62 appears to be in the tip of the N-terminal part of L1 (Figure 48A) and contacts the sugar-phosphate backbone and other amino acids through van der Waals and hydrogen bonds (cysteine residues with such properties also interact with the phosphate backbone of DNA in Jun and Fos complexes). These cysteine residues though, in the absence of DNA and in oxidative conditions can form inter-strand disulphide bonds. This would lock the N-terminal "jaws", preventing the clamping of DNA and thus inhibiting DNA binding activity. Interestingly, NF- κ B is induced by oxidative signals, provided that the cell is able to retain reducing conditions in the intracellular environment (e.g. through the glutathione system).

The ability of the N-termini to move through the hinge, also proves the high degree of flexibility of the binding of the two N-terminal domains to the C-terminal dimerisation domains through the L3 loop. This flexibility is also demonstrated in the apparent difference of the angle of the axis of the N-terminus which is dictated by the sequence of the DNA target (and especially from the size of the half-site spacing).

4.2.7 The role of the C-terminus of the L1 loop in DNA binding

Previous biochemical experiments suggested an interaction between the C-terminus of the AB loop of (p50)₂ and DNA (Bell et al., 1996; Hay and Nicholson, 1993; Matthews et al., 1995). Although residues K77, K79 and K80 were thought to be involved, the resolution of the techniques employed were such that the contribution of individual residues to the interaction could not be determined. Site directed

mutagenesis of this region was therefore employed to remove functional groups (K to A changes) and thus identify lysine residues in contact with DNA. The various mutants were placed in a rank order of DNA binding affinity using Surface Plasmon Resonance (SPR). These experiments demonstrated that the residue involved in the DNA contact is predominantly K80, although K77 may also play a role (Michalopoulos and Hay, 1999). Partial proteolysis analysis of the wt p50 and the triple mutant showed that the defect of the binding is not due to major conformational changes induced by introduction of the K to A changes.

The first piece of evidence supporting the role of K80 in this interaction is its conservation among all of the NF- κ B/Rel/Dorsal molecules. The only change is in human c-Rel where there is an Arginine instead of a Lysine, suggesting that the interaction is due to the positive charge of the amino acid (the chicken c-Rel contains Lysine) (Michalopoulos and Hay, 1999) (Figure 52). The only other NF- κ B protein containing three positively charged amino acids in that loop is the p50-related protein p52.

The crystallographic data demonstrate how the lysine cluster on the C-terminus of L1 loop could interact with DNA, since there are another two lysine clusters in the tips of L2 and L4 loops. The DNA contacts mentioned in section 4.2.2, determine DNA sequence specificity of the different NF- κ B molecules, and play an important role in the stabilisation of the NF- κ B•DNA complexes. The other type of contacts which are significant for the affinity of Rel related proteins to DNA are the non sequence specific sugar-phosphate backbone contacts (2/3 of the phosphates of the κ B site are contacted by at least one hydrogen bond. Almost all these contacts are preserved among the Rel family members: p50 Y60, C62 in L1, K147, K148 in L2, Q309 in L5 and the homologous residues in p65 and p52 interact with the sugar/phosphate backbone through hydrogen bonds or salt bridges. Similarly, p50 R308 in L5 and its p65 homolog make contacts to the DNA backbone, while their homologs in p52 do not make any DNA interactions. p50 K275 and Q277 in L4 and their p52 homologs interact with DNA, while p65 L4 interacts with DNA backbone through K221 which

Figure 52: Conservation of K80 among NF- κ B/Rel/Dorsal proteins. Multiple sequence alignment of p50, p52, c-Rel (human and chicken), v-Rel, RelA, RelB, Dorsal and Dif. The arrow points to the lysine residue in NF- κ B/Rel/Dorsal proteins (K80 in p50).

p50
 p52
 Human c-Rel
 Chicken c-Rel
 v-Rel
 RelA
 RelB
 Dorsal
 Dif

D	G	P	Y	L	L	O	I	L	E	Q	P	K	Q	R	G	F	R	F	F	Y	V	C	E	G	P	S	H	G	G	L	P	G	-	A	S	S	E	K	N	K	K	S	Y	P	Q	V	A	I	C	N		
D	G	P	Y	L	L	V	I	V	E	Q	P	K	Q	R	G	F	R	F	F	Y	V	C	E	G	P	S	H	G	G	L	P	G	-	A	S	S	E	K	N	K	K	S	Y	P	Q	V	A	I	C	N		
Y	N	P	Y	I	I	E	I	I	E	Q	P	R	Q	R	G	M	R	F	F	Y	K	C	E	G	R	S	A	G	S	I	P	G	-	E	H	S	T	D	N	N	N	R	T	F	P	S	I	Q	I	L	N	
S	E	P	Y	I	E	E	I	F	E	Q	P	R	Q	R	G	T	R	F	F	Y	K	C	E	G	R	S	A	G	S	I	P	G	-	E	H	S	T	D	N	N	N	R	T	F	P	S	I	Q	I	L	N	
L	F	P	L	I	F	P	A	E	E	Q	P	K	Q	R	G	M	R	F	F	Y	K	C	E	G	R	S	A	G	S	I	P	G	-	E	H	S	T	D	N	N	N	R	T	F	P	S	I	Q	I	L	N	
P	R	P	Y	L	V	I	I	T	E	Q	P	K	Q	R	G	M	R	F	F	Y	E	C	E	G	R	S	A	G	S	I	P	G	-	V	N	S	T	D	N	N	N	R	T	F	P	S	I	Q	I	L	N	
K	K	P	Y	V	W	I	I	T	E	Q	P	A	G	K	A	L	R	F	F	Y	E	C	E	G	R	S	A	G	S	I	P	G	-	V	N	S	T	D	N	N	N	R	T	F	P	S	I	Q	I	L	N	
S	G	P	H	L	F	I	V	I	V	E	P	T	S	N	I	L	I	R	F	F	Y	K	C	E	G	R	S	A	G	S	I	P	G	-	M	N	S	S	E	K	N	K	K	S	Y	P	Q	V	A	I	C	N



