PRODUCTION AND CHARACTERISATION OF MONOCLONAL AND POLYCLONAL ANTIBODIES AGAINST THE TRANSCRIPTION FACTOR NF-KB AND ITS INHIBITOR I-KB

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A Thesis Submitted for the Degree of MPhil
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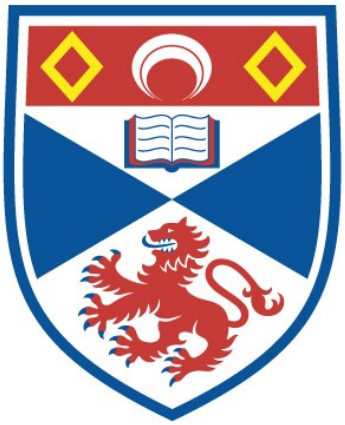

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PRODUCTION AND CHARACTERISATION OF MONOCLONAL AND POLYCLONAL ANTIBODIES AGAINST THE TRANSCRIPTION FACTOR NF-kB AND ITS INHIBITOR I-kB.

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ABSTRACT

The activation of the transcription factor NF-kB and its inhibition by I-kB has been the subject of much research. However, there are many questions which remain unanswered regarding the functional properties of, and relationship between, the factor and the inhibitor. In this study, antibodies directed against the protein components of NF-kB and I-kB have been produced and used as tools to determine the subcellular localisation of both proteins in different cell lines both before and after activation with various agents. In particular the production and characterisation of monoclonal antibody 3B6 will be described. This antibody was raised against the p50 subunit of NF-kB and recognises an epitope contained within the nuclear localisation signal of the protein. Polyclonal antisera raised against p50 and I-kB were used in Western blots and immunofluorescence experiments and show changes in the localisation of NF-kB subunits and I-kB following activation of cells for various times.

I, Elizabeth Ann Watson, hereby certify that this thesis has been written by me, that it is the record of work carried out by me, and that it has not been submitted in any previous application for a higher degree.

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I was admitted as a part time research assistant under Ordinance No. 12 in October 1990 and as a candidate for the degree of M. Phil. in June 1993: the higher study for which this is a record was carried out in the University of St. Andrews between 1990 and 1993.

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Finally I am indebted to my parents for all their help and support, especially over this last year of writing up.

Publications Arising from this Work

Matthews, J.R., Watson, E.A., Buckley,S. and Hay, R.T. (1993). Interaction of the C-terminal region of p105 with the Nuclear Localisation Signal of p50 is required for Inhibition of NF-kB DNA-Binding Activity. Nucleic Acids Research **21**, 4516-4523.

Matthews, J.R., Crawford, L., Watson, E.A., Hay, R.T. (1993). Mapping of the Interactive Regions of the Transcription Factor NF-kB with its Inhibitor Protein I-kB. Journal of Cellular Biochemistry,**S17A**, 47.

In addition, the antisera produced in the course of this project have been used in numerous publications both by R.T. Hay's laboratory, Department of Cell and Molecular Biology, University of St.Andrews, and many other laboratories throughout Europe.

List of Abbreviations

NF-kB	nuclear factor kappa B
HIV	human immunodeficiency virus
DNA	deoxyribonucleic acid
LPS	bacterial lipopolysaccharide
IgM	immunoglobulin M
EMSA	electrophoretic mobility shift assay
PMA	phorbol 12-myristyl 13-acetate
PLC	phospholipase C
IP_3	inositol triphosphate
DAG	diacyl glycerol
Il-1	interleukin -1
TNFx	tumour necrosis factor alpha
Il -2	interleukin -2
PHA	phytohaemagglutinin
TPA	13-0-tetrodecanoyl 12-phorbolacetate
KCl	potassium chloride
HPLC	high performance liquid chromatography
NLS	nuclear localisation signal
PKA	protein kinase A
EBP 1	enhancer binding protein 1
KBF 1	kappa binding factor 1
PCR	polymerase chain reaction
RHD	rel homology domain
CAT	chloramphenicol acetyl transferase
cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate

SV40	simian virus 40
PKA	protein kinase A
Con A	concanavalin A
ROI	reactive oxygen intermediates
NAC	N-acetyl-L-cysteine
H_2O_2	hydrogen peroxide
RNA	ribonucleic acid
UV	ultraviolet
TLCK	Na-p-Tosyl-L-Lysine chloromethyl ketone
TPCK	Na-p-Tosyl-Phenylalanine chloromethyl ketone
PDTC	pyrrolidinedithiocarbonate
AIDS	acquired immune deficiency syndrome
LTR	long terminal repeat
FPLC	fast protein liquid chromatography
HSA	human serum albumin
SPDP	3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester
DTT	dithiothreitol
PBS	phosphate buffered saline
ELISA	enzyme linked immunosorbent assay
TMB	3,3',5,5'-tetramethylbenzidine
OPD	o-phenyline-diamine
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
APS	ammonium persulphate
PTM	PBS/Tween 20/Marvel

ECL	enhanced chemiluminescence
HCl	hydrochloric acid
Ab	antibody
Ag	antigen
NaCl	sodium chloride
NaN_3	sodium azide
FITC	flourescein isothiocyanate
IgG	immunoglobulin G
NH_4Cl	ammonium chloride
FCS	foetal calf serum
rhIl-6	recombinant human interleukin 6
HAT	hypoxanthine aminoptorine thymidine
HT	hypoxanthine thymidine
PFHM 11	protein free hybridoma medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
PMSF	phenylmethylsulfonyl fluoride
BSA	bovine serum albumin
mAb	monoclonal antibody
GST	glutathione-S-transferase
Tween 20	polyoxyethylenesorbitan monolaurate

CONTENTS

INTRODUCTION

The transcription factor NF-kB (nuclear factor kappa B) is a transcription factor which, on activation, induces transcription of a variety of genes including the kappa Ig light chain gene, many cytokines, cell surface receptors and some viruses, including HIV-1. NF-kB belongs to a family of proteins which are controlled by a family of inhibitor proteins characterised by the possession of "ankyrin repeats". The factor itself, its subunits, and the inhibitor family will be discussed in detail below.

Identification of NF-kB

Nuclear factor kappa B was first identified in 1986 (1) as a factor which interacts with a sequence in the large intron of the k light chain gene, identified as a B cell specific enhancer element in the Ig kappa chain gene (2,3). Binding of this factor to the enhancer DNA could be competed for by addition of the complete k enhancer or the SV40 enhancer. This result suggested that the binding site for NF-kB could be an 11 nucleotide sequence - GGGGACTTTCC - common to both enhancers. This prediction was confirmed by methylation interference analysis of the nucleoprotein complex. Analysis of factor binding in nuclear extracts from a variety of cell lines showed NF-kB to be found only in cells of the B lymphoid lineage which had differentiated to mature B cells or plasma cells : the factor was not found in pre B cells or T cells.

If NF-kB was critical for activation of the k enhancer, it

would have to be inducible in pre B cell lines following treatment with an appropriate stimulus. The cell line 70Z/3 has the phenotype of a pre B cell, since it synthesises cytoplasmic u chain but no light chain (4,5). The k chain gene in these cells is functionally rearranged but is not transcriptionally active (6,7) unless cells are treated with bacterial lipopolysaccharide (LPS) which results in initiation of k transcription, synthesis of k chain protein and appearance of IgM on the cell surface. This treatment with LPS also induces a DNAase I-hypersensitive site associated with the k enhancer and on this basis it was suggested that k transcription is due to activation of the k enhancer (8).

Studies using this 70Z/3 line and another pre B cell line, PD (k chain in the process of rearrangement), showed that NF-kB activity in these cells was inducible by LPS as detected by electrophoretic mobility shift assay (EMSA) (9). The phorbol ester phorbol 12-myristyl 13-acetate (PMA) was also shown to induce NF-kB activity in 70Z/3 cells but the time course of activation by the two agents was different. Treatment with LPS results in detection of NF-kB after 0.5 hour stimulation, with NF-kB levels increasing until 2 hours after stimulation when the level plateaus out, whereas in PMA stimulated cells the maximum detection of NF-kB is at 0.5 hours after stimulation, remaining constant for 2 - 3 hours then rapidly decreasing until non detectable 8 hours after stimulation. This rapid decline of NF-kB was

postulated to be due to decreased activity of endogenous protein kinase C (PKC) due to prolonged exposure of the cells to phorbol esters, suggesting that PKC activity is necessary to maintain NF-kB activity. The faster response to PMA than LPS could be explained by the direct action of PMA on PKC, whereas LPS activates phospholipase C (PLC) which in turn generates inositol triphosphate (IP_3) and diacyl glycerol (DAG), which in turn activate PKC (10).

Treatment of 70Z/3 B cells with inhibitors of translation (cycloheximide or anisomysin) for 10 minutes followed by stimulation with LPS also induced expression of the k chain gene, as did treatment with cycloheximide alone (11). Further analysis of cells treated with cycloheximide alone, anisomysin alone or cycloheximide plus LPS showed that such treatments also induced NF-kB and that treatment with both agents results in a superinduction of the factor (9). These results show that induction of NF-kB in 70Z/3 cells does not require new protein synthesis and so must involve post translational modification of an existing protein.

Further agents have been shown to induce NF-kB activity in the 70Z/3 line. These include interleukin 1 (I1-1), tumour necrosis factor α (TNFα) and virus infection - all of which stimulated transcription of the k locus thus reinforcing the correlation between NF-kB induction and k light chain gene expression (9,12,13,14). Confirmation of this correlation was further provided by direct demonstration of chromatin changes and occupancy of the NF-kB site in the nuclei of LPS

treated 70Z/3 cells (15,16). Additional studies have shown that genetically altered 70Z/3 cells, which have defective k gene expession following LPS treatment, are also abnormal in their ability to activate NF-kB (18,19).

As previously mentioned, initial studies on pre B cells, non B cells and T cells failed to detect NF-kB activity but following the reports of the inducibility of NF-kB in pre B cells, further studies were done to determine whether NF-kB is also inducible in other cells such as T cells and nonlymphoid cells. The human T leukaemia cell line, Jurkat, had previously been shown to produce interleukin2 (I1-2) on stimulation with phytohaemagglutinin (PHA) plus PMA (20,21). Extracts were made from these cells stimulated with either or both agents, and analysed for NF-kB activity. NF-kB was induced in all cases with the costimulated cells exhibiting a higher level of activation. In PMA treated Hela cell extracts NF-kB activity was also induced (9).

This supported the idea that NF-kB must exist as an inactive precursor, or complexed with an inhibitor molecule, with certain stimuli resulting in release of active NF-kB after modification of the precursor or the protein-inhibitor complex. To investigate the state of NF-kB in unstimulated cells nuclear and cytosolic extracts from unstimulated or 13-0-tetrodecanoyl 12-phorbolacetate (TPA) stimulated 70Z/3 cells were examined for NF-kB binding activity (22). Both nuclear and cytosolic extracts from unstimulated cells exhibited very little kB specific binding activity, but

following treatment with TPA for 30 minutes the nuclear NF-kB activity increased dramatically with no increase in activity seen in the cytosolic fraction. The extracts from unstimulated cells were then subjected to denaturation, fractionation and renaturation - after which the majority of in vitro NF-kB activity was detected in the cytosolic fraction, suggesting that the factor may be bound to an inhibitor. Treatment of the cytosolic fraction with dissociating agents resulted in activation of the cytosolic kB binding activity. In experiments using equal cell equivalents of protein the amount of cytosolic NF-kB in unstimulated 70Z/3 cells was shown to be the same as the amount of nuclear NF-kB in stimulated cells (22). The same studies using Hela cells also showed the presence of inactive cytosolic NF-kB, with the total cellular NF-kB activity remaining constant before and after TPA activation, although activation by TPA in Hela cells is less complete. The transcription factor NF-kB has now been found in virtually all cell types examined, and it has been shown that its activation involves some form of post translational modification of a pre-existing cytoplasmic form.

Purification of NF-kB

Following the identification and partial characterisation of NF-kB, DNA affinity purification was carried out using double stranded multimers of the previously identified binding site 5'GGGACTTTCC 3'. Different groups purified the

factor from different sources - namely human cell lines (23,24), human placenta (25) and rabbit lung (26).

NF-kB was purified from two human cell lines : Namalwa cells (a human Burkitt lymphoma cell line) and Hela cells. In both cases a DNA binding activity of about 50kD was isolated by affinity chromatography - though nuclear extracts from Namalwa cells (23) and deoxycholate treated cystolic extracts from Hela cells (24) were used. The purification from Hela cell extracts also yielded a 65kD protein which did not bind DNA.

In the purification from human placenta a much more stringent washing procedure using chloroquine and potassium chloride (KCl) was employed (25) to reduce the purification to a much more cost effective one step process, rather than the multiple rounds of affinity chromatography that were required previously. Again the DNA binding activity of NF-kB was isolated.

The DNA binding p50 subunit of NF-kB was also purified from rabbit lungs (26). In this case the purification process was modified from multiple rounds of affinity chromatography by use of KCl elutions and a negative selection step using a mutant kB site. Using this procedure a high yield of good purity NF-kB was obtained - consisting of a 50kD and a 65kD fraction which displayed the characteristics of the p50 and p65 NF-kB subunits.

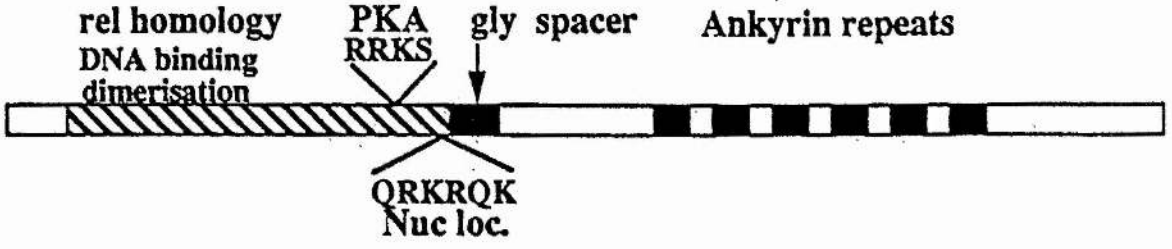
Cloning and Characterisation of the p50 Subunit

From this preparation the p50 was sequenced and then cloned. Sequencing of tryptic peptides of p50 purified by high performance liquid chromatography (HPLC) yielded a primary protein sequence of 166 amino acids. (26).

Polymerase chain reaction (PCR) amplification using primers from 2 of the tryptic peptides and a template of cDNA from total rabbit spleen and lung was performed. Screening of a mouse cDNA library identified 5 clones each containing inserts of about 4.0kb which were identical by restriction mapping. Sequencing of one of the clones showed a long open reading frame which encodes a 107kD protein. Presumably this is then processed to yield the 50kD p50, and a glycine rich region around amino acid 400 may be a cleavage site.

A database search looking for homologies with the predicted amino acid sequence for p50 showed a remarkable similarity to C-rel (a turkey proto-oncogene) (27), v-rel (the viral oncogenic counterpart of C-rel) (28) and dorsal (a maternal effect gene in Drosophila) (29). See Figure 1.

Figure 1.



Common to all rel related proteins is the property of selective nucleo/cytoplasmic distribution in response to different factors. A nuclear localisation signal (NLS) is found between amino acids 358-363 (30). All the proteins also have a potential site for phosphorylation by protein kinase A (PKA) (29) although its position is unknown.

Also described in the literature around this time were two other factors which bound to the same 10 base pair sequence as NF-kB. These factors were known as (Enhancer Binding Protein 1) EBP1 (31) and KBF1 (32). EBP1 was shown to bind to an NF-kB site in the HIV-1 enhancer, and DNAase footprinting showed that in fact both NF-kB binding sites in the enhancer could be occupied by EBP1 (33). DNA protein complexes formed by inducible NF-kB from Jurkat cells and target DNA have the same mobility as those formed by EBP1 and target DNA, and binding of both factors can be competed out by the same double stranded oligonucleotides. Further evidence also supported the notion that EBP1 is in fact the activated form of NF-kB (p50) and it is now accepted as such (34,35). Cloning of KBF1 purified from Hela cells showed that KBF1 cDNA encodes a 105kD protein, with KBF1 located in the N terminal region (32). Strong homology with rel family proteins was also noted with the DNA binding domain and a dimerisation domain being located in the homologous region. The C terminal region was shown to contain eight repeats of 33 amino acids which are similar to those found in the human erythrocyte ankyrin protein. KBF1 was shown to

be identical to p50 of NF-kB by molecular weight, protein-DNA complex mobility in a band shift assay, binding site affinity, DNA contact points, association with p65, cross reactivity with antisera and partial amino acid sequence (32). Again, KBF1 is now accepted as one and the same as NF-kB p50 (34,35).

Another group also purified p50 from another human cell line, HL60 (36). The cells were activated with TNFα, and nuclear extracts prepared then purified using the protocol described briefly above (23). The purest fraction obtained gave rise to two protein species - one of 51kD and one of 65kD - and these showed all the characteristics of p50 and p65 respectively. The p51 fraction was then sequenced, again by HPLC of tryptic peptides and oligonucleotides prepared from peptide sequences were used to screen a human cDNA library. Once again, an open reading frame encoding a 105kD protein was isolated, with the peptides obtained from p50 all mapping to the N terminus - thus suggesting this to encode p51. Compared with the KBF1/p50 sequence (32), there is one codon missing from this sequence which is explained as a possible allelic difference (36). The cloned p51 was also found to have a high level of homology with rel family proteins.

Fragments of the cloned cDNA's were used to probe cells for the 3-8kb transcript before and after stimulation with either TNFα or phorbol ester. Following activation by either agent, the transcript levels were greatly increased in both

HL60 and Hela cells suggesting that a more long term activation of NF-kB may involve induction of NF-kB mRNA (36).

Cloning of p65.

In the process of purifying p50, some groups also purified a 65kD protein, known as p65 (24,26,36). Following the successful cloning of p50 protein purified from rabbit lung (26), p65 was cloned in a similar manner (37) using primers from rabbit derived peptides to PCR mRNA from both rabbit lung and a murine pre B cell line 2206. The rabbit and murine clones obtained shared more than 97% amino acid homology, and the murine PCR insert was then used to probe a 2206 cDNA library.

The open reading frame obtained encodes a 60.2kD protein which encompasses the sequences of all the peptides obtained from purified p65. The predicted sequence, like p50, shows strong homology to members of the rel family of proteins with greatest similarity to c-rel. The carboxyl terminus of c-rel has a transcription activation domain (38,39), which shares charge distribution similarity with p65. The role of this region will be discussed in more detail later.

