ROLE OF SUMO-1 MODIFICATION IN TRANSCRIPTIONAL ACTIVATION

Maria Joana Pinto Desterro

A Thesis Submitted for the Degree of PhD at the University of St. Andrews

1999

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Role of SUMO-1 modification in transcripational activation

Maria Joana Pinto Deisterro
School of Biology
University of St. Andrews

A thesis submitted for the degree of Doctor of Philosophy

October 1999
DECLARATION

I. Maria Joana Pinto Desterro, hereby certify that this thesis, which is approximately 40,000 words in length, 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 higher degree.

Date 8/10/1999   Signature of candidate

I was admitted as research student in January 1996 and as a candidate for the degree of Doctor of Philosophy in January 1996; the higher study for which this is a record was carried out in the Faculty of Sciences of the University of St Andrews between 1996 and 1999.

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I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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<tbody>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>APC</td>
<td>Anaphase promoting complex</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>D-MEM</td>
<td>Dulbecco’s modified essential medium</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>DUB</td>
<td>Deubiquitination enzymes</td>
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<td>E. coli</td>
<td><em>Eschericia coli</em></td>
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<td>E1</td>
<td>Ubiquitin activating enzyme</td>
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<td>E2</td>
<td>Ubiquitin conjugating enzyme</td>
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<td>E3</td>
<td>Ubiquitin ligase</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>FCS</td>
<td>Foetal calf serum</td>
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<td>GST</td>
<td>Glutathione S-transferase</td>
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<td>HA</td>
<td>Haemagglutinin</td>
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<td>HCl</td>
<td>Hydrochloric acid</td>
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<td>HECT</td>
<td>Homology to E6-AP C-terminus</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IKAP</td>
<td>IKK complex associated protein</td>
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<td>IxB</td>
<td>Inhibitor kappa B</td>
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<td>IKK1 (α)</td>
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<td>IKK2 (β)</td>
<td>IxB kinase 2 (β)</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin 1β</td>
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<td>IPTG</td>
<td>Isopropyl-b-D-thiogalactopyranoside</td>
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<tr>
<td>IVTT</td>
<td><em>In vitro</em> transcription translated</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
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<tr>
<td>KDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LTR</td>
<td>Long terminal repeat sequence</td>
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<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>NEMO</td>
<td>NF-kappa B essential modulator</td>
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<tr>
<td>NES</td>
<td>Nuclear export sequence</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>NLS</td>
<td>Nuclear localisation signal</td>
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<tr>
<td>NP-40</td>
<td>Nonidet P-40</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
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<td>RHD</td>
<td>Rel homology domain</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
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<tr>
<td>SAE1</td>
<td>SUMO-1 activating enzyme subunit 1</td>
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<td>SAE2</td>
<td>SUMO-1 activating enzyme subunit 2</td>
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<tr>
<td>SCF</td>
<td>Skp1-Cdc53/ Cul1-F-box protein E3 complex</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SSPs</td>
<td>SUMO-1 specific proteases</td>
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<tr>
<td>SUMO-1</td>
<td>Small ubiquitin modifier</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
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<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)propane-1,3-diol</td>
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<tr>
<td>UCH</td>
<td>Ubiquitin C-terminal hydrolase</td>
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<tr>
<td>UV</td>
<td>Ultra violet</td>
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<td>WT</td>
<td>Wild type</td>
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### Abbreviations for amino acids

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<td>Tryptophan</td>
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Tyrosine  tyr  Y
Valine   val  V

Genetic Code

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To ALL I love, a HUGE thanks and to ALL my Family I have no words...

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To my Mum and Dad,
Abstract

In unstimulated cells, the transcription factor NF-κB is held in the cytoplasm in an inactive state by IκB inhibitor proteins. Activation of NF-κB is mediated by signal induced degradation of IκBα via the ubiquitin proteasome-dependent pathway. Targeting the proteins for ubiquitin-mediated proteolysis is an irrevocable decision, and as such, the process needs to be highly specific and tightly regulated. This task is achieved by conjugation and deconjugation enzymes that act in a dynamic and coordinated mechanism.

In a yeast two hybrid screen designed to identify proteins involved in IκBα signalling Ubch9 was found to interact with the N-terminal regulatory region of IκBα. Although Ubch9 is an enzyme homologous to E2 ubiquitin conjugating enzymes we have shown that is unable to form a thioester with ubiquitin but it is capable to form a thioester with the small ubiquitin-like protein SUMO-1. To fully characterise the SUMO-1 modification reaction we have purified the proteins and cloned the genes encoding the SUMO-1 activating enzyme (SAE1/SAE2) and shown that it is homologous to enzymes involved in the activation of ubiquitin, Smt3p, the yeast SUMO-1 homologue, and Rub1p/Nedd8, another ubiquitin-like protein. SUMO-1 is conjugated to target proteins by a pathway that is distinct from, but analogous to, ubiquitin conjugation.

SUMO-1 was efficiently conjugated, both in vivo and in vitro, to IκBα on lysine 21, which is also utilised for ubiquitin modification. Thus, by blocking ubiquitination SUMO-1 modification acts antagonistically to generate a pool of IκBα resistant to proteasome-mediated degradation which consequently inhibits NF-κB dependent transcription activation.

In view of several lines of similarity between NF-kB and p53, the involvement of SUMO-1 modification in the metabolism of the tumour supressor p53 was investigated. We have shown that p53 is modified by SUMO-1 at a single
site, lysine 386 in the C-terminus of p53. Although p53 is regulated by ubiquitination, SUMO-1 and ubiquitin modification do not compete for the same lysine in p53. However, overexpression of SUMO-1 activates the transcriptional activity of wild type p53, but not K386R p53 where the SUMO-1 acceptor site has been mutated.

A consensus sequence was obtained by comparison of the sequences surrounding the SUMO-1 acceptor lysine in proteins that have been shown to be modified by SUMO-1 and revealed a possible recognition site for SUMO-1 conjugation machinery.

Tagging of proteins with SUMO-1 regulates transcriptional activation, either by interfering with subcellular location or with the ubiquitination pathway. The pathway may represent a novel target for drug development.
1. INTRODUCTION
1.1. Rel/NF-κB family of transcription factors

Regulated eukaryotic gene transcription involves the assembly of an initiation complex at the core promoter region and a coordinated binding of multiple transcription factors and regulatory complexes to the promoter-enhancer region; many of these transcription factors are regulated by distinct signal transduction pathways. The activity of a particular transcription factor can be modulated in a number of different ways: by changing the rate of synthesis or degradation of the protein; by post-translational modification of the transcription factor or by altering its subcellular localisation. Rel/ NF-κB is a ubiquitous transcription factor subject to regulatory mechanisms that involves each one of the mentioned levels: protein-protein interaction, covalent modification, proteolytic degradation and nucleocytoplasmic translocation.

Although the transcription factor NF-κB was originally identified as a tissue specific enhancer-binding protein involved in the activation of κ light chain gene expression in mature B lymphocytes (Sen and Baltimore, 1986) subsequent studies demonstrated that it is one member of a ubiquitously expressed family of Rel-related transcription factors that serve as critical regulators of the inducible expression of many genes. The Rel/NF-κB transcription factors regulate the expression of genes involved in immune, acute phase, inflammatory and survival response. In addition, the early promoters of many viral genomes such as the HIV-1 provirus are regulated by Rel/NF-κB.

In humans the Rel/ NF-κB family comprises NF-κB1 (p50/p105), NF-κB2 (p52/p100), p65 (also known as RelA), C-Rel and RelB. Rel A, C-Rel and RelB are produced as transcriptionally active proteins, whereas NF-κB1 (Palombella et al., 1994) and NF-κB2 are synthesised as longer precursor molecules of 105 KDa and 100 KDa respectively, which are unable to bind DNA but are processed to smaller DNA binding forms. All members of this family contain a conserved 300 residue amino-terminal region called Rel-homology domain (RHD) which is
required for sequence specific DNA-binding, dimerisation, nuclear localisation (NLS) and interaction with inhibitory proteins. NF-κB binds to DNA as dimers composed of different combinations of the family members. Crystal structures of p50, p52 and p65 homodimers and p65/p50 heterodimers bound to DNA have been determined (Chen et al., 1998; Cramer et al., 1997; Ghosh et al., 1995; Muller et al., 1995). The structures of the RHDs of p50 and p65 bound to DNA show that each consists of two domains with immunoglobulin-like folds. C-terminal domains are responsible for dimer formation with other Rel family proteins and although both domains are involved in DNA binding, sequence-specific interactions come primarily from loops in the N-terminal domain (Chen et al., 1998). While almost all combinations of homo- and heterodimer can exist, the typical form of NF-κB that is activated in response to extracellular signals is a heterodimer p50 and p65. Each NF-κB complex displays distinct affinities for the different DNA binding sites present in the promoters of NF-κB-regulated genes, and this may contribute to some of the specificity exhibited by this family. (Baeuerle and Baltimore, 1996; Baldwin, 1996; May and Ghosh, 1998; Mercurio and Manning, 1999).

1.1.1. IκB family

NF-κB exists in the cytoplasm of unstimulated cells in an inactive form associated with the IκB family of inhibitor proteins which include, to date, seven different but structurally and functionally related, molecules: IκBα (Haskill et al., 1991), IκBβ (Thompson et al., 1995), IκBε (Whiteside et al., 1997a), Bcl3 (Ohno et al., 1990), p100, p105 and the drosophilia protein cactus. p100 and p105, the precursors of p50 and p52, respectively, contain IκB-like regions in the C-terminal half of the polypeptide and are known as IκBγ (Blank et al., 1991; Inoue et al., 1992; Liou et al., 1992) and IκBδ (Mercurio et al., 1993) respectively. All IκB family members share six or more ankyrin-like repeat domains (30-33 amino acid) and regulate the DNA binding (Zabel and Baeuerle, 1990) and subcellular
localisation of Rel-NF-κB proteins. This is accomplished by masking the nuclear localisation signals of NF-κB subunits which prevents their nuclear translocation. (Beg et al., 1992; Henkel et al., 1992). Specificity of inhibition is due to different IκB family members inhibiting NF-κB/Rel family members to varying degree. For example, IκBα and IκBβ associate predominantly with p50/p65 and p50/C-Rel heterodimers (Thompson et al., 1995), whereas IκBe binds to p65 and C-Rel homodimers (Whiteside et al., 1997b), and Bcl-3 interacts with p50 and p52 homodimers (Franzoso et al., 1992; Nolan et al., 1993).

The best-characterised IκB protein is IκBα, composed of three main regions (Jaffray et al., 1995): an N-terminal region, which regulates signal-dependent degradation; an ankyrin repeat domain; and the C-terminal PEST region, which is a target for casein kinase II (CKII) phosphorylation (Barroga et al., 1995) and regulates basal degradation (Ernst et al., 1995; Lin et al., 1996; Schwarz et al., 1996). Both amino- and carboxyl-terminal regions are required for signal-induced degradation of IκBα (Brown et al., 1997; Kroll et al., 1997; Rodriguez et al., 1995). The structure of the IκBα ankyrin repeat domain bound to NF-κB p50/p65 heterodimer has been determined by X-ray crystallography (Huxford et al., 1998; Jacobs and Harrison, 1998) and clarified the structural basis by which both nuclear entry and DNA binding of NF-κB are inhibited by IκBα. The contact area of the ankyrin repeats with NF-κB is large, with the most significant contribution from the dimerised Ig-like domain of the p50/p65 RHD and repeats 3 to 5 of IκBα. Cytoplasmic retention is achieved by the first two ankyrin repeats of IκBα which recognise the sequences of the NF-κB NLSs and appear to inhibit nuclear localisation by sterically impeding NLS access to the nuclear import machinery. In the presence of IκBα, the p65 subunit of NF-κB adopts a remarkable change in conformation which impedes NF-κB DNA binding through the occlusion of basic DNA-contacting surfaces and permits optimal IκBα/ NF-κB complex formation (Huxford et al., 1998; Jacobs and Harrison, 1998).
1.1.2. NF-κB activation pathway

The range of stimuli that activate members of Rel family is extensive and growing, giving emphasis to their central role in transcriptional responses. Potent inducers of NF-κB include: cytokines such as interleukin (IL)-1β and tumour necrosis factor (TNF)α; bacterial and viral products such as lipopolysaccharide (LPS), sphingomyelinase, double strand RNA and the Tax protein from human T-cell leukemia virus 1 (HTLV-1); and pro-apoptotic and necrotic stimuli such as oxygen free radicals, ultraviolet light and γ-irradiation. These diverse incoming signals, originating from different signal transduction pathways, converge in a key molecular structure - the cytosolic NF-κB-IκB complex.

Activation of NF-κB is mediated by signal induced degradation of IκBα in the cytoplasm, which allows the active transcription factor to translocate into the nucleus and activate transcription of a large number of responsive genes. As part of an autoregulatory loop, NF-κB induces efficient resynthesis of IκBα through the activation of IκBα mRNA transcription (LeBail et al., 1993; Sun et al., 1993). Newly synthesised IκBα accumulates transiently in the nucleus, where it is resistant to signal-induced degradation (Rodriguez et al., 1999) and negatively regulates NF-κB dependent transcription (Arenzana-Seisdedos et al., 1995). IκBα mediates transport of the NF-κB/ IκBα complex back to the cytoplasm (Arenzana-Seisdedos et al., 1997) in a nuclear export sequence (NES) -dependent process that is sensitive to Leptomycine B (LMB) (Rodriguez et al., 1999). Nuclear export of IκBα and many other proteins that shuttle between the nucleus and the cytoplasm is mediated by a leucine-rich NES that is recognised by CRM1/exportin 1 (Fornerod et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997). The formation of CRM1/NES complex is facilitated by the presence of Ran in its GTP-bound form. It has been proposed that this ternary complex is transported through
the nuclear pore complex and dissociates in the cytoplasm due to GTP hydrolysis by Ran-GAP (Figure 1A) (Fornerod et al., 1997).

Nuclear import of NF-κB-free IκBα does not result from passive diffusion but from a specific energy-dependent transport process that requires the ankyrin repeats of IκBα, importins α and β as well as the small GTPase Ran (Sachdev et al., 1998; Turpin et al., 1999). Importin α binds to NLS-containing proteins and, through interaction with importin β, mediates the docking of the NLS-containing protein to nucleoporins and the subsequent translocation of the NLS-containing protein to the nucleus (Figure 1B).

IκBα lacks a classical basic nuclear localisation sequence (NLS) involved in the importin α-β-mediated import pathway but a novel class of cis-acting nuclear import sequences was identified within the second ankyrin repeat of IκBα which mediates the nuclear localisation of newly synthesised IκBα (Sachdev et al., 1998). Although the second ankyrin repeat is the predominant nuclear import sequence, ankyrin repeats also contribute to the nuclear localisation of IκBα by interaction with additional protein (s) containing a basic NLS (Turpin et al., 1999). NF-κB retains IκBα in the cytoplasm by masking its ankyrin repeats, which are essential for nuclear import. In unstimulated cells, cytoplasmic retention of NF-κB. IκBα complexes are therefore caused by a mutual masking of the sequences responsible for the nuclear import of both proteins. IκBα, shuttling between the cytoplasm and the nucleus regulates both the activation and inactivation of gene transcription through its association with NF-κB.

The signal transduction cascades from the cell surface to NF-κB activation has been subject of intense investigation and they have been further elucidated with the identification of the IκB Kinases IKKα (or IKK1) and IKKβ (or IKK2), IKK signalsome, which mediate signal induced phosphorylation of IκBα on Ser 32 and Ser 36 (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Zandi et al., 1997). Mutation of these residues by site-directed mutagenesis was found to
Figure 1. Mechanism of IκBα shuttling between the cytoplasm and the nucleus.

A In the nucleus NF-κB/IκBα complex binds to CRM1 through the nuclear export sequence of IκBα. The formation of CRM1/NES complex is facilitated by the presence of RanGTP. This ternary complex is subsequently translocated through the nuclear pore complex. In the cytoplasm the GTP in the RanGTP-CRM1-NES complex is hydrolysed, causing the NES to dissociate from CRM1. Empty CRM1 can then be reimported to the nucleus. B In the cytoplasm NF-κB-free IκBα, interacts indirectly with Importin β via the adapter Importin α and another as yet unidentified protein. Via a “piggy back” mechanism this complex is translocated to the nucleus. In the nucleus the complex dissociates and both RanGTP bound to importin β and Importin α are exported back to the cytoplasm.
block phosphorylation of IκBα in response to activating signals, thereby preventing subsequent degradation of the protein (Brockman et al., 1995; Brown et al., 1995; Traenckner et al., 1995). Specific inhibition of the proteolytic activity of the proteasome prevents NF-κB activation and results in the accumulation of ubiquitinated forms of IκBα, indicating that IκBα is targeted for degradation by a phosphorylation-dependent ubiquitination process which occurs in two adjacent-N-terminal lysine residues at positions 21 and 22 (Alkalay et al., 1995; Chen et al., 1995; Rodriguez et al., 1996; Roff et al., 1996). Phosphorylation of IκBα serves as a molecular tag leading to rapid ubiquitination and degradation of IκBα by the recently identified SCFβTrCP ligase complex and other components of the ubiquitin-proteasome pathway (discussed later). Neither phosphorylation, nor ubiquitination alone is sufficient to dissociate the NF-κB/ IκBα complex. Free NF-κB is only released after degradation of IκBα by the 26S proteasome (Didonato et al., 1995). This process exposes the NLS, thereby freeing NF-κB to interact with the nuclear import machinery and translocate to the nucleus (Henkel et al., 1992), where it binds its target genes to initiate transcription.

1.1.3. IKK signalsome complex

1.1.3.1. Identification

It is well established that the critical event which targets the polyubiquitination and degradation of IκBs via the 26S proteasome is their stimulus-dependent phosphorylation at two serine residues that are located within their conserved N-terminal regulatory region (DiDonato et al., 1996). A protein kinase with such substrate requirements remained unidentified for many years and many candidates were identified, such as protein kinase C (Diaz-Meco et al., 1994), casein kinase II (Barroga et al., 1995), and ribosomal S6 kinase (pp90Rsk) (Schouten et al., 1997). Although most of these kinases phosphorylate IκB proteins in vitro on different serine, threonine or tyrosine residues, they were all unable to
phosphorylate the two regulatory serines that target the degradation of IκBαs in a stimulus-dependent manner. DiDonato et al. (DiDonato et al., 1997) purified a cytokine-activated protein kinase complex of 900 KDa, named IKK for IκB kinase, with such requirements. Two components of IKK were molecularly cloned and identified as two closely related protein serine kinases, IKKα (IKK1) and IKKβ (IKK2) (DiDonato et al., 1997; Woronicz et al., 1997). IKKα is identical in sequence to the putative serine/threonine kinase CHUCK, whose function and mechanism of regulation were previously unknown (Connelly and Marcu, 1995). In this case however the purified components were able to phosphorylate specifically IκBα (Ser32 and Ser36) and IκBβ (Ser19 and Ser23) (Zandi et al., 1997). IKKα was also isolated by a two hybrid screen as a NIK (NF-κB inducing kinase) interacting protein (Regnier et al., 1997). NIK is a member of the MEK kinase (MEKK) family that was identified as a TRAF2-interacting protein which is required for TNF-induced NF-κB activation (Rothe et al., 1995). NIK has the ability to potently stimulate NF-κB activity in transiently transfected cells (Malinin et al., 1997) and catalytically inactive mutants of NIK behave as dominant-negative inhibitors that suppress TNF-, IL-1-, TRADD-1-, TRAF2-, TRAF5-, TRAF6-induced NF-κB activation. NIK seems to be a common mediator in the NF-κB signalling cascades triggered by TNF and IL-1 downstream of TRAFs and other receptor-associated signalling proteins (Song et al., 1997). The inhibition of IKKα expression by the use of an antisense RNA expression vector and a catalytic inactive mutant of this kinase resulted in inhibition of NF-κB activation by TNF or IL1 (DiDonato et al., 1997; Regnier et al., 1997). The same effect was observed with a kinase-defective mutant of IKKβ (Mercurio et al., 1997) consistent with a critical role for both IKKα and IKKβ in the NF-κB pathway.
1.1.3.2. Composition

The main components of the IKK complex - IKKα and IKKβ - have a similar overall structure and are 51% identical. They contain a Ser/Thr kinase domain in their N-terminal half and a leucine zipper as well as a helix-loop-helix protein interaction motif in their C-terminal region. Immunoprecipitation studies have demonstrated that IKKα and IKKβ can form homo- or heterodimers and mutational analysis has shown that these interactions are mediated by the leucine zipper motif (Mercurio et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). By contrast, the helix loop helix motif does not affect the interaction between the two subunits but seems to be important for the kinase activity (Zandi et al., 1997). Although heterodimers seems to be the favoured conformation, the precise nature of the interactions between IKKα and IKKβ in vivo are not yet clear.

Using a monoclonal antibody against IKKα, a new subunit of the IKK complex was purified and identified as a regulatory protein required for the activation of the IKK complex (Rothwarf et al., 1998). This subunit denominated IKKγ binds preferentially to IKKβ and is a glutamine-rich protein that lacks a known catalytic domain but contains several coiled-coil interaction motifs, including a Leucine zipper (LZ) next to the C-terminus. A C-terminally truncated form of IKKγ lacking the LZ can still bind to IKKβ but blocks activation of IKK and NF-κB (Rothwarf et al., 1998). This subunit was named IKKAP1 by a different group, for IKK associated protein-1, and identified in two different IKK complexes in vivo- one containing a heterodimer of IKKα and IKKβ, the other containing an homodimer of IKKβ (Mercurio et al., 1999). IKKγ was also identified in a screen for genes that are able to complement a cell line that is unresponsive to multiple NF-κB activating stimuli including TNF, IL-1, LPS, phorbol myristate acetate (PMA), dsRNA and the Tax transactivator protein of human T cell leukemia virus (HTLV). A cDNA was found which could reconstitute activation of NF-κB by all of these inducers upon its stimulation. Consistent with its essential role as a NF-κB
modulator it was named NEMO (NF-κB essential modulator) and is the mouse homologue of IKKγ (Yamaoka et al., 1998).

Another component of the IKK signalsome was identified and named IKAP (IKK-complex associated protein). It is a 150 KDa protein containing several WD protein-interaction motifs which can bind NIK and IKKs and assemble them into an active kinase complex (Cohen et al., 1998). The interaction of IKAP with IKKα and IKKβ seems to be transient and it remains to be determined whether IKAP is required for IKK activation. As a scaffolding protein more biochemical analysis will be needed to determine whether IKAP assembles the same complex in response to different IKK-inducing stimuli.