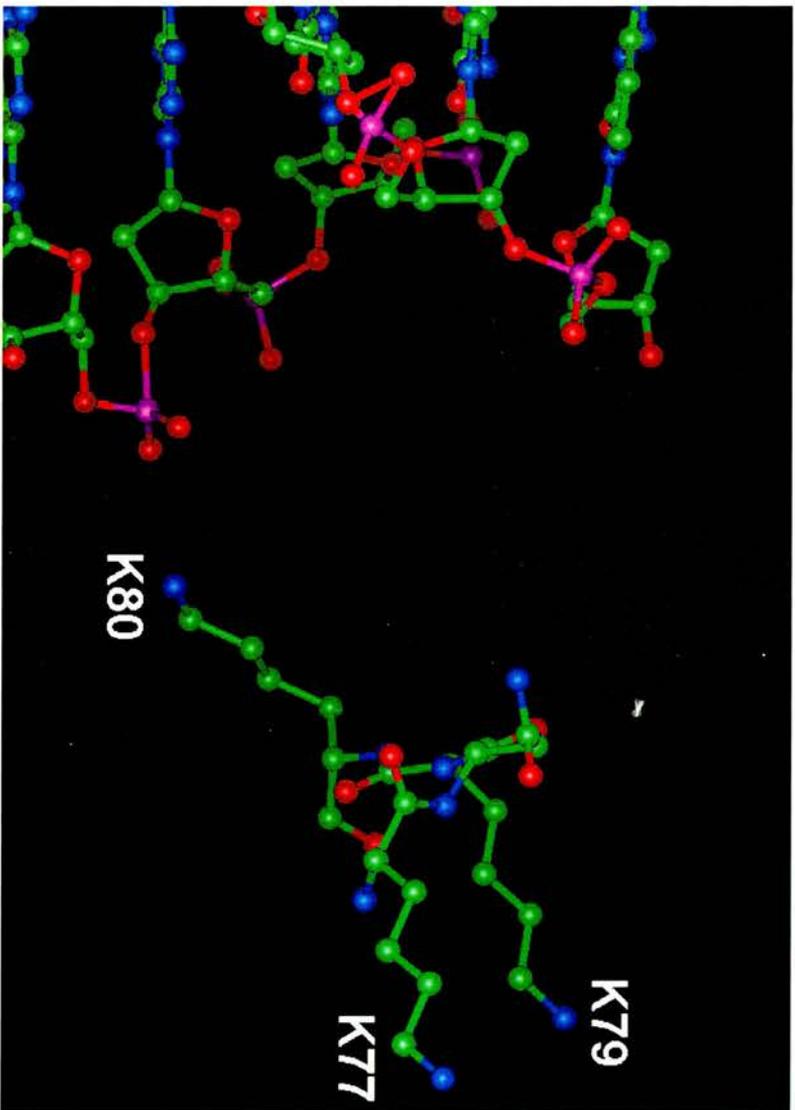
is homologous to p50 K278. p50 also contacts DNA through H144 in L2. This contact is not shown in the homologous His residue of p52, while the equivalent amino acid residue in p65 is conserved among Rel-related NF- κ B proteins C120 which does not make any interaction with DNA. All these data imply a role for the L1 loop lysine cluster in contacting the phosphate backbone of the target DNA. Furthermore, the observation that some NF- κ B molecules use the first and others the second of the two lysine residues of L4 to contact the DNA backbone, shows that neighbouring lysine residues can play similar roles one in the place of the other, thus demonstrating how K77 can partly substitute K80 in the K80A mutant, through adjustments.

The crystallographic data are also consistent with a DNA contact beyond the consensus sequence. However this interaction was not clearly resolved because the C-terminal part of the L1 loop is not highly ordered, and because of the small size of double stranded part of the oligonucleotides that were used to form the co-crystals (Chen et al., 1998a; Ghosh et al., 1995; Müller et al., 1995). More precisely, a double stranded 10-mer was used for the murine (p50)₂•DNA complex (Ghosh et al., 1995), a double stranded 11-mer for the p50•p65•DNA complex (Chen et al., 1998a) and a double stranded 11-mer for the human (p50)₂•DNA complex which was flanked by 4 mismatched base pair sequences (Table 5). Furthermore, it was shown that p50 affinity for a long oligonucleotide (16mer) was higher than of that for a shorter one (12mer), which corresponded to the oligonucleotide used in the crystallographic data (Bell et al., 1996). Analysis of the crystal structure of (p50)₂•DNA (Ghosh et al., 1995; Müller et al., 1995), indicated that although the two crystal structures were quite similar, they had a fundamentally different relationship to their DNA targets (Baltimore and Beg, 1995), as a consequence of the flexibility of the NRD contacts with DNA. This flexibility is due to the adjustability of the loops and the hinge (which links the two domains of each subunit) which allows recognition of different but related DNA sequences. One explanation for the apparent differences between the two structures is the slight differences of the DNA sequences of the double stranded parts of the two oligonucleotides: 5'-GGGAATTCCC-3' (Ghosh et al., 1995) and

5'-GGGGAATCCCC-3' (Müller and Harrison, 1995). Another reason is the slight difference of the length of the double stranded parts of the oligonucleotides: 10mer (Ghosh et al., 1995) and 11mer (Müller et al., 1995). This small difference of the size of the oligos played a dramatic role on the conformation of the AB (L1) loop in the two structures. While the 5' end of the double stranded 10mer is far from the C terminus of AB loop, the phosphate backbone beyond the duplex of the 19mer is in close proximity (2.87Å) with K80 of the loop (Figure 50). This interaction affects the conformation of the lysine cluster of the loop. In the complex of human p50 with the longer double stranded oligonucleotide, the lysines of the loop have different orientations than in that with the shorter one. In both structures the side chain of K79 points away from the target DNA molecule and K80 is ideally positioned to directly contact it. While the K77 side chain is positioned facing DNA in the complex with the 10mer, it has the opposite orientation in the complex with the 11 base pairs.