The cloned p65 subunit satisfied several criteria to be recognised as NF-kB p65. Peptides from affinity purified size separated NF-kB p65 were used to isolate cDNA which included peptides not used in the cloning. The protein obtained by <u>in vitro</u> translation runs at about 65kD and forms a DNA binding complex with p50 - the DNA binding of

which is inhibited by I-kB. Finally, the expected broad tissue distribution of the p65 gene is confirmed by Northern blot analysis.

The Structure and Function of p50 and p65.

Structure

As already described briefly, cloning of both p50 and p65 showed that both proteins share homology with members of the rel family of proteins (26,32,38). This homology is contained in a 300 amino acid sequence which functions as a DNA binding and dimerization domain and also contains sequences responsible for inhibitor binding and nuclear localisation. For simplicity, this region will be referred to as the rel homology domain (RHD). Sequences which lie to the C terminus of the RHD are unrelated in these proteins (40).

The RHD is unusual as a DNA binding and dimerization domain in that it requires the entire 300 amino acids to function (41) - this is unusually long. Another unusual feature is the lack of DNA binding motif normally seen in the equivalent domain of other transcription factors such as leucine zipper or helix-loop-helix. It has been shown that members of this family can form both homodimers and heterodimers.

Further dissection of the RHD of p50 has been done using deletion mutants created by restriction enzyme digestion of full length cDNA (26,32,42). These studies showed that DNA binding, detected by band shift assay, was abolished in any

protein longer than 670 amino acids from the N terminus. Internal deletions mapped the DNA binding domain to amino acids 19 to 399, with the C terminal region of the precursor 105kD protein preventing DNA binding. Co-immune precipitation experiments were then performed, using deletion mutants, to map the region involved in dimerization, and to establish whether the previously observed inability to bind DNA was linked to an inability to form dimers. The results show that the dimerization domain of p50 is located between amino acids 201 and 367, and that this region is responsible for homodimer and heterodimer formation. It also suggests that the N terminal region of the RHD is involved in interacting with DNA and some preliminary data from S. Grimm and P.A. Bauerle indicates that less than 30 amino acids at the N terminal domain of the RHD are sufficient when swapped over to confer p50 DNA binding specificity on p65 and vice versa (41). There is some published data (43) concerning this same region of the RHD in which radiolabelled iodoacetate was used to identify which of 5 conserved cysteine residues is involved in p50 DNA binding (43). This procedure identified a cysteine at position 62 which is protected from modification by binding to an oligonucleotide containing the binding site. Further work in which the five conserved cysteines were mutated in turn to a serine confirmed this cysteine 62 to be important in the recognition of DNA by p50 (44). The involvement of a conserved cysteine in binding of v-rel has also been shown

by mutation to a serine residue (45).

It has already been mentioned that the C terminal end RHD contains a sequence which is composed of positively charged amino acids. Using cells transfected with cDNA containing mutations in this region, it has been shown that the construct became exclusively cytoplasmic with the wild type being exclusively nuclear (46). Another study also looked at the effect of mutating this Arg-Lys-Arg-Gln-Lys sequence in p50 and found that mutations in this short chain of amino acids block nuclear translocation in transfected cells (47). The corresponding sequence in p65 -Lys-Arg-Lys-Arg- was mutated by another group such that the first three residues were replaced by uncharged threonine residues. While over expression of wild type p65 gave a predominantly nuclear localisation, over expression of the mutant did not result in any shift of protein from cytoplasm to nucleus (48). These results confirm the prediction that these short sequences of positively charged amino acids act as a nuclear localisation signal (NLS) in both p50 and p65, as has already been demonstrated with v-rel in other proteins (49).

Although the exact cleavage site of the precursor molecule, p105, is not known (location of the C terminus of p50 is an estimate) it has been noted that p65 has an extra 200 amino acids in its C terminal region (37,50). Since it is established that transcription factors require trans-activation domains as well as DNA binding ability, the

C termini of both p50 and p65 have been tested for trans-activation properties. Using a chloramphenicol acetyltransferase (CAT) reporter construct under the control of two kB sites it has been shown that expression of p65 leads to a potent transactivation whereas p50 has no effect. When p50 and p65 were coexpressed the p50 appeared to reduce the transactivation by p65 (35,42). p65 sequences were then fused to the DNA binding dommain of GAL4 (a yeast transcription factor) to identify the exact region(s) involved in the transactivation (35,51). Two transactivation domains were identified - TA_1 and TA_2 - with the principal domain TA_1 located in the 30 amino acid sequence at the C terminal and TA_2 in the 90 amino acids located adjacent to this. Sequence analysis of TA_1 by two different methods predicts an amphipathic α helical structure, with five hydrophobic amino acids on one face and five hydrophilic serine residues on the opposing face with this structure conserved between mouse, human and Xenopus p65. The TA_2 domain contains a sequence which could be a leucine zipper - but neither domain contains a functionally important proline rich region which is common in other transcription factors (52).

The information gained on the properties of various structural domains suggests that the p50 subunit of NF-kB provides the DNA binding activity of the factor and dimerises with p65 which then functions as a transcriptional activator.

Function of p50 and p65

Identification of the transcription factor NF-kB originally came about through its DNA binding properties. Purification of the factor resulted in two proteins being isolated - p50 and p65. Reconstitution experiments carried out using the two purified proteins (purified from Hela cells) showed that a complex was formed when p65 was added to p50 (24). Further renaturation experiments showed that p50 could form homodimers in solution and the 250kD size of NF-kB DNA complex determined by gel filtration could be explained by complex formation involving both p50 and p65 subunits.

Initial experiments looking at DNA binding by ultraviolet (UV) crosslinking and renaturation experiments show that only p50 is able to bind DNA (23,24). In these studies the DNA sequence used was GGGACTTTCC, and the subunits were purified from SDS gels. Then came some observations that suggested a possible involvement of p65 in DNA binding. Firstly it was found that when purified p50 and p65 co-renatured, the resulting heterodimer had a two fold higher binding affinity for the kB binding site than a p50 homodimer (53). It was then suggested that since *in vivo* most NF-kB binding sites are of low symmetry and also tend to consist of pentameric half sites (25) rather than being a homodimer binding site, both p50 and p65 are involved. Artificial motifs consisting of duplicated pentameric half sites were created (Figure 2) and the DNA binding of these plus the decameric site was compared between the p50

homodimer and the heterodimer.

Figure 2

consensus kB site	GGGACTTTCC
site AA	GGGAC-GTCCC
site BB	GGAAA-TTTCC

The p50 homodimer could recognise all three sites but with different affinities - recognising the AA site best, followed by AB and finally BB. The heterodimer however only recognised AB, but with an affinity constant more than two times higher than that for the homodimer binding the site. It was also found that the A half-site is more highly conserved than B (25,53) with a consensus of 5'GGGpuN-3' compared to 5'G(GPu)N-3'. Therefore it would appear that the p50 homodimer binds with higher affinity to the more conserved of the symmetrical sequences.

Studies comparing the effects of different mutations in the 11 base pair sequence, GGGGATTCCCC, on p50 dimer and p50-p65 heterodimer binding showed that mutations in positions 1 or 11 of this motif reduced binding of the p50 homodimer much more than that of the heterodimer. If the motif was mutated to GGGGACTTTCC, homodimer recognition was reduced 70 fold but heterodimer binding only fell 3 fold. This data suggests that the presence of p65 lowers the requirement of p50 for the complete 11 base pair motif and for symmetrical motifs (113). Binding competition experiments show that, in

contrast to p50, p65 binds preferentially to the BB motif described previously. This suggested that when NF-kB binds to DNA the p50 contacts the highly conserved A half site while p65 contacts the less conserved B site. UV crosslinking experiments were then carried out and the results show a direct participation of p65 in the DNA binding of NF-kB. Also shown is a preferential binding of p50 to the A half site and p65 to the B half site.

Further evidence of p65 binding to DNA was obtained in experiments using p65 from Baculovirus infected insect cells (55) and <u>in vitro</u> translated p65 (50). Homodimers of p65 have been shown to bind a different consensus sequence to that preferred by p50 homodimers - namely GGGPuNTTTCC (56). The 10th and 11th base pairs are not essential, and the TTTCC region is highly conserved.

The differential binding requirements of the 2 subunits composing the dimeric transcription factor, NF-kB, allows evolution of regulatory elements which are recognised by p50-p65 heterodimers but not homodimers. The role of homodimer specific binding sites in gene regulation is as yet unclear. (41)

The I-kB Inhibitor System.

<u>Purification and Specificity</u>

As well as the structural similarities between the rel family of proteins, and their common property of cytosolic to nuclear translocation, they also have inhibitor proteins associated with them. For NF-kB, this inhibitor protein is

I-kB, which is now recognised as belonging to a different family of proteins, the ankyrin family.

As already discussed, in non stimulated cells, NF-kB DNA binding activity is not detectable in nuclear or cytosolic fractions. However if the cytosolic fraction is treated with deoxycholate followed by NP-40, then a kB specific DNA-binding activity is seen, which has been shown to be identical to that seen in the nuclei of activated cells (24,25,26,32). This deoxycholate treatment results in release of a protein which was termed I-kB (57). Its inhibition of the NF-kB complex is reversible, saturable, and specific : I-kB does not interfere with DNA binding of any other factor tested so far. In subcellular fractionation experiments, the NF-kB/I-kB complex shows a cytosolic localisation, but this could be an artefact of the fractionation procedure and so enucleated cells were examined for the NF-kB/I-kB complex.These experiments showed that a large proportion of the complex was indeed cytoplasmic.

The cytosol of human placenta was used as the source for the first purification of I-kB to homogeneity (58). I-kB released from its complex with NF-kB by deoxycholate treatment was then purified further by chromatography to yield two peaks of activity. The majority of I-kB activity (~80%) is referred to as I-kBα and the remaining 20% as I-kBβ. Renaturation experiments show I-kBα to be a protein of 37kD while I-kBβ is of higher molecular weight. Multiple

chromatography steps were used in a separate purification process (59), resulting in a 35kD I-kB protein purified from rabbit lung. This presumably corresponds to the I-kBα from human placenta. In a glycerol gradient, purified I-kBα sediments with a size of 35 - 40kD, and in gel filtration elutes at 70kD which suggests it may form labile dimers.

A modified purification procedure was developed and used to further purify I-kBβ (60). Previously its molecular weight had been narrowed down to between 40-43kD (58) but the purification step used to further isolate I-kBα could not be used due to the proximity of the I-kBβ peak to that of non specific contaminants. Once finally purified, the I-kBβ was found to have molecular size of 43kD. Both I-kBα and I-kBβ have similar isoelectric points : between 4.8 and 5.0.

The 35kD I-kBβ purified from rabbit lung (59) was shown to be inactivated by treatment with protein kinase C and cAMP dependent protein kinase. When the same treatment was used on both I-kBα and I-kBβ from the human placenta, their inhibition of DNA binding by NF-kB was abolished in the presence of ATP. Treatment with immobilised alkaline phosphatase also abolished inhibition by I-kBβ, but had no effect on I-kBα suggesting that I-kBβ has been purified as a phosphoprotein, and that phosphoryl group(s) are necessary for its inhibitory activity. Phosphatase treatment also activates DNA binding of NF-kB in complex with I-kBβ.

Both I-kB species preferentially bind p65 rather than p50, as indicated by a number of observations. The homodimer of

p50 cannot be inactivated by I-kB, whereas heterodimeric NF-kB can (58) which suggests I-kB interacts with p65. However the presence of p65 in the heterodimer could influence p50 in some way that enabled it to bind I-kB, or p65 could bind I-kB directly. Preincubation of I-kBα with p65 abolishes inhibition of DNA binding by the heterodimer, but preincubation of I-kBα with p50 has no effect. The complex formed between p65 and I-kBα does not react with p50 homodimers or inhibit DNA binding of the homodimers (53). The same study also showed that a complex of p50-p65-IkB could be activated in a dose-dependent manner by the addition of increasing amounts of p65. The resultant activation was more effective than that seen with deoxycholate.

Further evidence for p65 being a receptor for I-kB came from two studies which show that I-kB can inhibit DNA binding of NF-kB and p65 homodimers but not p50 homodimers (54,57). This was shown by titration and kinetic analysis in which increasing amounts of I-kB were added to excess NF-kB resulting in dose-dependent inhibition of DNA binding. Addition of I-kB to p50 homodimers had no effect.

As mentioned previously, p65 has a unique C terminal region, and these results may suggest that the site for I-kB binding is located in this region, rather than in the RHD. However, it has been shown that DNA binding of truncated p65 can be inhibited by I-kB and so the I-kB binding site on p65 must map to the RHD (37). This was confirmed by mapping of the

binding site to the C terminal domain of the RHD (61).

Cloning of I-kB Proteins

The first I-kB cloned was identified unintentionally - in that the C terminal domain of p105 is now recognised as I-kBγ.

The C terminal region of this precursor protein has been shown to contain eight ankyrin repeats and to exert a trans-inhibitory effect on NF-kB DNA binding (62,63). In contrast to the other I-kB proteins, this I-kBγ shows a high affinity for p50, and can displace p50 DNA binding in less than 5 minutes. As with the other proteins, the ankyrin repeats are necessary for the inhibitory activity of I-kBγ.

As well as being identified as the C terminal region of p105, I-kBγ has also been expressed from a separate 2.6kb mRNA which is expressed in murine lymphoid cells (63). The 70kD I-kBγ is identical to the protein encoded by the C terminus of p105. As found in p105, the p70 protein contains a glycine rich domain and a series of ankyrin repeats.

Comparison of the homologous ankyrin region in different I-kB's shows that the sequences of these repeats align best when comparison is made between the first repeat of each gene, second is compared with second, and so on.

Studies on the adherence of monocytes to plastic led to the discovery of a gene, MAD-3, expressed early after adherence (64). The transcripts obtained are 1.6kb in size, with a predicted polypeptide sequence of 317 amino acids which should encode a 34kD protein. Structural predictions suggest

an arrangement in three domains. A stretch of 72 hydrophilic amino acids with consensus sequence DEEYEQMVK is found in the N terminal domain while the C terminal has a consensus sequence for PKC phosphorylation (RPSTR) and a region rich in PEST sequences which are associated with rapid protein turnover. The internal domain is made up of 5 repeats of the ankyrin motif (65).

The MAD-3 translation product was shown to specifically inhibit NF-kB DNA binding activity but to have no effect on the DNA binding of p50 homodimers. Partial amino acid sequence showed MAD-3 to be highly related if not identical to the 35kD I-kB purified from rabbit lung (59).

Due to the presence of ankyrin repeats, the proto-oncogene Bcl-3 (associated with chronic lymphocytic leukaemia)(66), was tested for I-kB activity. In band shift and gel retardation assays (62,67) Bcl-3 has been shown to inhibit DNA binding by p50, and to a lesser extent, by p65. This inhibition is due to the ankyrin repeat domain of Bcl-3. Phosphopeptide mapping experiments (68) using Bcl-3, IkBβ, and I-kBα show that I-kBα and Bcl-3 share phosphopeptides while I-kBβ and MAD-3 are not related. N terminal truncation of Bcl-3 alters its inhibition such that this shortened form is a potent inhibitor of p65 but not p50, which is the same specificity as that of I-kBα (69). This truncation may also occur <u>in vivo</u>, since immunoprecipitation with anti Bcl3 antibodies yields a 37kD protein, rather than its expected 50kD size. It has also been shown that rabbit polyclonal

antiserum raised against MAD-3 can reduce inhibition of NF-kB DNA binding by I-kBα, but not I-kBβ (43).

Functions of I-kB.

Since I-kB was originally identified due to its inhibition of DNA binding of NF-kB, at least one of its functions was known at the start. The cytoplasmic NF-kB/I-kB complex showed a size of around 150kD in both gel filtration and glycerol gradients (57,58) and this suggests a heterotrimer of p50, p65, and I-kB. Inactivation or removal of I-kB from the complex would then release p50-p65 heterodimer which form active complexes able to enter the nucleus.

The mechanism of inhibition by I-kB is not well understood. MAD-3 and I-kBα bind complexes that contain p65 and inhibit DNA binding of these complexes, but cannot associate with p50 homodimers (24,53). This binding to p65 has been mapped to the RHD of p65 (37). I-kBβ can also inactivate NF-kB containing the p65 subunit but will also inactivate complexes containing C-rel (58). In contrast, I-kBα inhibits DNA binding of p50 homodimers as well as that of p50-p65 and p50-C-rel (63).

In NF-kB/I-kB complexes containing I-kBα or I-kBβ, phosphorylation of the I-kB is required to activate the NF-kB DNA binding (59). For I-kBβ, phosphorylation by either PKA or PKC will remove inhibition, but with I-kBα only PKC phosphorylation will suffice (69). Phosphatase treatment of I-kBβ inactivates its inhibitory activity, whereas it has no effect on I-kBα. The size and phosphorylation

characteristics of I-kBβ suggest homology with the rel associated pp40 protein isolated by immunoprecipitation from avian cell extracts (69).

Investigation of the kinetics of inhibition of I-kBα and I-kBβ on preformed NF-kB DNA complexes show that addition of I-kBα reduces the half life of the complex to 3 minutes and I-kBβ reduces it to 7 minutes (58). The dissociation reaction displayed higher order reaction kinetics, therefore both inhibitors dissociate NF-kB already bound to DNA and, if found in the nucleus, could function as inhibitors of NF-kB mediated transcription.

Another function of I-kB is to control nuclear uptake of NF-kB. The complex of NF-kB and I-kB was first identified in the cytosol of cells, and could not be removed by enucleation - therefore showing it to be a truly cytoplasmic complex (57). Studies on transfection systems show that co-transfection of p50 and p65 encoding plasmids with a MAD-3 expression plasmid results in accumulation of p50 and p65 in the cytoplasm rather than the nuclear distribution seen in the absence of MAD-3 (61). Another similar study looked at the effect of MAD-3 on the distribution of over-expressed p50 and found that addition of MAD-3 resulted in only a partial redistribution of p50 to the cytoplasm (48). In the same study, over-expressed MAD-3 was observed in both the nucleus and cytoplasm when microinjected into Vero cells. This was shown to occur even at a very low level of expression. However no NLS sequence has been found in

MAD-3 as yet, so it is possible that this distribution is due to passive nuclear uptake of the 35kD protein. Co-expression of plasmids containing MAD-3 and p65 show that each could inhibit nuclear uptake of the other, although p65 was less efficient at preventing uptake of MAD-3.