Although the composition of the IKK signalsome is becoming elucidated, a definitive function for each of its subunit is still not clear.

1.1.3.4. Regulation

Although the specific roles of each IKK subunit in the regulation of IKK activity are not well understood, IKK is activated by phosphorylation, since treatment with phosphatase PP2A results in its inactivation (DiDonato et al., 1997). Analysis of the protein kinase domains of IKKα and IKKβ reveals potential phosphorylation sites in the activation loop. Mutations at those sites to alanine in IKKβ results in a kinase-inactive form and mutation of these residues to glutamate, which mimic to some degree the phosphoserine, yields a constitutively active kinase. However, elimination of equivalent sites in IKKα, did not interfere with IKK activation (Ling et al., 1998; Mercurio et al., 1997). So far these sites have not been shown to be phosphorylated in a cytokine mediated fashion and it is not clear whether phosphorylation of these sites occurs through autophosphorylation or by one or more upstream activating kinases, which are likely to be members of the mitogen-activating protein kinase kinase kinase (MAPKKK or MEKKS) family of enzymes. NIK and MEKK1, a protein involved in the activation of c-Jun N-terminal kinase (JNK)(Lee et al., 1997) appear to be involved in the IKK activation
and overexpression of either of these kinases in cells leads to activation of both IKKα and IKKβ, with NIK displaying a preference for IKKα and MEKK-1 a preference for IKKβ (Nakano et al., 1998). It has also been shown that IKK activity can be stimulated by Tax, a transforming and transactivating protein of Human T cell leukemia virus (HTLV) (Geleziunas et al., 1998; Yin et al., 1998). Although the mechanism is not well understood Tax apparently binds to the N-terminus of MEKK1 and stimulates its kinase activity (Yin et al., 1998).

If it is important to activate IKK and NF-κB in response to infection it is also crucial to rapidly terminate their activities once the inflammatory stimulus has disappeared. The phosphorylation of the C-terminal serine cluster of IKKβ by autophosphorylation has a negative regulatory function and seems to be involved in preventing prolonged IKK activation (Delhase et al., 1999). The C-terminal serine cluster is located next to the HLH motif, mutations in which diminish kinase activity (Zandi et al., 1998) suggesting that this motif is required for IKK activation through an intramolecular interaction with the kinase domain. It is likely that the C-terminal portion of IKKβ, including the HLH motif interacts with the kinase domain and that this interaction is required for full activity of the IKK in addition to phosphorylation of the T-loop. Phosphorylation of the C-terminal serine cluster may weaken this interaction, causing a conformational change which results in lower kinase activity (Delhase et al., 1999). Structural data will help to understand these possible mechanisms. Also, the identification of new signalling molecules that directly or indirectly interact with the IKK complex will help to understand better the signalling pathways that activate NF-κB. Very recently, Akt, a serine/threonine kinase that is activated in response to platelet-derived growth-factor (PDGF) treatment of cells by a mechanism involving phosphoinositide 3-OH kinase PI(3)K signalling pathway (Marte and Downward, 1997), was shown to be involved in the activation of NF-κB through controversial mechanisms. Romashkova and Marakov (Romashkova and Makarov, 1999) showed a direct association between Akt and IKK and using
dominant-negative mutants of the IKK subunits, IKKβ was shown to be the subunit involved in the PDGF-induced activation of NF-κB (Romashkova and Makarov, 1999). NF-κB was shown to be a target of the anti-apoptotic Ras/PI(3)K/Akt pathway (Kauffmann-Zeh et al., 1997; Kennedy et al., 1997) which does not seem to be activated after TNF stimulation (Romashkova and Makarov, 1999). By contrast, Ozes et al. (Ozes et al., 1999), in epithelial cells showed that TNF activates phosphatidylinositol-3-OH kinase (PI(3)K) and its downstream target Akt (protein kinase B) (Ozes et al., 1999) which promotes NF-κB activation by phosphorylation of threonine 23 in IKKa. These authors suggested that TNF activates NF-κB by two different signalling pathways that converge on IKKa. Akt, via PI(3)K, and NIK, via TRAF2, phosphorylate IKKa at threonine 23 and serine 176, respectively (Ozes et al. nature, 1999)(Ling et al., 1998). While there is an agreement on the activation of NF-kB by Akt serine threonine kinase via IKK, there is less agreement on the mechanism involved in this activation. A cell-type specific process could explain the controversial nature of these data.

To better elucidate the biological functions of IKKa and IKKβ, mouse mutants lacking either IKKa or IKKβ were generated (Hu et al., 1999; Li et al., 1999; Takeda et al., 1999). Most of the biochemical results already described were confirmed: IKKa is not required for activation of IKK in response to proinflammmatory stimuli, where as IKKβ is absolutely essential for this response. IKKa -/- mice are born alive but die within 30 minutes. These animals demonstrate striking development abnormalities: defective proliferation and differentiation of skin epidermal cells (keratinocytes) and defective limb and skeletal patterning (Hu et al., 1999; Takeda et al., 1999). These phenotypes although never observed in mice lacking individual members of the NF-kB/Rel transcription factor family (Ghosh et al., 1998), suggest that NF-κB is important for embryonic development. The activation of IKK and NF-κB by TNF or IL-1 in embryonic fibroblasts and
thymocytes of IKKα -/- mice seems to be normal indicating that IKKα is not essential for cytokine-induced activation of NF-κB (Hu et al., 1999; Takeda et al., 1999). By contrast IKKβ -/- mice die in utero at day 12 to 13 of gestation from extensive loss of liver cells due to apoptosis (Li et al., 1999). Their phenotype is similar to that observed for p65 (RelA) - deficient mice (Beg et al., 1995b). Mouse embryonic fibroblast cells that were isolated from IKKβ -/- embryos were defective in activation of IKK and NF-κB in response to TNF and IL-1, which confirms the importance of IKKβ for IKK activation. Although IKKα and IKKβ have a very similar overall structure and they are tightly associated in most cell types, these two protein kinases play different regulatory and functional roles in the NF-κB pathway.

It remains to be clarified if the complex which contains IKKα and IKKβ is the unique integrator of all signals that activate NF-κB or if different stimuli utilise alternative pathways in which the IKK complex is not involved. In certain cases NF-κB activation does not seem to involve IκB phosphorylation by IKK or even IκB degradation. Short-wavelength UV radiation (UV-C) activates NF-κB through IκBα degradation, however neither IKK activation nor the phosphorylation of IκBα on Ser32 and Ser36 was observed after UV-irradiation (Bender et al., 1998; Li and Karin, 1998). Phosphorylation of tyrosine 42 in IκBα, either by treatment with pervanadate or upon reoxygenation of hypoxic cells, has been linked to aiding the release of IκBα from NF-κB by a mechanism which does not require degradation (Imbert et al., 1996). The role of tyrosine phosphorylation of IκBα is somewhat controversial however. Imbert et al. (Imbert et al., 1996) showed that if IκBα is phosphorylated on tyrosine 42 it dissociates from NF-κB thus releasing the DNA binding form of NF-κB. However a subsequent report by Singh et al showed that NF-κB is not transcriptionally activated under these conditions (Singh et al., 1996).
Overall regulation of NF-κB is a complex situation with many stimuli and many pathways contributing to the activation and deactivation of a single transcription factor. The role of many proteins involved in these processes are not yet fully understood and although the discovery of the IKK signalosome and the IκBα E3 ligase in the last couple of years gave an amazing contribution to the area, there are still many open questions to be answered to further elucidate the NF-κB activation.

A general mechanism of NF-κB activation can be described as shown in figure 2 and figure 5 where extracellular stimuli cause phosphorylation of IκB on serines 32 and 36 via the IKK complex, targeting the protein for specific ubiquitination on lysines 21 and 22 by the SCFβTrCP complex followed by degradation of polyubiquitinated IκBα by the 26S proteasome and translocation of active NF-κB into the nucleus allowing transcription of responsive genes.

1.1.4. Clinical relevance

It has been well documented that activation of Rel-NF-κB plays an important role in many cellular processes, including inflammation, cell proliferation and apoptosis (Foo and Nolan, 1999). Rel-NF-κB family of transcription factors coordinately controls the expression of genes involved in disease processes and thus NF-κB has been linked to a variety of diseases such as autoimmunity, dysplasias or even oncogenesis. Knockout mouse studies have also confirmed the key role of this transcription factor family in broad physiological processes, including limb development, immune function and metabolic processes such as bone remodelling (Baeuerle and Baltimore, 1996; Baldwin, 1996).

There is growing evidence that NF-κB is involved in regulating apoptosis. p65 and IKKβ knockout mice die from extensive loss of liver cells due to apoptosis (Beg et al., 1995b; Li et al., 1999). The Rel- NF-κB family of transcription factors upregulates the expression of genes that directly rescue cells from apoptosis - anti-
Figure 2. Schematic overview of components of the NF-κB signal transduction pathway.
See text for details. Abbreviations of signaling components: IL-1, interleukin 1; IL-1R, IL-1 type 1 receptor; IL-1RACP, IL-1R accessory protein; IRAK, IL-1 receptor-associated kinase; CD4L, CD4 ligand; TNF, tumor necrosis factor; TNFR1, TNF receptor subtype 1; TRADD, TNFR1-associated death domain protein; FADD, Fas-associated protein death domain protein; TRAF, TNFR-associated factor; RIP, ring finger interacting protein; PDGF, platelet-derived growth factor; PI(3)k, phosphatidylinositol-3-OH kinase; NIK, NF-κB-inducing kinase; MEKK1, mitogen-activated protein kinase; Akt, protein kinase B; IKKα/IKKβ, IκB-kinase α- and β- subunits; IKAP, IKK-complex-associated protein; NEMO, NF-κB essential factor.
apoptotic genes, such as FasL (Kasibhatla et al., 1999), IEX1 (Wu et al., 1998) and IAP (Chu et al., 1997; You et al., 1997). Persistent NF-κB activity protect cells from apoptosis and thereby contributes to the dysregulation of growth that is essential to oncogenesis. Pro-apoptotic extracellular signals can also induce NF-κB which in turn will induce expression of anti-apoptotic genes (Wang et al., 1998). It is not clear whether NF-κB normally functions as a pro-apoptotic factor or if it protects against cell death. The anti-apoptotic effect of NF-κB may be more general and inhibition of NF-κB could be used to make cancer therapies more effective although this process must be accompanied by a procedure that directs cell death only in specific cells. It is also now clear that several human cancers contain rearrangements, amplifications or mutations that alter the expression or function of cellular Rel family transcription factors (Cabannes et al., 1999; Sovak et al., 1997; Wood et al., 1998).

Given the impact of NF-κB in physiological processes, the development of new modalities for chemotherapeutic, immuno-modulatory and anti-inflammatory drugs, involving the NF-κB pathway is an area of increasing pharmaceutical interest. Commercially available glucocorticoids, well known for their anti-inflammatory and immunosuppressive properties have a repressive effect on NF-κB-mediated gene expression. Two possible mechanisms can be involved in this process. Glucocorticoids can either act via direct interaction of the glucocorticoide receptors with NF-κB, inhibiting transactivation capacity of both proteins or inducing the synthesis of IκBα, which will remove NF-κB from its DNA binding site. These mechanisms are not mutually exclusive but it remains to investigated which mechanism predominates under particular conditions or in certain cell types (Dumont et al., 1998).

Understanding the mechanisms which regulate NF-κB activity and the identification of specific Rel/ NF-κB-regulated target genes that control various processes will provide new insights into signalling pathway. This will be of
particular importance in the development of selective inhibitors of the NF-κB pathway. At present, the most specific targets for the inhibition of NF-κB activity are the components of the IKK complex since the recently identified IκBα ligase - SCF
\textsuperscript{31}CP, can ubiquitinate several proteins (Maniatis, 1999).
1.2. The ubiquitin-proteasome system

Cellular proteins exist in a dynamic equilibrium. Their steady-state levels are maintained by a tightly controlled and highly regulated balance of synthesis and degradation. A selective and programmed way of targeting many proteins for degradation, in eukaryotic cells, is carried out by the ubiquitin proteasome system.

Ubiquitin, one of the most highly conserved proteins, is a 76-residue protein that exists in cells either free or covalently linked to other proteins. It was first isolated by Goldstein and co-workers from the thymus and was thought to be a thymic hormone (Goldstein et al., 1975) but in subsequent work it was found in all tissues and eukaryotic organism and hence its name. In addition to degrading damaged, misfold, or misassembled proteins, the ubiquitin system targets many cellular proteins, including transcription factors, cell growth modulators, signal transducers, cell cycle regulators, tumour suppressors, oncoproteins, short-lived enzymes, viral gene products, membrane polypeptide receptors for degradation (Hershko and Ciechanover, 1998). With the multiple cellular targets, it is not surprising that the system is involved in the regulation of many basic cellular processes such as cell cycle and division, differentiation and development, the response to stress and extracellular modulators, morphogenesis of neuronal networks, modulation of cell surface receptors, DNA repair, regulation of the immune and inflammatory responses and apoptosis.

Degradation of a protein via the ubiquitin pathway involves two distinct and successive steps: covalent attachment of multiple ubiquitin molecules to the protein substrate, and degradation of the tagged protein by the 26S proteasome. Ubiquitin conjugation is catalysed by an enzymatic cascade that begins with the ATP-dependent activation of the C-terminus of ubiquitin by the ubiquitin-activating enzyme (E1). The C-terminus of ubiquitin is first adenylated with release of pyrophosphate. This is followed by formation of a high energy thioester linkage between the side chain of a cysteine residue in E1 and the C-terminal carboxyl
group of the ubiquitin protein with release of AMP. In a transesterification reaction, the ubiquitin is transferred from the E1 to a conserved cysteine residue in a family of ubiquitin-conjugating enzymes (E2). Ubiquitin is then transferred from the E2 enzyme to the ultimate protein acceptor via an isopeptide linkage with the ε amino group of a lysine in the target protein. In many cases this final step requires the participation of ubiquitin protein ligase (E3) which may act either as the ultimate ubiquitin donor or in substrate recognition (Hershko and Ciechanover, 1998). Proteins destined for degradation or processing via the 26S proteasome are coupled to multiple copies of ubiquitin by formation of further isopeptide bonds between additional ubiquitin molecules and lysine residues in previously conjugated ubiquitin (see Figure 3).

The structure of this system appears to be hierarchical: a single E1 carries out activation of ubiquitin required for all modifications. Several major species of E2 enzymes were characterised in mammalian cells, plants and yeast. The E2s act in concert with E3s, and it appears that each E2 can act with one or more E3. Finally, the E3 ligases belong to a large, still growing family of enzymes. The mechanisms that underlie the high specificity and selectivity of the ubiquitin system are still an unsolved problem but the exponential increase of information on this system has demonstrated that E3 ligases are probably the main family of enzymes responsible for these properties. Nevertheless post-translational modifications such as phosphorylation of either the target protein or involved enzymes also play an important role in the recognition process.

An additional conjugation factor, named E4 was recently described as being involved in the multiubiquitin chain assembly (Koegl et al., 1999). Although E4 function does not participate in the ubiquitin-enzyme thioester cascade and in contrast to E3s, does not interact with substrate directly it may be part of a large, not yet identified family of regulatory factors which together with E3s give more specificity to the ubiquitin degradation pathway.
Figure 3. The Ubiquitin-proteasome degradation pathway.

1. Activation of ubiquitin (Ub) by the Ubiquitin activating enzyme -E1- in an ATP dependent fashion.
2. Transfer of activated ubiquitin from E1 to the active cysteine residue of a member of the ubiquitin conjugating enzymes -E2.
3. Transfer of activated ubiquitin directly or indirectly from E2 to a protein-substrate via ubiquitin protein ligase -E3.
   - In some cases, E3 accepts the activated ubiquitin from an E2 and binds it as a thioester intermediate prior to transfer to protein, while in others the E3 helps to transfer ubiquitin directly from E2 to a protein, by tight binding of E2 and the protein substrate. Repeated conjugation of ubiquitin to Lysine residues of formerly bound ubiquitin moieties leads to the formation of multi-ubiquitin chains.
4. Binding of the polyubiquitinated substrate to the ubiquitin receptor subunit in the 19S complex of the 26S proteasome and degradation of the substrate to short peptides by the 20S complex.
5. Recycling of ubiquitin via the action of isopeptidases.
1.2.1. E1

E1 catalyses the first step in ubiquitin conjugation. A single E1 enzyme has been characterised and has been detected in both cytoplasm and nucleus of eukaryotic cells but is phosphorylated and localised to the nucleus in a cell cycle-dependent manner (Stephen et al., 1996). It is possible that E1 and other components of ubiquitin system may perform distinct, essential roles by localising to different subcellular compartments within the cell (Trausch et al., 1993). The E1 gene is essential for cell viability (McGrath et al., 1991). Because the E1 is unique, it does not appear to play a role in selecting protein substrates for ubiquitination. However, ubiquitin co-exists with a number of ubiquitin-like molecules, and the E1 enzyme must distinguish between these molecules.

1.2.2. E2/UBC

E2s are relatively small molecules, mostly 14-25 kDa, that share a conserved ubiquitin conjugating domain (UBC) which contains a catalytic cysteine residue that accepts the activated ubiquitin from E1. In addition to the core UBC domain, some E2s have unique C- or N-terminal extensions that may be involved respectively in the substrate recognition and E1 interaction (Liu et al., 1999).

The genome of Saccharomyces cerevisiae encodes for 13 E2s and E2-like proteins, and many more have been described in mammals. Specific functions of this class of enzymes may be the result of their association with specific E3s, which in turn bind their specific protein substrates. There is not much experimental evidence for the direct binding of E2s to protein substrates with the exception of the E2-like Ubc9. Ubc9 was originally described as an essential yeast protein required for cell-cycle progression at the G2- or early M-phase and it was proposed that the proteolytic pathway that degrades B-type cyclins involves Ubc9 (Seufert et al., 1995). However in subsequent work it was demonstrated that the conjugation of cyclin B to ubiquitin in a cell-free system from Xenopus eggs could not be supported by a Xenopus homologue of ubc9 (King et al., 1995). Ubc9 was
identified as an interacting protein in yeast two-hybrid screens with a surprisingly large number of proteins, but evidence that the protein has ubiquitin conjugating activity in a thioester assay was never reported.

1.2.3. E3

Given the range of substrates and the requirement for specificity, the E3 enzymes or ubiquitin protein ligases, which mediate substrate recognition must be highly selective.

The mechanism of transfer of activated ubiquitin from a thioester intermediate to the amino group of a lysine acceptor in the target protein appear to differ in various types of E3s. In some cases, E3 accepts the activated ubiquitin from an E2 and binds it as a thioester intermediate prior to transfer to protein, while in others a ligase may help to transfer ubiquitin directly from E2 to a protein, by tight binding of E2 and the protein substrate. Thus, the definition of E3 has been changing with the discovering of new members of this class of enzymes and although it remains unclear, the best definition so far is that E3 is an enzyme that binds, directly or indirectly, specific protein substrates and promotes the transfer of ubiquitin, directly or indirectly, from a thioester intermediate to the ultimate protein acceptor via an isopeptide linkage with the ε amino group of a lysine in the target protein. Specificity derives from the high-fidelity protein-protein interactions between the E3 and substrate but details of E3-substrate interaction and how the activity of an E3 is modulated and what controls E2-E3 interactions are still an open door in the ubiquitin field. So far, four different classes of E3 ubiquitin ligases have been defined: Ubr1 (Bartel et al., 1990), the HECT domain family (Huibregtse et al., 1995), the SCF family (Feldman et al., 1997; Skowyra et al., 1997) and the anaphase promoting complex (APC)/cyclosome (King et al., 1995).
1.2.3.1. E3α/UBR1

Although the majority of cellular proteins are targeted through different signals a few proteins may be recognised by their free and “destabilising” N-terminal residue. E3α, the mammalian homologue of UBR1 and E3β recognise protein substrates via their N-terminal residue (Varshavsky, 1995). E3α recognises basic or bulky-hydrophobic N-terminal amino acid residues and E3β may be specific for proteins with small and uncharged N-terminal amino acid residues (Hershko and Ciechanover, 1998). The N-end rule recognition mechanism is highly conserved, but its cellular substrates and physiological roles are still obscure.

1.2.3.2. HECT family

The HECT (homologous to E6-AP C-terminus) domain proteins have E3 activity and they are characterised by sequence similarity of their C-terminal regions to the C terminus of E6-AP (E6 associated protein), the first member of this family involved in the human papillomavirus E6-induced ubiquitination of p53. E6-AP does not bind directly to p53 but rather binds indirectly via E6, which binds to both p53 and E6-AP. The mechanism used by E6-AP involves the formation of a thioester complex between E6-AP and ubiquitin in the presence of E1 and distinct E2 ubiquitin-conjugating enzymes including human ubcH5 or Arabidopsis thaliana AtUBC8. Ubiquitin is then transferred directly from E6-AP to the substrate. E6-AP was the first E3 ubiquitin ligase identified with ability to form a thioester with ubiquitin (Scheffner et al., 1995). A large family of proteins that contain an approximately 350 amino-acid C-terminal region homologous to that of E6-AP, the HECT domain family, has been identified in many eukaryotic organisms and they all contain a conserved active site Cys residue near the C-terminus (Huibregtse et al., 1995). Although the function of these proteins is unknown most HECT domain proteins are likely E3 enzymes with ability to form thioesters with ubiquitin.
1.2.3.3. The SCF (Skp1-Cdc53/CUL1-F-box protein) family

Different members of this family of multisubunit ubiquitin-protein ligases use different adapters that recruit the target protein to a complex containing an E2 enzyme. This E3 family is not involved directly in ubiquitin transfer and targets different substrates such as cell cycle regulators, including mitotic cyclins (Cln2p) and CDK inhibitors (Sic1p) and transcription regulators such as IxB and β-catenin, among many others, to proteasome degradation (Maniatis, 1999; Patton et al., 1998). The common feature of all these substrates is that phosphorylation converts the substrate to a form susceptible to the action of the E3 complex.