Even if the interaction of the L1 C-terminus was shown, in the case of the human (p50)₂•DNA complex, the significance of those data was not clear as the interaction occurred in a region of heavy DNA distortion, where DNA is not properly annealed (Figure 50). Using molecular modelling, the region of the oligo used in that crystallographic study (Müller et al., 1995) with these 4 distorted base pairs was replaced with a 4 base pair canonical B-form DNA. This clearly showed that the nitrogen atom of the positively charged K80 -NH₂ group is in close proximity (2.7Å) to the oxygen atom of the negatively charged phosphate backbone of DNA. While K80 was ideally positioned to contact the phosphate backbone, K77 and K79 were pointing away from the DNA (Michalopoulos and Hay, 1999) (Figure 53). In the other report (Ghosh et al., 1995), K77 is not ideally placed to make an interaction but, it could also make a phosphate back bone contact with minor adjustments. Therefore, molecular modelling suggested the interaction between K80 and DNA backbone, beyond the consensus sequence, since the positioning and the distance of K80 from the DNA backbone was ideal. The apparent differences in the positioning of K77 in

Figure 53: Structural representation of the interaction of the p50 sequence 77-80 (Lysine cluster of the C-terminal AB Loop) with the 4 base pair computer extended double stranded DNA. Orientation of the Lysine cluster: K80 is ideally positioned, because its ϵ -NH₂ group is 2.7Å from the DNA phosphate backbone. K77 and K79 point away from the DNA backbone.



the two studies also explained the important but not critical role of K77 in the interaction with DNA.

While the C-terminus of the p52 L1 loop has a very similar structure (Cramer et al., 1997) to its p50 homologue (the homologue to p50 K80 lysine residue of p52 points towards the 5' free phosphate, since the oligonucleotide used was short again), the same loop in p65 homodimers bound to a palindromic DNA 20-mer is at least 11Å from the DNA backbone (Chen et al., 1998c), although this may be a reflection of the complex and novel way that (p65)₂ binds to DNA. This loop could have other functions that are not related to DNA binding but may serve as a target for IκBα recognition, in the same way that the p50 homologous loop serves as a target for IκBγ recognition (Bell et al., 1996).

4.3 NF- κ B-I κ B interactions

After the discovery of the interaction of p50 K80 with the phosphate backbone of DNA, our objective was to clarify the role of the L1 C-terminus in the interaction between p50 and its inhibitor protein I κ B γ . Protein footprinting by chemical modification (Bell et al., 1996) suggested that the p50 L1 lysine cluster (K77, K79 and K80) could contact I κ B γ . Once again, although the C-terminus of L1 loop was implicated the techniques employed did not have sufficient resolution to identify the lysine residue(s) making the contact with I κ B γ (Bell et al., 1996). One of the objectives of this study was therefore to use site directed mutagenesis to investigate the role of each lysine in the sequence between 77 and 80. Our experiments demonstrated that K77, together with the conserved K80, interact with I κ B γ . We also determined the stoichiometry of the p50•I κ B γ complex and suggested how the complex might behave in solution. These findings can be interpreted in the light of the discovery of the structure of p50•p65•I κ B α complex (Huxford et al., 1998; Jacobs and Harrison, 1998) and by combining various biochemical data on different NF- κ B•I κ B complexes.

4.3.1 Ankyrin repeat domain structure

As mentioned elsewhere, members of the I κ B family of proteins contain a homologous region of 6 or 7 tandem ankyrin repeats (Ghosh et al., 1990; Kieran et al., 1990) (Figure 8). An ankyrin repeat is a 33 amino acids motif which is present in erythrocyte membrane-associated protein ankyrin, in Notch transmembrane protein of *Drosophila melanogaster* and in the proteins cdc10/SWI6 that control the yeast cell-cycle (Bennett, 1992). The periodicity of the structure of ankyrin was already known even before its sequence analysis, as electron microscopy of erythrocyte membranes uncovered that ankyrin was a long banded molecule (Margaritis et al., 1977). The size of the repetitive black and white bands corresponded to the size of an ankyrin repeat. Crystal structure analysis of the first ankyrin repeat containing proteins (p53-binding protein 2 (53BP2) (Gorina and Pavletich, 1996), the β subunit of GA-binding protein