The control of nuclear uptake of p50 and p65 by MAD-3 suggested that MAD-3 may interact with or block the NLS of these proteins. Antisera were raised against peptides overlapping these NLS's (47,48) and used to investigate whether they could block I-kB binding or vice versa. Immunoprecipitations show that only nanogram amounts of MAD-3 are sufficient to block immunoprecipitation of p65 by antibodies against the p65 NLS. However MAD-3 had no effect on precipitation of p50 by anti-p50 NLS antibodies. In the presence of p65, immunoprecipitation of p50 by anti-p50 NLS antibodies was inhibited by MAD-3, suggesting that in the p50-p65 complex, binding of MAD-3 to p65 masks the NLS of both proteins.

A further two observations support NLS masking by I-kB (61). Fusion of the NLS of SV40 to p65 expression vectors gave nuclear expression of p65 which remained unaffected on the addition of MAD-3. Deletion or mutation of four NLS amino acids in p65 resulted in a protein which could no longer bind MAD-3, as determined by gel shift and coimmunoprecipitation. It seems very probable therefore that MAD-3 binds directly to the p65 NLS.

The precursor molecule p105 which contains both a DNA

binding domain (p50) and an inhibitor domain (I-kBɣ) shows an exclusively cytoplasmic distribution when over-expressed in Vero cells and Cos cells (47). It has been shown that p105 cannot bind to DNA (70). Further studies showed that addition of p105 to a DNA binding assay inhibits DNA binding by p50 (71) and p65, with the inhibition of p65 requiring a similar concentration of p105 or MAD-3. The 70kD I-kBɣ isolated from murine cells also inhibits DNA binding of p50 heterodimers (63). When p50 and p105 are mixed a complex of about 170kD is obtained, suggesting a direct interaction between p105 and p50 to form a heterodimeric complex.

Cotransfection of p105 and p50 or p65 results in a shift from nuclear to cytoplasmic localisation of p50 or p65 (71). Again using antisera raised against a peptide encompassing the NLS of p105, it has been shown that in p105 the NLS sequence is masked (47). Truncations at the C terminal end of p105 enabled recognition of the NLS, as did denaturation of p105.

Therefore, it seems likely that in p105 the C terminal I-kBɣ folds over to mask the NLS of p50 and thus retain the molecule in the cytoplasm. This association can also be maintained after cleavage of the molecule, thereby retaining p50 in the cytoplasm.

Activation of NF-kB and Its Control

The transcription factor NF-kB is composed of and controlled by several subunits belonging to two protein families : the rel and ankyrin families. Activation of the factor is

mediated by a wide range of stimuli in many different cell types and results in induction of many inducible genes.

The activation of NF-kB *in vitro* can be effected by various reagents which release I-kB from the cytoplasmic complex. These include sodium deoxycholate, protein phosphatases, low pH and protein kinases - which have all been mentioned already. Protein kinases appear to have a significant role to play, both in their own right and in their role as second messengers. Both PKA and PKC have been shown to activate DNA binding activity of NF-kB in the cytosol of pre B cells (72). Agents which activate protein kinase C have also, not surprisingly, been shown to induce DNA binding of NF-kB. These include active phorbol esters such as TPA, PMA, (9), PDB (73) and bacterial LPS (9). A recent report shows that activation of NF-kB in *Xenopus* oocytes through ras p21 or phospholipase C (PLC)-mediated phosphatidylcholine (PC) hydrolysis involves PKC_{β} (74). NF-kB activation only occurs when the *Xenopus* oocytes are stimulated into their maturation programme by insulin and not progesterone, showing that NF-kB only transmits signals to specific pathways.

Lectins, including PHA and Con A, can also activate NF-kB in T cells when used in conjunction with a phorbol ester. This pathway is also thought to be PKC mediated and results in expression of genes under the control of kB elements. In T cells, these include the HIV enhancer (74), the cytokine Il-2 (75), and the alpha chain of its high affinity receptor

(76).

The ability of cytokines to activate NF-kB has elicited much research. Both interleukin-1 (Il-1) and tumour necrosis factor α (TNFα) have been shown to activate NF-kB (77,78,79). The mechanism by which these agents activate NF-kB is still not completely clear, although reactive oxygen intermediates (ROI) have been shown to play a role in so far as counteracting the effect of ROI's using N-acetyl-L-cysteine (NAC) prevents activation of NF-kB by H_2O_2. It has also been shown that PKA and PKC can both trigger NF-kB activation and nuclear translocation in vitro (72), so this seems a likely activation pathway. Il-1 binding to cells has also been shown to lead to production of diacylglycerol (80), which is an activator of PKC thus suggesting Il-1 activation of NF-kB may be mediated through either protein kinase.

Both TNFα and TNFβ can activate NF-kB, via a pathway that does not require an increase in cellular cAMP levels (81). Neither cytokine has any effect on cAMP levels in HL60 cells. NF-kB activation occurs while the level of cAMP stays the same and the use of agents which increase levels of cAMP does not lead to NF-kB activation in these cells. The mechanism of NF-kB activation by TNFα does not involve activation of PKC as shown by studies on the cell lines Jurkat and K562 (another human leukaemic cell line) (82). The use of the protein kinase inhibitors H7 and staurosporine completely blocked activation of NF-kB by PMA

but had no effect on TNFα mediated activation. In Jurkat cells depleted of PKC, TNFα could still induce NF-kB activity. TNFα activation of NF-kB occurs very quickly, with high levels of NF-kB activity seen after only 2 - 4 minutes of addition to cells (83). This activity could be maintained for more than 3 hours but required continual protein synthesis and continual TNFα stimulation. In the same cell line (HL60), induction by PMA took 30 - 45 minutes and was totally dependent on protein synthesis, while in 70Z/3 cells induction was more rapid, and independent of protein synthesis. Both TNFα and PMA also induce NF-kB mRNA in Hela cells (36). Other cytokines such as Il-2, Il-4 and IFNγ do not activate NF-kB.

Other factors which can lead to induction of NF-kB activity are varied in type and include protein synthesis inhibitors (mentioned previously), double stranded RNA (84,85), damage to DNA (by UV light treatment with maximum induction after 2 hours) (86), viral transactivators and the parasite Theileria parva (reviewed in 87).

It appears that multiple pathways and signalling systems are involved in the induction of NF-kB in different cell types. Many do not require de novo protein synthesis thereby activation involves post-translational modification.

This post-translational modification is thought to involve phosphorylation of I-kB. It has been shown in vitro that purified I-kB's can be phosphorylated by PKC and PKA (63), but treatment of I-kBα with PKC abolishes inhibition while

treatment with PKA has no effect (59). The PKC preparation used was an impure preparation consisting of a mixture of different isotypes. Both forms of I-kB purified from human placenta can be inhibited by both PKA and PKC (60) but phosphorylation of pp40 (homologue of I-kBα from chicken cells) by PKA results in inactivation whereas PKC has no effect (69). It has now been shown that LPS activation of THP-1 cells and cytokine stimulation of Hela, Jurkat, or 70Z/3 cells all result in phosphorylation of I-kBα, providing evidence that this does take place in vivo. Following dissociation of I-kBα from the complex, mediated by phosphorylation, the inhibitor is degraded by proteases similar to chymotrypsin, namely TLCK (Na-p-Tosyl-L-Lysine Chloromethyl Ketone) and TPCK (Na-p-Tosyl-Phenylalanine Chloromethyl Ketone) (88). Inhibitors of these proteases block activation of NF-kB - as shown in PMA activated 70Z/3 cells or TNFα activated Jurkat cells. This argues that phosphorylation of I-kBα is not sufficient for activation, but that degradation is also required.

Following cell activation, phosphorylation and degradation of I-kBα, the inhibitor protein reappears in the cytoplasm about 2 hours later (89,90). Induction of NF-kB DNA binding activity, degradation of I-kBα and induction of I-kBα mRNA gene expression were all blocked by use of pyrrolidinedithiocarbamate (PDTC) which is a metal chelator and free radical scavenger. This inhibition has been shown in many studies (90,91,92,93) using various antioxidants,

thus suggesting a role for reactive oxygen intermediates in the activation of NF-kB.

In transfected cos-7 cells, p65 expression is required to induce I-kBα mRNA expression and protein production. This is also the case in transfected Jurkat cells. Association of I-kBα with p65 increases the half life of I-kBα from 40 minutes to 4 hours (89). This has been shown in other transfection systems (94,95) indicating the existence of an autoregulatory loop which maintains NF-kB complexed with I-kB in the cytoplasm until a specific activation signal induces translocation to the nucleus.

NF-kB Activation of HIV

Human Immunodeficiency Virus (HIV) is a retrovirus which infects cells expressing surface CD4, including T lymphocytes and cells of the macrophage lineage. This virus is the causative agent of acquired immunodeficiency syndrome (AIDS) (96), and so is the subject of much research, not least to investigate events involved in cell-virus interactions at the molecular level.

T cell activation can lead to production of HIV virus from latently infected cells. The long terminal repeat (LTR) of the HIV virus contains two NF-kB binding sites, and mutation of these sites abolished induction of virus production (23,97). Jurkat cells transfected with the viral tat 111 gene (encoding a potent activator of viral gene expression) (98) were used to investigate the effect of NF-kB on viral transcription and showed that the two factors act

synergistically to increase viral gene expression. In cells of monocyte/macrophage lineage, promonocytes do not constitutively express NF-kB until activated by agents such as TPA (73) or LPS (99). TPA stimulation of promonocytes results in transactivation of the HIV enhancer while in differentiated cells there is a high basal level of activity that is not affected by TPA stimulation.

HIV transcription in T cells is barely detectable in vivo, (100) with very little basal activity of the HIV enhancer and low nuclear NF-kB expression (101). In tissue macrophages continual virus transcription results in detectable levels of HIV proteins (102) and it has been shown that chronic HIV infection leads to induction of NF-kB and a permanent increase in HIV enhancer activity (103). This chronic infection also induces nuclear translocation of NF-kB - suggesting that in monocytes, HIV infection can also induce its own LTR transactivation. This HIV induced translocation does not involve a PKC pathway, as shown by PKC inhibitors (104). Analysis of the nuclear complex shows that it is a heterodimer of p50 and p65. Activity of the p105 promoter is also increased in chronically infected cells in a kB dependent manner. This shows that HIV exploits the characteristic of autoregulation of NF-kB functions.

Since PKC is not involved in the HIV mediated NF-kB activation, other mechanisms such as those discussed earlier for TNF or Il-1 stimulation may be involved, althogh neither cytokine is produced by HIV infection (103).

In T cells, it has been shown that HIV proviral DNA can produce infectious virus within 9 days in the absence of both kB sites from the enhancer, while the wild type provirus virus is produced within 5 days (105). In these cells, deletion of Sp-1 sites abolishes viral production - suggesting that, in the absence of NF-kB, Sp-1 can activate the HIV enhancer with lower efficiency than NF-kB. The importance of NF-kB in HIV activation varies from cell to cell and is markedly cell type specific.

Aims of study

The aims of this study were to produce polyclonal ond monoclonal antibodies against different subunits of the NF-kB family and against the cloned I-kB, MAD-3, and to use these antibodies to determine the subcellular localisation of these proteins in different cell types before and after activation by various stimuli.

Ultimately it is hoped that the work reported herein may contribute to the understanding of the events involved in the induction of latent HIV-1 to infectious virus in cells of the immune system, especially the role of NF-kB in this activation and the potential inhibition of this by the family of I-kB proteins.

MATERIALS AND METHODS

1. Cells and Tissue Culture Media

Jurkat cells (T cell leukaemia cell line) were grown as a suspension in RPMI medium (GIBCO) containing 10% foetal calf serum (Sera Lab Ltd). A549 cells (human lung carcinoma cell line) and Hela cells (human cervical carcinoma cell line) were grown as monolayers in Glasgow modified Eagle's Medium containing 50 units/ml of penicillin and 50 µg/ml of streptomycin (G-Mem GIBCO) supplemented with 5% newborn calf serum (Sera Lab Ltd) while MG63 cells (human osteosarcoma cell line) were also grown as monolayers in the same medium as above but supplemented with 10% FCS. All cell lines were maintained at 37°C.

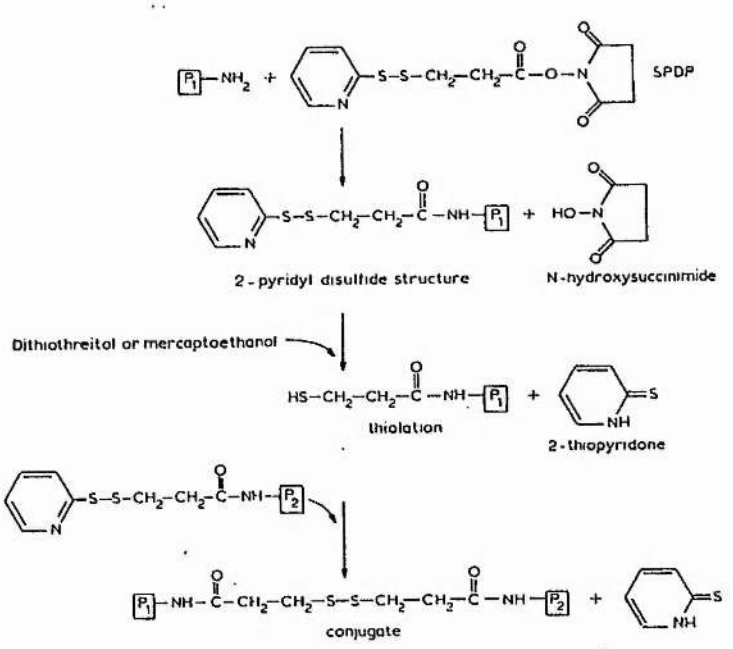
2. Production of Polyclonal Antisera.

a. Against Peptides

Peptides corresponding to the following regions of proteins were synthesised by Paul Talbot (Department of Cell and Molecular Biology, University of St. Andrews) : N and C termini of NF-kB p105, N and C termini of MAD-3, C terminal of p65 rel, and a region of ankyrin. Each peptide was synthesised using solid phase fluorenylmethoxycarbonyl polyamide chemistry on a CRB Pepsynthesiser 11 with a terminal cysteine residue to enable coupling to the linker molecule - this cysteine was at the C terminus of N terminal peptides and the N terminus of C terminal peptides. The peptide purity was determined by FPLC. The linker molecule SPDP (SIGMA,106) was coupled to a carrier protein Human

serum albumin (HSA, SIGMA), the carrier plus linker was then coupled to the peptide and efficiency of coupling checked by spectrophotometry. The coupling reaction is shown below in Figure 3.

Figure 3.



This method of coupling was chosen for many reasons. Its advantages include its speed and efficiency at pH 5 to 9, and most importantly, the fact that the amount of coupling can be quantified by spectrophotometry. The reaction is done in two stages, with the coupling of the carrier protein to SPDP being done first, and the efficiency checked by measuring the amount of pyridine-2-thione released on addition of dithiothreitol (DTT). Assuming a reasonable amount of coupling has been achieved (greater than 50%)

coupling of the peptide is then done. Again the efficiency of the coupling is checked before the preparation is aliquoted and stored at $-20^{o}C$. An example of the calculations done to check the level of coupling at both stages is shown below.

Efficiency of coupling is calculated using the formula:

$$A = Ec$$

where A = absorbance at 343nm

E = molar extinction coefficient for pyridine-2-thione

c = concentration

1. Coupling of HSA to SPDP

$A = 6.6$ $E = 8.08 \times 10^{3}$

$c = 6.6/8.08 \times 10^{3}$

$= 8.2 \times 10^{-4}$ => 0.82mM

concentration of lysine in HSA = molarity of HSA x no. of lysine residues

= 20 x 0.06

= 1.2mM

efficiency of derivatisation = 0.82/1.2 x 100%

= <u>68%</u>

2. coupling of peptide of HSA-SPDP

$A = 3.6$

$c = 3.6/8.08 \times 10^{3}$

$= 0.44$mM

efficiency of coupling = 0.44/0.68 x 100%

= <u>65%</u>

200µg of coupled product in 1ml PBS was mixed with 1ml Freunds complete adjuvant (SIGMA) and sonicated until a viscous white emulsion was formed. Two Dutch female rabbits (Hyline) were each subcutaneously injected at 4 sites with 1ml of this emulsion. The rabbits were boosted every two weeks with the emulsion containing Freund's incomplete adjuvant (SIGMA). 5ml of blood was removed by ear bleed from each rabbit prior to immunisation (preimmune bleed) and one week after each subsequent immunisation (test bleed).

b. Against Proteins

Rabbit polyclonal antisera were raised against cloned, bacterially expressed purified NF-kB p50, MAD-3, ankyrin repeat protein and p65-GST fusion protein (all obtained from E. Jaffray or J. Nicholson, both Department of Cell and Molecular Biology, University of St. Andrews.). The region of each protein expressed is shown below:

protein	amino acids expressed
p50	35 - 381
p65	12 - 317
I-kB	1 - 317
ankyrin	509 - 307

100µg of purified protein or fusion protein in 1ml PBS was mixed with 1ml Freund's complete adjuvant (SIGMA) and sonicated to form an emulsion as above. Two Dutch dwarf female rabbits (Hyline) were each subcutaneously injected with 1ml of emulsion at four sites. The rabbits were then given a booster injection two weeks later with emulsion

prepared as before but this time with Freund's incomplete adjuvant. Subsequent boosts, all prepared with Freund's incomplete adjuvant, were given at four weekly intervals. Preimmune and test bleeds were collected as above. A guinea pig was also boosted using the same protocol as for the rabbits, but only two test bleeds were taken, by heart puncture, after 3 boosts and 5 boosts. In accordance with Home Office regulations the guinea pig was then sacrificed. The blood was allowed to clot at room temperature for one to two hours then the clot loosened and the sera separated by centrifugation at 1200 rpm for 10 minutes. The sera was then stored in 0.5ml aliquots at -70°C. Once removed from -70° sodium azide was added to 0.1%, and the serum further aliquoted and stored in 50µl volumes at -20°C.

Once the titre of the test sera had plateaued, as determined by ELISA, the rabbits were sacrificed and bled out. The sera was separated as above but stored at -70°C in 1ml aliquots.

3. Enzyme Linked Immunosorbent Assay (ELISA)

a. To Determine Titre of Peptide Antisera.

Immulon 2 96 well plates (DYNATECH) were coated with 1% gluteraldehyde in PBS overnight at 4°C. Plates were then washed once with PBS and the peptide plated out at 10µg/ml in PBS. The carrier protein and an unrelated peptide were also included as controls. After incubation at room temperature for 1-2 hours, the peptides were removed and the plates dried at 37°C for 15 minutes. Wells were then blocked by incubating with 10%(w/v) non-fat powdered milk (Marvel)

and 0.1%(v/v) Tween 20 (SIGMA) at 37°C. The wells were washed three times with 1%(w/v) Marvel, 0.1%(v/v) Tween 20 in PBS before addition of the sera to be tested. Sera were assayed in duplicate in doubling dilutions from $^1/_{10}$ to $^1/_{10240}$, leaving the 1st column of each plate blank. The primary Ab was left for 1 hour at room temperature, the wells then washed three times as before and the second antibody, donkey anti-rabbit horseradish peroxidase (SIGMA), was added to all wells at a $^1/_{10000}$ dilution. This was incubated for 1 hour again at room temperature, the plates washed three times as before and the substrate TMB (DYNATECH) added. Plates were left in the dark for 20 minutes then read on an MR2000 plate reader (DYNATECH) at 630nm. Some of the ELISA's were performed using OPD (o-phenylene-diamine,SIGMA) as a substrate - these plates were read at 493nm.

b. Protein.