The SCF is named for three of its core components, Skp1, Cdc53/cullin and an F-box containing protein but they may also have other components specific for certain protein substrates. Skp1 and F-box proteins interact through the F-box motif. Cdc53 functions as a bridging molecule to bind the Skp1/F box complex to the E2 (Figure 4). The mechanism of the SCF ubiquitin ligase system was initially discovered in studies of the cell cycle in yeast for the Cdc34-dependent degradation of phosphorylated Sic1p and G1 cyclins, Cln1p and Cln2p. Sic1p is a B-type cyclin kinase inhibitor, the degradation of which is essential for the G1-S transition in the budding yeast. Sic1p after being phosphorylated by a G1 Cyclin-activated protein kinase (Cln/Cdc28) (Schneider et al., 1996) is targeted for degradation by a complex including Cdc4p, Skp1p and Cdc53. Cdc4p binds to the phosphorylated substrate Sic1p through its WD repeats and an F-box motif in Cdc4p mediates the connection to the ubiquitin-proteasome machinery by interacting with Skp1p. Grr1p is the F-box protein involved in the Cln1p and Cln2p ubiquitination. Association of Grr1 with Clns is mediated by autophosphorylation that allows activation of Cln kinases to eventually turn themselves off to reset the balance for the next G1 (Feldman et al., 1997; King et al., 1996a; Skowyra et al., 1997; Verma et al., 1997; Willems et al., 1996).

Skp1, Cdc53/cullin and F Box proteins are not the only essential components of SCF complexes. In yeast, the recently identified protein Rbx1/Roc1,
Figure 4. SCF complex, an E3 ubiquitin ligase superfamily.

The complex is composed of a set of adapter proteins that recruit different binding partners through specific protein-protein interactions. Skp1 and F-box proteins interact through the F-box motif. Cdc53/CUL1 functions as a bridging molecule to bind the Skp1/F-box complex to the E2 (Cdc34). The newly discovered Rbx1/Roc1 plays an integral role in tethering components to each other and maybe involved in the E2 recruitment.
which contains a RING-H2 fingerlike motif termed the R box, is a subunit and potent activator of the SCF complex required for Sic1 and Cln ubiquitination in vitro (Kamura et al., 1999; Seol et al., 1999; Skowyra et al., 1999). Mammalian Rbx1/Roc1 was discovered as part of the von Hippel-Lindau (VHL) tumour suppressor complex. This complex includes Elongin B, Elongin C, and Cullin-2, proteins associated with transcriptional elongation and ubiquitination (Kamura et al., 1999). The core structure of Elongin B is highly similar to that of ubiquitin, Elongin C is a Skp1 homolog and Cullin-2 is a homolog of Cdc53 and therefore the VHL is structurally analogous to SCF complex. Rbx1/Roc1 is a component of VHL, SCF<sub>Cdc4</sub> and SCF<sup>Grr1</sup> complexes (Kamura et al., 1999; Skowyra et al., 1999) and since they have very high structural similarity it is tempting to speculate that the VHL complex may function as ubiquitin ligase for as yet unidentified target proteins (Tyers and Willems, 1999). Rbx1/Roc1 may define a new class of E3-associated proteins which may act as an adapter to recruit the E2 Cdc34 to Cdc53 or SCF complexes by bridging or stabilising the Cdc34-Cdc53 interaction (Seol et al., 1999; Skowyra et al., 1999; Tyers and Willems, 1999). In vitro reconstitution of the phosphorylation-dependent ubiquitination of I<sub>x</sub>B<sub>α</sub> have demonstrated that Roc1 is recruited by cullin1/Cdc53 to form a complex with SCF<sup>HOS<sub>HOS</sub>TrCP</sup> (Tan et al., 1999).

Although the mechanism of this family of E3 ubiquitin ligases is mainly based on studies in yeast, the components of these machineries are conserved in evolution. Over the past few years, F-box proteins and other components of the SCF/Cdc34 pathway have been identified in many eukaryotes and linked to diverse cellular processes. Recently a <em>Drosophila</em> F-box protein, Slimb, and its mammalian homolog β-TrCP (β-transducin repeat-containing-protein) have been identified as a subunit of a SCF complex (SCF<sup>βTrCP</sup>) implicated in the regulation of three different signal transduction pathways: NF-κB, Wnt/ Wingless (Wnt/Wg) and Hedgehog (Maniatis, 1999). SCF complexes couple protein kinase signalling pathways to the control of the stability of substrate proteins (Skowyra et al., 1997). Given the large
number of protein kinases and the possible SCFs, this may be one of the main pathways in the control of protein abundance. The dynamic nature of this type of complex is not well defined and it is not known whether and how these multisubunit complexes are regulated. Their assembly and disassembly may represent another possible mechanism to control the rate of degradation of their target proteins.

1.2.3.4. Cyclosome/anaphase-promoting complex (APC)

Cyclosome(Sudakin et al., 1995) or anaphase-promoting complex (King et al., 1995) is another E3 ubiquitin ligase that uses different adapters to target different substrates specific for cell-cycle regulatory proteins including mitotic cyclins and other proteins that regulate mitosis (Peters, 1998). Many of these substrates contain a conserved 9-residue motif called the “destruction box” and all of them are degraded during mitosis. This ubiquitin ligase is a high-molecular-weight complex composed of 12 subunits in budding yeast (Zachariae et al., 1998a; Zachariae et al., 1996) and at least 10 subunits in mammals and Xenopus (Yu et al., 1998). Among many core subunits of unknown function, some turned out to be homologous to subunits of the SCF complexes supporting a possible parallel mechanism between APC and SCF complexes with a major difference in the type of substrate. SCF substrates are recognised after substrate phosphorylation which does not occur with APC substrates. Apc2, subunit of the APC is homolog to Cdc53 (Zachariae et al., 1998b) and the APC also contains an Rbx1/Roc 1 related protein, Apc11, which is required for Clb2 ubiquitination in vitro (Zachariae et al., 1998b) and interacts with cullin Apc2 suggesting its possible involvement in the E2 recruitment (Ohta et al., 1999).

1.2.4. IκBα Ubiquitination - The SCF$^{BTCP}$ complex

Until very recently the E3 responsible for the ubiquitination of IκBα has remained unknown and just a partially purified activity had been isolated which was
able to support the phosphorylation and ubiquitination of IκBα in an in vitro system in presence of ATP, ubiquitin, E1 and an E2 protein (ubcH5 or UbcH7) (Alkalay et al., 1995; Chen et al., 1995; Chen et al., 1996; Kroll et al., 1997).

Using different strategies, independent groups identified β-TrCP as a specific component of the IκB ubiquitin ligase complex (Hatakeyama et al., 1999; Spencer et al., 1999; Vuillard et al., 1999; Winston et al., 1999; Yaron et al., 1998). β-TrCP is a mammalian homolog of the Drosophila Slimb protein (Jiang and Struhl, 1998) and is an F-box/WD40 repeat protein that appears to act as the receptor for recruitment of phosphorylated substrates. Slimb negatively regulates both the Hedgehog and Wnt/Wingless pathways (Jiang and Struhl, 1998). Human β-TrCP was isolated as a protein interacting with Vpu, a small human immunodeficiency virus (HIV)-encoded protein that interacts with CD4 in the endoplasmic reticulum and targets it for degradation (Margottin et al., 1998). β-TrCP also interacts directly with β-catenin, a component of the Wingless/Wnt pathway, in a phosphorylation dependent manner, (Hart et al., 1999; Kitagawa et al., 1999; Latres et al., 1999; Winston et al., 1999). There is a common structural feature among IκBα, β-catenin and Vpu: signal induced phosphorylation occurs on two closely located serines at positions 32 and 36 (IκBα), 33 and 37 (β-catenin) and 52 and 56 (Vpu) suggesting that β-TrCP recognises a very similar motif, with minimal sequence consensus of DS*GX-S*, where S* represents phosphoserine and X a hydrophobic residue. Further studies are required to determine whether any of the many proteins containing the above motif are also substrates of β-TrCP.

The first group that identified β-TrCP as a specific component of the IκB ubiquitin ligase took advantage of its high affinity for phosphorylated IκBα (pIκBα) and devised an immunoaffinity purification method to isolate the protein from HeLa cells (Yaron et al., 1998). Later studies showed that β-TrCP protein functions as the F-box protein of an SCF complex containing Skp1 and Cdc53/Cul1, which recognises pIκBα bound to NF-κB (Spencer et al., 1999;
Winston et al., 1999) or a phosphorylated peptide substrate representing residues 20-43 of IκBα (Vuillard et al., 1999). While there is general agreement in the subunits that composed the SCFβTrCP complex, there is less agreement on the E2 involved in the polyubiquitination of IκBα. Many SCF complexes involved in cell cycle protein ubiquitination appear to utilise Cdc34 as the E2 for ubiquitination and this is consistent with data indicating that Cdc34 is responsible for ubiquitination of pIκBα (Tan et al., 1999; Vuillard et al., 1999). However, in other studies in vitro ubiquitination of pIκBα was achieved using either UbcH5 (Spencer et al., 1999; Yaron et al., 1998) or yeast extracts as a source of E2 activity (Winston et al., 1999). The recently identified protein Rbx1/Roc1 as a novel subunit of SCFβ-TrCP complex (Tan et al., 1999) and possible involvement in the recruitment of Cdc34 to the SCF complexes strengthens the hypothesis that Cdc34 is the E2 required for the phosphorylation dependent ubiquitination of IκBα in vitro and raises the possibility of others factors being involved in the specificity of the SCF complexes. Nevertheless it remains to be determined which E2 (or E2s) functions in the signal-induced ubiquitination of IκBα in vivo.

In β-TrCP, both the F-box and the WD domain are protein-protein interaction motifs. WD repeats at the C-terminus mediate binding to the substrate, and an F-box near the N-terminus is involved in interaction with Skp1p, which in turn is bound to Cdc53. The β-TrCP F-box is necessary for IκBα ubiquitination in vitro and an F-box deletion mutant of β-TrCP, which is still able to bind to pIκBα but fails to promote its ubiquitination in vitro, acts in vivo as a dominant-negative, inhibiting the degradation of pIκBα and consequently NF-κB activation (Spencer et al., 1999; Yaron et al., 1998). The F-box deleted form of β-TrCP has the same effect on Vpu-mediated CD4 degradation (Margottin et al., 1998) and blocks β-catenin degradation in human cells (Hart et al., 1999; Kitagawa et al., 1999; Latres et al., 1999). The SCFβTrCP is involved in the phosphorylation dependent ubiquitin-mediated proteolysis of IκBα, β-catenin and CD4 (through HIV Vpu) suggesting
that a single F-box is capable of recognising different substrate with identical destruction motifs.

The model proposed for signal induced ubiquitination of IκBα through the SCFβTrCP complex is shown in figure 5.

1.2.5. The 26S proteasome

The proteasome is an ATP-dependent multicatalytic protease complex which recognises and specifically degrades cytosolic and nuclear proteins, most of which are ubiquitin tagged. It is composed of the 20S core catalytic complex flanked on both sides by the 19S regulatory complexes which seem to be responsible for recognition and unfolding of the ubiquitin-tagged target protein.

Groll et al. solved the crystal structure of the Saccharomyces cerevisiae 20S proteasome to 2.4 Å resolution giving an important development in studies of this complex structure. The eukaryotic 20S core catalytic is composed of 14 pairs of different, but related, protein subunits which assemble into a single structure arranged into four seven-membered rings with the α-type subunits forming the two outer rings guarding an inner pair the β-type subunit rings, α₁₋₇ β₁₋₇ β₁₋₇ α₁₋₇ (Groll et al., 1997). The α subunits do not seem to be catalytic, but they can self assemble while the β subunits which cannot self assemble possess proteolytic activity. The three major peptidase activities of eukaryotic proteasomes, present on some of the β subunits, are: a chymotrypsin-like activity, which cleaves after hydrophobic residues, a trypsin-like activity, which cleaves after basic residues and a peptidylglutamyl peptide hydrolysing activity which cleaves after acidic residues (Baumeister et al., 1998). The proteolytic mechanism of the 20S complex involves the utilisation of the hydroxyl group of the terminal threonine residue of the β subunit as the catalytic nucleophile that attacks peptide bounds.

Knowledge about the sizes of peptides generated by proteasomes during protein degradation is essential to fully understand their degradative mechanisms.
A variety of signals lead to the activation of an IκB kinase complex which specifically phosphorylates Ser 32 and Ser 36 at the amino terminus of IκBα. This phosphorylated form associated to NF-κB (p50/p65) is recognised by an E3 ubiquitin ligase complex (SCFβTrCP), described in figure 4. This complex promotes the site-specific ubiquitination of IκBα at lysines 21 and 22 in an E1 and E2 dependent reaction. Polyubiquitinated IκBα is then targeted to the 26S proteasome for degradation, resulting in the release and nuclear translocation of NF-κB.
and the subsequent steps in protein turnover and generation of major histocompatibility complex class I antigenic peptides. The size of peptides generated ranges in length from 3 to 22 residues. (Kisselev et al., 1998; Kisselev et al., 1999). Other peptidases must function after the proteasome to complete the turnover of cell proteins to amino acids.

The main role of the α subunits seems to be involved in the stabilisation of the two-ring structure of the β chains and in the binding of the 19S “cap” regulatory complexes (Groll et al., 1997). The openings that give access to the inner cavities of the proteasome, involved in the entry of substrates and exit of proteolysis products, are apparently different in the yeast and Thermoplasma proteasome structure. In the Thermoplasma proteasome there are two entry pores at the ends of the cylinder which in the yeast 20S proteasome seem to be occluded by the N-terminal residues of the α subunits. On the other hand, the yeast proteasome has small side orifices at the interface between α and β rings which are unlikely to be used for the entry of the substrate but they may have a role in discharging degradation products. Entry from the ends is probably only possible after ATP-dependent rearrangements that may occur in the 19S complexes rendering ubiquitinated proteins into a form competent for degradation by the 20S core complex.

The 19S complex is composed by at least 15 subunits, six of which are ATPases and members of the AAA-ATPase family (ATPases associated with a variety of cellular activities) (Baumeister et al., 1998). The precise role of these ATPases, that is, the energy dependent step in the degradation of proteins, and the functions of the most of the non-ATPase subunits are still unknown but they may be involved in the recognition and unfolding of the target proteins helping in their translocation into the 20S inner proteolytic complex.

An additional regulator that associates with the 20S proteasome in an ATP-independent fashion is the 11S complex. This complex is a ring-shaped hexamer, composed of alternating α and β subunits. It does not stimulate the degradation of proteins or of ubiquitin-protein conjugates but is γ-interferon inducible and is
apparently involved in the immune response, more specifically in the antigen processing (Groettrup et al., 1995). Under the influence of γ-interferon there is no indications for an influence on the subunit composition of the 19S complex but three proteolytically active β-subunits of the 20S proteasome are replaced (Groettrup et al., 1996a; Groettrup et al., 1996b). The subcomponents of the 26S proteasome are dynamic structures and its is likely that changes in the subunit composition reflect adaptation to current cellular conditions and influence protease specificity.

Specific proteasome inhibitors have been developed and they have become powerful research tools in probing the structure and function of the proteasome and ubiquitin pathway. Most of them act as pseudosubstrates that become linked covalently to the active site hydroxyl groups in threonine of the β subunits and as a result the chymotryptic and trypic-like activities are inactivated. Examples of these inhibitors most widely used are: peptide aldehydes like MG132, MG115, ALLN and natural products like lactacystin (Lee and Goldberg, 1998). In addition to being useful research tools, proteasome inhibitors have also potential use in the treatment of human diseases in which the ubiquitin proteolytic pathway is involved.

1.2.6. Deubiquitination enzymes

Ubiquitination of target proteins is a dynamic process and appears to be a reversible reaction that is catalyzed by processing proteases known as deubiquitination enzymes (DUB). Although the main regulation of this modification occurs at the level of the ubiquitin conjugation, deubiquitination, or removal of this modification, is being recognised as an important regulatory strategy. Deubiquitination enzymes are cysteine proteases that specifically hydrolyse ester, thiol ester and amido bonds to the carboxyl group of G76 of ubiquitin. This family of enzymes can be sub-classified into at least two gene families that are structurally unrelated: the UCH (ubiquitin C-terminal hydrolases) family and the UBP (ubiquitin processing proteases) family (Wilkinson, 1997). More than 60 full length
DUB sequences have been identified so far and although little is known about their biological role, they are the largest family of enzymes in the ubiquitin system suggesting that they may be involved in the recognition of different types of ubiquitin conjugates. These enzymes act at different levels in the ubiquitin pathway: in the generation of free ubiquitin by processing of polyubiquitin chains either from linear precursor fusion proteins or from already branched-chain polyubiquitin, in the removal of ubiquitin from ubiquitinated target protein avoiding protein degradation by the 26S proteasome and finally in clearing the proteasome of peptide remnants conjugated to ubiquitin chains (D'Andrea and Pellman, 1998).

In addition to ubiquitin, all eukaryotic cells contain several other ubiquitin-like proteins that can also be conjugated to proteins and they are synthesised as fusion proteins that must be processed by enzymes similar to DUBs. It is likely that some of the known deubiquitinating enzymes will be responsible for processing these fusions proteins and disassembling the conjugated ubiquitin like proteins.

1.2.7. Ubiquitin and Human diseases

Given the broad range of substrates and processes in which the ubiquitin proteolytic pathway is involved, it is not surprising that aberrations in the process have been implicated in the pathogenesis of several diseases either by stabilisation or accelerated degradation of the protein target. Regulated proteolysis by the ubiquitin-proteasome system has a critical function in memory storage and is part of the molecular switch that converts short-term synaptic plasticity to long term. Learning induces a neuron-specific ubiquitin C-terminal hydrolase which associates with the proteasome and increases its proteolytic activity. The resulting up-regulation of the ubiquitin pathway enhances the degradation of proteins that inhibit long-term memory storage (Hegde et al., 1997).
1.2.8. Ubiquitin-like modifications

In some instances protein ubiquitination functions not as a signal for degradation, but to alter the properties of the linked protein. Thus histone ubiquitination alters chromatin structure (Bradbury, 1992) while ubiquitination of a plasma membrane receptor modifies ligand-stimulated endocytosis (Hicke and Riezman, 1996). Whereas addition of multiple copies of ubiquitin targets proteins for degradation it is now widely recognised that covalent attachment of other ubiquitin related molecules does not result in degradation of the modified protein. The protein UCRP, which contains two ubiquitin-like domains is conjugated to a number of intracellular proteins by a series of reactions that are separate from ubiquitination (Haas et al., 1987; Narasimhan et al., 1996). Recently a small ubiquitin-like protein variously known as sentrin, GMP1, SUMO-1, UBL1 and PIC1 has been found covalently linked to Ran GTPase activating protein 1 (RanGAP1) and associated with a variety of other proteins (Boddy et al., 1996; Kamitani et al., 1997b; Mahajan et al., 1997; Matunis et al., 1996; Shen et al., 1996a). Covalent modification of RanGAP1 appears to be necessary for its interaction with the Ran-GTP-binding protein RanBP2 at the cytoplasmic face of the nuclear pore complex (Mahajan et al., 1997; Saitoh et al., 1997) while SUMO-1 modification of PML targets the protein to PML nuclear bodies (Muller et al., 1998; Sternsdorf et al., 1997). SUMO-1 and Smt3p, a yeast homologue of SUMO-1, are conjugated to target proteins by a pathway that is distinct from, but analogous to, ubiquitin conjugation. Recently an additional protein modification pathway involving the ubiquitin-like protein Rublp has been characterised in Saccharomyces cerevisiae (Lammer et al., 1998; Liakopoulos et al., 1998). A major substrate for Rublp is CDC53/cullin which is a common component of SCF ubiquitin ligase complexes. In this system the products of the ULA1 and UBA3 genes act as a heterodimeric E1 enzyme while the product of the UBC12 gene acts as the E2 conjugating enzyme. It also appears that this conjugation system is active in higher
eukaryotes with the Rub1p homologue Nedd8 also being conjugated to CDC53 (Hochstrasser, 1998; Kamitani et al., 1997a).

SUMO-1/Smt3p and NEDD8/Rub1p are processed proteolytically at the C-terminus to generate a free C-terminal -Gly-Gly, which like the C-terminal -Gly76 of ubiquitin is essential for conjugation (Kamitani et al., 1997b).

In all of the above cases the protein conjugated has been similar to ubiquitin. However a separate protein conjugation system, which is required for autophagy in *Saccharomyces cerevisiae*, involves conjugation of a protein unrelated to ubiquitin. In this case the C-terminus of Apg12, a 186 residue protein, is conjugated to a lysine side chain in Apg5. Although Apg12 is unrelated to ubiquitin it appears that it is activated by Apg7, which is homologous to ubiquitin E1 enzymes, and conjugated by Apg10, an E2 equivalent (Mizushima et al., 1998).

UCH-L3, a putative ubiquitin C-terminal hydrolase, was recently identify as a NEDD8 (Kamitani et al., 1997a)-interacting protein, able to cleave the C terminus of NEDD8 but not bind to sentrin-1, sentrin-2 or sentrin-3 (Wada et al., 1998).

Conjugation of the ubiquitin-like proteins raises several questions related to the chemical nature of the adduct, the identity of the conjugating and deconjugating enzyme(s) and the specificity of substrate targeting. The expanding number of ubiquitin-like proteins and intracellular targets suggest that the understanding of how much ubiquitin and ubiquitin-like proteins can do, it is just in the beginning of an intriguing field.

### 1.3 Aims of the project

Inside the cell, post-translational protein modifications modulate protein function by altering protein activity, subcellular localisation and/or ability to interact with other proteins. Ubiquitination is one of the most well known of these modifications and provides a versatile way of cellular regulation.

This modification has a well-established role in the degradation of IκBα, which occurs during NF-κB activation. However the involvement of ubiquitin-like
modifications in the NF-κB pathway had not been reported. To identify and purify proteins involved in the IkBα signalling pathway a Yeast Two Hybrid Screen was used as primary strategy. Proteins able to interact with the N-terminal regulatory domain of IkBα were isolated. One of the isolated proteins, Ubch9, turned out to be involved in a novel ubiquitin-like modification, SUMO-1 conjugation. Further in vivo and in vitro characterisation of this specific interaction was carried out to investigate a possible role for SUMO-1 modification in the regulation of the NF-κB pathway.

Specific objectives of this work involved the identification and characterisation of the enzymes responsible for SUMO-1 conjugation and the definition of the biological relevance for this novel ubiquitin-like modification in vivo.

In order to achieve the last objective, the involvement of SUMO-1 modification in the metabolism of another transcription factor, p53, which is also regulated by ubiquitin mediated proteosomal degradation was investigated.
2. Materials and Methods
2.1. Materials

Human recombinant TNFα was provided by the MRC ADP reagent program. Human recombinant Interleukin 1β (IL1) was purchased from Sigma and Okadaic acid (Oka) was obtained from Calbiochem. Ubiquitin was purchased from Sigma.

2.2. Antibodies

IκBα (C-21, Santa Cruz) is a rabbit polyclonal antibody raised against a peptide (amino acids 297-317) corresponding to carboxyl terminus of IκBα of human origin and used in western blots at 1:2000 dilution.