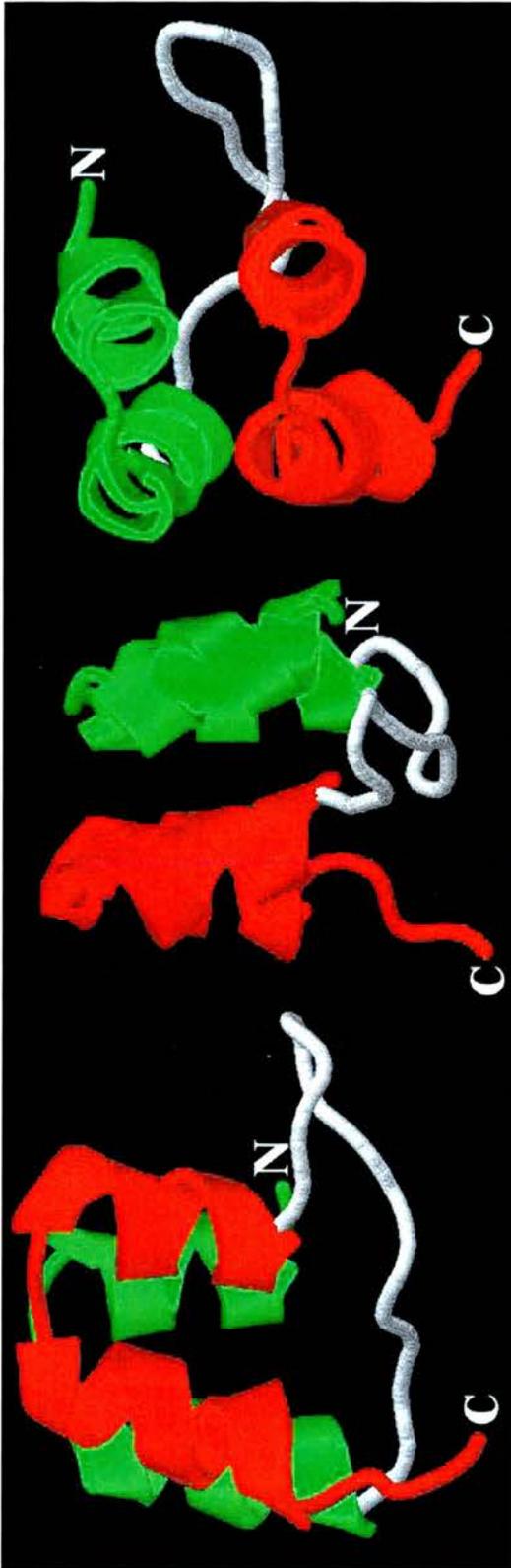
(GABP β) (Batchelor et al., 1998) and the CDK4/6 inhibitory protein p18^{INK4c} (Venkataramani et al., 1998)) revealed that its L-shaped structure consists of a β hairpin (an extended loop that contains a type I β turn in its tip) which is perpendicularly related to two α helices that form an antiparallel coiled-coil (Figure 54). The β hairpin is linked to the first helix with one amino acid. The packed repeats form a continuous antiparallel β sheet via their hairpins and helix bundles via their coiled-coils. The plain of the β sheet is perpendicular to the axes of the α helices. It is not likely that an ankyrin motif can fold stably in an isolated peptide sequence, as its folding also depends of the neighbouring repeats (the hydrophobic residues of the α helices form intra- and inter-repeat van der Waals interactions, while the conserved histidine residues contribute to the inter-repeat stabilisation through a network of hydrogen bonds). Other conserved amino acids are glycines (they facilitate the formation of sharp turns in the tips of β hairpins and the termination of α helices), threonines (they often initiate α helices), leucines and alanines (contribute to the stabilisation of the helix bundles). Although these amino acids are conserved among some repeat units of a variety of ankyrin repeat containing proteins, they are not uniformly conserved in each ankyrin repeat, which proves that the structures containing ankyrin repeat stacks tolerate small deviations from the consensus sequence.

The vertical spacing of the helices in close proximity to the β hairpins is smaller than that of the helices that are away from the β hairpins, causing a curvature of the ankyrin repeat stack. All the crystallographic data show that ankyrin repeats mediate protein interactions through the β hairpin protruding tips, although the helix bundles may also offer possibilities for various macromolecular interactions.

4.3.2 *NF- κ B/I κ B α complex structure*

The revelation of the novel L-shaped structure of the individual ankyrin repeat, together with sequence alignment analysis, prompted the prediction of the structure of I κ B molecules, so that even before the crystallographic data of I κ B α , experiments of swapping ankyrin repeats between the I κ Bs were performed and demonstrated that the

Figure 54: An ankyrin repeat is an L-shaped structure which consists of a β hairpin loop which is perpendicularly related to two α helices that form an antiparallel coiled-coil.



first ankyrin repeat of I κ B α confers strong inhibitory properties on I κ B β and I κ B ϵ (Simeonidis et al., 1999). Data like that should be treated though with extreme caution, as swapping of these highly packed modules might abolish highly important inter-repeat interactions (the recombination technique is far more reliable when distinct domains are swapped). The regions of NF- κ B molecules that were required for interaction with I κ Bs were already known before crystal structure analysis of I κ B α /NF- κ B: The NLS peptide of various NF- κ B molecules (Beg et al., 1992; Inoue et al., 1993; Malek et al., 1998; Matthews et al., 1993b), the same surface of NF- κ B p50 that is occupied, according to crystal structure data, by specific DNA (Bell et al., 1996) and the C-Terminus of AB loop (Bell et al., 1996; Malek et al., 1998) whose interaction with the DNA backbone our results demonstrated (Michalopoulos and Hay, 1999) (see section 4.2.7).

Two independent crystallographic studies of the complex between "classical" NF- κ B heterodimer and I κ B α (p50•p65•I κ B α) (Huxford et al., 1998; Jacobs and Harrison, 1998) (Figure 55) revealed mechanisms of the interaction between I κ B α •NF- κ B. Since obtaining crystals of the complexes was a complicated aim, the two laboratories selected the participating protein constructs, based on results of various proteolysis (Jaffray et al., 1995) and protein binding studies (Malek et al., 1998). The lengths of I κ B α , p50, and p65 constructs the two groups selected were similar but not identical. More precisely, using the numbering of the human homologue (in order to have direct comparisons):

Protein	(Jacobs and Harrison, 1998)	(Huxford et al., 1998)
p65	20-320 (human)	19-304 (murine)
p50	248-354 (human)	247-365 (murine)
I κ B α	70-282 (human)	67-302 (human)

The NF- κ B constructs had almost identical N-termini, in both studies. The C-termini of the murine constructs were exactly the NF- κ B NLS sequences. On the contrary, human p65 construct extended another 16 amino acids in the C-terminus of NLS, while in human p50 construct NLS was omitted. In the study of the murine

Figure 55: Different views of the crystal structure of p65•p50•IκBα. p65 RHR (in blue), p50 C-terminal (dimerisation) domain (in light green) and IκBα ARD (in green) were co-crystallised. The first two ankyrin repeats of IκBα interact with p65 NLS.