96 well plates (Immulon 2, DYNATECH) were coated with protein at a concentration of 10µg/ml in buffer containing 0.035M $NaHCO_3$ and 0.015M Na_2CO_3 (pH 9.6) and incubated at room temperature for 1 hour or overnight at 4°C. The protein solution was then removed and the wells dried and blocked as before. The procedure was continued as above.

4. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Biorad Mini Protean System Gel Rigs were assembled and used in accordance with the manufacturers instructions. 10% running gels were prepared using a 30% acrylamide stock

(29.25% acrylamide, 0.75% N,N'-methylene-bis-acrylamide) and also contained 0.375M Tris-HCL, pH8.9, 0.1% SDS, and $^{1}/_{250}$ volume of 25%(w/v) ammonium persulphate. Immediately before pouring the gel, $^{1}/_{2500}$ volume of TEMED (KODAK) was added to catalyse polymerisation. Once poured, the running gels were overlaid with isopropanol to prevent air bubbles and maintain an even surface - this was then removed and the gel surface rinsed with H_2O once the gel had polymerised. The stacking gel contained 5% acrylamide, 0.105M TrisHCl (pH 6.7), 0.1% SDS and APS as before. Again TEMED was added immediately prior to pouring the gel. Samples were denatured prior to loading on the gel by heating at 100°C for 2 minutes in 30%(v/v) glycerol, 15%(v/v) β-mercaptoethanol, 6%(w/v) SDS, 0.128M Tris-HCl pH 6.7 and 0.05% bromophenol blue. Electrophoresis buffer contained 0.025M Tris-HCl, 0.19M glycine and 0.1% SDS, and electrophoresis was done at 150 Volts.

5. Western Blotting

Separated proteins were transferred from SDS-PAGE gels to nitrocellulose membrane (Amersham) using a semidry blotter (Ancos) in a 20% methanol (v/v), 0.02M Tris pH8.0 transfer buffer by applying a current of 80mA for 60 minutes. The nitrocellulose membrane was blocked for 1 hour at 20°C in PBS, 0.1% Tween-20 (v/v), 5%(w/v) non fat powdered milk (Marvel), PTM, then incubated with antibody appropriately diluted in PTM for 1 hour at 20°C. The membrane was then washed twice in PTM before addition of horse radish

peroxidase conjugated second antibody - either donkey anti-rabbit immunoglobulin or sheep anti-mouse immunoglobulin (both Amersham, diluted 1:3000 in PTM) for 30 minutes at 20°C. The membrane was again twice washed in PTM, then finally once in PBS, 0.1% Tween before incubating the membrane in enhanced chemiluminescence (ECL) detection reagent (Amersham) and visualisation on X-ray film.

In some cases, after the primary antibody, and subsequent washes the antigen antibody complex was detected by addition of ^{125}I-Protein A diluted in 5ml PTM for 60 minutes at 20°C The nitrocellulose was then washed extensively in PBS until the background count dropped to 2-5 cps then exposed to X-ray film overnight at -70°C. Prestained molecular markers (Biorad) were run but were too faint to photograph, so the size of the bands was marked on the autorads.

6. Affinity Purification of Polyclonal Antisera.

Affinity chromatography was carried out using 1ml HiTrap NHS activated columns (Pharmacia). The appropriate purified protein was coupled to the NHS activated Sepharose as described in the manufacturer's instructions.

Firstly, the antibody solution was passed over a resin with bound GST and the flow through collected, then the affinity column pre-equilibrated with 10mM Tris-HCl (pH7.5). 1ml of polyclonal antisera was spun in a microfuge for 2 minutes to clear any debris and then diluted 1 in 10 in 10mM Tris-HCL (pH7.5). This was loaded onto the column in 1ml volumes - each being left on the column for 20 minutes to maximise Ab

binding. The flow through was collected and then loaded again in 1ml fractions, this time being left on the column for 10 minutes. The column was then washed with 10ml~10mM Tris (pH 7.5), then with 10ml 10mM Tris, 500mM NaCl (pH 7.5). Bound antibody was eluted using twenty 0.5ml aliquots of 100mM glycine-HCl (pH 2.25) and collected in 50µl 1M Tris (pH 8.0).

The fractions were tested for specific antibody activity in dot blot and Western blot analysis.

7. Immunofluorescence

Cells, either A549, Hela, or MG63, were grown on circular glass coverslips in wells on 24 well plates to about 80-90% confluence. The cells were then activated for the stipulated time using TNFα (SIGMA) at 10ng/ml (in the case of MG63 the cells were serum starved overnight prior to activation). After the appropriate activation time, cells were immediately washed once quickly in ice cold PBS then fixed for 10 minutes in 5%(v/v) formaldehyde, 2%(w/v) sucrose in PBS, followed by 2 washes in 1% normal donkey serum (SeraLab) in PBS. If not to be used immediately, the coverslips were stored at 4°C in PBS, 1% normal donkey serum, 0.1% NaN_3, and rinsed once in PBS before use. The cells were permeabilised for 5 minutes at 20°C in PBS, 1% normal donkey serum, 10%(w/v) sucrose, and 0.5% NP40 then washed twice in PBS, 1% normal donkey serum and once in PBS.

The primary antibodies were diluted as required in PBS, 1%

normal donkey serum and pipetted onto a glass slide in a 6µl drop. Coverslips were carefully inverted onto the antibody drop, the slides transferred to a moist chamber and incubated overnight at 4°C. The coverslips were then washed for 1 minute in permeabilisation buffer, 3 minutes in 3 changes of wash buffer and 1 minute in dH_2O.

Bound proteins were then visualised using FITC-conjugated donkey antirabbit IgG (if rabbit primary antisera were used) or Texas Red conjugated donkey anti-mouse IgG (both from Jackson ImmunoResearch Laboratories Inc.) diluted $^1/_{100}$ in wash buffer. The antibodies were applied as before and incubated for 60 minutes at 20°C, then cells were washed as before.

The cells were mounted in a glycerol/PBS solution (Citifluor Ltd) - the mount was pipetted onto a glass slide in a 1:5µl drop and the coverslip inverted onto it, taking care to avoid air bubbles. The upper (non cell) side of the coverslip was dried carefully and the coverslip sealed with nail varnish before viewing under a Nikon Microphot conventional immunofluorescence microscope or a laser scanning confocal microscope (Bio-Rad 500).

8. Production of Monoclonal Antibody

a. Immunisation

1mg of purified p50 was diluted to 800µl in PBS then denatured by heating to 70°C for 15 minutes. This was then mixed with 1mg of native purified p50 in 100µl PBS, and 1ml of Freund's complete adjuvant (SIGMA). The mixture was

sonicated until a viscous white emulsion was formed, then 200µl of this emulsion was injected into each of 10 Balb/c mice intraperitoneally. The mice were each boosted again two weeks later but this time the emulsion contained Freund's incomplete adjuvant (SIGMA). 7 days later, a test bleed of 50µl was taken from each mouse by tail vein bleed, and the sera tested by dot blot and Western blot analysis against both native and heat denatured p50.

The 4 mice giving the best response were boosted once more each with 100µl of a 50/50 mix of native and heat denatured p50 in PBS intravenously (into the tail vein). 4 days later 2 of the mice were sacrificed and their spleens removed for the fusion.

b. Fusion

Spleen cells were removed and washed, red blood cells were lysed by addition of 8% NH_4Cl for 10 minutes, then fused with the myeloma cell line SP2/0 using polyethylene glycol (PEG,SIGMA) at 37°C. Both cell types were in serum-free medium and were fused at a ratio of 1 myeloma : 3 spleen. After fusion, the cells were plated out at various dilutions (neat, 1:5, 1:25) in Dulbecco's modified Eagle's medium (D-Mem) containing 50µg/ml streptomycin plus 20%(v/v) foetal calf serum (Sera Labs Inc) and 150µg/ml recombinant human interleukin 6 (rhI1-6, Boehringer) supplemented with HAT (hypoxanthine aminoptorine thymidine [SIGMA]). After 10 days this medium was removed and replaced with the same, except the HAT supplement was replaced by HT supplement (SIGMA).

Wells were checked daily both macro and microscopically and once cells reached 70% confluence, the medium from the well was removed for testing in dot blot assay and replaced with fresh medium. If the medium tested positive the cells from that well were split into one well of a 24 well plate and the medium tested again, once confluence was reached. If still positive, the cells were split to give enough for freezing and subcloning. Each original well of the 96 well plates was tested twice before discarding as negative.

c. Subcloning

Once established in a 24 well plate, positive cultures were subcloned using the chequerboard method of dilution. This involved transferring 50µl of tissue culture medium containing hybridoma cells into the top left well of a 96 well tissue culture plate. This suspension was doubly diluted across the rest of the top row of wells in tissue culture medium, and then each of these wells was doubly diluted down the columns of the plate. Again wells were checked daily for the appearance of colonies - any arising from a single cell were noted then grown up and the medium tested.

Positive clones were again transferred to wells of a 24 well plate, a stock was frozen down and the remainder split to be grown in tissue culture and to inject into mice to produce ascitic fluid.

Once established, the tissue culture cell lines were transferred to protein-free hybridoma medium (PFHM11 :

GIBCO) supplemented with rh Il-6 at 50units/ml.

d. Freezing Stocks

Cells to be frozen were pelleted by centrifugation at 1200rpm for 10 minutes, washed twice in medium then resuspended in D-Mem containing 30% FCS and 10% DMSO (SIGMA) on ice. Cells were aliquoted into cryovials (1ml/vial) and immediately tranferred to $-70^{o}C$ for 24 hours after which they were transferred to liquid N_2.

e. Ascitic Fluid

Cells to be injected into mice for ascitic fluid production were transferred to serum-free medium, then resuspended in PBS and washed once.

The cells were then injected intraperitoneally into Balb/c mice which had been primed one week previously with Pristane (Sigma). The mice were checked daily for appearance of peritoneal swelling, and the ascitic fluid was tapped when the mice showed any discomfort.

Collected fluid was centrifuged at 1500rpm for 10 minutes, the serum was removed and left overnight at $4^{o}C$, then the clot removed and 1ml aliquots stored at $-70^{o}C$. The cells were resuspended in 7ml NH_4Cl for 15 minutes, then centrifuged as above, resuspended in PBS and left to stand for 10 minutes, after which the supernatant was removed, underlaid with 2ml FCS and centrifuged at 1500rpm for 10 minutes. The cells were then frozen or cultured at $37^{o}C$ in RPM1/10%FCS.

9. Dot Blot Assay

Sheets of nitrocellulose membrane (Amersham) were incubated with 200µg of protein for 15 minutes at 20°C, and the nitrocellulose then blocked with 10%(w/v) non-fat powdered milk (Marvel) for 30 minutes again at 20°C. If required, membranes could then be stored at 4°C, after addition of 0.1% NaN_3. Test sera or tissue culture media was added in 10µl volumes at appropriate dilution to the wells of a Terisaki plate. The antigen-soaked nitrocellulose membrane was blotted dry using a sheet of 3mm filter paper (Whatman) then placed over the wells of the plate, with a sheet of damp filter paper then placed on top of the membrane. A clean Teraski plate was inverted onto the filter paper and the wells of the two plates lined up to form a sealed channel. The 'sandwich' was then clamped at all four sides with bulldog clips, and inverted to drop the serum onto the nitrocellulose, then incubated for 60 minutes at 20°C. Plates were again inverted, the membrane removed, and washed three times in PBS containing 1%(w/v) non-fat milk powder (Marvel).

Antibody-protein complexes were detected using 10uCi ^{125}I-Protein A in PBS for 60 minutes at 20°C and the membrane then washed extensively in PBS until the background counts dropped to 2-5cps. The membrane was then exposed to X-ray film overnight at -70°C.

10. Preparation of Nuclear and Cytoplasmic Cell Extracts.

A549, Hela, or MG63 cells were grown as monolayers in 75cm^2

flasks to 90% confluence. The MG63 cells were serum-starved overnight before activation. Cells were activated with 5ng/ml tumour necrosis factor α (TNFα,SIGMA) for various times then the TNF-containing medium was poured off immediately and the cells harvested by scraping into ice cold PBS. Cells were pelleted by centrifugation at 1200rpm for 10 minutes and washed twice in ice cold PBS then lysed on ice. Lysis was done in 400µl of buffer containing 50mM NaCl, 10mM HEPES pH8.0, 500mM sucrose,1mM EDTA, 500µM spermidine, 150µM spermine, 0.2% Triton X, 200µM phenylmethylsulfonyl fluoride (PMSF), 2mM β mercaptoethanol and 50µM Na-p-Tosyl-phenylalanine chloromethyl Ketone (TPCK) on ice for 2 minutes followed by centrifugation in a microfuge at 6500rpm for 2 minutes and collection of the supernatant as cytoplasmic extract. This process was repeated and the supernatants from each time point pooled. The cell pellet was then washed very gently (rocking the tube - no resuspension) in 200µl of 50mM NaCl, 10mM HEPES pH8.0, 25% glycerol, 100µM EDTA, 500µM spermidine, 150µM spermine, 200µM PMSF, 2mM β mercaptoethanol and 50µM TPCK three times, and the nuclei then lysed on ice for 60 minutes with agitation in 80µl of 350mM NaCl, 10mM HEPES (pH8.0), 25% glycerol, 100µM EDTA, 500µM spermidine, 150µM spermine, 200µM PMSF, 2mM β- mercaptoethanol, and 50µM TPKC. The cell debris was removed by centrifugation in a microfuge at 13,000rpm, and the supernatant removed as nuclear extract. The method of Bradford (1970) was used to determine the

protein concentration of the extracts.

11. Production of Photographs

Negatives were produced from autoradiographs by Bill Blythe (Department of Cell and Molecular Biology, University of St. Andrews.) and then photographs made. The negative was put in an enlarger and focussed onto photographic paper. This was exposed for 3 - 5 seconds, put into developer for 1 minute (or until a clear image was obtained) then fixed for 5 minutes and finally washed under running water for 20 minutes. The prints were left to dry in a safety cabinet. For the immunofluorescence experiments negatives and photographs were produced by Bill Blythe.

RESULTS

Characterisation of Monoclonal Antibodies against p50

The first attempt to produce monoclonal antibodies against p50 was unsuccessful, since none of the clones tested reacted with p50 in a Western blot. Fifteen different clones were tested with the positive control, a polyclonal rabbit antisera against p50, giving a strong reaction but the others giving no reaction at all. The clones had all previously tested positive in a dot blot against native p50 using an anti-mouse immunoglobulin but failed to detect denatured p50 using the same detection system. Isotyping of the clones showed them all to be IgM. Since one of the major uses of the monoclones would be to detect proteins in Western blots it was decided to try the fusion again.

Figure 5 shows the results of dot blots performed to test the tail bleeds from the mice immunised with a mix of denatured and native p50. Bleeds from mice number 1, 2, 8, and 10 all reacted strongly with denatured p50 (Figure 5a) as did the polyclonal rabbit antisera. Against native p50 (Figure 5b) the same sera gave a positive, but slightly weaker response with the control polyclonal sera again giving a positive result. No reaction was seen against the control protein, BSA. The sera from the tail bleeds was then tested in a Western blot against the insect cell line (SF9) infected with Baculovirus expressing the p50 precursor p105. As shown in Figure 6, it was again the sera from mice 1, 2, 8, and 10 that gave a positive result, with no reaction seen

against uninfected SF9 cells. From these results, mice 1, 2, 8, and 10 were given a further tail vein boost as described in the Methods section.

Following the fusion of spleen and myeloma cells, and selection of fused cells using HAT medium, (see Methods), the wells were checked daily for cell growth, and medium tested when cells reached 70% confluence. Testing was done by dot blot with the medium being tested in 3 blots - one against native p50, another against denatured p50 and finally against BSA, a control to check for non-specific binding. Figure 7 shows the result of one such dot blot which identified one positive culture out of the thirty six tested to be positive against both native and denatured p50, with no reaction against BSA. A total of 12 positive cultures were identified in this way and medium was then tested in a Western blot against SF9 cells infected with Baculovirus expressing p105 and uninfected SF9 cells. Figure 8a shows the results obtained with three of these culture media, with 3B6 giving a positive reaction, lane 1, both 6F2 and 8B5 not giving any reaction, lanes 2 and 3 respectively, and the positive control rabbit polyclonal serum giving a positive result as expected. None reacts with the control uninfected SF9 cells, suggesting the result to be specific. Both 6F2 and 8B5 only reacted with native p50 on a dot blot, so this result serves to confirm their non reaction with denatured protein and confirms the recognition of denatured protein by 3B6. As in previous results, the band detected is

lower than that expected for p105, suggesting that proteolysis has occurred to produce p50.

All the positive cultures were then subcloned but problems were encountered at this stage . Medium from wells containing cultures which had grown up from a single cell was tested in dot blots as before (data not shown). From 5 subclones grown up from 3B6, only 3B6A and 3B6B gave a positive result with both recognising native and denatured p50. None of the other eleven cultures subcloned gave rise to antibody producing clones, with some failing to grow altogether. This does occur with hybridoma cells which tend to shed chromosomes as they replicate to reduce their chromosome number. Thus they may lose genes essential for growth, or they may lose genes encoding the antibody. The two subclones obtained from 3B6 were the only monoclones obtained from the second fusion - and were injected into mice to produce ascitic fluid, and grown up in tissue culture to produce antibody containing medium and stocks of cells to freeze down. The ascitic fluid production was not a success since the mice formed solid tumours and had to be destroyed. However, the tissue culture cells grew successfully, and it was decided not to pursue ascitic fluid production further, as it was not deemed to be necessary. Samples of medium from cultures of both 3B6A and B were tested regularly by Western blot against purified p50. An example of such a blot is shown in Figure 8b - with lanes 1-8 blotted with medium from 3B6A cultures at various times

after splitting, and lanes 9-16 with medium from 3B6B cultures. All of the samples tested positive at all times tested and were then tested at various dilutions against SF9 cells infected with Baculovirus expressing p105. Figure 9 shows the dilution of 3B6A (lanes 2-8) and 3B6B (lanes 9-15). The band seen in lane 1 is probably due to leakage of antibody out of the manifold used in the blot. The activity of the antibody is seen to dilute out down the doubling dilution series, with 3B6A giving a stronger result throughout. This is probably due to variation in the cell numbers and medium volume between the two cultures.