Rabbit polyclonal antibody raised against purified human recombinant IκBα was described previously (Arenzana-Seisdedos et al., 1995) and used at 1:2000 dilution in western blotting.

21C7 (Matunis et al., 1996) is a mouse monoclonal antibody which recognises GMP1 (SUMO-1) and was purchased from Zymed. It was used in western blots at 1:2000 dilution.

SV5 Pk tag monoclonal antibody (Hanke et al., 1992) was obtained from R.E. Randall, University of St. Andrews and was used to immunodetect (1:2000) and immunoprecipitate proteins containing either an N-terminal or C-terminal SV5 epitope tag of 14 aminoacids (GKIPNPPLLGLDST) of the protein P of Simian Virus 5 (SV5).

HA-tagged proteins were detected using a monoclonal antibody 12CA5, specific for a 9 amino acid HA peptide sequence (YPYDVPDYA) from influenza HA, obtained from Babco. It was used in western blots at 1:5000 dilution.

Monoclonal antibody DO-1 (Vojtesek et al., 1995) recognises the N-terminus of human p53 and used at 1:1000 dilution in western blotting.
Polyclonal rabbit serum CM-1 was raised against human p53 expressed in *E. coli* (Midgley et al., 1992). Both DO-1 and CM-1 antibodies were obtained from D.P. Lane, University of Dundee.

2.3. Bacterial Strains

*E. coli* DH5α (genotype: Δ80dlacZΔM15, rec A1, end A1, gyr A96, thi-1, hsd R17 (rK-, mK+), sup E44, rel A1, deo R, Δ(lacZYA-argF)U169) was used for routine DNA preparation. *E. coli* B834 (F, ompT, hsdSB, (rB-, mB-), dcm, gal) was used for protein expression. Bacteria were grown in Luria-Bertani (LB) broth with antibiotics added when required.

2.4. Plasmids and expression vectors

pGEXT-2T was obtained from Pharmacia. The transient mammalian expression vector pcDNA3 was purchased from Invitrogen Corporation. The luciferase reporter vectors 3EnhConALuc, ConALuc, HIVLTRLuc and ΔKBHV LTRLuc were a kind gift from F. Arenzana-Seisdedos (Institut Pasteur). pG13-luc reporter plasmid was a kind gift from David P. Lane (University of Dundee). pV44ER.LexA and pACT/pACT-cDNA were received from Colin Goding (Marie Curie Research Institute, Oxted, United Kingdom) and Stephen Elledge (Baylor College of Medicine, Houston, Texas), respectively, and both have been described previously (Durfee et al., 1993; Jayaraman et al., 1994).

Vector maps of all constructs generated during this work are shown in Appendix I.

2.4.1. DNA preparation

All the DNA preparations (minipreps, maxi-preps and gel extraction) used for cloning and transfections were prepared with Qiagen kits in accordance with manufacturers instructions. DNA restriction enzymes for cloning were obtained from
New England Biolabs (NEB) and Promega. The Vent DNA polymerase used for PCR (polymerase chain reaction) was obtained from NEB. A Boeringher "Titan™ one tube RT-PCR System" was used for reverse transcription followed by PCR-amplification (RT-PCR). Quality and quantity of DNA was analysed by spectrophotometric readings at 260 nm and 280 nm and by electrophoresis in an agarose gel in the presence of ethidium bromide, followed by U.V.(Sambrook et al., 1989).

2.4.2. cDNA Cloning

2.4.2.1. Preparation of Electrocompetent Bacteria (DH5α)

A 10 ml overnight culture grown in LB at 37°C was used to inoculate one liter of LB which was maintained in culture at 25°C until an OD_{600} of approximately 0.5. Cells were then chilled on ice for 30 minutes and centrifuged 15 minutes at 4000 rpm. Bacteria were resuspended in one liter of cold 1mM Hepes pH 7 and then centrifuged as described previously. Bacteria were resuspended in 500 ml of cold 1 mM Hepes pH 7 and centrifuged at 4000 rpm for 15 minutes. Bacteria were then resuspended in 20 ml of cold 10% Glycerol and centrifuged as described previously. Finally the bacteria were resuspended in a final volume of 2 to 3 ml of cold 10% glycerol, frozen in dry ice and kept at -70°C in small aliquots. All solutions were previously autoclaved and kept at 4°C.

2.4.2.2. Transformation of electrocompetent bacteria

40 µl of electrocompetent bacteria were incubated with either plasmid DNA or ligation mixtures, on ice for at least 1 minute. 1 ng of known plasmid DNA or no DNA were added to electrocompetent cells as positive and negative controls. After incubation the transformation mixture was transferred to a 2 mm electroporation cuvette (Flowgen), previously chilled on ice and the following electroporation conditions were used: V= 2500; C= 201; T= 5 msec. 1 ml of LB/20 mM Glucose
was immediately added to the transformed bacteria and the cells were incubated one hour at 37 °C before plated on LB-Agar containing the relevant antibiotic.

**Two Hybrid screen plasmids**

The yeast two hybrid system (Fields and Song, 1989) was used to screen a human cDNA expression library for proteins that interact with N-terminal regulatory domain of IκBα (amino acids 1-74). The pV44ER.LexA expression vector was used to generate a fusion of the LexA DNA binding domain with the N-terminus of IκBα (1-74). A second vector pACT was used to express a fusion of Gal4 activation domain with proteins encoded by cDNAs in a library generated from human lymphocytes (Durfee et al., 1993). Interacting species were identified in yeast L40a displaying histidine independent growth and β-galactosidase activity.

**pcDNA3/SV5-N**

The DNA sequence encoding the Pk-SV5 peptide (IPNPLLGLG) was inserted into pcDNA3 using KpnI and BamHI cloning sites and the following oligonucleotides

\[ 5'-\text{CATGGGAAAGCCGATCCCAAAAACCTTTGCTGGGATTTGGACTCCACCG-3'} \text{ and 5'}-\text{GATCCGGTGGAGTCCAATCCCAGCAAGGGTTTGGGATCGGCTTTCCCATGGTAC-3'} \].

**pcDNA3/HA-N**

The DNA sequence encoding the HA peptide (YPYDVPDYA) was inserted into pcDNA3 using KpnI and BamHI cloning sites and the following oligonucleotides

\[ 5'\text{-CATGGGCTTCCATATCTTACGATTTCCAGACCTACCGCCTTTCCCTTGG-3'} \text{ and 5'}-\text{GATCCGCTGGAGTCCAATCCCAGCAAGGGTTTGGGATCGGCTTTCCCATGGTAC-3'} \].

**pcDNA3/6xHis-N**

DNA encoding 6 histidines was inserted into pcDNA3 using KpnI and BamHI cloning sites and the following oligonucleotides: 5'.
CATGGCTCATCATCATCATCATCATGGTG-3' and 5'-
GATCCACCAGATGATGATGATGATGAGCCATGGTAC-3'

Ubch9

A full length Ubch9 cDNA was isolated from a human cDNA library. The entire open reading frame of 474 nucleotides was amplified by polymerase chain reaction (PCR) using primers (5'-ACAAACGGATCCATGTCGGGGATCGCCCTCAGC-3' and 5'-GCCGCGGAATTCTTTATGAGGGCGCAAACTTCTTGCC-3') with additional restriction enzyme cleavage sites to facilitate insertion into pGEX-2T such that a glutathione-S-transferase-Ubch9 fusion protein can be produced in E. coli. Ubch9 cDNA was cloned with a C-terminal SV5 tag in BamHI and XhoI cloning sites of pcDNA3 after PCR using the following primers, 5'-ACAAACCATGGATCCATGTCGGGGATCGCCCTCAGC-3' and 5'-TCCGAGCTCGAGTTAGTCCAATCCCAGCAAAGGGTTGAGGGGAGCGCGCGCGCAAACTTCTTGCC-3'.

Ubch5

cDNA from human Jurkat cells was produced by reverse transcription of total RNA and the complete open reading frame of Ubch5 isolated by PCR-amplification with specific primers (5'-ACAAACGGATCCATGGCGCTGAAGAGGATTCAG-3' and 5'-GCGCGGGATCCTTACATFGCATATTTCTGAGTCC-3') and inserted into pGEX-2T as described above.

SUMO-1

A cDNA encoding the complete open reading frame of SUMO-1 was obtained by reverse transcription followed by PCR-amplification (5'-GCCGCGGGATCCCTAAACTGTTGAATGACC-3' and 5'-ACAAACGGATCCATGTCGGGGATCGCCCTCAGC-3') and inserted into pGEX-2T. PCR amplification, using a downstream primer containing a stop codon after nucleotide 291 of the open reading frame (5'-GCCGAGGGATCCCTAAACCCCTCTTTGTCG-3') was used to create a modified
form of SUMO-1 in which the C-terminus of the protein is G97. To express NH₂-
terminal hemagglutinin (HA)-tagged SUMO-1, SUMO-1 cDNA (Desterro et al.,
1997) was inserted into the BamHI cloning site of the constructed pcDNA3/HA-N
vector in frame with the HA sequence. To generate a histine tagged version of
SUMO-1, SUMO-1 cDNA (Desterro et al., 1997) was inserted into the BamHI
cloning site of the constructed pcDNA3/6XHIS-N vector.

**SAE1**

A cDNA encoding the complete open reading frame of SAE1 was obtained
as a single fragment by reverse transcription followed by PCR (RT-PCR) using
Boeringher "Titan™ one tube RT-PCR System". The following primer containing
an EcoRI restriction site was used as the downstream primer for reverse
transcription (5'-GCGGGAATTCTTCATCTGCCCACCAAAGGCACTCCACAA-3'). The
upstream primer used for PCR amplification (5'-
AACGGATCCCATGGTGGAGAAGGAGGAGGCTGGC-3') contains a BamHI site at the 5'
end. PCR products were cloned as a BamHI/EcoRI insert in pGEX-2T
(Pharmacia), or in pcDNA3 (Invitrogen) with an N-terminal Pk-SV5 tag antibody
(pcDNA3/SV5-N) which is recognised by the 336 monoclonal (Hanke et al.,

**SAE2**

The cDNA of SAE2 was obtained from two different RT-PCR reactions and
a final PCR. An intermediate upstream and downstream primer (5'-
GATATCAATCAATGCGAGGGAAC-3' and 5'-GTTCCTGCCCATGATTGATATC-3')
were used in each RT-PCR reaction to generate two different fragments which were
later used in the final PCR reaction as template with the following primers (5'-
GAGGAATTCATGGCAGTTGCGGTTGCTG-3' and
GAGGAATTCATCTCAATCTGCTATGACATC-3') both containing an EcoRI restriction
site. The final PCR fragment was inserted into the EcoRI restriction site of
pcDNA3.
p53 mutants

PCR was used to generate a wild type version of the p53 coding sequence containing a BamHI site at the sequence corresponding to G361/S362. Full length p53 was cloned into HindIII-EcoRI sites of pcDNA3 vector. All p53 mutants were generated by site-directed mutagenesis using PCR strategy. The wild type p53 BamHI-EcoRI cassette was replaced to generate each K to R mutant.

Others

IkBα ctag and mutants in pcDNA3, ConALuc, 3EnhConALuc, HIV1LTRLuc and ΔkBHIV1LTRLuc reporter vectors were as previously described (Rodriguez et al., 1996).

2.4.3. DNA sequencing

All constructions were used to transform E. coli DH5α to ampicillin resistance. Plasmid DNA of all constructs was isolated and inserts sequenced (ABI377) by Alex Houston of the University of St Andrews DNA sequencing facility.

2.5. Expression and purification of recombinant proteins

GST-Ubch9, GST-Ubch5, GST-SAE1,GST-IkBα and both GST-SUMO-1, full length and 1-97 a.a., constructions were expressed in Escherichia coli strain B834. Induction of expression, glutathione agarose affinity chromatography and thrombin cleavage of fusion proteins was as described (Jaffray et al., 1995). Both SUMO-1 proteins were further purified over an anionic exchange column (FPLC, Mono Q H5/R5) equilibrated with 50 mM Tris pH7.5 and eluted with a 0 to 1 M KCl gradient in 50 mM Tris pH7.5. Human E1 ubiquitin activating enzyme was purified from HeLa cell extracts or from recombinant baculovirus infected insect cells by covalent affinity chromatography on ubiquitin-Sepharose as described (Haas and Bright, 1988; Rolfe et al., 1995). Arabidopsis thaliana E1 was produced in E. coli
BL21DE3 using the pET expression system (Hershko et al., 1983) and purified by ubiquitin affinity chromatography as described (Haas and Bright, 1988). Human Mdm2 (residues 6-491) was expressed in bacteria and purified as described for p53 (Midgley et al., 1992) and was obtained from Carol Midgley, University of Dundee.

2.6. Quantitation of protein

Protein concentrations were determined using Bradford’s method (Bradford, 1976). Protein samples were mixed with Bradford’s reagent (Biorad) and the absorbance at 595 nm was measured on a spectrophotometer. Protein absorbances were converted to mg/ml concentrations using a standard curve constructed by measuring the absorbances of a range of bovine serum albumin (BSA) concentrations.

2.7. SDS-PAGE and Western blot analysis

Protein samples were resuspended in disruption buffer (1X: 20 mM Tris/HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 2.5% glycerol and 2.5% bromophenol blue) and denatured at 100°C for 5 minutes before loading on a 8.5-12.5% SDS-polyacrylamide gel (acrylamide percentage appropriate for the size of proteins to be separated). Bio-Rad mini gel equipment was used in accordance with the manufacturer’s instructions. New England Biolabs protein molecular weight markers were used as standards to establish the apparent molecular weights of proteins resolved on SDS-polyacrylamide gels. Separated polypeptides were either stained with Coomassie Blue (0.2% Coomassie brilliant blue R250; 50% methanol; 10% acetic acid) for 30 minutes and then destained (20% methanol; 10% acetic acid) or transferred to a polyvinylidene difluoride membrane (Sigma) using a wet blotter (Biorad Systems). The membranes were blocked with PBS containing 5% skimmed milk powder and 0.1% Tween 20 then incubated with monoclonal or polyclonal antibodies diluted in blocking buffer. Horseradish peroxidase conjugated anti-mouse IgG and anti-rabbit IgG (Amersham) were used as secondary antibodies.
Western blotting was performed using ECL detection system. After ECL detection and when necessary western blots were stripped as described (Roff et al., 1996).

2.8. N-terminal peptide sequencing

The protein sample was fractionated by SDS-PAGE using freshly prepared acrylamide: piperazine diacrylamide solution (30:0.8) and with the addition of sodium thioglycolate to 0.1 mM in the upper electrophoresis buffer. Proteins were electrophoretically transferred to PVDF membrane (Amershan) and proteins stained with 0.1% Amido Black in 40% methanol, 1% acetic acid, for 30 seconds before bands were excised. Excised membrane bands were extensively washed in distilled water and the peptide sequence determined using a Procise microsequencer (Applied Biosystems) with on line phenylthiohydantoin analysis. Protein sequence analysis was carried out by Paul Talbot and Graham Kemp at the University of St. Andrews Protein Sequence Facility.

2.9. “In gel” trypsin digestion

Samples to be digested in the gel were fractionated by SDS-PAGE as described for N-terminal peptide sequencing. The gel was stained in 0.1% Coomassie Brilliant Blue R250/ 20% Methanol/ 0.5% Acetic acid and then destained in 30% Methanol until the bands were visible above a clear background. After excision, gel slices were washed for at least one hour in 100 mM NH₄HCO₃ pH 8.0 and 30 minutes at 60°C in 100mM NH₄HCO₃ pH8/ 3 mM DTT. Iodoacetamide was added to a final concentration of 6 mM and after 30 minutes of incubation at room temperature in the dark, gel slices were washed with 50% acetonitrile/ 100 mM NH₄HCO₃ pH 8.0 for one hour with shaking. Acetonitrile was added to shrink gel pieces and after 10-15 minutes of incubation the solvent was removed and the samples dried in a rotatory evaporator. Gel slices were reswollen with 25 mM NH₄HCO₃ pH 8.0 containing modified trypsin (Promega)
and incubated for 4 hours at 37°C. The supernatant was acidified by adding TFA to a final concentration of 1% and peptides extracted from gel slices twice with 60% acetonitrile/0.1% TFA for 20 minutes. All supernatants were combined and, after evaporation to near dryness, peptides fragments were reconstituted in 20μl of 0.1% TFA and separated by HPLC using a microbore HPLC System. The amino acid sequence of selected peptides was determined as described for N-terminal peptide sequencing.

2.10. In vitro transcription translation

In vitro transcription/translation was performed using 1-2 μg of plasmid DNAs and a TNT Coupled Wheat Germ Extract System (Promega) according to the instructions provided by the manufacturer. 35S-methionine (Amersham) was used in the reactions to generate radiolabelled proteins.

2.11. Iodination of Ubiquitin and SUMO-1

Human Ubiquitin from Sigma and recombinant SUMO-1-97 expressed in Escherichia coli, were radiolabeled with carrier-free Na 125I (Amersham) by the Chloramine-T method (Ciechanover et al., 1980). 125I-SUMO was fully reduced by incubation with 100 mM DTT and after removal of the reducing agent by passing through a Biogel P6 spin-column, free sulphhydryl groups were acetylated by incubation with 10 mM Iodoacetamide. After 10 minutes at room temperature the Iodoacetamide was quenched by the addition of DTT to 20 mM.

2.12. Thioester assay

Formation of thioester adducts between recombinant E2s (GST-Ubch5 and Ubch9) and Ubiquitin was determined essentially as previously described (Haas et al., 1982). Reactions contained the indicated amount of Arabidopsis thaliana E1 or human E1, 1U of inorganic pyrophosphatase (Sigma), 0.5 μg of 125I-ubiquitin and
recombinant E2s in a final volume of 20 μl of 50 mM Tris/HCl pH 7.6, 10 mM MgCl₂, 1 mM ATP. Reactions were incubated at 30°C for 10 minutes and terminated either by boiling for 10 minutes in the presence of 2% (w/v) sodium dodecyl sulphate and 4% (v/v) 2-mercaptoethanol, or by incubating the samples at 30°C for 15 minutes in the same buffer containing 4M Urea instead of 2-mercaptoethanol. Samples were subjected to SDS-PAGE (12.5%) and dried gels analysed by phosphorimaging (Fujix BAS1000, MacBAS software). Formation of thioester adducts between recombinant ubch9 and ¹²⁵I-SUMO-1 was determined using the above procedure with 0.5 μg of ¹²⁵I-SUMO-1 and instead of purified E1 enzyme, either SAE immunoprecipitated on protein A beads (rSAE) or 5 μl of HeLa cell fraction containing SAE activity (FrII.4). To detect the activity of SUMO-1 activating enzyme, formation of thioester adducts between either immunoprecipitated or affinity purified SAE and SUMOGG-1 was determined as described above in absence of Ubch9 activity.

2.13. Preparation of HeLa cell fractions

HeLa cell extracts were prepared by lysis in hypotonic buffer containing 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM DTT, phosphatase inhibitors (5mM NaF, 1mM sodium orthovanadate) and protease inhibitors (1mM PMSF, 2 mM Benazamidine, 5mg/ml Leupeptin, 10mg/ml pepsatin). Following incubation on ice for 15 minutes the cells were disrupted with a dounce homogeniser. After centrifugation at 40 000 rpm for 30 minutes at 4°C the supernatant was loaded onto a Q-sepharose column equilibrated with 50mM Tris pH 7.5/0.5 mM DTT. The flowthrough was collected (Fr I) and bound proteins were eluted stepwise with 0.1, 0.2, 0.3, 0.4 and 0.5M KCl in 50mM Tris pH 7.5/0.5 mM DTT (Fr II.1-FrII.5). All fractions were further concentrated and fractionated by ammonium sulphate (40%) precipitation. After overnight dialysis against 50 mMTris pH 7.5/0.5 mM DTT the fractions were stored at -70°C.
2.14. SUMO-1 affinity chromatography

Five milligrams of recombinant purified SUMO_GG -1 (1-97 a.a.) were coupled to a 1 ml NHS-activated Hi-Trap column (Pharmacia) according to the manufacturer’s instructions. 40% of the added protein was coupled to the beads. Following the affinity chromatography procedure, the column was regenerated by washing with 10 column volumes of a buffer containing 50 mM Tris/HCl pH 9.0, 1 M KCl, 2 mM dithiothreitol, followed by 10 column volumes of 50 mM Tris-HCl pH 7.2 buffer containing 0.02% NaN₃. The affinity column was stored at 4°C.

HeLa Fr II.4 and Fr II.5 (eluted with 0.4 M and 0.5 M of KCl) containing SUMO-1 activating enzyme activity (tested by a thioester assay) were dialysed overnight against 50 mM Tris pH 6.8/0.2 mM DTT and affinity chromatography carried out essentially as described for ubiquitin (Ciechanover et al., 1982). Following dialysis, fractions were adjusted to 50 mM Tris pH 6.8, 2 mM ATP, 5 mM MgCl₂, 0.2 mM DTT, 2.5 U/ml of yeast inorganic pyrophosphatase, 10 mM creatine phosphate, 2 U/ml of creatine kinase and applied, at a flow rate of approximately 0.5 ml/min, to the SUMO_GG-1 Sepharose column pre-equilibrated with 3 column volumes of a buffer containing 50 mM Tris/HCl pH 6.8, 2 mM ATP, 5 mM MgCl₂, 0.2 mM DTT (Buffer A). The column was washed with 3 column volumes of buffer A followed by 5 column volumes of 50 mM Tris/HCl pH 7.5, 1 M KCl and then 50 mM Tris/HCl pH 7.5. SUMO-1 E1 activity was eluted with 3 column volumes of 50 mM Tris/HCl pH 7.5, 0.2 mM DTT, 2 mM AMP, 2 mM sodium pyrophosphate (AMP eluate) and finally with 3 column volumes of 50 mM Tris/HCl pH 9.0 containing 10 mM DTT and 1 M NaCl. Column eluates were 10 fold concentrated using Centricon 30 microconcentrators which had been pre-treated with BSA to reduce non-specific adsorption. The buffers were changed by 3 successive 10 fold dilutions in 50 mM Tris/HCl pH 7.5, 1 mM DTT followed by 10 fold reconcentration using the same microconcentrator. The final volume of the column eluates was brought to 10% of the starting volume of loading fraction.
Column operations were carried out at room temperature but all fractions were collected on ice. The enzymatic activity present in each fraction was determined using a thioester assay.