NF- κ B•human I κ B α , the I κ B α construct was 4 amino acid N-terminally, and 20 amino acid C-terminally longer, than the I κ B α molecule used in the study of the entirely human complex. So, it was rather the difference in size than the difference of the origin of the molecules, that explained the apparent major differences on the intermolecular interactions reported in the two studies: While totally 87 residues were reported to participate in protein-protein interactions, only 32 of them were common in both studies.

The combination of the structural data of the two studies revealed the nature of NF- κ B-I κ B interactions: The stoichiometry of the complex is 1 I κ B α :1 NF- κ B dimer and their chains run in an antiparallel fashion, so that the N-terminus of I κ B α is in the side of the C-terminal transactivation domain of p65 and the C-terminal PEST sequence of I κ B α interacts with the N-terminal p65 domain. This topology is consistent with the hypothesis of the autoinhibition of p105 and p100, where the part of p105 or p100 which comprises a p50 and p52 respectively, is bound to an NF- κ B subunit and the part of p105 or p100 which comprises I κ B γ or I κ B δ respectively, is folded backwards (probably in the glycine rich region) and masks the NLSs sequestering the complex in the cytoplasm. The binding interface of NF- κ B-I κ B α is extensive, but not continuous. It consists of three predominately polar patches: Ankyrin repeats 1 and 2 interact with p65 NLS and the sequence which is C-terminal to it. The β -loops of the ankyrin repeats 4-6 interact with the dimerisation domain of p50 and the C-termini of the inner helices of the ankyrin repeats 5 and 6 interact predominantly with p65, giving the complex its asymmetry. Ankyrin repeat six and the serpentine-structured C-terminal PEST-like domain of I κ B α make contacts with the N-terminus of p65. Since most of the NF- κ B residues which contact I κ B α are conserved, it was assumed that subtle differences in molecular contacts are responsible for the I κ B/NF- κ B specificity.

4.3.3 Interaction with NLS

Biochemical data had already uncovered the role of NLS in the NF- κ B-I κ B interactions, long before the discovery of the crystal structure of an NF- κ B•I κ B complex. NF- κ B NLS is required for interactions with I κ B molecules. Both I κ B α and

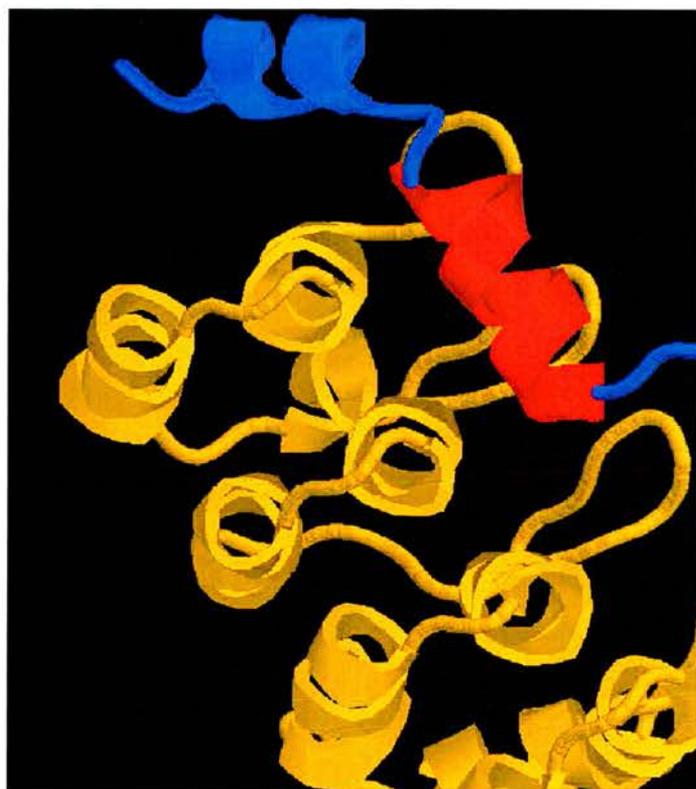
I κ B γ interact with p50 NLS (Matthews et al., 1993a). Although p50 and p65 NLS are both involved in direct interactions with I κ B α , p65 NLS•I κ B α interaction is more important (Malek et al., 1998). What was unclear before the solution of the crystal structure of the NF- κ B•I κ B complexes was the mechanism that was employed for the masking. It was eventually discovered that p65 NLS forms an α -helix when it interacts with and is masked by, the ankyrin repeats 1 and 2 (Jacobs and Harrison, 1998). Another sequence located C-terminally to the NLS which also forms an α -helix, capping ankyrin repeat 1, is necessary for the stabilisation of the helical form of NLS (Figure 56A). Interestingly, a sequence very similar to the NF- κ B NLS (from SV40) appears as an extended chain when bound to karyopherin- α (Figure 56B) and NF- κ B NLS which does not interact with DNA, is not structured in NF- κ B•DNA complexes. Therefore, it is accepted that NLS formation depends on the molecular context. On the other hand, the NLS of p50 appeared unstructured in both structural studies. Since a dimer needs at least one NLS for nuclear entry, and therefore I κ B α must mask both NLSs (Latimer et al., 1998), the crystallographic data failed to explain how p50 NLS is masked and therefore NF- κ B remains sequestered in the cytoplasm. What is proposed is that either steric hindrance by ankyrin repeat 1 and 2 and p65 NLS is sufficient for blocking p50 NLS to interact with import factors, or the N-terminal sequence of I κ B α folds back and masks p50 NLS (Latimer et al., 1998). Interestingly, our data on p50-Bcl-3 interactions and previous data on p50-I κ B γ interactions (Matthews et al., 1993a) showed that p50 NLS is necessary for DNA binding inhibition. This interaction occurs far from the site of DNA binding, therefore, it is necessary for stable anchoring of the I κ B molecule to NF- κ B, a step which is apparently a precondition for effective DNA inhibition.