An ELISA was performed to determine the strength of reaction of the antibodies against native p50 and the control protein BSA. Similar results were obtained with medium from both antibodies, and the results obtained with 3B6A are summarised in Figure 10. Both antibodies reacted strongly with p50 and gave no reaction against BSA. Following this result, the 3B6 antibodies were used in immunofluorescence experiments on both A549 and Hela cells either inactivated or activated with PMA and PHA or TNF-α. Both antibodies failed to detect anything in any experiments carried out (data not shown) - suggesting that the epitope recognised by the antibodies <u>in vitro</u> was not accessible <u>in vivo</u>. Finally on these preliminary characterisation experiments both antibodies were isotyped and identified as IgG1.

All the results obtained up to this stage are identical with both antibodies, suggesting that they may be very similar,

if not the same. Functionally, they do not show any difference in behaviour at all, and are likely to be indistinguishable.

Mapping of the epitope of 3B6A and B.

The recognition of both native and denatured p50 by the monoclonal antibodies suggested that they reacted with a linear rather than conformational epitope. To try to identify the epitope on p50 recognised by the antibody, trypsin was used to digest p50. This digestion generated two products which could be separated by SDS-Page and visualised by staining with Coomassie Brilliant Blue, as shown in Figure 11a. The result seen when digested p50 was Western blotted with 3B6A is shown in Figure 11b. Neither of the trypsin digestion products was recognised by the antibody but a strong reaction with the uncleaved p50 was obtained. The site of trypsin cleavage of p50 had been determined previously in our laboratory (107) and lies within the NLS of p50, shown in Figure 11c. The same results were obtained with 3B6B, and thus show that at least part of the epitope recognised by the mAbs is found within the NLS of p50.

The next approach to further define the region on p50 which binds the mAbs was to synthesise two peptides : a 10mer which starts at the beginning of the NLS (amino acid 362) just upstream of trypsin cleavage site and a 20mer which extends from amino acid 352, further upstream of the NLS and trypsin cleavage site. The sequences and localisation of the peptides are shown in Figure 12a. The mAbs were incubated

with the peptides before being used in a Western blot with p50. Incubation of the mAbs with the 20mer peptide blocked binding of 3B6A to p50 while the 10mer had no effect as shown in figure 12b, with the same result seen with 3B6B (Figure 14c). Even at a concentration of 0.05mg/ml the 20mer inhibited binding of p50 by more than 50% while the 10mer had no effect even at concentrations of 1mg/ml. These results indicate that the binding site of the mAbs on p50 is located between amino acids 352-371, and includes the trypsin cleavage site at residue 362. Since the epitope for the antibody binding seems to include the NLS of p50, a Western blot was performed using 3B6A on p50 and a protein including the NLS of p65. As can be seen in Figure 12c there is no cross reaction between 3B6A and p65.

Production of Polyclonal Antisera.

Polyclonal antisera have been raised against many proteins and peptides of the rel and ankyrin family of proteins by immunisation of rabbits or guinea pigs. In rabbits, antisera against p50, p65, ppank (a protein corresponding to 7 ankyrin repeats of p105) and MAD-3 have been produced as well as antisera against N and C terminal of p105, N and C terminal of I-kB, and the C terminal of rel. The sequences of these peptides are shown in Figure 13. Most peptides alone are not large enough to elicit an immune response and so these peptides were coupled through a C terminal cysteine residue to the linker SPDP, and then to a carrier protein - HSA. A guinea pig was also immunised with MAD-3. Test bleeds

from the rabbits and guinea pig were taken and tested by ELISA. The results from some of these ELISA's are shown in Figure 14. As shown, the p50 antibody titre from each rabbit (P & M) is good and it stayed like this until the final bleed - bleed 17. The peptide antisera typically show a high response to the carrier protein but usually also a good response to the peptide too. With the C terminal p105 peptide, this is seen in sera from both rabbits (E & F) with no cross reaction with the N terminal peptide. However the N terminal peptide antisera from both rabbits (T & V) reacts well with HSA, but shows no reaction with the peptide. The epitope recognised by antisera against this peptide must be blocked in the ELISA since the anti - N terminal sera detected p50 in a Western blot Figure 15b). The N terminal peptide contains a Lysine residue which may bind to the gluteraldehyde used to coat the ELISA plate - this residue may constitute part of the epitope recognised by the sera, and so be inaccessible for Ab binding in the ELISA. The varying activity of sera collected at different times is shown in Figure 14d. This shows a marked increase in activity at bleed 2 while the response in rabbit D is much higher in bleed 3 than any other. Rabbit G shows a much more typical profile - with the titre being maintained in later bleeds. All the antisera that have been produced and tested to date have given strong reactions with the protein used for immunisation (data not shown).

Some of the antisera produced have been used further to

elucidate the subcellular localisation of different subunits of NF-kB and I-kB in unactivated and activated cells.

Localisation of p105.

Early experiments made use of the antisera raised against the N and C terminal peptides of p105. Western blots using nuclear and cytoplasmic extracts of Jurkat cells before and after activation with PMA and PHA were probed with these peptide antisera. Figure 15a shows the C terminal peptide antisera detects a band in cytoplasmic extracts of unactivated cells but nothing in cytoplasmic extracts of activated cells or in nuclear extracts of unactivated or activated cells, although there is a mark on the blot in the activated nuclear extract lane. However, the edges and corners of the blot often show some marking due to handling of the nitrocellulose membrane with tweezers. Since it is generally accepted that p105 is not found in the nucleus of activated cells and the mark is more like two dots than a band, this would seem the most likely explanation. This band corresponds to p105 - as seen by its size and the expected distribution. The N terminal peptide serum detects a smaller size band, generated by proteolytic cleavage of p105 - this is p50 (Figure 15b). Blocking of the sera by incubation with the corresponding peptide abolished recognition by the antisera.

Immunofluorescence experiments were also carried out using the peptide antisera, but the results obtained were not consistent and so were difficult to interpret. The results

are not shown since they did not photograph clearly .

Further Characterisation of Antisera.

More Western blots were performed using nuclear and cytoplasmic extracts from PMA-activated Jurkat cells probed with the polyclonal p50 antibody (data not shown). This antibody detects a range of bands with the strongest in the activated nuclear extracts and corresponding to about 50kD size. This band is also seen in the cytoplasm where it is less intense. There are also higher molecular weight bands corresponding to p105 seen in the cytoplasm and decreasing in intensity following activation.

Western blots were then done with the I-kB-GST fusion protein or purified I-kB. The blots were probed with preimmune sera, polyclonal I-kB rabbit sera, and polyclonal sera from both the guinea pig bleeds. The blot results are shown in Figure 16. The preimmune sera do not detect anything in either blot, while all three polyclonal sera pick up 3 bands against the fusion protein, and only the smallest band in the I-kB blot. The smallest band is likely to be I-kB, while the larger bands in the fusion protein blot will be fusion protein and GST.

Localisation of p50 and I-kB

To increase the specificity of reaction and decrease background reactions, the polyclonal antisera against p50, I-kB, and p65 were affinity purified. Since I-kB was produced as a fusion protein with GST, the Ab was purified first over a GST column, then a Hitrap I-kB column. The

purification was tested by dot blot since I-kB does not bind well to Coomassie and so cannot be assayed by the method of Bradford. The results of the purification were good with the Ab eluting in fractions 1-8 (Figure 17). The other antisera - against p50 and p65 - were purified by J Bosher and F Arenzana-Seisdedos in our laboratory.

Work on refining the protocol for preparation of nuclear and cytoplasmic extracts was performed in our laboratory by F Arenzana-Seisdedos and R T Hay (Department of Cell and Molecular Biology, University of St. Andrews.). This protocol was then used to prepare nuclear and cytoplasmic extracts from A549, Hela, and MG63 cells to be used in Western blots to determine the localisation of I-kB and p105/p50 at different times after activation with TNFx. Firstly, a Western blot was performed using p50 Ab to determine efficiency of the new extraction protocol. As shown in Figure 18a, p105 is observed in the cytoplasmic extracts of MG63 cells at all times after activation, while p50, but not p105, was present in the nuclear extracts - suggesting no contamination of nuclear extracts by cytoplasm.

Blots were then performed on the three different cell types using the purified p50 and I-kB polyclonal antisera. In A549 cells, I-kB was shown to be present in the nucleus before TNF activation but no longer there 30 minutes later with a faint band seen at 2 hours after activation (Figure 19a). In cytoplasmic extracts, I-kB was seen before activation but

had disappeared at 30 minutes and 1 hour after activation, reappearing at 2 hours post activation (Figure 19b). In both Figure 19a and b, no reaction is seen with the purified I-kB Ab against 10ng GST, and 10ng Bcl-3, with very strong activity against 10ng I-kB and one clear band seen against 1ng I-kB. This shows the specificity and purity of the Ab.

In MG63 cells, no nuclear I-kB was detected - only a non-specific band which is often observed (Figure 20 lanes 1-5), but in the cytoplasm, I-kB is seen before activation (Figure 20 lane 6), very faintly after 5 minutes activation (lane 7) and 2 hours after activation (lane 10). As shown in Figure 18, the levels of p50 increased in the nucleus 5 minutes after activation and stayed at an elevated level through 15 and 45 minutes after activation, decreasing back to that seen before activation by 2 hours. In the cytoplasmic extracts, a slight decrease in p105 levels was seen after TNF activation returning to basal level by 2 hours after activation. Very little change was observed in cytoplasmic p50 levels.

Finally, Western blot analysis of Hela cell nuclear extracts again showed a decrease in nuclear I-kB 10 minutes after TNF activation, with no I-kB seen 50 minutes after activation and a reappearance beginning at 2 hours post-activation (Figure 21a lanes 1-4). An increase in nuclear p50 is observed 10 minutes after TNF activation and this is maintained throughout (Figure 21b lanes 1-4). The corresponding blot on cytoplasmic extracts showed no

degradation of cytoplasmic I-kB and showed no change in p50 levels (Figure 21b lanes 5-8). However, on testing the batch of TNF used for activity, very low levels of activity were found, so the experiment was repeated using extracts activated with a new batch of TNFα. The results of this blot on extracts activated for 0 minutes, 50 minutes and 2 hours are shown in Figure 21c. These results show a low level of I-kB present before activation, with none seen after 50 minutes and reappearance after 2 hours.

In all of these experiments a higher molecular weight band (about 80kD) is often detected by the I-kB antisera - this has been observed consistently by many workers in the laboratory, and is likely due to cross-reaction with another protein. However, the fact that this band shows an inverse relationship with the I-kB band suggests that I-kB itself may form part of a complex which is detected by this antibody. After prolonged cell activation this complex breaks up and releases the I-kB. The p50 Ab detects p50 as a doublet, which decreases slightly in size immediately after activation.

Since the presence of I-kB in the nucleus of cells remains a controversial issue, further studies were performed by immunofluorescence on Hela and MG63 cells at different times following TNFα activation. The time course used for each cell type was the same as that used for the corresponding cell type in the Western blots.

In MG63 cells a broad distribution of I-kB in both nucleus

and cytoplasm of cells before activation by TNFα (Figure 22a). At 5 and 15 minutes after activation there is no staining of cells in either nucleus or cytoplasm (Figure 22b and c) but I-kB reappears in cells at 45 minutes and 2 hours after activation, again staining in both nucleus and cytoplasm (Figure 22d and e). In Hela cells however, there is no detectable change throughout the time course of activation (Figure 23a,c,e,g) with I-kB maintaining a nuclear and cytoplasmic distribution throughout. The corresponding time course of activation using preimmune rabbit serum is shown in Figure 23(b,d,f,h) - very little staining is observed in these cells, suggesting the staining seen using the I-kB antibody is specific. Hela cells stained with p50 antibody are shown in Figure 24(a-d). A shift from general cell staining with more cytoplasmic p50/p105 to predominantly nuclear staining in TNFα activated cells is seen, with a return to more overall cell staining by 50 minutes and 2 hours, although the cytoplasmic staining is still not as pronounced as before activation. The lack of staining seen with pre-immune serum is shown in Figure 24 e and f. In Hela cells the effect of TNF seemed minimal, with no major differences seen in the staining pattern of the proteins. This illustrates the difficulties that can be encountered in trying to detect relatively low levels of cellular proteins with polyclonal antisera and also explains the lack of published immunofluorescence data except in transfected cells. Much time was put into trying to optimise

the fluorescence procedure and following a few further modifications a clear data set should be obtained on Hela cells as well as the MG63 cells.

In some of the immunofluorescence experiments the staining of the protein is diffuse while in others a more speckled pattern is seen. It could be that the more speckled staining illustrates proteins localised to distinct subcellular sites. The nonspeckled staining may be due to diffusion of proteins to or from these specific sites in response to activation signals. Further work will be required to elucidate the nature of these protein pockets.

DISCUSSION

Using the polyclonal antisera against p50 and I-kB it has been possible to demonstrate the subcellular localisation of these proteins. As has been shown previously, indirect immunofluorescence studies illustrate that on activation of cells with PMA, p50 moves from predominantly cytoplasmic to a predominantly nuclear staining.

Antibodies to the MAD-3/I-kBα protein show that this inhibitor can be located in both the nucleus and cytoplasm of cells. The results obtained in fluorescence correlate with other observations (48) from transfection experiments that show MAD-3 can be located in the nucleus of cells. It is not clear whether MAD-3 has its own NLS or passively enters the nucleus due to its size of only 37kD (37) but the presence of the inhibitor in the nucleus is not particularly surprising. There it could facilitate the inactivation of NF-kB released from DNA or could actively remove NF-kB from DNA, thus halting transcription induced by NF-kB which otherwise could continue uncontrolled. This potential role for I-kBα is supported by an early observation that inactivation and redistribution of NF-kB from nucleus to cytoplasm 8 hours after phorbol ester stimulation was inhibited by cycloheximide - which suggests the involvement of a newly synthesised protein. As p65 has since been shown to induce I-kBα mRNA expression, extracellular stimulation of cells does lead to production of I-kBα about 2 hours after stimulation.

The data seen in Western blots carried out on TNFα activated cells confirm that nuclear I-kB is seen before activation, then disappears and reappears about 2 hours after stimulation. Degradation of I-kB in both nucleus and cytoplasm was seen in all cell lines tested, both here and in other experiments carried out in our laboratory, following activation, reappearing some time after activation. However, in immunofluorescence experiments different results were seen in Hela cells and MG63 cells, with disappearance of I-kB following activation only detected in MG63 cells.

In MG63 cells, the time points examined are 5, 15 and 45 minutes post-activation, with the MAD-3 reappearing by 45 minutes. Further refining of the immunofluorescence protocol may also be required, and the condition of the cells when the fixing, permeabilisation, and staining is done may prove critical to the result. The experiment shown on MG63 cells used cells activated that same day, whereas the Hela cells were stored overnight at $4^{o}C$ after fixing, although this is unlikely to make protein 'reappear'! The MAD-3 in Hela cells after activation is maintained in the cytoplasm - as seen in Western blots - and may leech back into the nucleus following fixation, especially if the cells are stored at $4^{o}C$ for a length of time. The nucleus does stain deceptively strongly due to its depth on the coverslips. Cytoplasm is spread out thinly on the glass but the nuclei remain in their rounded form and so give a much stronger staining.

Therefore a relatively small amount of MAD-3 (or any protein) leeching from cytoplasm to nucleus would give quite a visible staining. However, formaldehyde fixation should be sufficient to prevent this. Fluorescence studies on cells transfected with MAD-3 have shown the protein to be present in the nucleus of cells (48) but the system used over-expressed proteins and so may not be an accurate reflection of the <u>in vivo</u> situation. The presence of residual MAD-3 in the nucleus has recently been shown in cells treated with TNFα in the presence of TLCK (a serine protease inhibitor) (108). In these cells nuclear translocation of p50 is not completely blocked but there is no detectable DNA binding activity. This residual MAD-3 may not be sufficient to give the observed staining in Hela cells - but could contribute to the leeching MAD-3 - such that small amounts of MAD-3 which may not be detectable in their own right may add together to give a detectable level.

Demonstration of p50 and I-kB in the nucleus of cells before activation, and the subsequent disappearance of I-kB with p50 remaining, would give the best evidence for I-kB in the nucleus. This is difficult to show due to the lack of any pattern to the staining in the nucleus. The use of laser scanning confocal microscopy may aid this investigation since it 'slices' the nucleus so that single layers can be examined, as has been shown for p50. The guinea pig I-kB Ab and rabbit p50 Ab could be used to enable differential

staining of the two proteins.

The production of monoclonal antibodies againt p50 has for some reason proved very difficult. Attempts by many laboratories have proved fruitless while the yield of productive clones from successful fusions has been very low. The method described in Chapter Two was modified from a first unsuccessful attempt and incorporated several changes. First time round, mice were injected with native p50 only - not a mixture of native and denatured protein. The detection method was changed from ELISA using anti mouse immunoglobulin - thereby detecting all classes of antibody produced against native p50 - to two dot blots (one against native, the other denatured p50) detected by [^{125}I]-Protein A, which only detects IgG antibodies. The reasoning behind the changes was simple : the ELISA used originally was not sensitive enough, with very little difference in readings between background and positive values and it required at least 50µl of tissue culture medium. The dot blot is quicker, much more sensitive, and allows testing against both native and denatured protein. The injection of a mixture of native and denatured protein was used to produce antibodies which would be useful for Western blotting - since the first fusion using native p50 only - did not produce any clones reactive against denatured p50. All the positive clones from the first fusion were found to be IgM, and so proved difficult to use - giving very poor detection levels in ELISA and immunofluorescence - so it was decided

to use [^{125}I]-Protein A to identify positive clones in the second fusion, so that only antibodies of IgG subclass would be selected.

Finally, from two fusions and many subclonings, a positive monoclonal antibody was obtained and grown up in bulk in serum-free medium (to maintain purity and remove need for purification). The original dot blot screening showed it to recognise both native and denatured p50 and it picks up a single band corresponding to p50 when used in Western blots. However, it failed to detect anything in immunofluorescence experiments, suggesting that in vivo its epitope on the protein might be masked. Since the antibody could recognise both native and denatured protein, the epitope was likely to be linear and in Western blots only recognised p50, not p105. Recognition of p50 but not BSA in ELISA confirms that the antibody specifically recognises native p50. The results of the cleavage experiments using trypsin on p50 narrowed down the location of the antibody binding site and competition experiments with synthesised peptides showed that the antibody binds to a region within the NLS of p50. Further studies (109) illustrated that binding of the mAb 3B6-A to p50 is blocked by addition of a protein mentioned briefly before, consisting of seven ankyrin repeats, and by the C terminal region of p105 - confirming the result seen in Western blots of insect cells expressing p105.