2.15. Cell Culture and transfections

COS7 (Monkey African Green kidney cells containing the SV40 large T-antigen gene) were maintained in exponential growth in Dulbecco’s modified Eagle’s medium, containing 10% foetal calf serum. 1-2 µg of plasmid DNAs were transfected for 14 hours in subconfluent 75 cm$^3$ flasks using Lipofectamine™ according to instructions provided by the manufacturer (Gibco). After 36 hours of expression, cells were washed in PBS and cellular extracts prepared for further analysis.

Wild type p53 U2OS (osteosarcome cells) and p53 null Saos-2 cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS). Cells were transfected by electroporation (950mF, 200V, Equibio Easyject plus) as described (Arenzana-Seisdedos et al., 1997). A total of 10 µg of plasmid DNA encoding the 6- His-SUMO-1 and p53 were co-transfected into 5 X10$^6$ cells. After transfection, cells were seeded in 75 cm$^3$ flasks and incubation continued for a further 36h.

2.16. Luciferase assays

2.16.1. NF-κB transcriptional activity

HeLa (Human Negroid cervix epitheloid carcinoma), COS7 and human embryo kidney 293 cell lines were maintained in exponential growth in Dulbecco’s-modified Eagle’s medium, containing 10% foetal calf serum. 1µg-2µg of plasmid DNAs (an NF-kB dependent, 3EnhConALuc or HIVLTRLuc or independent, ConALuc or ΔκBHVLTRLuc, luciferase reporter vector) were co-transfected with described plasmids for 14 h in subconfluent cells seeded in 25 cm$^3$ flasks using
Lipofectamine® according to instructions provided by the manufacturer (Gibco). After transfection, cells were trypsinized, aliquots were seeded into six-well plates and cultured for an additional 36 hr. Cells from a single transfection were incubated for 8 hr. with medium containing IL-1β (10 ng/ml), TNFα (10 ng/ml), okadaic acid (75 nM) or control medium and processed for luciferase reporter activity as described (Rodriguez et al., 1996).

2.16.2. p53 transcriptional activity

To measure p53 transcriptional activity 10⁶ Saos-2 cells were electroporated with 1 μg of pG13-luc reporter plasmid together with 1 μg of SUMO-1 expression plasmid and 1 ng of p53 (wild type and K386R) expression plasmid. pCDNA3 empty vector was used to bring the total amount of DNA to 10 μg. After electroporation cells were grown in 6 well plates for 24 hours and processed for luciferase reporter activity as described (Rodriguez et al., 1996). Results are the mean of 4 independent transfections, with error bars representing one standard deviation

2.17. Preparation of cell extracts

Cell extracts were prepared either by lysis in 20 mM Na phosphate buffer pH 7.5, 50 mM NaF, 2 mM EDTA, 0.5% NP40, 5 mM tetra-sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, containing complete® protease inhibitor cocktail (Boehringer Mannheim) as described (Roff et al., 1996) or in SDS sample buffer (5% SDS, 0.15 M Tris HCl pH 6.7, 30% glycerol) diluted 1:3 in RIPA buffer ( 25 mM Tris/pH8.2, 50 mM NaCl, 0.5% Nonidet P40, 0.5% Deoxycholate, 0.1% SDS, 0.1% Azide), containing 10 mM Iodoacetamide and complete® protease inhibitor cocktail (Boehringer Mannheim). Both lysates were sonicated briefly and cleared by centrifugation. Supernatants were diluted 1:10 in PBS/0.5% NP40 plus complete®
protease inhibitor cocktail and used for immunoprecipitations. 10 μl of each lysate was fractionated on either 10% or 12.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Sigma). Protein expression was checked by Western blotting.

2.18. Protein interaction assays

2.18.1. SAE Immunoprecipitations

Extracts from COS 7 transfected cells or in vitro transcribed/translated SAE proteins were incubated for 1 hour at 4°C with 336 anti-SV5 antibody either in lysis buffer or 50 mM Tris pH 7.5, 10 mM MgCl₂ buffer containing 1mM ATP and complete® protease inhibitor cocktail, respectively. Antigen-antibody complexes were collected by adding 20 μl of protein A-Sepharose beads and incubation continued for an additional 2 hours on a rotating shaker. The beads were collected by brief centrifugation and after extensive washing with ice cold 50 mM Tris/HCl pH 7.5, 10 mM MgCl₂ containing complete® protease inhibitor cocktail, antigen-antibody complexes were used, as source of SUMO-1 activating enzyme in thioester or conjugation assays.

2.18.2. SAE pull downs

Glutathione S-transferase (GST) or GST-SAE1 beads were incubated with ³⁵S-methionine labelled in vitro transcribed/ translated SAE2 for 1 hour at room temperature. Beads were collected by centrifugation and washed 4 times with 50 mM Tris/HCl pH 7.5, 10 mM MgCl₂, 1mg/ml BSA containing complete® protease inhibitor cocktail and 0.5% NP40. Washing buffer was removed and beads, after resuspension in 3X disruption buffer (5% SDS, 0.15 M Tris HCl pH6.7, 30% glycerol, 0.72 M β-mercaptoethanol, were fractionated in a 10 % polyacrylamide gel. Dried gels were exposed to a phosphorimager screen to detect ³⁵S radioactivity.
In vitro transcribed/translated SAE proteins were incubated for 1 hour at 4°C with 336 anti-SV5 in 50 mM Tris pH7.5, 10 mM MgCl₂ containing 1mM ATP and complete® protease inhibitor cocktail. Antigen-antibody complexes were collected on protein A-Sepharose beads and analysed as described for GST pull downs.

2.18.3. SUMO-1-IκBα Immunoprecipitation

Immunoglobulins from a preimmune rabbit serum or from anti-IκBα rabbit antisera were covalently cross-linked to protein A-Sepharose beads (Sigma) using dimethylpimelimidate as described (Arenzana-Seisdedos et al., 1995). Cells were lysed in SDS sample buffer diluted in RIPA buffer containing 10 mM Iodoacetamide and complete® protease inhibitor cocktail (Boehringer Mannheim). The lysates were sonicated briefly and cleared by centrifugation. Supernatants were diluted 1:10 in PBS/0.5% NP40 plus complete® protease inhibitor cocktail and incubated for 3 hr. at room temperature with the antibody coated beads. The beads were sedimented by brief centrifugation, washed 5 times with ice-cold PBS/0.5% NP40 plus complete® protease inhibitor cocktail and the antigen-antibody complexes were recovered by boiling in SDS sample buffer.

2.18.4. SUMO-1-IκBα/p65 pull downs

Glutathione agarose beads were added to an in vitro SUMO-1 conjugation assay (purified proteins) containing either ¹²⁵I SUMO-1 and GST-p65 or GST instead of p65 recombinant protein. After 10 minutes of incubation at room temperature the beads were collected by centrifugation and washed 5 times with PBS/0.1% Tween 20. Affinity purified proteins were recovered by heating to 100°C in SDS sample buffer containing β-mercaptoethanol, fractionated in a 12.5% polyacrylamide gel and the dried gel analysed by phosphorimaging.
2.19. Purification of 6XHIS-tagged SUMO-1-p53 conjugates

36 hours after transfection cells from a 75 cm² flask were lysed in 4 ml of 6 M guanidinium-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris/HCl, pH 8.0 plus 5mM imidazole and 10 mM β-mercaptoethanol. After sonication, to reduce viscosity, the lysates were mixed with 50 µl of Ni²⁺-NTA-agarose beads prewashed with lysis buffer and incubated for 2 hours at room temperature. The beads were successively washed with the following: 6 M guanidinium-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris/HCl, pH 8.0 plus 10 mM β-mercaptoethanol; 8 M Urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris/HCl, pH 8.0, 10 mM β-mercaptoethanol; 8 M Urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris/HCl, pH 6.3, 10 mM β-mercaptoethanol (buffer A) plus 0.2% Triton X-100; buffer A and then buffer A plus 0.1% Triton x-100. After the last wash with buffer A the beads were eluted with 200 mM imidazole in 5% SDS, 0.15 M Tris HCl pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol. The eluates were subjected to SDS-PAGE (10%) and the proteins transferred to a polyvinylidene difluoride membrane (Sigma). Western blotting was performed with a monoclonal antibody against p53, DO-1.

2.20. In vitro SUMO-1 conjugation assay

³⁵S-methionine labelled in vitro transcribed/translated proteins (1 µl) were incubated with 2 µl of HeLa cell fraction containing SUMO-1 E1 activity (Fr. II.4) in a 10 µl reaction including an ATP regenerating system (50 mM Tris pH 7.6, 5 mM MgCl₂, 2 mM ATP, 10mM creatine phosphate, 3.5 U/ml of creatine kinase and 0.6 U/ml of inorganic pyrophosphatase), SUMO-1 (1 µg/µl), Ubc9 (60 µg/ml). Reactions were incubated at 37°C for 2 h. After terminating the reactions with SDS sample buffer containing β-mercaptoethanol, reaction products were fractionated by
SDS-PAGE (8.5%) and the dried gels analysed by phosphorimaging (Fujix BAS 1500, MacBAS software).

SUMO-1 conjugation using purified proteins was accomplished in a 10 µl reaction containing 50 mM Tris pH 7.6, 5 mM MgCl₂, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/ml of creatine kinase and 0.6 U/ml of inorganic pyrophosphatase, 0.6 µg of human recombinant IκBα (or mutants), 0.5 µg ¹²⁵I-SUMO-1, 0.5 µg Ubch9 and either SAE immunoprecipitated protein A beads (rSAE) or 90 ng of SAE purified by affinity chromatography on immobilised SUMO-1. Reactions were incubated at 37°C for 2 h and terminated by boiling in SDS sample buffer containing β-mercaptoethanol. Samples were subjected to SDS-PAGE (12.5%) and dried gels analysed by phosphorimaging.

2.21. In vitro Ubiquitination assay

³⁵S-methionine labelled in vitro transcribed/translated p53 proteins (1 µl) were incubated in a 10 µl reaction including an ATP regenerating system (50 mM Tris pH 7.6, 5 mM MgCl₂, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/ml of creatine kinase and 0.6 U/ml of inorganic pyrophosphatase), 10 µg ubiquitin, 10 ng human E1, 60 ng Ubch5 and 60 ng Mdm2. After incubation at 37°C for 2 h the reactions products were treated as mentioned for the SUMO-1 conjugation assay.
3. RESULTS
3.1. Ubch9 conjugates SUMO-1 but not Ubiquitin
3.1.1. Summary

Modification of proteins by ubiquitin is the critical step in targeting proteins for degradation via the proteasome. Ubiquitin conjugating enzymes participate in the thioester cascade that leads to protein ubiquitination. Ubc9 is homologous to E2 ubiquitin conjugating enzymes and has been isolated repeatedly from yeast two hybrid screens in association with a wide variety of proteins. However, it has not been possible to demonstrate that ubc9 has the enzymatic activities that would be expected of a ubiquitin conjugating enzyme. We demonstrate that while ubc9 is unable to form a thioester with ubiquitin it is capable of forming a thioester with the small ubiquitin-like protein SUMO-1. Thus, Ubc9 is a SUMO-1 conjugating enzyme rather than a ubiquitin conjugating enzyme. Furthermore, we show that transacetylation of Ubch9 by SUMO-1 is not mediated by the E1 ubiquitin activating enzyme, but by a distinct enzymatic activity.

The X-ray crystal structure of recombinant Ubch9 has been obtained to a resolution 2.8 Å. The differences observed in the structure surrounding the reactive cysteine of Ubch9 may reflect the specificity of Ubch9 for SUMO-1 and its distinct E1 activity. SUMO-1 conjugation to target proteins is mediated by a different, but parallel pathway to ubiquitination.
3.1.2. **Ubch9 is unable to form a thioester with ubiquitin**

The NF-κB/Rel proteins are sequestered in the cytoplasm of unstimulated cells in association with IκBα. In response to external signals, the N-terminal region of IκBα is phosphorylated and ubiquitinated prior to degradation by the proteasome. Released NF-κB translocates to the nucleus and activates a large number of responsive genes. To identify proteins involved in IκBα signalling, a yeast two hybrid screen was used to isolate human cDNAs encoding proteins that could interact with the N-terminal regulatory domain of IκBα (1-74). Such a human cDNA was isolated and sequenced to reveal an open reading frame of 474 nucleotides encoding a 158 amino acid protein, identified as the human protein Ubch9, which is homologous to ubiquitin conjugating enzymes. Although this protein has been repeatedly isolated in yeast two hybrid screens evidence that the protein has ubiquitin conjugating activity has not been reported. To characterise the enzymatic activity of Ubch9 the protein was expressed as a GST fusion in bacteria and purified. A known ubiquitin conjugating E2 enzyme, Ubch5 (Scheffner et al., 1994), was expressed and purified in similar fashion (Figure 6). The ability of purified Ubch9 and GST-Ubch5 to form a thioester with 125I-ubiquitin in the presence of MgATP and recombinant E1 from A. thaliana was analysed by SDS-PAGE under non-reducing conditions. While a thioester was formed between GST-Ubch5 and 125I-ubiquitin none could be detected using Ubch9 (Figure 7A). To rule out the possibility that this result was a consequence of incompatibility between plant E1 and human E2 enzymes, the human E1 enzyme was affinity purified from HeLa cells and tested in the same assay with identical results (Figure 7B). Thus, under the conditions employed Ubch9 did not display ubiquitin conjugating activity.

3.1.3. **Ubch9 forms a thioester with iodinated SUMO-1**

Recently a small ubiquitin-like protein known variously as UBL1, SUMO-1, Sentrin and PIC1 has been found covalently linked to RanGAP1 and associated
Figure 6. Purification of recombinant proteins.

Recombinant proteins were purified from *E. coli* as described in Materials and Methods, subjected to SDS-PAGE (12.5%) and stained with Coomassie Blue. Lane 1, 1.5 μg GST-Ubch9 (44 kDa); Lane 2, 1 μg Ubch9 (18 kDa); lane 3, 1 μg GST-Ubch5 (42 kDa); lane 4, 1.5 μg GST-SUMO-1,1 (38 kDa); lane 5, 1 μg SUMO-1,1 (12 kDa); lane 6, 1 μg *A. thaliana* E1 (123 kDa). The molecular weight of protein markers (M) is shown at the left of the figure.
Figure 7. Ubch9 is unable to form a thioester with ubiquitin.

Assay for thioester formation between $^{125}$I-ubiquitin and Ubch9 (0.9 μg lane 2, 1.8 μg lane 3) or GST-Ubch5 (0.7 μg lane 5, 1.4 μg lane 6) in the presence of recombinant A. thaliana E1 (A) or human E1 from HeLa cells (B). After 10 minutes at 30°C reactions were terminated and products fractionated by SDS-PAGE under non-reducing conditions. $^{125}$I radioactivity in the dried gel was detected by phosphorimaging. The positions of $^{125}$I- ubiquitin and thioester adducts with E1 and E2 proteins are indicated.
with Ubc9 (Boddy et al., 1996; Kamitani et al., 1997b; Mahajan et al., 1997; Matunis et al., 1996; Saitoh et al., 1997; Shen et al., 1996b). We therefore investigated whether Ubch9 might be a SUMO-1 conjugating enzyme rather than a ubiquitin conjugating enzyme. Full length SUMO-1 or a modified form lacking four amino acids from the C-terminus, which is thought to be the active form of SUMO-1 (Kamitani et al., 1997b), were produced in bacteria and purified. To determine if ubiquitin activating enzyme could activate SUMO-1, human and A. thaliana E1 were used in a thioester assay with Ubch9 but were unable to promote thioester formation between Ubch9 and SUMO-1 (data not shown), although these E1 enzymes were active for the formation of a thioester between ubiquitin and GST-Ubch5 (Figure 7). Thus it seemed likely that SUMO-1 activation would require a distinct E1 activity, distinct from that involved in ubiquitin activation. To explore this possibility a cytoplasmic extratct from HeLa cells was incubated with \(^{125}\text{I}-\text{SUMO-1}\) in the presence or absence of Ubch9. An activity capable of catalysing formation of a Ubch9-\(^{125}\text{I}-\text{SUMO-1}\) thioester in an ATP dependent fashion was detected with the 97 amino acid form of SUMO-1 (Figure 8) but not with full length SUMO-1 (data not shown). Fractionation of the HeLa extract by anion exchange chromatography revealed that the SUMO-1 E1 activity was eluted with 0.4 and 0.5 M KCl (Figure 8) and was distinct from the ubiquitin E1 activity which elutes with 0.3 M KCl (data not shown). An apparent background activity detected in the absence of ATP could be explained by formation of a linkage between Cys52 in SUMO-1 and Ubch9. This activity was eliminated by acetylation of Cys52 by treatment of SUMO-1 with iodoacetamide. The ability of Ubch9 to form a thioester with acetylated \(^{125}\text{I}-\text{SUMO-1}\) in the presence of the SUMO-1 E1 activity contained in Fr II.4 was determined. In presence of ATP, Ubch9 forms a conjugate with \(^{125}\text{I}-\text{SUMO-1}\) that is labile to reducing agents, such as 2-mercaptoethanol (Figure 9), indicating that the two molecules are linked by a thioester bond. Under the same conditions GST-Ubch5 was unable to form conjugates with \(^{125}\text{I}-\text{SUMO-1}\). Thus Ubch9 has the properties
Figure 8. Detection of a SUMO-1 activating enzyme that transacetylates Ubch9.

Unfractionated extract from HeLa cells (Total) or extract fractionated by Q-Sepharose chromatography (Fractions I, II.1, II.2, II.3, II.4, II.5), was assayed for the ability to form thioester adducts with $^{125}$I-SUMO-1 either alone (-) or in the presence of 1 µg Ubch9 (+). ATP was omitted from the lane marked with an asterisk and control lanes (A.mix) did not contain any HeLa extract fraction. Reactions products were analysed as described in the legend to Figure 7.
Figure 9. Ubch9 is a SUMO-1 conjugating enzyme. Ubch9 (1 μg) and GST-Ubch5 (1 μg) were assayed for their ability to be transacetylated by $^{125}$I-SUMO-1 (treated with iodoacetamide) in the presence of the SUMO-1 activating enzyme present in HeLa fraction II.4. Assays were conducted either in the absence (-) or presence (+) of ATP and reaction products analysed as described in the legend to Figure 7. The indicated samples (+SH) were analysed under reducing conditions after treatment with 2-mercaptoethanol.
of a SUMO-1 conjugating enzyme that can act in concert with a unique SUMO-1 activating enzyme.

### 3.1.4. Structure of Ubch9

To determine the structure of Ubch9 a large batch of Ubch9 was purified to homogeneity by a combination of affinity chromatography of the fusion protein on glutathione agarose and a second affinity chromatography after thrombin cleavage. Using the purified protein, crystallisation trials were set up and conditions developed for crystal growth. The three-dimensional structure of recombinant Ubch9 was obtained in collaboration with Marie-France Giraud and Jim Naismith using molecular replacement followed by a combination of automated refinement and graphical intervention. The protein appears to be a monomer in the crystal and consists of four $\alpha$ helices (H1, Ile4 - Lys18; H2, Ile109 - Asn121; H3, Ala131 - Asn139; H4, Arg141 - Lys154) and an anti-parallel $\beta$-sheet composed of four strands (S1, Val25 - Pro28; S2, Asn40 - Pro46; S3, Leu57 - Leu63; S4, Lys74 - Phe77) (Figure 10). The N-terminus is located on the opposite face from the C-terminus. Helices H1, H2 and H3 form one side of the protein, while the other side is composed mostly by one face of the $\beta$-sheet. The reactive cysteine (Cys93) is located in a region of the molecule (residues 78 - 108) consisting of four turns and a $3_{10}$ helix (Glu98 - Leu97). Residues Thr91 to Leu97 form a small depression in which Cys93 lies.

Sequence alignments show that Ubch9 has two small insertions compared with the other ubiquitin-conjugating enzymes. These two insertions consist of residues Pro32 - Met36 and Asp100 - Lys101 and create the main differences with the other ubiquitin-conjugating enzymes. The two-residue insertion (Asp 100 and Lys101) is located as a lip near to the active site Cys93. This insertion forms a small accessible loop. Interestingly, insertions at this position are also found in other ubiquitin conjugating enzymes but they are much longer and structurally different to that in Ubch9. The region of the five-residue insertion Pro32 - Met36 is a flexible loop and
Figure 10. Schematic representation of Ubch9.
The four α-helices are represented in red (H1, H2, H3, H4) and the four β-strands (S1, S2, S3, S4) in yellow. The $3_{10}$ helix is symbolised by a pink ribbon. Turns are indicated in blue. The N- and C-terminus and the reactive cysteine are labelled.
the main chain of the loop must form a protruding region near the N-terminal α-helix. Residues near the reactive cysteine such as His83, Pro84, Asn85, Ile96 and Leu97 are highly conserved suggesting that they may be important to maintain the cysteine in the appropriate conformation. A noticeable feature of the active site of Ubch9 is the presence of Lys101, which is located close to the S atom of Cys93 (Figure 11).

The non-catalytic face of Ubch9 is much more positively charged than other Ubc enzymes and is a reflection of the much higher pI of Ubch9 compared with ubiquitin conjugating enzymes (8.7 versus <6.5) (Figure 11). In contrast to the positive non-catalytic face, Uch9 has a negatively charged ridge close to the reactive cysteine (Figure 11). The ridge is composed of Asp67, Glu98, Asp100, Asp102 and Asp127. Thus, Lys101 in Ubch9 generates a positively charged lip close to the reactive cysteine in the midst of the negatively charged ridge (Figure 11). This lip may discriminate between SUMO-1 (pI 5.4) and ubiquitin pI (7.2), since without Lys101, the active cysteine of Ubch9 sits in a very negative pocket which could favour ubiquitin binding.

3.1.5. Discussion

Ubch9 was isolated in a yeast two hybrid screen as a protein that could interact with the N-terminal region of IκBα. As IκBα undergoes signal induced ubiquitination within the N-terminal domain our expectation was that Ubch9 could participate in the ubiquitination of IκBα. Using an in vitro system for the phosphorylation and ubiquitination of IκBα (Chen et al., 1996; Kroll et al., 1997) we were unable to demonstrate a role for Ubch9 (data not shown). Furthermore Ubch9 was unable to form a thioester with ubiquitin, in the presence of the E1 ubiquitin activating enzyme, under conditions where this activity could be clearly demonstrated with Ubch5 (Figure 7).

As it has been reported (Shen et al., 1996b) that the ubiquitin like protein SUMO-1 is found in complexes containing Ubc9 it has been suggested that Ubc9
The SG atom of the reactive cysteine is shown in yellow. The right pictures correspond to the back face of the left pictures (180° rotation about a vertical axis in the plane of the paper). The x, y and z axes are shown. The colour spectrum from red to blue corresponds to changes from negative to positive potential.