4.3.4 Interaction with the NF- κ B dimerisation domain

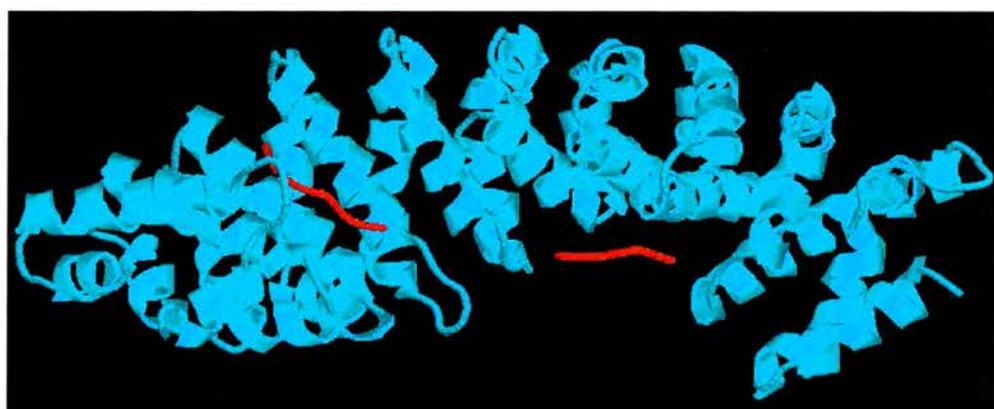
Although the overall architecture of the dimerisation domains of the p50•p65 complex is the same when NF- κ B interacts with DNA or I κ B α , there are some interesting alterations. E.g. p50 R305 and its homologous p65 R246 make non-specific DNA backbone contacts in the presence of DNA. When I κ B α is bound to p50•p65, p50

Figure 56: NLS conformation depends on its protein context. **(A)** p65 NLS (in red) forms an α -helix when it interacts with the I κ B α (in orange). **(B)** SV40 NLS appears as a linear fragment when it interacts with karyopherin- α . NF- κ B NLS is not structured when it does not interact with any other protein (e.g. when NF- κ B is bound to DNA).