The monoclonal antibody 3B6-A has already proved to be useful in identifying proteins which bind to the NLS of p50.

Already other studies in our laboratory (109) have shown that 3B6A does not inhibit DNA binding of p50 - confirming that the NLS is not involved. No cross reaction is seen between 3B6A and p65, suggesting the monoclone recognises a unique epitope on p50. This should also prove useful since the high degree of homology between rel family proteins always means the possibility of cross reactions. Unfortunately, the monoclone does not detect protein in immunofluorescence studies. This is to be expected in the cytoplasm since the NLS is masked, presumably by the C terminal of p105. However it also suggests masking of the epitope once p50 has been translocated to the nucleus. The next problem is to identify the protein which masks the epitope. It is very unlikely to be p65, since 3B6A will immunoprecipitate a heterodimer of p50-p65. Again, MAD-3 could be a candidate given that it is detected in the nucleus of cells, and that it does partially protect the NLS of p50 from trypsin cleavage. However, the monoclone cannot immunoprecipitate complexes of MAD-3 and p50 (109). Further investigation is required into the interactions of p50 with other proteins once it is translocated to the nucleus. It had been hoped that a panel of monoclones recognising different regions of p50 would be obtained to help elucidate further the areas involved in interaction with other proteins - unfortunately this was not accomplished. The non reactivity of the antibody in immunofluorescence is frustrating since it could have been used in double

labelling experiments to attempt to determine the relative localisation of p50 and MAD-3. However a guinea pig polyclonal antiserum has been raised against MAD-3, so perhaps studies with this in the future will further elucidate the interaction between these two proteins, since the rabbit p50 Ab and guinea pig I-kB Ab could be detected by different fluorochromes.

Many other antisera have been raised - but not as yet put to much use. These include antibodies against p65, the partial ankyrin protein (seven ankyrin repeats), and peptide antisera to the N and C termini of I-kB and p105. The polyclonal p65 antiserum has been used extensively by F. Arenzana-Seisdedos and R. T. Hay. For each protein or peptide, two rabbits were immunised and in most cases both produced serum which reacted strongly with the immunogen. However, with p65 only one rabbit gave reactive serum and only one bleed from this rabbit was of good reactivity. Usually the titre of the antibody (as measured in ELISA) increased at each bleed then plateaued off, at which time the rabbits were sacrificed and bled out. Some rabbits continued to produce high titre sera until about bleed 17 (p50), and the sera increased in affinity as the immune response continued to select for high affinity clones.The case with p65 was surprising and the reason behind it not clear unless the protein injected became less pure such that the response was raised against a wider range of epitopes.

The production of peptide antisera was successful to a large

extent, although the sera have to be purified due to the large response elicited against the carrier proteins. Because of the need for careful purification, and the range of cross reactions which may occur, the peptide antisera have as yet not been used routinely with the exception of the N and C terminal p105 sera. These were used early on in the project in both Western blots and immunofluorescence to determine the subcellular localisation of p50 and p105. Surprisingly, it was observed that following cell activation p105 was detected in the nucleus of Jurkat cells, but using an improved extraction procedure this was no longer observed, with p105 only detected in cytoplasmic extracts, therefore the original detection was probably due to contamination of nuclear extracts with cytoplasm. It is still controversial as to whether p105 can enter the nucleus of activated cells although it has been detected in the nuclei of unactivated cells. A complex formed with the HTLV-1 Tax protein (in its active form) and p105 has been shown to translocate into the nucleus (110), and in Jurkat cells a complex between hc-Rel and p105 is also found in the nucleus (111). However, immunofluorescence studies on a GAL4-p105 fusion protein transfected in CEF cells showed the p105 fusion protein to have a cytoplasmic localisation (112). The first two studies reflect a more in vivo situation, but there is considerable variation in results from different groups using different cell types. It would seem that p105 maintains a cytoplasmic localisation unless

in complex with another protein which may result in a conformational change to unmask its NLS, or to provide another NLS. It does seem possible that p105 can enter the nucleus of cells : recent evidence that MAD-3, one of the I-kB family, can be located in the nucleus would lend support to this, since the C terminal of p105 encodes on I-kB protein.

It is hoped that the work described in this thesis will contribute to the elucidation of the role of NF-kB and I-kB proteins in cellular activation, and that a further understanding of these events will aid work towards the prevention and / or treatment of diseases such as viral infection and cancer.

Figure 5

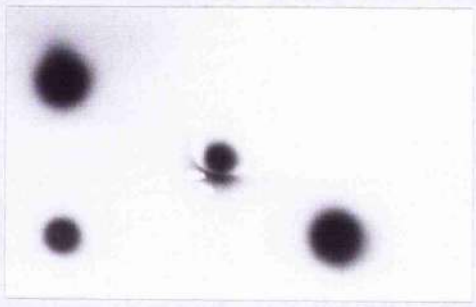
Dot blot showing the reactivity of sera from tail bleeds of mice immunised with a mix of native and denatured p50. Sheets of nitrocellulose paper were incubated with native or denatured p50 then serum was applied as described in Methods. Bound antibodies were detected by ^{125}I-Protein A and autoradiography. Activity against denatured p50 is shown in A, against native p50 in B, and against BSA as a control in C.

The layout of the sera on the blot is as follows:-

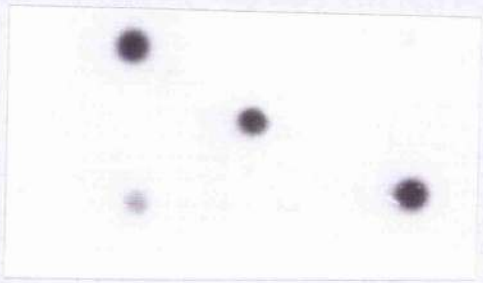
1	2
8	
10	+ve C

+ve C is rabbit polyclonal anti-p50 Ab

A



B



C

Figure 6

Western blot showing the reactivities of sera from tail bleeds of mice immunised with a mixture of native and denatured p50. SF9 cells infected with Baculovirus expressing p105 or control cells infected with non-transfected Baculovirus were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Bound antibodies were detected by ^{125}I-Protein A and autoradiography. The number above each lane corresponds to the number of the mouse from which the serum was taken, with the sera used at $^{1}/_{200}$ dilution. The blot with SF9 cells expressing p105 is shown in A - with sera 1, 2, 8, and 10 giving a positive reaction to a p50 size band while B shows none of the sera react with SF9 cells not expressing p105.

A

1 2 3 4 5 6 7 8 9 10

B

1 2 3 4 5 6 7 8 9 10

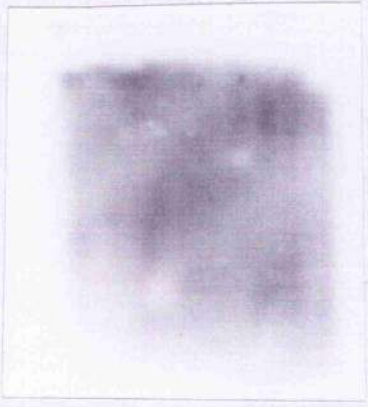


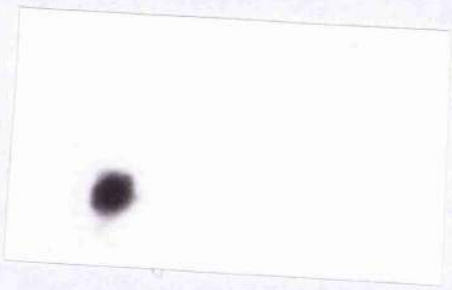
Figure 7

Testing of hybridoma culture medium by dot blot against native or denatured p50 or BSA. Blot was performed as described previously (Figure 5). Panel A shows the result using denatured p50, panel B native p50, and panel C BSA. One positive is seen in both A and B - this is the same hybridoma in both - 3B6. No reaction is seen with BSA.

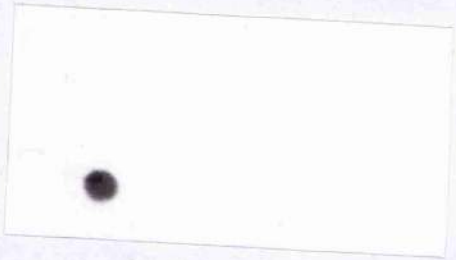
Layout of the blot is as follows:-

3B6	8B5	6F2

A



B



C

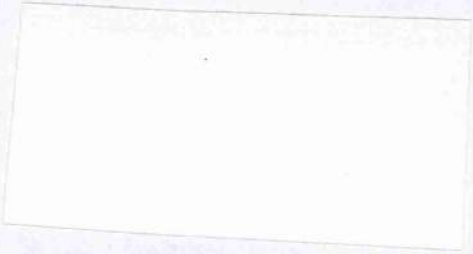
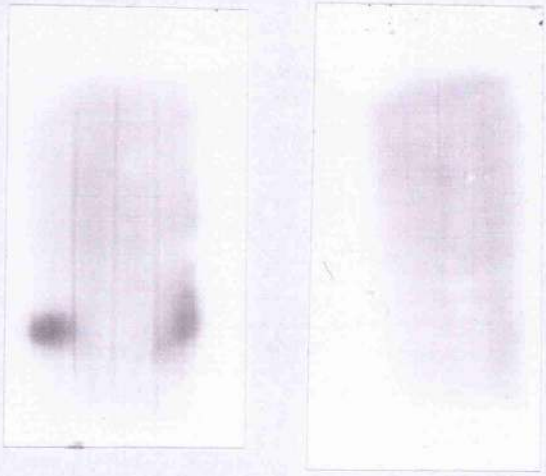


Figure 8

A. Western blot of culture medium from hybridomas tested positive in dot blot. Blot performed as in Figure 6 with Baculovirus infected SF9 cells expressing (Panel A), or not expressing (Panel B), p105. The medium tested is from hybridoma 3B6 (lane 1), 6F2 (lane 2), 8B5 (lane 3), and anti-p50 rabbit polyclonal antisera is used in lane 4 as a positive control. Only 3B6 and the control Ab give a positive reaction - none reacts with the control cells which do not contain p105.

B. Western blot with p50 run on SDS-PAGE then transferred to nitrocellulose membrane. Detection is by ECL (chemiluminescence system) for a 30 second exposure. The blot is probed with tissue culture medium from various 3B6A cultures: 1 (lane 1), 2 (lane), 5 (lane 3), 7 (lane 4), 8 (lane 5), 12 (lane 6), 16 (lane 7),and 20 (lane 8) : or 3B6B cultures : 2 (lane 9), 4 (lane 10), 8 (lane 11), 11 (lane 12), 14 (lane 13), 20 (lane 14), 21 (lane 15), and 23 (lane 16).

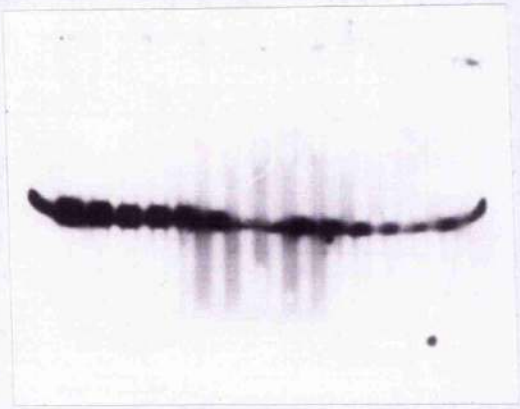
A



B

Figure 9

Western blot of Bacterially expressed p50 separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Detection is by ECL for 1 minute exposure. Both antibodies 3B6A and 3B6B are used in a series of doubling dilutions from 1 in 2 to 1 in 128 with 3B6A in lanes 2-8, and 3B6B in lanes 9-15.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 10

Graph showing results of an ELISA to determine the level of binding of 3B6A to p50. 3B6A culture medium was added in doubling dilutions to wells of a 96 well plate coated with native p50. Bound antibody was detected by anti-mouse IgG and TMB substrate. Each point on the graph represents the average of 4 OD readings obtained for each dilution at 630nm. The first point represents neat tissue culture medium, and the last is $^{1}/_{1024}$.

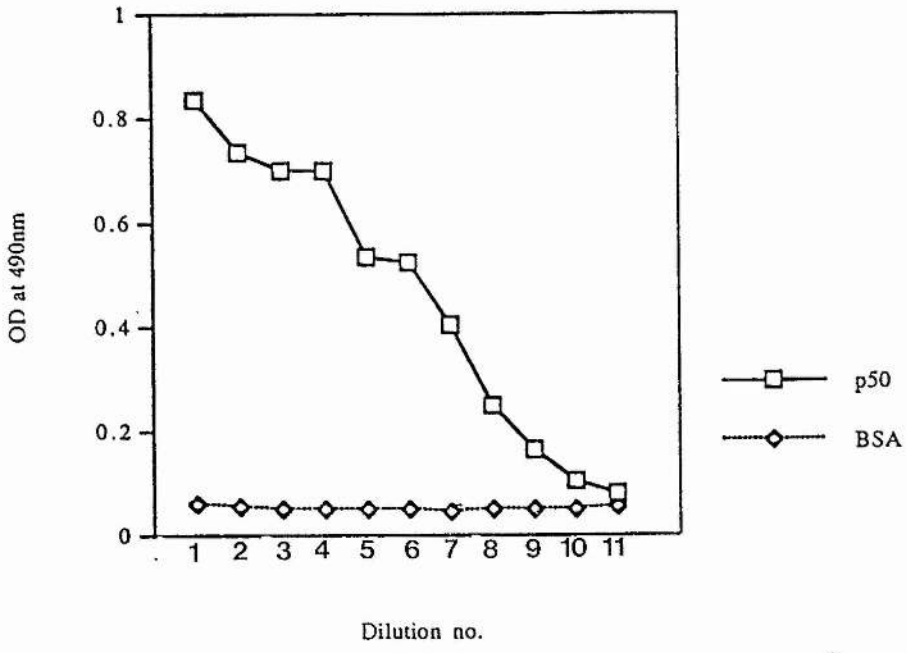
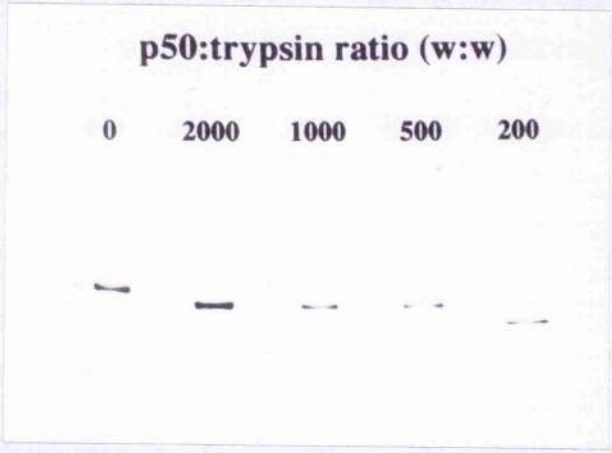

OD at 490nm
1
0.8
0.6
0.4
0.2
0
1 2 3 4 5 6 7 8 9 10 11
Dilution no.
p50
BSA

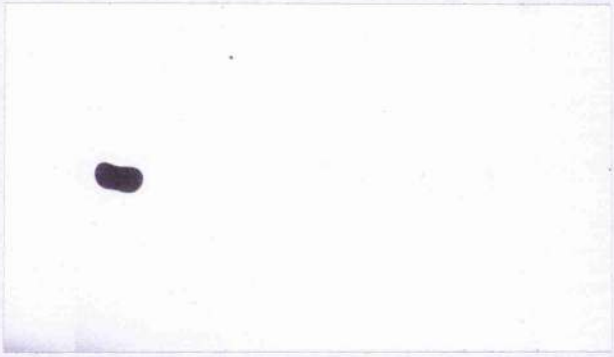
Figure 11.

Purified p50 was mixed with trypsin at various ratios for 3 hours then the reaction stopped by addition of PMSF to 1Mm. The products of digestion were separated by SDS-PAGE then stained with Coomassie Brilliant Blue (A) or Western blotted with 3B6A (B). The ratio of p50 : trypsin (w:w) in both is as follows: 0 (lane 1), 2000 (lane 2), 1000 (lane 3), 500 (lane 4), 200 (lane 5). Panel C shows the site of trypsin cleavage on the NLS.

A


p50:trypsin ratio (w:w)
0
2000
1000
500
200

B



C

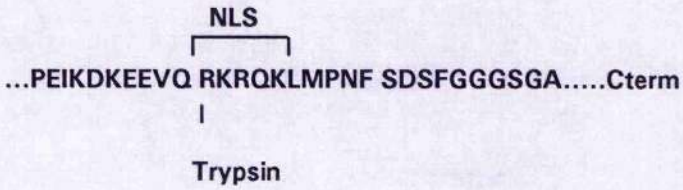

NLS
...PEIKDKEEVQ RKRQKLMPNF SDSFGGGSGA.....Cterm
Trypsin

Figure 12.

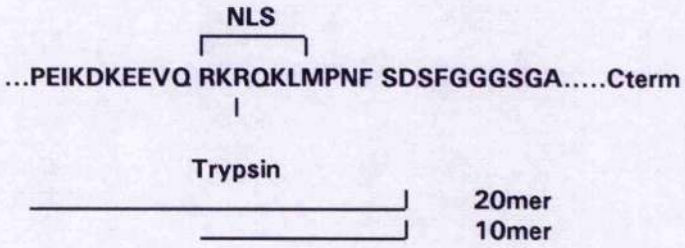
A. Sequence of the p50 NLS, the site of the trypsin cleavage, and the composition of the 10mer and 20mer peptides.

B. 3B6A was mixed with varying amounts of 10mer and 20mer peptide and then used to probe for p50 on a Western blot. Lanes 2-7 show incubation with the 10mer at concentrations of 0, 0.05, 0.1, 0.2, 0.5, and 1.0mg/ml respectively, while lanes 10-15 show incubation with the 20mer at the same range of concentrations.