Figure 11. Electric surface potentials of Ubc9 and plant Ubc1 (Ubc1p).
might be involved in SUMO-1 conjugation rather than ubiquitination (Saitoh et al., 1997). Here we demonstrate that this is indeed the case as Ubch9 can form a thioester with SUMO-1 (Figures 8 and 9) provided that a SUMO-1 activating enzyme is present. It seems likely that SUMO-1 conjugation is carried out by a series of transacetylation reactions that is similar but distinct from the ubiquitination pathway. Others have reported an interaction between Ubch9 and IκBα in yeast (Tashiro et al., 1997) and interpreted this as an involvement in IκBα ubiquitination and degradation. Given the inability of Ubch9 to conjugate ubiquitin in vitro it seems unlikely that Ubc9 is involved in IκBα degradation, but a role for SUMO-1 modification in other aspects of IκBα metabolism, such as nuclear import (Arenzana-Seisdedos et al., 1995) can not be ruled out.

A remaining puzzle is why Ubc9 is isolated from yeast two hybrid screens by such a wide variety of proteins. One possible explanation is that “bait” proteins are modified by SUMO-1 in yeast and it is the association between SUMO-1 and Ubc9 that mediates the apparent interaction between Ubc9 and bait proteins. However, Ubch9 possesses a distinct electrostatic potential distribution that may provide another explanation to its remarkable ability to interact with other proteins (Figure 11). Since Ubch9 can recognise a wide range of substrate proteins, adjustments of its conformation may also be required to fit a range of different contact surfaces. Thus, the substrate binding site on Ubch9 is likely to have high conformational flexibility. The flexible regions of Ubch9 are localised at the N- and C-terminus (Liu et al., 1999) which are located at opposite sides of the active site Cys93. The region near the N-terminus of Arabidopsis UBC1 has been shown to interact with E1-ubiquitin conjugate (Sullivan and Vierstra, 1991). The five residue insertion of Ubch9 which forms a flexible loop close to the N-terminal helix may be important for the ability of Ubch9 to discriminate between ubiquitin-like proteins and/or may participate in interactions with the SUMO-1 activating enzyme. The region near the C-terminus, which has a positive electrostatic potential may be
important for substrate binding and catalytic activity given the proximity to the active site Cys93.
3.2. Identification of the Enzyme required for Activation of the Small Ubiquitin-like modifier SUMO-1
3.2.1. Summary

SUMO-1 and Smt3p, a yeast homologue of SUMO-1, are conjugated to target proteins by a pathway that is distinct from, but analogous to, ubiquitin conjugation. A separate E1-like enzyme is responsible for SUMO-1 modification (Desterro et al., 1997) and in yeast the enzyme responsible for Smt3p activation has been shown to consist of a heterodimer of Uba2p and Aos1p (Johnson et al., 1997). We have purified by affinity chromatography the SUMO-1 activating enzyme from human cells and shown that it contains two subunits of 38 and 72 kDa. Isolation of cDNAs for each subunit indicates that they are homologous to ubiquitin activating enzymes and to the *Saccharomyces cerevisiae* enzymes responsible for conjugation of Smt3p and Rub-1p. *In vitro*, recombinant SAE1/SAE2 (SUMO-1 Activating Enzyme) was capable of catalysing the ATP dependent formation of a thioester linkage between SUMO-1 and SAE2. Addition of the SUMO-1 conjugating enzyme Ubch9 results in efficient transfer of the thioester linked SUMO-1 from SAE2 to Ubch9. Thus, SAE1/SAE2 is the enzyme responsible for the activation of the small ubiquitin modifier SUMO-1.

Identification of the human SAE should allow a more detailed characterisation of the SUMO-1 conjugating pathway.
3.2.2. Purification of SUMO-1 activating enzyme

The purification procedure for the SUMO-1 activating enzyme (SAE) used an affinity procedure previously described (Ciechanover et al., 1982); (Hershko et al., 1983), for isolation of ubiquitin activating enzymes, based on covalent binding of the enzyme to ubiquitin Sepharose beads in the presence of ATP. FrII.4 containing SUMO-1 activating activity from HeLa cells (Figure 8) (Desterro et al., 1997) was thus applied to SUMO-1 Sepharose beads in the presence of ATP and inorganic pyrophosphatase, to suppress the reverse reaction. Under these conditions SAE activates the immobilised SUMO-1 forming a thioester bond which retains the SAE on the column. The affinity column was washed sequentially with buffers containing ATP, 1M KCl, Tris buffer alone and AMP + pyrophosphate. Column eluates were assayed for their ability to form a thioester with 125I-SUMO-1 (Figure 12). SAE activity was detected in the column load and was substantially reduced in the column flow through, indicating that the activity was bound to the column (Figure 12A). The bulk of the SAE activity was eluted with AMP and pyrophosphate, which reverses the activation reaction (Figure 12A). Eluates were fractionated by SDS PAGE and proteins detected by staining with Coomassie Brilliant Blue. Two polypeptide species with apparent molecular weights of 40,000 and 90,000 were detected in the AMP eluate, in addition to BSA which was added to the fractions to prevent non-specific adsorption to surfaces during concentration (Figure 12B).

3.2.3. cDNA cloning of human SUMO-1 activating enzyme

Proteins present in the AMP eluate were fractionated by SDS PAGE, transferred to PVDF membrane and the location of bound species detected by Amido Black staining. Both the 90 kDa and 40 kDa species were excised and subjected to direct N-terminal sequencing. Although the N-terminus of the 90 kDa species appeared to be blocked a unique sequence was obtained from the N-terminus of the 40 kDa species. To obtain sequence from the 90 kDa species and
Figure 12. Purification of SUMO-1 Activating Enzyme by SUMO-1 covalent affinity chromatography.

HeLa cell extracts were initially fractionated on a Q Sepharose column. FrII.4, containing SAE activity, was supplemented with ATP and inorganic pyrophosphatase and fractionated on a SUMOGG-1 Hi-trap column. (A) Equivalent amounts of SUMO-1 column load (Fr II.4, lane 1, 2), flow-through (F/T lane 3, 4) and AMP eluate (lane 5, 6) were assayed for the ability to form thioester adducts with $^{125}$I-SUMO-1 either alone (-) or in the presence of recombinant Ubch9. After 10 min at 30°C reactions were stopped and products subjected to SDS-PAGE (12.5%) under non reducing conditions. Dried gels were analysed by phosphorimaging. The positions of $^{125}$I-SUMO-1 and thioester adducts with SAE and Ubch9 are indicated. (B) Fractions indicated in A were fractionated by SDS-PAGE (10%) and stained with Coomassie blue. Prior to concentration the AMP eluate was supplemented with BSA which was also loaded on lane 6 as a control. The molecular weight of protein markers (M) and SAE components are indicated.
additional sequence from the 40 kDa species, peptides generated by in-gel trypsin digestion were fractionated by microbore HPLC. Unambiguous sequence was obtained from three 40 kDa, and eight 90 kDa derived peptides (Figure 13, underlined sequences). BLAST searching of the EST public database allowed the identification of ATCC clones which were used, in combination with RT-PCR, to construct full length cDNAs for each species. The 40 kDa species was designated as SAE1 and has a predicted molecular weight of 38 kDa while the 90 KDa species was designated as SAE2 and has a predicted molecular weight of 72 kDa (Figure 13).

Amino acid sequence alignments of different E1s demonstrate that SAE is similar to Smt3p (Aos1p/Uba2p) and Rublp (Ula1p/Uba3p) activating enzymes (Johnson et al., 1997) (Liakopoulos et al., 1998). Although SAE1 displays significant similarities to the N-terminus of human and Saccharomyces cerevisiae Uba1, the SAE2 protein contains the putative active-Cys (Cys 173) and is homologous to the C-terminus of Uba1. The apparent molecular mass of the $^{125}$I-SUMO-SAE thioester conjugate of 100 kDa (Figure 12) confirms the presence of the active-site cysteine in SAE2. Most of the sequence similarity (Figure 14) is concentrated in a limited number of previously identified domains (Liakopoulos et al., 1998) (Johnson et al., 1997).

### 3.2.4. SUMO-1 is activated by an E1-like enzyme containing two subunits

cDNA corresponding to SAE1 and SAE2 obtained by RT-PCR was cloned into a eukaryotic expression vector permitting proteins to be produced and $^{35}$S-labelled by coupled in vitro transcription and translation (Figure 15A). SAE1 was fused at the N-terminus to an epitope from Simian virus 5 that is recognised by the previously characterised (SV5 Pk Tag, 336) monoclonal antibody (Hanke et al., 1992). Although SAE1 and SAE2 copurified in equimolar amounts (Figure 12B) during SUMO-1 affinity chromatography it was not clear that they were associated.
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SAE2

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Figure 13. SAE1/SAE2 sequence.
Protein sequences of SAE1 and SAE2. The putative active site cysteine residue (Cys173) in SAE2 is shown in bold. Peptide sequences obtained either by direct Edman degradation N-terminal sequencing or after in-gel trypsin digestion are shown underlined.
Figure 14. SAE1/SAE2 sequence analysis.
Schematic representation of homologous domains in human ubiquitin E1 enzyme, hUba1 (Handley et al., 1991); *S. cerevisiae* ubiquitin E1, S.c. Uba1p (McGrath et al., 1991); *S. cerevisiae* Smt3p activating enzyme, Aoslp (Johnson et al., 1997) and Uba2p (Dohmen et al., 1995); RUB1 activating enzyme, Ula1p and Uba3p (Liakopoulos et al., 1998) and SUMO-1 activating enzyme, SAE1 and SAE2. The domains shown correspond to those of Johnson et al. (1997).
To obtain direct evidence for such an interaction different mixtures of $^{35}$S-labelled \textit{in vitro} transcribed translated proteins were immunoprecipitated with a specific (336) and a non-specific (214) monoclonal antibody. In presence of SV5 tagged SAE1, SAE2 was co-immunoprecipitated with anti-SV5 antibody (336). Labelled SAE2 protein was not immunoprecipitated with either the control antibody (214) or when SAE2 was \textit{in vitro} transcribed translated in the absence of SAE1 (Figure 15C). Confirmation of the interaction between SAE1 and SAE2 was obtained when glutathione-S-transferase SAE1 beads were used to pull down $^{35}$S-labelled \textit{in vitro} transcribed translated SAE2. (Figure 15B). To determine the composition of the SAE complex, COS7 cell extracts co-transfected with SAE1 and SAE2 were analysed by gel filtration chromatography on a Superose 6\textsuperscript{TM}, FPLC (Pharmacia Biotech). SAE1 and SAE2 co-eluted at approximately 130 KDa (data not shown) suggesting that the SUMO-1 activating enzyme is a SAE1/SAE2 heterodimer.

To demonstrate that SAE1 and SAE2 are required for SUMO-1 activation \textit{in vitro}, its ability to form a stable adduct with radioactively labelled SUMO-1 in an ATP-dependent reaction was tested. SAE proteins were either \textit{in vitro} transcribed translated or expressed in transfected COS7 cells. In both situations, the activity was immunoprecipitated using anti-SV5 protein A beads. The ability of recombinant SAE to form a thioester with $^{125}$I-SUMO-1 in presence of MgATP was analysed by SDS-PAGE under non-reducing conditions. This immunoprecipitated activity was capable of catalysing formation of a Ubch9-$^{125}$I-SUMO-1 thioester when recombinant Ubch9 was added to the reaction (Figure 16A and 16B). The linkages formed between SAE2 and $^{125}$I-SUMO-1 or Ubch9 and $^{125}$I-SUMO-1 were labile to reducing agents, such as DTT (Figure 16A), indicating that they are likely to be thioester bonds. Neither SAE1 nor SAE2 alone were capable of forming a thioester complex with SUMO-1. Some activity is detected when SAE1 is immunoprecipitated from COS7 SAE1 transfected extracts, probably due to endogenous SAE2 activity (Figure 16B). These results together suggest that the SUMO-1 activating enzyme is composed of SAE1/SAE2.
Figure 15. Interaction of SAE1 with SAE2.

(A) SV5 tagged SAE1 (lane1) and SAE2 (lane2) were either separately in vitro transcribed, translated (IVTT) and labelled with $^{35}$S-methionine or labelled cotranslationaly (lane 3). (B) Glutathione S-Transferase (GST) or GST-SEAE1 beads were used to pull down $^{35}$S-methionine labelled IVTT SAE2. After extensive washing bound proteins were analysed in a 10% SDS-PAGE and $^{35}$S radioactivity in the dried gel detected by phosphorimaging. (C) $^{35}$S-methionine labelled IVTT SV5 tagged SAE1, SAE2 or cotranslated SV5 tagged SAE1 and SAE2 were immunoprecipitated either with 336 anti-SV5 monoclonal antibody or with an unrelated antibody (214). After extensive washing with 50 mM Tris HCl pH7.5, 10 mM MgCl$_2$, 0.5% NP40 immunoprecipitates were analysed as described in panel B. The IVTT protein present and antibody used for the immunoprecipitation are shown for each reaction. $^{35}$S-methionine labelled SAE1tag and SAE2 proteins are indicated.
Figure 16. Both SAE1 and SAE2 are required for SUMO-I activating activity.

(A) IVT SV5 tagged SAE1 or cotranslated SV5 tagged SAE1 and SAE2 were incubated with 336 anti-SV5tag monoclonal antibody and immunoprecipitated with protein A beads. The ability of these complexes to form a thioester bond with $^{125}$I-SUMO-I was tested either in absence (-) or presence of 0.6 µg of Ubch9. Samples treated with 100 mM DTT before SDS-PAGE fractionation are indicated (+DTT). (B) COS7 cells were either transfected with control pcDNA3, SV5 tagged SAE1, SAE2 or co-transfected with SV5 tagged SAE1 and SAE2. After 36 hours of expression cell extracts were prepared and immunoprecipitated with anti-336 SV5 monoclonal antibody. Immunoprecipitates were assayed for the ability to form thioester adducts with $^{125}$I-SUMO-I either alone (-) or in the presence of 0.6 µg of Ubch9. SUMO-I column AMP fraction was tested in the same thioester assay as a positive control. Reactions products were analysed as described in the legend to Figure 12A.
3.2.5. Discussion

Targeting of proteins for ubiquitin mediated proteolysis is an irrevocable decision and as such the process needs to be highly specific and tightly regulated. This specificity appears to be accomplished by a combination of E2 ubiquitin conjugating enzymes and E3 ubiquitin protein ligases. In many cases the E3 appears to consist of a multiprotein complex that recognises the substrate and brings it in to intimate contact with the E2 which catalyses addition of ubiquitin to the substrate. As the E1, ubiquitin activating enzyme is unique it does not appear to play a role in selecting protein substrates for ubiquitination. However, ubiquitin co-exists with a number of ubiquitin-like molecules and the E1 enzymes must distinguish between these molecules. As distinct E1 activities have been described for ubiquitin (Handley et al., 1991), Smt3p (Johnson et al., 1997) and Rub1p (Lammer et al., 1998; Liakopoulos et al., 1998) we undertook the isolation and characterisation of the SUMO-1 activating enzyme (SAE).

By taking advantage of the mechanism of ubiquitin activating and conjugating enzymes, which involves formation of a thioester intermediate with ubiquitin, we have used SUMO-1 affinity chromatography to isolate a novel enzyme that catalyses the ATP-dependent activation of SUMO-1, the first step in the conjugation pathway. Furthermore this enzyme could also transfer activated SUMO-1 to Ubch9, the conjugating enzyme involved in this process (Desterro et al., 1997; Johnson and Blobel, 1997; Saitoh et al., 1998; Schwarz et al., 1998) (Figure 16). Although the E1 activity for ubiquitin is contained within a single large polypeptide the E1 activity of SUMO-1, like that of Smt3p and Rub1p is partitioned between two smaller polypeptides, SAE1 and SAE2. Sequence comparisons between the E1 enzymes indicates that SAE1 is homologous to Aos1p, Ula1p and the N-terminus of the ubiquitin activating enzymes (Figure 14) while SAE2 is homologous to Uba2p, Uba3p and the C-terminus of the ubiquitin activating enzymes. The association between SAE1 and SAE2, confirmed by
immunoprecipitations and GST pull downs (Figure 15), brings together conserved domains present in each subunit. As purified SAE contains equimolar amounts of SAE1 and SAE2 and the two proteins associate *in vitro* it is probable that, like the Smt3p E1, the activating enzyme is a heterodimer. The heterodimeric nature of SAE was confirmed by gel filtration. Each SAE subunit contains a conserved nucleotide binding motif, GXGXXG, (positions 24-29 in SAE2 and positions 43-48 in SAE1, Domain I, figure 14). The putative cysteine (Cys173) which forms a thioester bond with the C-terminal glycine of SUMO-1 is in an active site consensus sequence (KXXPZCXCTXXXP) found in conserved domain III. Conserved domain II is present in SAE1, while conserved domain IV is found in SAE2. The function of conserved domains II and IV has yet to be determined. The C-terminal extension of SAE2 contains a region that matches with two consensus sequences for nuclear localisation signals (RKRR, 610-613 and RKRLDEKAKSLLR, 610-626) which are also present in the C-terminal region of Uba2p (Dohmen *et al.*, 1995).

Within the cell it appears that virtually all of the SUMO-1 is present in protein conjugates and there is a very low concentration of free SUMO-1 (Matunis *et al.*, 1996). Thus it is likely that the availability of free SUMO-1 is tightly controlled by a dynamic equilibrium between SAE/Ubc9 mediated conjugation of SUMO-1 and deconjugation mediated by the highly active, but as yet uncharacterised, SUMO-1 deconjugating and processing enzymes. However the cellular signals which regulate this process have yet to be defined.

The large number of ubiquitin-specific proteases (UBP) and ubiquitin C-terminal hydrolases (UCH) already identified (Wilkinson, 1997) suggest that they may be involved in the recognition of different types of ubiquitin conjugates but little is known about their biological roles. It is likely that some of the known UBPs and UCHs will be responsible for processing of ubiquitin like proteins. UCH-L3, a putative ubiquitin C-terminal hydrolase, was recently identify as a NEDD8 (Kamitani *et al.*, 1997a) interacting protein and able to cleave the C-terminus of NEDD8 but the same did not bind to sentrin-1, sentrin-2 or sentrin-3 (Wada *et al.*, 1984).
The first ubiquitin-like specific protease, Ulp1, was very recently identified in yeast and able to cleave proteins from Smt3 and SUMO-1 but not from ubiquitin. This novel protease is unrelated to any known deubiquitinating enzymes and is required for cell-cycle progression in yeast (Li and Hochstrasser, 1999).

The availability of the genes for the SUMO-1 activating and conjugating enzymes SAE1/2 and Ubc9 will facilitate further biochemical and cell biological studies aimed at defining the role of these proteins in vivo.
3.3. SUMO-1 Modification of IκBα inhibits NF-κB activation.
3.3.1. Summary

Activation of NF-κB is achieved by ubiquitination and proteasome mediated degradation of IκBα. As we had previously isolated Ubch9 as an IκBα interacting protein in a yeast two hybrid screen we searched for physiological situations in which IκBα is modified by SUMO-1. In a number of cell types IκBα is modified by SUMO-1 to a form that is resistant to TNFα induced degradation. SUMO-1 modified IκBα remains associated with NF-κB. In the presence of SUMO-1 activating enzyme, Ubch9 and ATP SUMO-1 was efficiently conjugated to IκBα primarily on K21, which is also utilised for ubiquitin modification. Thus SUMO-1 modified IκBα cannot be ubiquitinated and is resistant to proteasome mediated degradation. As a result overexpression of SUMO-1 inhibits signal induced activation of NF-κB dependent transcription. Unlike ubiquitin modification, which requires phosphorylation of S32 and S36, SUMO-1 modification of IκBα is inhibited by phosphorylation. Thus, while ubiquitination targets proteins for rapid degradation, SUMO-1 modification creates a privileged pool of IκBα that is resistant to signal induced degradation.

In vitro reconstitution of the conjugation reaction with recombinant purified proteins revealed that in the presence of SUMO-1 activating enzyme, Ubch9 was capable of directly transferring SUMO-1 onto IκBα. Thus, it appears that SUMO-1 conjugation of IκBα in vitro does not require the equivalent of an E3 ubiquitin protein ligase.
3.3.2. Detection of a modified form of IκBα resistant to signal induced degradation

As described in the first chapter, a yeast two-hybrid screen, using the N-terminus of IκBα as “bait”, indicated a strong and highly specific interaction between IκBα and Ubch9. Although Ubch9 is homologous to ubiquitin conjugating enzymes the substrate for this enzyme is not ubiquitin but SUMO-1 (Desterro et al., 1997). While ubiquitinated IκBα can be detected in the presence of proteasome inhibitors there have been no reports of SUMO-1 modified IκBα. A number of cell lines were therefore surveyed for the presence of a more slowly migrating form of IκBα that would be consistent with SUMO-1 modification. Western blotting of cell extracts prepared using standard NP40 lysis procedures failed to show any SUMO-1 modified IκBα (Figure 17A). However it had been reported that SUMO-1 modification of RanGAP1 and PML (Mahajan et al., 1997; Matunis et al., 1996; Muller et al., 1998) was rather labile and the cells were therefore lysed directly in SDS before analysis by Western blotting. In this instance a more slowly migrating form of IκBα consistent with SUMO-1 modification (IκBα*) was detected in substantial amounts in 293, COS7 and HeLa cells with smaller amounts in Jurkat cells (Figure 17A). To rule out the possibility that the more slowly migrating form of IκBα detected in Figure 17A was a peculiarity of the antibody preparation another well characterised IκBα antibody raised against recombinant IκBα (Arenzana-Seisdedos et al., 1995; Mellits et al., 1993) was also employed. COS7, 293 and Jurkat cells revealed the same slowly migrating species (IκBα*) when Western blot analysis was conducted with the antibody to recombinant IκBα (Figure 17B). As SUMO-1 modified proteins do not appear to be targets for proteasome mediated degradation the fate of the modified form of IκBα was determined after treatment of 293 cells with TNFα. Prior to activation NF-κB is held in the cytoplasm by the inhibitor protein IκBα but exposure of cells
Figure 17. A modified form of IκBα, present in different cell lines, is resistant to signal induced degradation.