A



B

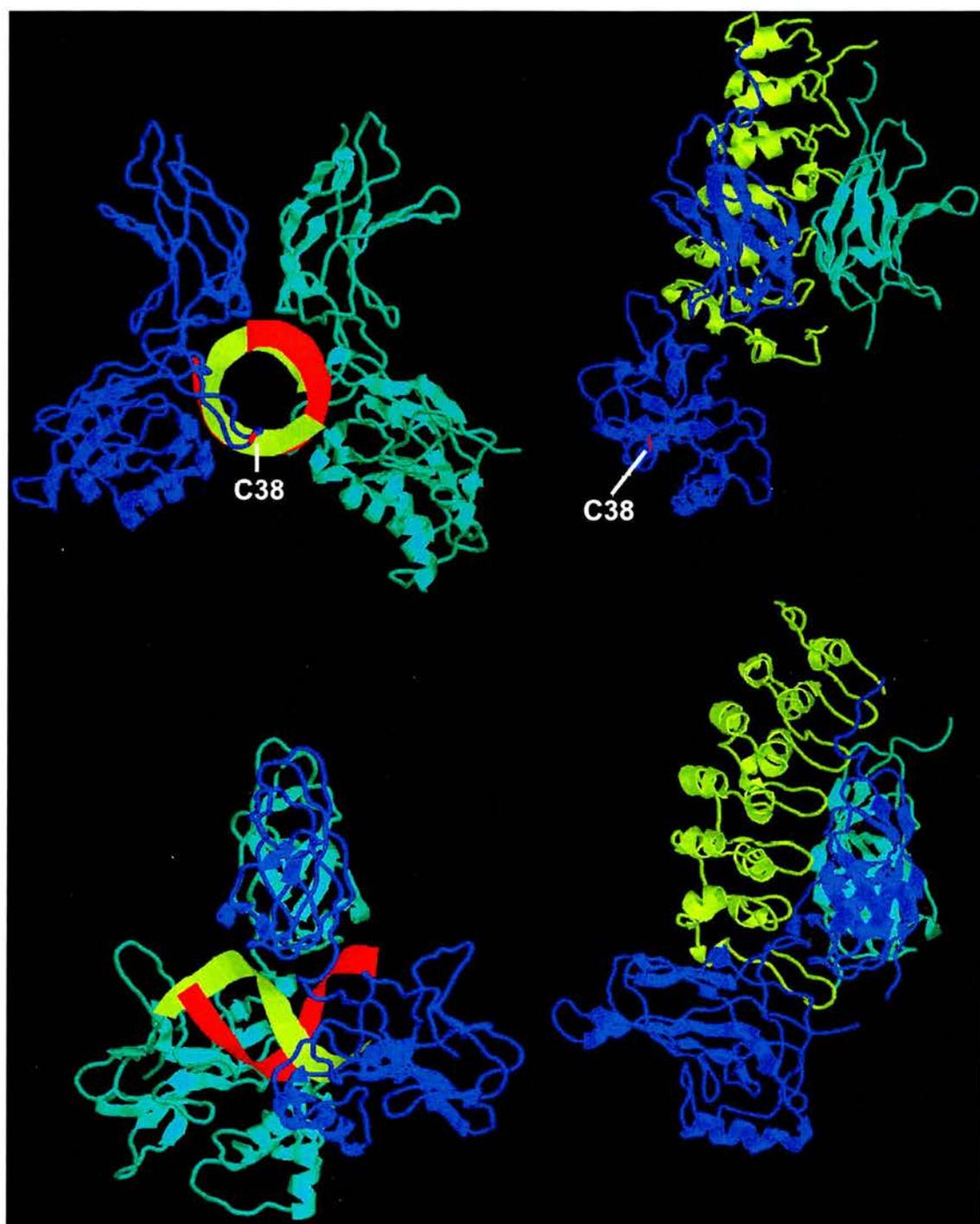


R305 and p65 R246 make salt bridges with p65 D217 and its homologous p50 D271 respectively. An opening of the dimer interface is also apparent. Finally, the interaction between p50 D254 and p65 N200 which only occurs in heterodimers (see section 4.2.3), is abolished upon I κ B α binding. I κ B α is aligned closer to p50, thus allowing more interactions between the ARD and the p50 subunit, although interactions like the salt bridges p65 R246-I κ B α E282 and p65 D243-I κ B α R246 contribute significantly to the I κ B α -p65 dimerisation domain interactions.

4.3.5 Domain movement and DNA binding inhibition

Comparison the structure of p50•p65•DNA and p50•p65•I κ B α complexes shows that p65 N-terminal domain orientation in relation to the homodimerisation p50 and p65 domains is almost opposite: In the NF- κ B•DNA complex conserved p65 C38 (homologous to p50 C62) which locates on the tip of the "recognition loop" points inside, towards the cleft of DNA. In the NF- κ B•I κ B α complex C38 points in the opposite direction (Figure 57). Apart from the almost 180° rotation, there is a translation of 40Å. This movement allows this domain to loosely associate with the ankyrin repeat 6. As it was previously mentioned, the NF- κ B•I κ B α complexes of both studies did not include the p50 N-terminal domain. Whether this apparent rotation/translation is due to the specific interactions between the serpentine PEST-like I κ B α C-terminus and the ankyrin repeat 6 with the p65 N-terminus or due to simply favourable crystal packing, has yet to be clarified. Nevertheless, such allosteric mechanism could disrupt NF- κ B DNA binding. Our data are consistent with this hypothesis. Our sedimentation velocity findings showed that (p50)₂, I κ B γ and (p50)₂•I κ B γ in solution are more extended than in the crystal structure. As far as I κ B γ alone is concerned, we can assume that a proportion of it is elongated/misfolded because free I κ B is unstable and is stabilised by forming complex with NF- κ B (Li et al., 1998). The "extended" form of (p50)₂ in solution explains the "paradox" that while full length p50 RHR NF- κ B alone failed to form crystals, a heterodimer of p50 and p65 dimerisation domains alone (Huang et al., 1997) and various NF- κ B•DNA complexes (Chen et al., 1998; Chen et al., 1998; Cramer et al., 1997; Ghosh et al.,

Figure 57: Comparison of the structure of the NF- κ B•DNA (left) and NF- κ B•I κ B α (right). p65 is shown in blue, p50 in light green, the two DNA strands are shown in yellow and orange, and I κ B α conserved C38 (homologous to C62 in p50) is showed in red, as a “marker”. In both pairs of pictures the dimerisation domains of NF- κ B have the same direction. It is apparent that p65 N-terminal domain points almost to opposite directions in the two conformations.



1995; Müller et al., 1995) could produce crystallographic data. That happened because the N-terminal domains can freely rotate and translate, due to the flexible hinge (L3 loop) that links them to their C-terminal dimerisation domains, unless they interact with other molecules which stabilise them (e.g. DNA). The 20 Å translation of p50 C62 during oxidative conditions also shows the flexibility given by L3 hinge (see section 4.2.6). In the same way, the (p50)₂•IκBγ appeared more extended because the 2 p50:1 IκBγ stoichiometry in which our data suggested that the molecules participate in the complex (in accordance with the crystallographic data for the NF-κB•IκBα complex) makes the complex NF-κB•IκBγ asymmetrical and IκBγ which is one ankyrin repeat longer and therefore can play roles similar to those of C-terminus, can only stabilise one of the two p50 N-termini, leaving the other N-terminus free to rotate. That explains why both the studies of the NF-κB•IκBα used p50 constructs that were lacking the N-terminus whose rotation was not stabilised by any interaction. If we accept that p50 N-terminus could not be co-crystallised due to lack of any interaction that would stabilise it, then we can assume that it would not make any contacts with the p65 N-terminus, and therefore the 180° rotated domain is not an artefact. Another suggested way of DNA binding inhibition, which is not necessarily mutually exclusive with the one previously proposed, is the competition between the C-terminal ankyrin repeats and/or the C-terminal domain of IκBs for the same binding sites on NF-κB with DNA. Indeed, protein footprinting using chemical modification and partial proteolysis showed that IκBγ and DNA interact with the K77-K80 of the C-terminus of p50 L1, K147-K149 of L2, K244 and K252 of L3, and K275 and K279 of L4, while IκBα does not. Many of the lysine residues that were contacted by DNA were also contacted by IκBγ, but not IκBα (Bell et al., 1996). Furthermore, this hypothesis is also based on the negative charge of IκB PEST-like C-termini, which form electrostatic bonds with the positively charged NF-κBs and on the finding that IκBβ and Bcl-3 C-termini are able to effectively inhibit DNA binding after phosphorylation (Bundy and McKeithan, 1997; Tran et al., 1997), which increases the negative charge of that region. Our finding of the “non classical” IκB property of Bcl-

3 ARD to form stable complexes with p50 and p52 bound to DNA, together with a similar finding on the formation of ternary complexes between I κ B β and NF- κ B bound to DNA (Tran et al., 1997), suggests that during I κ B-driven NF- κ B DNA binding inhibition, a ternary I κ B•NF- κ B•DNA complex is formed. Our data showed that the stability of such a ternary complex containing Bcl-3 ARD was DNA sequence dependent (the more symmetrical the DNA site, the more stable the ternary complex). Since it is known that p50 and p52 have stronger affinities for symmetrical κ B motifs, it seems that Bcl-3 ANK (or maybe full length Bcl-3 with unphosphorylated PEST-like sequence) is able to bind to the NLS and the dimerisation region but unable to compete with DNA for binding to specific NF- κ B residues, especially in the N-terminus, thus forming a ternary complex. The ability of Bcl-3 to compete with DNA and the affinity of an NF- κ B subunit for its half site depends on how its N-terminus and its flexible loops are related to DNA.