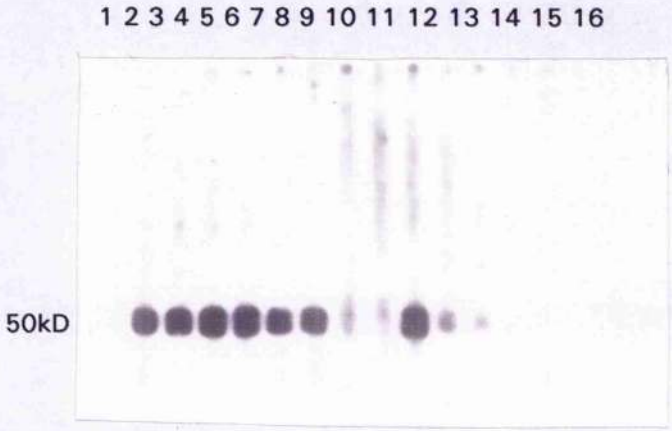
C. Lane 1. Western blot of p50 probed with 3B6A and detected by ECL for a 1 minute exposure.

Lane 2. Western blot of p65 probed with 3B6A detected as Lane 1.

A


NLS
...PEIKDKEEVQ RKRQKLMPNF SDSFGGGSGA.....Cterm
Trypsin
20mer
10mer

B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
50kD

C

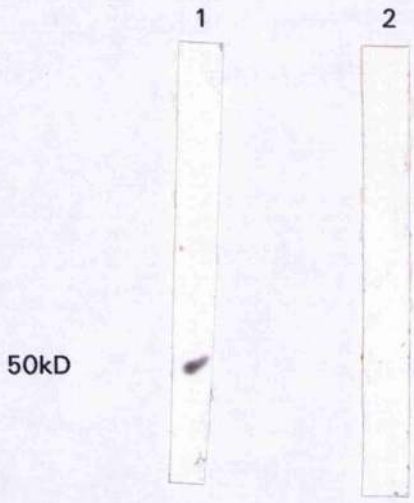

1
2
50kD

Figure 13

Sequences of synthetic peptides used in production of polyclonal antisera. A - N terminal p105. B - C terminal p105. C - C terminal p65 rel. D - N terminal I-kB. E - C terminal I-kB. All have a terminal cysteine residue to enable coupling to the linker molecule.

FIGURE 13.

N term p105 peptide

M A E D DP Y L G R P E C

C term p105 peptide

C D Y G Q E P L E G K I

C term p65 rel

C M D F S A L L S Q I S S

N term I-kB peptide (MAD-3)

M F Q A A E R P Q E W A C

C term I-kB peptide (MAD-3)

C D D C V F G G Q R L T L

Figure 14

Graphs showing results of ELISA's to test the reactivity of protein/peptide polyclonal antisera, against the immunogen and control peptides/proteins. Blots C and D are shown on the next page.

A. Antisera against C terminal of p105.

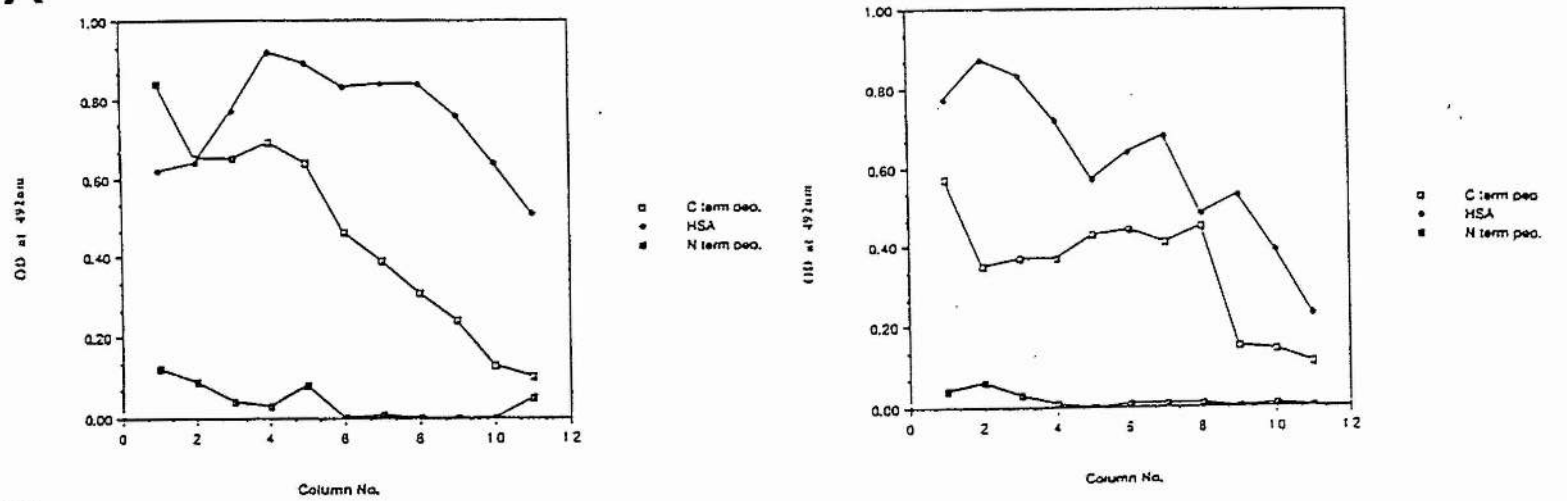
B. Antisera against N terminal of p105.

C. Antisera against p50 (DBD).

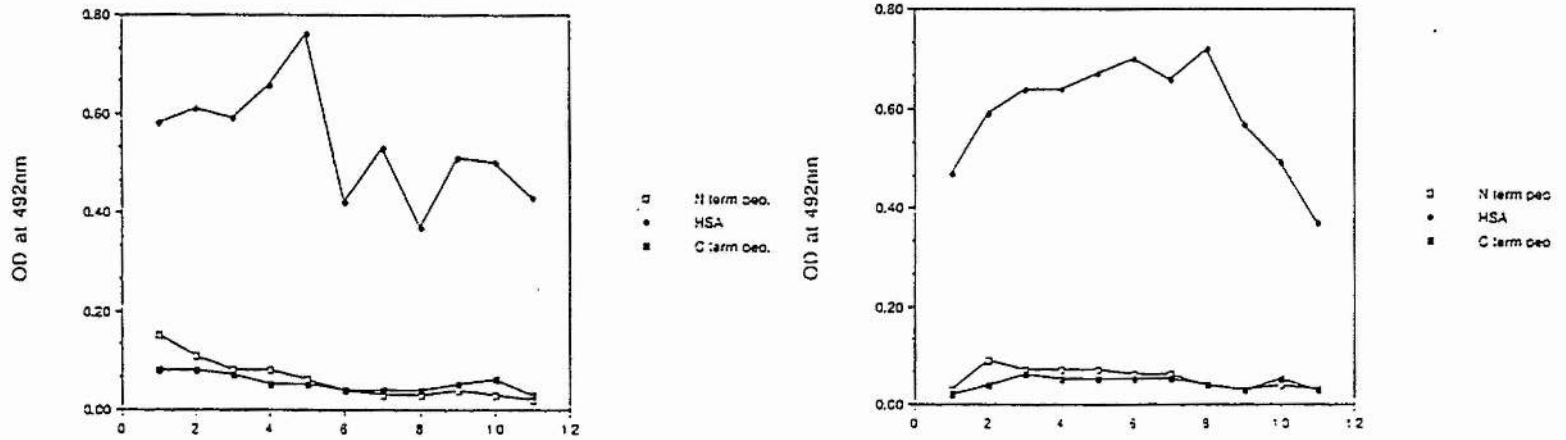
D. Graph showing response over time to p65 - from preimmune serum to final bleed

In panels A, B, and C the substrate used is OPD and OD is measured at 492nm, while in panel D, TMB substrate is used and measured at 630nm.

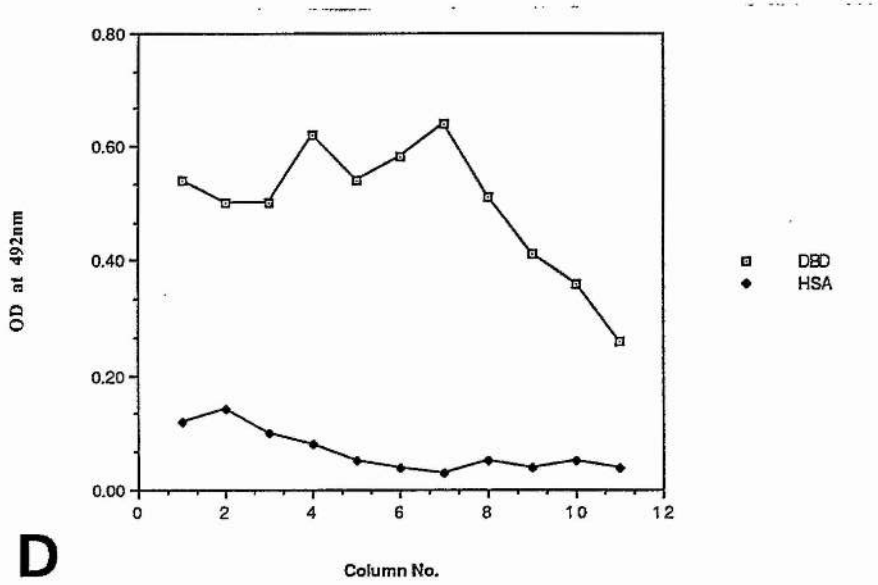
A

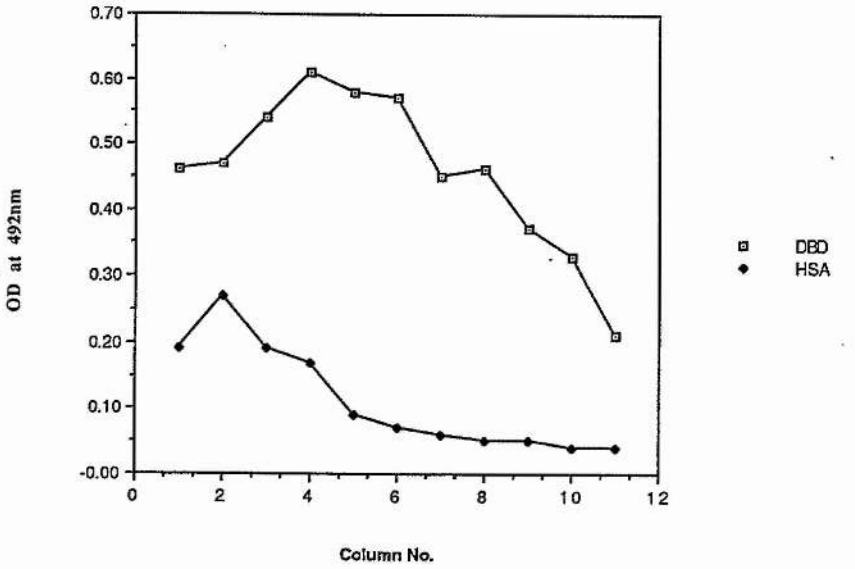

OD at 492nm
1.00
0.80
0.60
0.40
0.20
0.00
0
2
4
6
8
10
12
Column No.
C term peo.
HSA
N term peo.
OD at 492nm
1.00
0.80
0.60
0.40
0.20
0.00
0
2
4
6
8
10
12
Column No.
C term peo
HSA
N term peo.

B

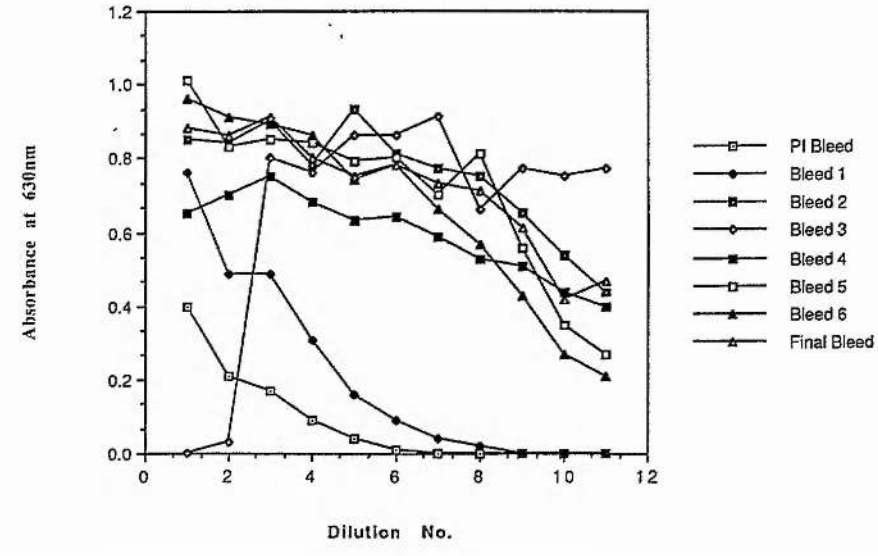

OD at 492nm
0.80
0.60
0.40
0.20
0.00
0
2
4
6
8
10
12
N term peo.
HSA
C term peo.
OD at 492nm
0.80
0.60
0.40
0.20
0.00
0
2
4
6
8
10
12
N term peo
HSA
C term peo

C


OD at 492nm
0.80
0.60
0.40
0.20
0.00
0
2
4
6
8
10
12
Column No.
DBD
HSA


OD at 492nm
0.70
0.60
0.50
0.40
0.30
0.20
0.10
-0.00
0
2
4
6
8
10
12
Column No.
DBD
HSA

D


Absorbance at 630nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
Dilution No.
PI Bleed
Bleed 1
Bleed 2
Bleed 3
Bleed 4
Bleed 5
Bleed 6
Final Bleed

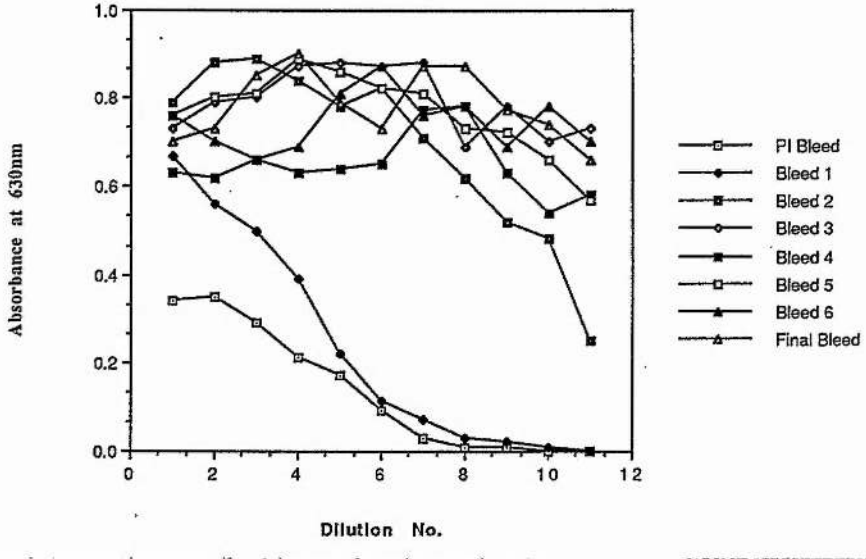

Absorbance at 630nm
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
Dilution No.
PI Bleed
Bleed 1
Bleed 2
Bleed 3
Bleed 4
Bleed 5
Bleed 6
Final Bleed

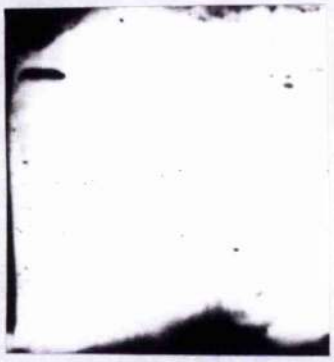
Figure 15

Western blots with nuclear and cytoplasmic extracts from Jurkat cells before and after stimulation with PMA and PHA for 30 minutes.

A. Unactivated cytoplasm (lane 1), activated cytoplasm (lane 2), unactivated nuclear (lane 3), activated nuclear (lane 4). Jurkat extracts probed with rabbit polyclonal anti-C terminal peptide antisera. Lanes 5-8 are as lanes 1-4, but probed with antibody preincubated with the peptide for 30 minutes at $37^{o}C$.

B. As A but probed with rabbit polyclonal anti-N terminal peptide antisera.

A



1 Un. 2 Act. 3 Un. 4 Act.
Cytoplasm Nucleus

5 Un. 6 Act. 7 Un. 8 Act.
Cytoplasm Nucleus

B

1 Un. 2 Act. 3 Un. 4 Act.
Cytoplasm Nucleus

5 Un. 6 Act. 7 Un. 8 Act.
Cytoplasm Nucleus

Figure 16.

A. Western blot with I-kB-GST fusion protein.

B. Western blot with I-kB.

Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. Detection was by ^{125}I-Protein A. Blots were probed with polyclonal antisera as follows:- preimmune rabbit serum (lane 1), rabbit anti-MAD-3 serum (lane 2), guinea pig anti-MAD-3 serum1 (lane 3), and guinea pig anti-MAD-3 serum2 (lane 4).

Band 1 corresponds to I-kB/GST fusion protein.

Band 2 corresponds to GST.

Band 3 corresponds to I-kB.

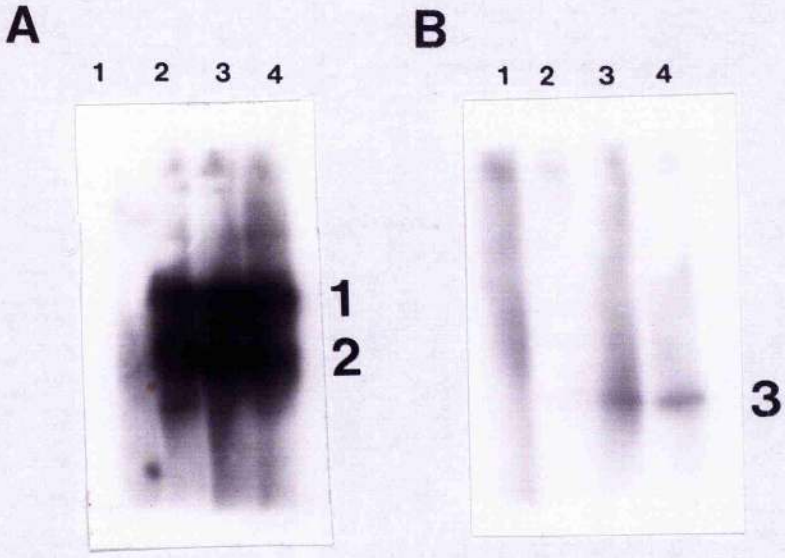

A
1 2 3 4
1
2
B
1 2 3 4
3

Figure 17.