(A) Extracts (20 μg protein) of different cell lines prepared either by standard NP40 lysis or by direct lysis in SDS buffer, were fractionated by SDS-PAGE and IκBα protein analysed by Western blotting using the IκBα* antibody C21. (B) Extracts lysed in SDS were analysed as in A using a rabbit polyclonal anti-IκBα antibody. (C) 293 cells were exposed to 10 ng/ml of TNFα for the indicated times. Extracts were lysed in SDS, separated by SDS-PAGE and proteins analysed by Western blotting using the C21 antibody. Positions of prestained molecular weight markers, IκBα and the modified form of the protein (IκBα*) are indicated.
to TNFα activates NF-κB by signal induced ubiquitination and proteasome mediated degradation of IκBα. Western blotting of SDS isolates of 293 cells reveals that while the unmodified form of IκBα undergoes signal induced phosphorylation and degradation as expected, the modified form of IκBα appears to be resistant to TNF induced degradation as its level is unchanged over the time course of the experiment (Figure 17C).

3.3.3. SUMO-1 is conjugated to IκBα in vivo

To demonstrate directly that IκBα was conjugated to SUMO-1, SDS cell lysates were immunoprecipitated with the antibody raised against recombinant IκBα and associated proteins analysed by Western blotting with the SUMO-1 specific mouse mAb 21C7 (Matunis et al., 1996). In HeLa, Jurkat, COS7 and 293 cells IκBα antibodies immunoprecipitated a polypeptide of about 55 kD, recognised by the SUMO-1-specific mAb, that is consistent with addition of a single molecule of SUMO-1 to IκBα. A more slowly migrating immunoreactive species was also detected with the SUMO-1 specific mAb. This species remains unidentified, and failed to react with antibodies to NF-κB family members (data not shown). Under identical conditions no SUMO-1 reactive species were immunoprecipitated with the preimmune serum (Figure 18A).

To confirm these findings pcDNA3 expression plasmids containing cDNAs for HA tagged SUMO-1, IκBα-ctag (Rodriguez et al., 1995) and Ubch9 were transfected in various combinations into COS7 cells and SDS lysates prepared as described. To monitor expression of HA-SUMO-1 a fraction of the lysate was analysed by Western blotting using the HA-specific mAb 12CA5. When HA-SUMO-1 alone is exogenously expressed a substantial amount of unconjugated protein is detected along with a series of higher molecular weight species that presumably represent conjugation to a range of polypeptides (Figure 18B). When IκBα-ctag is cotransfected less free HA-SUMO-1 is detected and this amount is
## Western Blot Analysis

### A. α-SUMO Immunoblotting

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**Western α-SUMO**

- **HeLa**
- **Jurkat**
- **COS7**
- **293**

### B. Exogenous Expression

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**Western αHA**

**Western αHA**

- **HeLa**
- **Jurkat**
- **COS7**
- **293**

### C. IP, IκBα

**Western αHA**

**Western αHA**

- **HeLa**
- **Jurkat**
- **COS7**
- **293**

### D. IP, IκBα

**Western αSUMO**

**Western αSUMO**

- **HeLa**
- **Jurkat**
- **COS7**
- **293**

### E. IP, IκBα

**Western αctag**

**Western αctag**

- **HeLa**
- **Jurkat**
- **COS7**
- **293**
further reduced by the additional presence of plasmid expressing Ubch9 (Figure 18B). To detect IκBα-SUMO-1 conjugates SDS cell lysates were subjected to immunoprecipitation with IκBα antibodies and the associated proteins analysed by Western blotting using either antibodies to HA, ctag or SUMO-1. When IκBα-ctag and HA-SUMO-1 are cotransfected, either in the presence or absence of Ubch9, conjugates between IκBα-ctag and SUMO-1 are detected using antibodies specific for HA (Figure 18C), SUMO-1 (Figure 18D) or ctag (SV5 PKtag) (Figure 18E). Thus SUMO-1 modification of IκBα is detected on endogenous, as well as exogenously expressed, proteins.

3.3.4 SUMO-1 expression inhibits NF-κB dependent transcription

As it was demonstrated that SUMO-1-modified IκBα was resistant to signal-induced degradation and that exogenously supplied SUMO-1 could be conjugated to IκBα, it was of interest to determine the effect of expressed SUMO-1 on signal induced activation of NF-κB. To measure NF-κB activation an NF-κB dependent luciferase reporter (3enhConALuc) (Rodriguez et al., 1996) was cotransfected into COS7 cells along with expression plasmids for SUMO-1, Ubch9 or IκBα and reporter activity measured after treatment of the cells with the NF-κB activators TNFα, IL-1β or okadaic acid. When the empty vector is cotransfected with the κB reporter substantial transcriptional activation is obtained with TNF (9.8 fold), IL-1 (98 fold) and okadaic acid (85 fold). As expected cotransfection of the reporter with a plasmid expressing IκBα reduced TNF, IL-1 and okadaic acid induced activation. When the reporter was cotransfected with a plasmid expressing SUMO-1, or the SUMO-1 conjugating enzyme Ubch9 the extent of activation was also reduced with TNF, IL-1, and okadaic acid. Cotransfection of the reporter plasmid with combinations of plasmids expressing IκBα, SUMO-1 and Ubch9
demonstrated a more complete inhibition of transcriptional activation than either plasmid alone. Thus the combination of IκBα and SUMO-1 expression results in an 80% inhibition of TNF induced transcriptional activation whereas expression of either IκBα or SUMO-1 alone only gives about 50% inhibition. Reporter activity from a plasmid lacking NF-κB binding sites (conALuc) was not increased by TNF, IL-1 or okadaic acid and the basal level of reporter activity was unchanged by exogenous expression of IκBα, SUMO-1 or Ubch9 (data not shown). To investigate the specificity of the SUMO-1 mediated inhibition of NF-κB dependent transcription an alternative reporter plasmid and an expression plasmid for ubiquitin were employed. While activation of the HIV-LTR by TNF and IL-1 is almost entirely dependent on NF-κB, activation of the HIV-LTR by okadaic acid has both NF-κB dependent and independent components. Thus ΔκBHIVLTR can still be activated by okadaic acid (Vlach et al., 1995). Like the 3enhConALuc reporter the HIV LTR is activated by TNF (12 fold), IL-1 (35 fold) and okadaic acid (11 fold) and the activation is reduced by cotransfection of plasmids expressing IκBα or SUMO-1. Cotransfection of a plasmid expressing ubiquitin has little inhibitory effect. Cotransfection of the combination of IκBα and SUMO-1 substantially inhibits transcriptional activation mediated by TNF, IL-1 or okadaic acid whereas cotransfection of both IκBα and ubiquitin expression plasmids has a relatively minor effect on transcriptional activity. The ΔκBHIVLTR reporter was activated by neither TNF nor IL-1 but was activated approximately 3 fold by okadaic acid. Cotransfection of the ΔκBHIVLTR reporter with IκBα, SUMO-1, ubiquitin or combinations of IκBα and SUMO-1 or IκBα and ubiquitin had no substantial effect on basal activity and did not influence the κB independent okadaic acid induced activation of the reporter (Figure 19). Thus SUMO-1 appears to specifically inhibit NF-κB dependent transcriptional activation.
3.3.5. Requirements for SUMO-1 conjugation to IκBα in vitro

To investigate the biochemical requirements for SUMO-1 modification of IκBα an *in vitro* system which could accurately recapitulate the *in vivo* phenomenon, was developed. 35S-Met-labelled IκBα generated by *in vitro* translation was used as substrate in the presence of recombinant SUMO-1, recombinant Ubch 9 and a fraction from HeLa cells containing SAE activity (Fr. II.4) (Desterro et al., 1997). SUMO-1 modification of IκBα required the presence of each of these components and was dependent on the addition of ATP (Figure 20). Other HeLa fractions such as II.1 failed to substitute for fractions II.4 and SUMO-1 was only fully active if it was truncated by four amino acids at the C-terminus to expose G97. In fact some conjugation activity was observed with the full length SUMO-1, but it is thought that this is due to the presence of a SUMO-1 C-terminal hydrolase in the column fractions which cleaves the C-terminus of SUMO-1 after G97 to generate the active form of the protein (Figure 20).

3.3.6 Identification of lysine residues in IκBα that are conjugated to SUMO-1

Prior to signal induced degradation, IκBα is ubiquitinated on residues K21 and K22 and to a lesser extent on residue K38 and K47 (Baldi et al., 1996; Rodriguez et al., 1995; Scherer et al., 1995). To determine the sites at which IκBα is modified by SUMO-1 the previously described (Rodriguez et al., 1996) series of molecules containing K to R changes in IκBα was employed. 35S labelled IκBα proteins were incubated *in vitro* under conditions which permit SUMO-1 modification of IκBα. The K21R mutant of IκBα is severely compromised as a substrate for SUMO-1 modification *in vitro*, while this is less so for the K22R mutant. When both lysines are changed to arginine (K21R, K22R) SUMO-1
Figure 20. Requirements for SUMO-1 conjugation of IκBα in vitro.

*In vitro* expressed IκBα 256 (aa 1-256) labelled with 35S-methionine was incubated at 37°C for 2 hours in a reaction mix containing an ATP regenerating system and in presence (+) or absence (-) of different components as indicated in the figure. Reactions products were fractionated by SDS-PAGE and the dried gel analysed by phosphorimaging. The positions of IκBα, the phosphorylated form of IκBα (IκBα-P) and IκBα-SUMO are indicated.
Figure 21. Identification of lysine residues in IκBα that are conjugated to SUMO-1

*In vitro* expressed IκBα (WT, K21R, K22R and K21,22R) labelled with 35S-methionine were incubated in an assay mix containing an ATP regenerating system, recombinant p65, Ubch9, SUMO₁₀₀ and HeLa FrII.4 containing SUMO E1 activity for 2 hours at 37°C. Reactions products were fractionated by SDS PAGE and the dried gel analysed by phosphorimaging.
modification is abolished (Figure 21). Thus the major site of SUMO-1 modification is K21, which is also used to conjugate ubiquitin.

3.3.7. Role of IκBα phosphorylation in SUMO-1 modification

As ubiquitination of IκBα is dependent on signal induced phosphorylation of S32 and S36 we investigated the role of these residues in SUMO-1 modification. Previously described mutants in IκBα where these serine residues were changed to either alanine, aspartic acid or glutamic acid (Rodriguez et al., 1996; Roff et al., 1996) were labelled with $^{35}$S methionine and tested for their ability to be modified by SUMO-1 in vitro. The S32A, S36A mutant of IκBα that is resistant to signal induced phosphorylation is efficiently modified by SUMO-1 as is the S32D, S36D mutant. However, SUMO-1 modification of the S32E, S36E mutant, which most closely mimics phosphorylated IκBα, is severely compromised, suggesting that phosphorylation of IκBα on S32 and S36 is inhibitory for SUMO-1 modification (Figure 22A). To demonstrate that phosphorylated IκBα is not a substrate for SUMO-1 modification residues 1-70 of IκBα fused to GST (GST- IκBαN) were phosphorylated in vitro with purified IκB kinase (Regnier et al., 1997) in the presence of γ- $^{32}$P ATP. $^{32}$P GST- IκBαN was recovered and tested for its ability to act as a substrate for SUMO-1 conjugation in vitro. Under conditions where unlabelled GST- IκBαN is efficiently conjugated to SUMO-1 (data not shown) the labelled material was not conjugated to SUMO-1. $^{35}$S labelled unphosphorylated IκBα$_{256}$ was efficiently modified in the same assay (Figure 22B).

In vivo the C-terminus of IκBα is required for signal induced degradation and is phosphorylated by CKII. A mutant of IκBα containing amino acids 1-256 which is resistant to signal induced degradation (Kroll et al., 1997) was efficiently modified by SUMO-1 in vitro, indicating that the C-terminal region of the protein
Figure 22. Role of IκBα phosphorylation in SUMO-1 modification.

(A) In vitro expressed and 35S-methionine labelled wild type IκBα (W/T) and mutants (256; S32,36A; S32,36D; S32,36E) were assayed for SUMO-1 conjugation in vitro as described in the legend of Figure 21. Assays of IκBα WT and 256 were conducted either in the presence (+) or absence (-) of recombinant p65. (B) The N-terminus of IκBα (1-70) fused to GST (GST- IκBαN) was phosphorylated in vitro with purified IκB kinase in the presence of γ32P ATP. 35S Met labelled in vitro expressed IκBα1-256 and 32P labelled GST- IκBα N were assayed for SUMO-1 conjugation in vitro.
(amino acids 257-317) is dispensable for SUMO-1 modification (Figure 22A). The HeLa extract used in these assays is capable of phosphorylating IκBα on S32 and S36 to a more slowly migrating species of IκBα that is detected after incubation with wild type IκBα and IκBα 1-256 but not with S32A, S36A or S32D, S36D. Under these conditions wild type IκBα, which undergoes phosphorylation in vitro, is a less efficient substrate for SUMO-1 modification than either IκBα S32A, S36A or S32D, S36D (Figure 22A). While most assays contained recombinant NF-κB p65, its omission was without consequence for SUMO-1 modification of IκBα (Figure 22A).

3.3.8. SUMO-1 modification of IκBα with purified proteins

Although SUMO-1 conjugation of IκBα is dependent on Ubch9, ATP and FrII.4 (Figure 20) (Desterro et al., 1998), a complete description of the biochemical activities present in the HeLa cell fraction or in the in vitro transcribed translated substrate was lacking. The SAE was affinity purified from Fr. II.4, as described in the second chapter, but by analogy with ubiquitin modification it is not clear if other activities, such as an E3 equivalent, would be required. In presence of recombinant, immunoprecipitated SAE, Ubch9 and ATP, 125I-SUMO-1 was efficiently conjugated to recombinant IκBα (Figure 23A). This reaction was dependent on SAE, Ubch9, ATP and IκBα substrate (Figure 23B). The enzymatic properties of rSAE were identical to those of the protein isolated from HeLa FrII.4. As SUMO-1, Ubch9 and IκBα are all homogeneous, recombinant proteins expressed in bacteria and the SAE is either highly purified (Figure 23B) or recombinant anti-SV5 immunoprecipitated (Figure 23A) it appears that SUMO-1 conjugation of IκBα in vitro does not require an equivalent E3 activity and that after SUMO-1 being activated by SAE, the SUMO-1 activating enzyme Ubch9 is capable of recognising,
then transferring SUMO-1 onto IκBα. To demonstrate specificity \textit{in vitro} two deleted forms of IκBα and GST were used as substrates in reactions also containing purified SAE, Ubch125I-SUMO-1 and ATP. SUMO-1 is efficiently conjugated to IκBα and IκBαΔC (lacks the C-terminal 61 amino acids) but is not conjugated to either ΔN IκBα (lacks the N-terminal 70 amino acids) or GST (Figure 23C). As the lysine involved in SUMO-1 modification is present in wild type IκBα and IκBαΔC, but absent in ΔN IκBα, this indicates that the purified conjugation system is displaying the expected specificity.

If SUMO-1 modified IκBα blocks NF-κB activation then it is likely that SUMO-1 modified IκBα remains associated with NF-κB. To address this question directly IκBα was conjugated to 125I-SUMO-1 \textit{in vitro} and incubated with either a GST-NF-κB fusion protein (GST-p65) or GST alone. A control SUMO-1 modification reaction which lacked IκBα substrate was analysed in parallel. GST-p65 efficiently bound 125I-SUMO-1 modified IκBα (Figure 24). This species was not bound by GST alone and was not present in reactions which did not contain IκBα substrate (Figure 24). Thus SUMO-1 modification does not interfere with the ability of IκBα to interact with NF-κB.

3.3.9. Discussion

In uninduced cells the transcription factor NF-κB is held in an inactive state by its inhibitor IκBα. After exposure of cells to activators such as IL-1β or TNFα the IκBα inhibitor is marked by phosphorylation and targeted for degradation by site specific ubiquitination. A specific quantity of NF-κB is thus released for transcriptional activation. In any particular cell type the amount of active NF-κB released is therefore determined simply by the amount of NF-κB bound to inducibly degradable forms of IκB. Here we demonstrate that IκBα is modified by SUMO-1
Figure 24. $^{125}$I-SUMO-1 Conjugated IκBα interacts with recombinant NF-κB p65. An in vitro $^{125}$I SUMO-1 conjugation assay was set up as described in the presence of either recombinant GST or GST-p65 protein. After 2 hours incubation at 37°C the reaction products were incubated with glutathione agarose beads for 10 minutes at room temperature. The beads were washed with PBS/0.1% Tween20 and bound proteins fractionated on a 12.5% acrylamide gel and analysed by phosphorimaging. The position of IκBα conjugated with $^{125}$I SUMO-1 is indicated.
(Figures 17 and 18) on lysine 21, which is also used for ubiquitin conjugation (Figure 21). Thus SUMO-1 modified IκBα cannot be ubiquitinated and is therefore resistant to proteasome mediated degradation. As SUMO-1 modified IκBα remains bound to NF-κB (Figure 24), this creates a "privileged" pool of NF-κB/IκBα-SUMO-1 complexes that do not respond to signal induction (Figure 17C). As a consequence exogenous expression of SUMO-1 has a strong inhibitory effect on NF-κB dependent transcription measured in reporter assays. The inhibitory effect of SUMO-1 appears to be specific to NF-κB dependent transcription as other promoters are not affected. As the amount of SUMO-1 modified IκBα appears to vary between different cell types this may provide a mechanism by which the cell can precisely regulate the quantity of NF-κB available for transcriptional activation. However, it is likely that SUMO-1 modification is controlled in a dynamic fashion with the overall level of SUMO-1-conjugated IκBα being determined by a balance between SUMO-1 modification and hydrolysis of the IκB-SUMO-1 conjugates. The existence of hydrolases which cleave the bond between the C-terminus of SUMO-1 and the lysine to which it is conjugated have been reported (Li and Hochstrasser, 1999; Mahajan et al., 1997; Matunis et al., 1996; Muller et al., 1998) and the activity of these enzymes is such that the detection of IκBα-SUMO-1 conjugates is difficult unless special precautions are taken to quickly inactivate these enzymes (Figure 17). Removal of the four C-terminal amino acids of SUMO-1 is required to expose G97 (Figure 20) the carboxyl terminus of which is directly coupled to the ε amino group of lysine in the target protein (Mahajan et al., 1998). Western blot analysis of endogenous SUMO-1 indicates that there are many cellular proteins that are conjugated to SUMO-1, and as no free SUMO-1 was detected this implies that virtually all of the endogenous SUMO-1 is conjugated to proteins (Matunis et al., 1996) and data not shown. This is also the case in yeast where all of the SUMO-1 homologue Smt3p is present in conjugates (Johnson et al., 1997).
These data indicate that SUMO-1 is limiting, suggesting that SUMO-1 deconjugation is required to release free SUMO-1 for further modification.

While SUMO-1 modification of \( \text{I} \kappa \text{B}\alpha \) can serve to block signal induced ubiquitination and thus degradation of \( \text{I} \kappa \text{B}\alpha \), SUMO-1 modification of RanGAP1 serves to direct the modified protein to the nuclear pore complex (Mahajan et al., 1997; Mahajan et al., 1998; Matunis and Blobel, 1997; Matunis et al., 1996). SUMO-1 modification of RanGAP1 creates, or exposes, a binding site for NUP358, a nucleoporin associated with the cytoplasmic fibres of the nuclear pore complex (Matunis et al., 1998). The only other reported substrates for SUMO-1 modification are the nuclear dot (ND) associated proteins PML and Sp100 and in this case SUMO-1 modification appears to regulate the subnuclear partitioning of these proteins (Muller et al., 1998; Sterndorf et al., 1997).

Although we have not shown that SUMO-1 modification alters the cellular location of \( \text{I} \kappa \text{B}\alpha \) one feature that the known SUMO-1 substrates (RanGAP1, PML, Sp100 and \( \text{I} \kappa \text{B}\alpha \)) have in common is that they undergo regulated transport between the cytoplasm and the nucleus. Nuclear transport is required for \( \text{I} \kappa \text{B}\alpha \) to mediate post-induction repression of NF-\( \kappa \)B dependent transcription (Arenzana-Seisdedos et al., 1995; Beg et al., 1995a; Cressman and Taub, 1993; Zabel et al., 1993). However mutation of K21 and K22 does not appear to affect the ability of \( \text{I} \kappa \text{B}\alpha \) to accumulate in the cell nucleus (Zabel et al., 1993). Thus it is unlikely that SUMO-1 modification has a major role in the nuclear import of \( \text{I} \kappa \text{B}\alpha \).

Although targeting of protein for ubiquitination appears to be highly specific it is often the case that multiple lysine residues can act as acceptor sites with modification of either residue being sufficient to target the protein for degradation (Hou et al., 1994; King et al., 1996b). In contrast, SUMO-1 modification appears to be highly specific. \( \text{I} \kappa \beta\alpha \) appears to be modified by SUMO-1 predominantly on K21 (Figure 21) while RanGAP1 is conjugated to SUMO-1 solely via K526 (Mahajan et al., 1998). To further investigate the enzymatic requirements of
the SUMO-1 conjugation reaction an in vitro system with recombinant components was developed. In the purified in vitro system SUMO-1 is efficiently conjugated to recombinant IκBα in the presence of only the SAE and Ubch9. Thus under the conditions employed in this assay SUMO-1 conjugation does not require the presence an E3 protein ligase. However, we can not rule out the possibility that in vivo such proteins may increase the efficiency of the conjugation process. As our initial yeast two hybrid screen and protein affinity chromatography experiments utilising immobilised GST-Ubch9 (data not shown) demonstrated a protein-protein interaction between Ubch9 and IκBα (Desterro et al., 1997), it is likely that substrate specificity is achieved by Ubch9. A diverse range of proteins have been shown to interact with Ubc9 in yeast two hybrid experiments and this may be a direct consequence of substrate recognition by Ubc9. Again it is not possible to rule out the participation of yeast proteins in these interactions.