On the other hand, I κ B γ ARD is able to effectively inhibit p50 DNA binding activity. The ability of the C-terminus of I κ B γ ARD to compete with DNA for the same binding areas of p50 (Bell et al., 1996) and/or to consequently rotate p50 N-terminus to an unfavourable angle for DNA binding, destabilises any ternary complex. We demonstrated that K77 and K80 of the C-terminus of p50 L1 loop interact with I κ B γ , confirming previous biochemical findings (Bell et al., 1996). In the case of the conserved K80, we have already shown that it forms a very important bond with the DNA phosphate backbone, therefore competition between I κ B γ and DNA for that residue, would result in loosening of this bond. Furthermore, our data show that I κ B γ makes another interaction of equal importance with K77. K77 can also, under certain circumstances, recognise the DNA backbone. Since K80 is conserved in all NF- κ B/Rel/Dorsal, but its p65 homologue K46 was 11Å from the phosphate backbone it was hypothesised that this lysine residue could be a specific target for I κ B α . The crystallographic data of NF- κ B•I κ B α did not confirm the interaction between p65 K46 and the C-terminus of I κ B α , either because there is no such specific interaction, or because the C-terminus of the study was very short.

Our data demonstrated that the deletion of the amino acids 257-264 could efficiently block NF- κ B DNA binding inhibition. At the time when this experiment was conducted, we considered this area of significant importance, as it was supposed that it belonged to the C-terminal serpentine domain and could be a recognition signal for phosphorylation by PKA. We, now, know that phosphorylation is due to constitutive casein kinase II (CKII) (MacKichan et al., 1996) and that this sequence is N-terminally adjacent to the I κ B α nuclear export signal (NES) which extends in the region 265-277 of I κ B α and is responsible for the fast export of I κ B α out of the nucleus (Arenzana-Seisdedos et al., 1997). In the light of the crystal structure, it was apparent that the deletion was not in the C-terminal domain but in the linker sequence of the two antiparallel α -helices of the imperfect ankyrin repeat 6 (Figure 58) and that this region was necessary for the interaction with the dimerisation domain of p50, p65 and probably the p65 N-terminus. Therefore, removal of such sequence would result in the abolition of this interaction and/or the destruction of the ankyrin repeat 6. Combining our data with those of the crystal structures, it can be suggested that the interaction between the area 257-264 of the I κ B α with NF- κ B is critical for effective DNA binding of p65 containing NF- κ B molecules because it stabilises the NF- κ B•I κ B α and/or stabilises the 180° rotation of the p65 N-terminus which is unfavourable for DNA recognition. If the latter is correct (and not a crystal packing artefact), then we can assume that our data suggest that the I κ B α region 257-264 contributes to the specificity of I κ B α for p65.

Figure 58: Location in I κ B α structure and sequence of the deletions in the mutants used in the experiments: The part that is common in all constructs (wt and mutants) is shown in green. The peptide which is removed in I κ B α Δ 257-264 is shown in red and the C-terminus of I κ B α which is clipped in I κ B α 1-256 is shown in red and purple. The C-terminal domain of p65 is shown in blue. The peptide missing in I κ B α Δ 257-264 mutant is the linking sequence between the inner and outer anti-parallel α -helix of the sixth ankyrin repeat which interacts with p65 c'd loop. I κ B α 1-256 even lacks the entire outer α -helix together with the PEST sequence.



Ank VI : 243

VNRV¹YGGYSPYQ²LTWGRPSTR³IQOO⁴LGQ⁵L⁶TE

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5 FUTURE DEVELOPMENTS

5.1 Bcl-3 as a transactivator

The model of the direct and indirect transactivation through the interaction of Bcl-3 with p50 bound to symmetrical and asymmetrical κ B sites has to be tested by a series of transfection experiments. More precisely, cotransfection in cell lines with genotype RelA^{+/+} and RelA^{-/-} of a plasmid with a reporter gene under the transcriptional control of various symmetrical and asymmetrical sites, a plasmid that constitutively expresses p50 (or this plasmid without p50 insert) and a plasmid which that constitutively expresses Bcl-3 (or this plasmid without Bcl-3 insert), would prove if the *in vitro* findings are correct. This experiment was tried under many different conditions but it failed, mainly because the commercially available reporter plasmid had distant sites that could be recognised by NF- κ B, as it was shown, once the precise mechanisms of NF- κ B DNA half-site recognition were uncovered by crystal structure analysis and the idea of the “NF- κ B consensus sequence” was abolished.

A more ambitious project would be the co-crystallisation of a p50•Bcl-3 ARD•DNA complex, provided that an efficient method for the production of notoriously insoluble Bcl-3 is found. Moreover, even if complexes between p50, Bcl-3 and asymmetrical κ B motifs are more unstable than those containing symmetrical κ B motifs, crystallisation of both ternary complexes would reveal the exact mechanisms that allow Bcl-3 to form ternary complexes with p50 bound to symmetrical sites and dissociate p50 bound to asymmetrical sites.

5.2 (p50)₂•I κ B γ in solution

Since we demonstrated that replacement of hydrogen with deuterium in p50 and I κ B γ does not influence the properties of these molecules and their complex, intend to find more on the structure on (p50)₂•I κ B γ in solution, using Neutron Scattering. This method will reveal more information on the native state of the entire complex.

6 REFERENCES

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