Dot blot to test fractions from affinity purification of antiserum against MAD-3 for anti-MAD-3 activity. Nitrocellulose membrane was incubated with MAD-3 then samples from various stages of the purification were ''dotted'' on. Detection of bound Ab was by ECL. Layout of the blot is as follows:

row 1	+ve C	1	2	3	4	
row 2	5	6	7	8	9	
row 3	10	11	12	13	14	15
row 4	16	17	18	19	20	
row 5	W_{1A}	W_{1B}	W_{2A}	F_W	F_T	

Numbers represent the elution number off the column (each represents a 50µl fraction). W is wash cycles with W_1 being the first wash cycle, and W_2 being the second wash cycle. F_W is the final wash and F_T is the flow through off the column. The positive control (+ve C) is unpurified polyclonal anti-MAD-3 Ab.

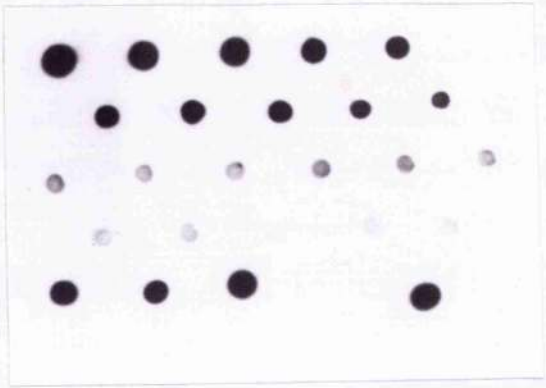


Figure 18.

Western blot of MG63 cells at various times after activation by TNFα probed with purified anti-p50 Ab. Nuclear and cytoplasmic extracts are prepared and separated by SDS-PAGE, then transferred electrophoretically to nitrocellulose membrane. Detection is by ECL for a 2 minute exposure. Lanes 1-5 show nuclear extracts at time 0, 5, 15, 45, and 120 minutes respectively after TNFα activation, while lanes 6-9 show cytoplasmic extracts at time 0, 5, 15 and 45 minutes after activation.

The size of the bands was marked at the time against prestained molecular weight markers. The markers were too faint to photograph and so are not shown for any of the blots.

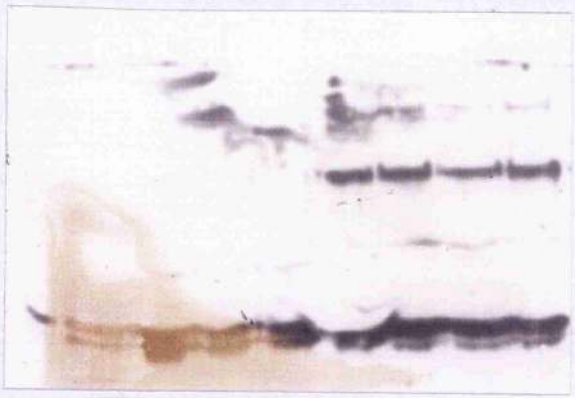

p105
p50
1 2 3 4 5 6 7 8 9

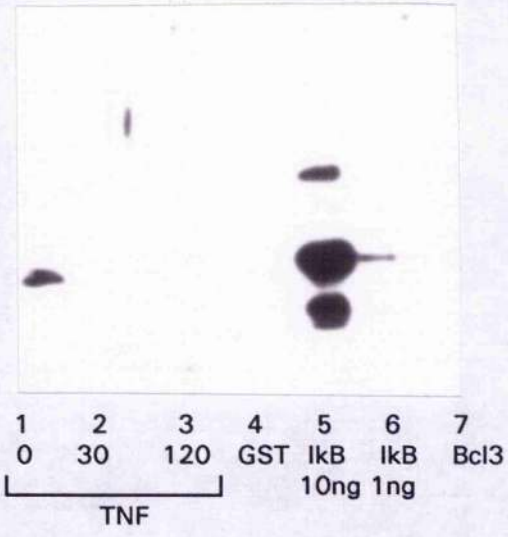
Figure 19.

Western blot of A549 cells at various times after activation by TNFα probed with purified I-kB Ab performed as above (Figure 18).

A. Nuclear extracts at time 0, 30 and 60 minutes after activation (lanes 1-3 respectively). Control proteins 10ng GST, 10ng I-kB, 1ng I-kB, 10ng Bcl-3 (lanes 4-7 respectively).

B. Cytoplasmic extracts at time 0, 30, 60 and 120 minutes after activation (Lanes 1-4 respectively). Control proteins 10ng GST, 10ng I-kB, 1ng I-kB, 10ng Bcl-3 (Lanes 5-8 respectively.

A


1 2 3 4 5 6 7
0 30 120 GST IkB IkB Bcl3
10ng 1ng
TNF

B

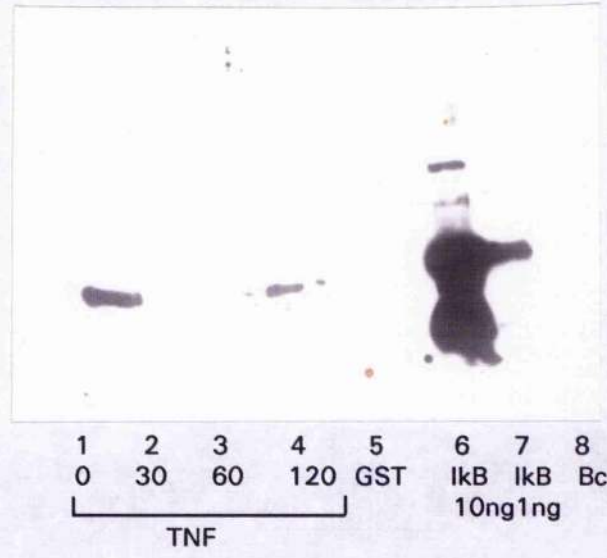

1 2 3 4 5 6 7 8
0 30 60 120 GST IkB IkB Bcl3
10ng1ng
TNF

Figure 20

Western blot of MG63 cells at various times after activation with TNFα, probed with purified I-kB Ab. Performed as in Figure 18. Lanes 1-5 show nuclear extracts at times 0, 5, 15, 45, and 120 minutes after activation, while lanes 6-10 show the same time points in cytoplasmic extracts.

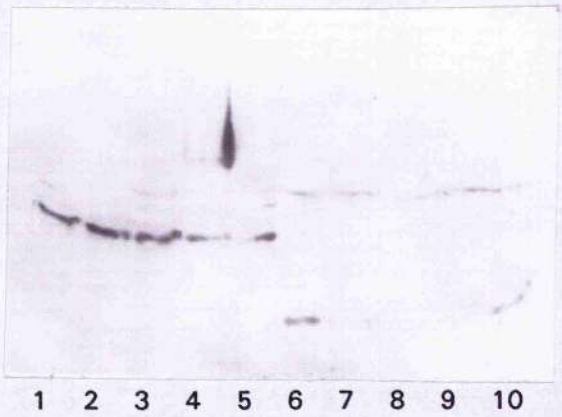
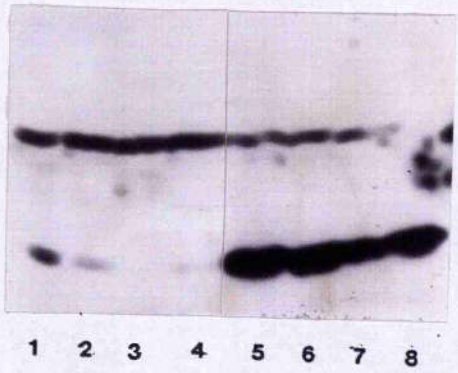

non-specific
non-specific
I-kB
1 2 3 4 5 6 7 8 9 10

Figure 21.

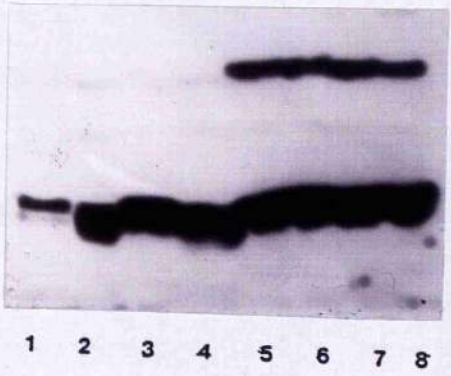
Western blot of Hela cells at various times after activation with TNFα, probed with purified I-kB Ab (Panel A and C), or purified p50 Ab (Panel B). In both A and B, lanes 1-4 show nuclear extracts at 0, 10, 50, and 120 minutes after activation respectively and lanes 5-8 show the same time points for cytoplasmic extracts.

Panel C shows a Western blot performed on cytoplasmic extracts activated using a new batch of TNFα for 0, 50, and 120 minutes (lanes 1 - 3 respectively).

A



B



C

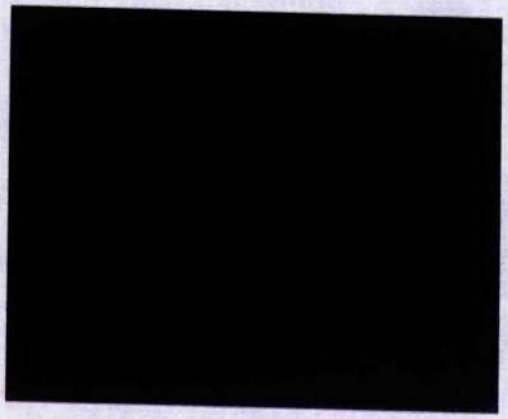
Figure 22.

Photographs of MG63 cells at various times after activation. After activation with TNFα for the appropriate time, cells were fixed with formaldehyde, permeabilised, and labelled with purified anti-MAD-3 rabbit polyclonal Ab used at $^{1}/_{1000}$ dilution. Bound antibody was visualised by use of FITC conjugated anti-rabbit IgG. Cells were examined microscopically and photographs taken. Panels A, B, C, D, and E show the cells after 0, 5, 15, 45, and 120 minutes activation respectively.

A

B

C



D

E

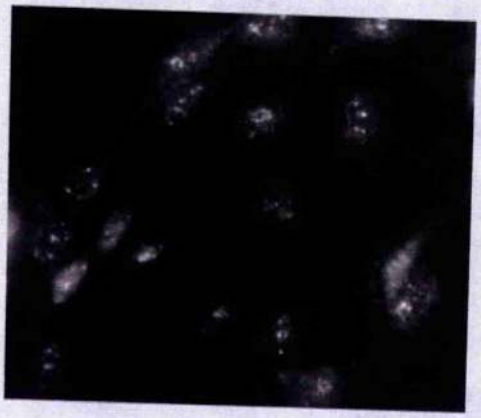


Figure 23.

Photographs of Hela cells treated as in Figure 22. Panels A, C, E, and G show the cells after 0, 10, 50 and 120 minutes activation respectively with TNFα, probed with purified I-kB Ab used at $^{1}/_{1000}$ dilution. Panels B, D, F, and H show corresponding time points, but the cells are labelled with preimmune serum.

Here there is no degradation of I-kB seen - maybe this is due to the TNF not working since there was very little degradation of I-kB seen in the corresponding Western blot on these cells (Figure 21a).

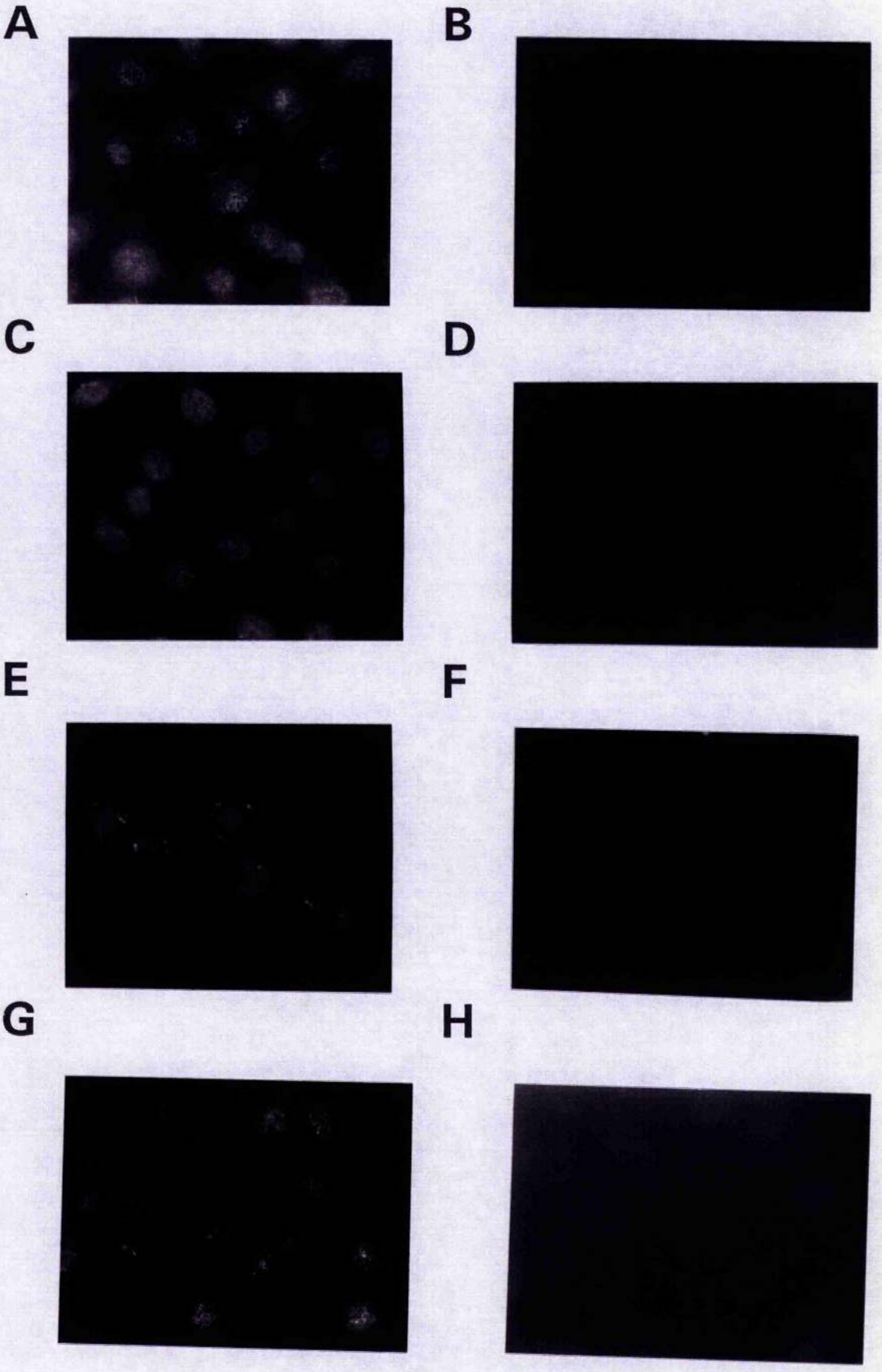
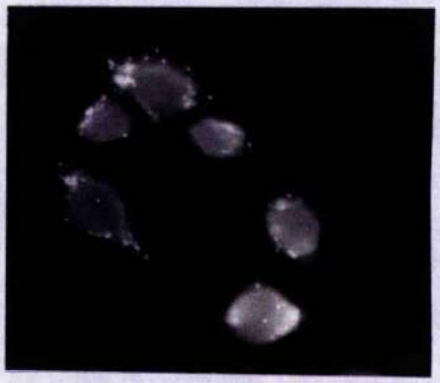

A
B
C
D
E
F
G
H

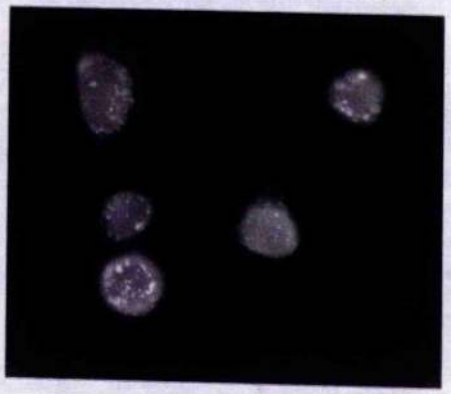
Figure 24.

Photographs showing Hela cells prepared as in Figure 22. Panels A-D show cells 0, 10, 50, and 120 minutes after activation, labelled with purified polyclonal rabbit anti-p50 Ab used at $^{1}/_{5000}$ dilution. Panels E and F show cells at 10 and 120 minutes after activation, labelled with preimmune rabbit serum.

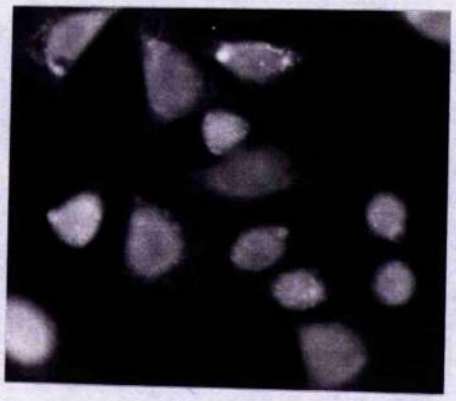
A



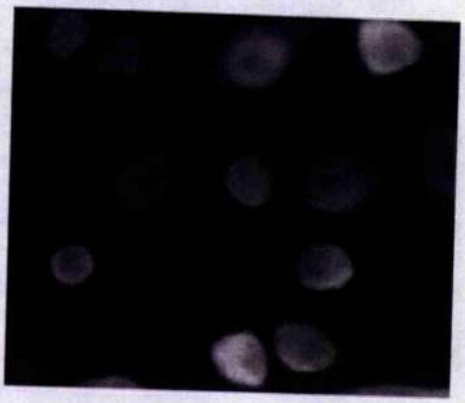
B



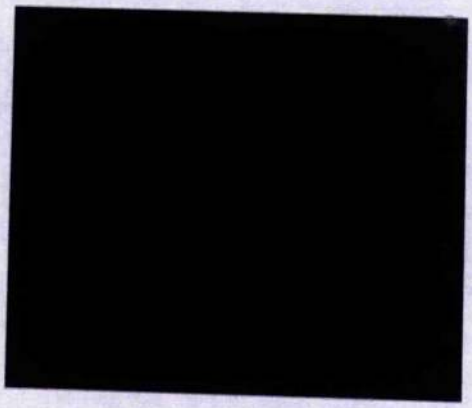
C



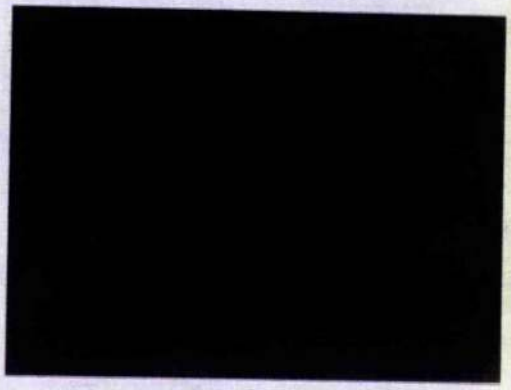
D



E



F



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