While signal induced ubiquitination of IκBα requires the phosphorylation of S32 and S36 this is not the case for SUMO-1 modification, as an S32A, S36A mutant is more efficiently conjugated to SUMO-1 than the wild type protein. In contrast an S32E, S36E mutant, which may mimic the phosphorylated protein, or IκBα phosphorylated in vitro on S32 and S36 are poor substrates for SUMO-1 conjugation. Thus, SUMO-1 acts antagonistically to ubiquitination: while multiubiquitination of IκBα targets the protein for destruction, SUMO-1 modification creates a pool of IκBα that is resistant to degradation. This function of SUMO is rather similar to that observed when mutations are introduced into ubiquitin in the lysine residues that are used for multiubiquitination. K29R and K48R mutants in ubiquitin generate proteins that can be conjugated to substrates but which cannot form multiubiquitin chains. As such the modified proteins are resistant to degradation (Johnson et al., 1995). Although only a few substrates for SUMO-1 modification have been identified it is evident that many cellular proteins are modified in such a fashion. The balance between ubiquitination and this newly
described activity of SUMO-1 may be a general mechanism for controlling the level of critical proteins within the cell.
3.4. SUMO-1 modification activates the transcriptional activity of p53
3.4.1. Summary

The p53 tumour suppressor plays a significant part in the response of the cell to genotoxic damage. The important role of p53 in maintenance of genome integrity is illustrated by the loss of p53 function in most human tumours (Harris and Hollstein, 1993) and the high rate of tumour development in p53 knockout mice (Armstrong et al., 1995; Donehower, 1996). p53 is a transcription factor that binds to specific sequences in the upstream region of many genes whose protein products regulate cell cycle progression and apoptosis (Ko and Prives, 1996). Thus cells with damaged DNA do not proliferate and the loss of this protective function allows the uncontrolled growth of cells containing oncogenic mutations (Lane, 1992).

The p53 tumour suppressor protein is regulated by ubiquitin mediated proteasomal degradation (Maki et al., 1996). In normal cells p53 is constitutively ubiquitinated by the Mdm2 ubiquitin ligase (Bottger et al., 1997; Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). Exposure of cells to a range of different stress signals, including DNA damage, hypoxia and heat shock activates the p53 response and p53 levels rise due to inhibition of this degradative pathway. Here we show that p53 is modified by the small ubiquitin like protein SUMO-1 at a single site, K386 in the C terminus of the protein. Modification in vitro requires only SUMO-1, the SUMO-1 activating enzyme and ubc9. SUMO-1 and ubiquitin modification do not compete for the same lysine acceptor sites in p53. Thus overexpression of SUMO-1 activates the transcriptional activity of wild type p53, but not K386R p53 where the SUMO-1 acceptor site has been mutated. The SUMO-1 modification pathway therefore acts as potential regulator of the p53 response and may represent a novel target for the development of therapeutically useful modulators of the p53 response.
3.4.2. p53 is modified by SUMO-1 \textit{in vivo}

As described in the third chapter ubiquitin mediated proteolysis of IκBα, could be antagonised by modification with the small ubiquitin-like protein SUMO-1 (Desterro et al., 1998). As the p53 response to genotoxic insult is also critically dependent on ubiquitin mediated proteolysis we investigated the metabolism of p53 in U2OS cells. Preservation of SUMO-1 modifications requires rapid inhibition of SUMO-1 specific isopeptidases and this is accomplished by direct lysis of cells into buffer containing SDS and iodoacetamide. In the absence of any stimulus p53 and more slowly migrating products, which we assume to be ubiquitin adducts, are detected. Also present is a species of 65 KDa which is consistent with a single SUMO-1 addition to p53. Exposure of U2OS cells to UV results in a time dependent increase in p53 levels and an accumulation of the 65 KDa species (Figure 25A). The lack of immunoreactive species in Saos-2 cells, which lack p53, indicates that the more slowly migrating species are indeed related to p53 (Figure 25A, right panel). To establish that the 65 KDa species is indeed the SUMO-1 modified form of p53, U2OS cells were transfected with a 6-His version of SUMO-1, either alone or with a p53 expression construct or empty vector as a control. Cells were lysed in guanidinium HCl and 6-His containing proteins eluted from Ni-agarose with imidazole. Western blotting with an antibody to p53 indicated that 6-His SUMO-1 linked to p53 could be detected in cells transfected with the 6-His SUMO-1 expression plasmid, but not in cells transfected with the empty vector. Cotransfection with a p53 expression construct strongly increased the quantity of 6-His SUMO-1 modified p53 (Figure 25B). Treatment of 6-His SUMO-1 transfected U2OS cells with UV results in a time dependent increase in the amount of 6-His SUMO-1 conjugated to p53 (Figure 25B, right panel). Thus p53 is covalently coupled to SUMO-1 \textit{in vivo} and this modified form of p53 accumulated after UV irradiation.
Figure 25. U.V. irradiation leads to accumulation of p53 and SUMO-1 conjugated form of p53 in U2OS cells

(A) U2OS cells were exposed to 25 J/m² of U.V. radiation and then collected by direct lysis in SDS buffer at the indicated times. Samples were fractionated by SDS-PAGE and p53 analysed by Western blotting using a p53-specific monoclonal antibody, DO-1. Saos-2 cells which lack p53 (right panel) were analysed to demonstrate antibody specificity. (B) 6His-tagged SUMO-1 is conjugated to p53 in vivo. U2OS cells were transfected with pcDNA3, 6-His-SUMO-1 and p53 as indicated and after 36 hours of expression were lysed in buffer containing guanidinium HCl. Proteins linked to 6His-SUMO-1 were purified using Ni²⁺-NTA-agarose beads and after extensive washing eluted with 200 mM imidazole. Eluted proteins were fractionated by SDS-PAGE and transferred to a nitro-cellulose membrane. 6His-SUMO-1 p53 conjugates were detected by Western blotting using anti-p53 antibody (DO-1). Molecular weight markers are shown on the right. The position of SUMO-1 conjugated p53 is indicated on the left. In the right panel 6His SUMO-1 transfected cells were exposed to UV and analysed as indicated above.
3.4.3. SUMO-1 modification of p53 takes place on K386

To investigate the site of SUMO-1 modification in p53 the in vitro system for SUMO-1 modification, previously described was employed (Desterro et al., 1998). $^{35}$S-labelled in vitro translated p53 was incubated with a source of SUMO-1 activating enzyme (SAE1/2) (Desterro et al., 1999) and SUMO-1 conjugating enzyme (Ubc9) (Desterro et al., 1997) in the presence of SUMO-1 and ATP. Under these conditions $^{35}$S-labelled p53 was converted to a more slowly migrating form that is consistent with SUMO-1 modification. To confirm that this species was indeed SUMO-1 modified p53, GST-SUMO-1 was substituted for SUMO-1 in the reaction, resulting in the detection of a modified species with altered electrophoretic mobility (Figure 26). SUMO-1 modification was abolished if either SUMO-1, SAE, Ubc9 or ATP was omitted from the reaction (Figure 26). A series of p53 molecules in which regions of the N- and C-termini had been deleted (Hansen et al., 1998) were used as substrates for SUMO-modification in vitro (Figure 27C). p53 molecules lacking parts of the N-terminus were efficiently SUMO-1 modified whereas p53 molecules lacking parts of the C-terminus were not modified by SUMO-1 (Figure 27A). Deletion 4M, which lacks the C-terminal 30 amino acids, was not modified thus indicating that this region is required for modification and may in fact be the site of modification. As ubiquitin-like proteins modify substrates on the ε-amino group of lysine a series of mutants was created in which lysines within the C-terminal 30 amino acids of p53 were changed to arginine. Modification of K370, K372, K373, K381 or K382 was without consequence for SUMO-1 modification whereas p53 containing the K386R mutation was not modified by SUMO-1 in vitro (Figure 27B). To establish that the in vitro results are an accurate representation of the situation in vivo, the same collection of p53 mutants was co-expressed with 6-His SUMO-1 in cells lacking endogenous p53. Proteins conjugated to 6-His SUMO-1 were isolated by chromatography on Ni-agarose and p53 detected by Western blotting. Again mutations in K370, K372, K373, K381 and K382 did not affect SUMO-1 modification, while the K386R mutation
Figure 26. Requirements for in vitro conjugation of SUMO-1 to p53.

In vitro expressed and $^{35}$S labelled p53 was incubated with ATP, recombinant SUMO-1 or GST-SUMO-1, Ubc9, and HeLa Fr II.4 containing SUMO-1 E1 activity (SAE) as indicated. Reactions products were fractionated by SDS-PAGE, and the dried gel was analysed by phosphorimaging. The positions of p53, the SUMO-1 conjugated form of p53 and the GST-SUMO-1 conjugated form of p53 are indicated.
Figure 27. The C-terminus of p53 is modified by SUMO-1 in vitro.

(A) In vitro SUMO-1 conjugation of C- and N-terminal p53 deletions. In vitro expressed and 35S labelled wild-type p53 (WT) and C-and N-terminal deletions were incubated with recombinant Ubch9, SUMO-1 and HeLa Fr II.4 containing SUMO-1 E1 activity. Reactions products were fractionated by SDS-PAGE, and the dried gel was analysed by phosphorimaging. The SUMO-1 conjugated form of p53 is indicated with an asterisk. (B) In vitro SUMO-1 conjugation of C-terminal lysine point mutants of p53. In vitro expressed and 35S-labelled WT P53 and mutants were assayed for SUMO-1 conjugation in vitro as in A. The positions of p53 and the SUMO-1 conjugated form of p53 are indicated. (C) Schematic representation of p53 deletions, C-terminal lysine to arginine point mutants and their ability to act as substrates for SUMO-1 conjugation activity.
abolished SUMO-1 modification in vivo (Figure 28A). Direct Western blotting of the cell lysates before Ni-agarose chromatography indicated that each of the p53 proteins was expressed at comparable levels. Although representing only a small proportion of the total, due to overexpression of p53, the SUMO-1 modified forms of p53 proteins containing K386 are detected. This SUMO-1 modified form of p53 is not detected in those proteins containing the K386R mutation (Figure 28B). Thus K386R is required for SUMO-1 modification and is likely to be the residue to which SUMO-1 is conjugated.

3.4.4. SUMO-1 and ubiquitin modification do not compete for the same lysine acceptor site in p53

It has recently been established (Honda and Yasuda, 1999; Kubbutat et al., 1998) that the C-terminal 30 amino acids are required for Mdm2 mediated degradation of p53. In light of observations with IκBα the effect of SUMO-1 modification on p53 ubiquitination was investigated. As expected, in vitro ubiquitination of 35S labelled p53 was dependent on the presence of Ubc5 and Mdm2 (Figure 29A) and required the C-terminus of p53 (Figure 29B, 4M deletion). To determine if ubiquitination takes place at the same site as SUMO-1 modification the same lysine to arginine point mutants (Figure 27) were used for in vitro ubiquitination. Unlike SUMO-1 modification K386R was competent for ubiquitination and none of the other point mutations had a substantial effect on ubiquitination. Thus, the SUMO-1 and ubiquitin modification systems do not compete for modification of the same lysine residue in p53.

3.4.5. SUMO-1 modification induces p53 dependent transcription

To assess the functional consequences of SUMO-1 conjugation to p53 we determined the effect of cotransfected SUMO-1 on the transcriptional activity of
Figure 28. K386 is required for SUMO-1 modification of p53 in vivo.

(A) Saos-2 cells were transfected with the indicated plasmids and after 36 hours of expression lysed in buffer containing guanidinium HCl. 6His linked proteins, eluted from Ni²⁺-NTA-agarose, were fractionated by SDS-PAGE and analysed by Western blotting with anti-p53 antibody (DO-1). Molecular weight markers and the position of His-tagged SUMO-1 conjugated to p53 are indicated. An asterisk indicates a non-specific cross reacting species which is independent of transfection. (B) Saos-2 cells transfected as indicated in A, were directly analysed by Western blotting with anti-p53 antibody (DO-1).
Figure 29. SUMO-I and ubiquitin modification do not compete for the same lysine residue in p53.

(A) $^{35}$S labelled p53 generated by in vitro transcription and translation was incubated with recombinant E1 in the presence (+) or absence (-) of different components, as indicated in the figure, in 50 mM Tris pH 7.5, 5 mM MgCl$_2$, and an ATP regenerating system at 37°C for 2 hours. Reactions products were analysed as described in legend of figure 26. (B) In vitro ubiquitination of $^{35}$S labelled p53, the indicated p53 point mutants and the C-terminal deletion 4M was carried out as described in A.
wild type p53 and K386R p53 where the SUMO-1 acceptor site has been mutated. Saos-2 cells, which lack endogenous p53, were cotransfected with the p53 responsive reporter plasmid pG13-luciferase and expression plasmids for p53 and SUMO-1. Wild type p53 alone activates transcriptional activity by 8 fold while the additional presence of SUMO-1 activates transcription by about 18 fold (Figure 30). In contrast K386R p53 has an inherently higher transcriptional activity and this is not further augmented by co-expression of SUMO-1 (Figure 30). Similar data was obtained over a wide range of input p53 expression vector amounts and with different p53 dependent reporters, such as the mdm2 promoter (data not shown). Western blotting indicated that neither wild type p53, nor K386R p53 levels were dramatically altered by coexpression of SUMO-1 (Figure 30, insert).

3.4.6. Discussion

In normal cells p53 is maintained at a low level by mdm2 mediated ubiquitin dependent proteolysis. Following genotoxic insult p53 is stabilised and as a consequence the protein accumulates and activates p53 dependent transcription. As no single unifying hypothesis can yet describe this phenomenon, it is likely that multiple pathways lead to activation of the p53 response (Lane, 1998). We demonstrate that p53 is modified by SUMO-1 and that the amount of SUMO-1 conjugated p53 increases after exposure of cells to ultraviolet radiation (Figure 25). SUMO-1 is conjugated to p53 on K386 (Figures 27 and 28) which is located in the C-terminus of the protein in a region that is known to be required for mdm2 mediated ubiquitin dependent degradation of p53 (Honda and Yasuda, 1999; Kubbutat et al., 1998). A role for SUMO-1 modification in p53 activation is consistent with the observation that overexpression of SUMO-1 leads to increased p53 dependent transcriptional activity. This increase is dependent on SUMO-1 modification of p53 as SUMO-1 overexpression does not influence the transcriptional activity of K386R p53 (Figure 30). It is possible to envisage a number of models by which SUMO-1 modification may increase the transcriptional
Figure 30. SUMO-1 conjugation on K386 stimulates the transcriptional activity of p53.
Saos-2 cells were electroporated with pG13-luc reporter plasmid and expression plasmids for SUMO-1, wild type p53 and K386R p53 as indicated. 24 hours after electroporation reporter activity was determined. Each point is the mean from 4 independent transfections with error bars representing one standard deviation. Shown in the insert is a representative experiment in which p53 levels were determined by Western blotting using antibody DO1.
response of p53. These include an increase in protein stability, a higher intrinsic transcriptional activity and alterations in subcellular localisation. Although the site of SUMO-1 modification may represent a potential site of ubiquitination, mutation K386R does not have a dramatic effect on ubiquitination in vitro (Figure 29). While the C-terminal region is required for ubiquitination it does not appear that a unique lysine is a target for mdm2 as no single lysine to arginine mutation of the 6 lysines in this region abolishes ubiquitination (Figure 29). This is different from the situation in IκBα where one of two lysine residues is the target for signal induced ubiquitination. As this also represents the site of SUMO-1 conjugation ubiquitination is inhibited simply because the acceptor lysine is already modified by SUMO-1 (Desterro et al., 1998). Although the major binding site for mdm2 is located in the N-terminal region of p53 (Bottger et al., 1997; Chen et al., 1993; Kussie et al., 1996; Midgley and Lane, 1997; Momand et al., 1992; Oliner et al., 1993; Picketsley et al., 1994) it has also been reported that the oligomerisation of p53 is required for efficient interaction with mdm2 (Marston et al., 1995). While the extreme C-terminus of p53 is not required for efficient interaction with mdm2, its deletion dramatically reduces mdm2 catalysed ubiquitination in vitro and increases the stability of p53 in vivo (Honda and Yasuda, 1999; Kubbutat et al., 1998). It is possible that the C-terminal region of p53 is required as the site of ubiquitination by mdm2, but ubiquitination can be targeted to any of the 6 lysines in this region. Thus while SUMO-1 modification of this region may not directly disrupt the interaction between p53 and mdm2 it could inhibit the ability of mdm2 to access the lysine residues in the C-terminus which are the substrates for ubiquitination. In addition to SUMO-1 modification it has recently been reported that phosphorylation of p53 with DNA dependent protein kinase reduces the substrate activity of p53 in an mdm2 dependent in vitro ubiquitination assay (Honda and Yasuda, 1999). The tumour suppressor p19ARF blocks ubiquitination of p53, but in this case the target appears to be mdm2 rather than p53 (Honda and Yasuda, 1999). Likewise RB has also been shown to block mdm2 induced degradation of p53 and in this instance a
trimeric, but inactive complex containing p53, RB and mdm2 was detected (Hsieh et al., 1999). As an alternative to effects on protein stability SUMO-1 modification could increase the inherent transcriptional activity of p53. This could result from a change in protein structure which increases DNA binding activity, allows more efficient access to p53 dependent promoters embedded in repressive chromatin, or has a greater capacity to recruit co-activators such as CBP/p300. In this respect K386R p53 elicits a greater transcriptional response than wild type p53 which may reflect an increased activity in any one of the above properties.

It is also possible that SUMO-1 modification alters the subcellular localisation of p53, as has been noted for other SUMO-1 modified proteins. SUMO-1 modified Ran GAP is recruited to the nuclear pore complex (Mahajan et al., 1997; Matunis and Blobel, 1997) while SUMO-1 modified PML and SP100 are present in PML oncogenic domains or PODs (Duprez et al., 1999; Muller et al., 1998; Sternsdorf et al., 1997). IκBα is predominantly cytoplasmic but shuttles rapidly between the nucleus and the cytoplasm and while the cellular localisation of SUMO-1 modified IκBα has not been determined it is worth noting that IκBα, trapped in the nucleus by leptomycin B, is resistant to signal induced degradation (Rodriguez et al., 1999). Mdm2 also shuttles between the nucleus and the cytoplasm (Roth et al., 1998) and blockage of nuclear export results in accumulation of p53, mdm2 complexes in subnuclear domains adjacent to the PODs (Lain et al., 1999) which are rich in SUMO-1 modified proteins. Like IκBα, nuclear p53 is resistant to degradation (Freedman and Levine, 1998) and it is possible that SUMO-1 modification takes place when these proteins are imported into the nucleus. The predominantly nuclear localisation of the SAE and Ubc9 enzymes involved in SUMO-1 modification (recent observations in the laboratory) lends supports to this hypothesis. Thus SUMO-1 modification could increase the nuclear content of p53 or alternatively concentrate the protein in distinct subnuclear domains that are active sites of transcription. Diverse stress stimuli activate the p53 response and are likely to act via separate signalling pathways with distinct
activation mechanisms. SUMO-1 modification represents an additional route to p53 activation that may link the p53 response to other aspects of cellular metabolism. Manipulation of the SUMO-1 modification pathway may therefore represent a novel target for the development of therapeutically useful modulators of the p53 response.
4. Conclusions
Ubiquitin modification of proteins provides an extremely versatile means of cellular regulation but over the past few years a family of ubiquitin-like proteins has been discovered that raise new and intriguing regulatory and mechanistic questions on cell biology. SUMO-1 is a member of this new family of ubiquitin-like proteins that is covalently linked to target proteins and we undertook the characterisation of this modification.

Several features of the ubiquitin pathway are conserved in the SUMO-1 conjugation pathway. Like ubiquitin, SUMO-1 is proteolytically processed to expose its mature C-terminus (Kamitani et al., 1997b). Then SUMO-1 is activated in an ATP-dependent manner by an activating enzyme composed of SAE1 and SAE2. The activated SUMO-1 is then transferred to the active-site cysteine of Ubc9. Although Ubc9 is able to recognise the substrate and directly transferred the activated SUMO-1 to a lysine residue in the target protein in vitro, we can not rule out the possibility of E3-like protein ligases increase the efficiency of the conjugation process in vivo. This is rather different from ubiquitin modification where an E3 activity is required for target recognition. As almost all identified targets for SUMO-1 modification are Ubc9 interacting proteins (see Table 1 and Table 2) it is likely that substrate specificity is achieved by Ubc9. To date, there is no evidence to suggest that an E3-like activity is involved in this conjugation pathway. The C-terminal region of Ubc9 which is thought to be involved in substrate binding (Liu et al., 1999) lies close to the catalytic site and favours the direct transfer of SUMO-1 to substrate proteins. While the SUMO-1 conjugation pathway is mechanistically similar to the ubiquitin pathway it utilises specific conjugating enzymes (Figure 31).

![Figure 31. SUMO-1 conjugation pathway.](image-url)
It remains to be seen which other proteins are modified by ligation to SUMO-1 and whether at least some of the many proteins that interact with Ubc9 (Table 1) are also substrates for SUMO-1 conjugation. Ubc9 interaction with so many proteins in the yeast two-hybrid screen justifies commentary. One possible explanation is that in yeast some of the “bait” proteins can be modified by SUMO-1 and SUMO-1 acts as a bridging protein mediating the interaction between Ubc9 and bait proteins. However, non-specific interactions between some proteins and the positively charged surface of Ubc9 (Giraud et al., 1998; Tong et al., 1997) may also explains the large number of Ubc9 interacting proteins.

A particularly large number of transcription factors interacts with Ubc9. This interaction has in many cases a stimulatory effect in transcription activation but is not in all the cases directly related to SUMO-1 modification. Ubc9 activates the transcriptional activity of ETS (E-26 transforming specific), TEL (ETS-related gene) and AR (androgen receptor) transcription factors but the stimulatory activity of Ubc9 cannot be inhibited by mutating the Cys93 (Chakrabarti et al., 1999; Hahn et al., 1997; Poukka et al., 1999; Tashiro et al., 1997). Thus, Ubc9 is able to act in a fashion independent of its ability to catalyse SUMO-1 conjugation. It would be interesting to speculate that Ubc9 has additional properties which are not dependent directly on its enzymatic activity. As a scaffolding protein giving specificity to different protein-protein interactions or by regulating nuclear transport. The regulated nuclear import of transcription factors provides an efficient mechanism to restrict their access to the transcriptional machinery. The involvement of a Ubc9 homologue in nuclear import during Drosophila embryogenesis has been reported (Epps and Tanda, 1998). Mutation of the Drosophila semushi (semi) gene, which encodes an E2 enzyme homologous to Ubc9, blocks nuclear import of the transcription factor Bcd during early embryogenesis and results in misregulation of the segmentation genes that are Bcd targets (Epps and Tanda, 1998). Ubc9 may either regulate transport by modification of pore proteins or modify nuclear proteins in a transport coupled manner.
One feature common to many of the transcription factors which interact with Ubc9 is the mechanism of regulation. ATF2, p53, C-Jun and NF-κB are transcription factors whose activity is regulated by ubiquitin-dependent proteolysis. IκBα, the inhibitor of NF-κB, and p53 are both modified by SUMO-1 (Table 2) and in both cases SUMO-1 conjugation increases protein stability. This evidence may be particularly pertinent in view of several lines of similarity between p53 and NF-κB.

<table>
<thead>
<tr>
<th>Ubc9 interacting protein</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clb2,Clb5</td>
<td>M- and S-phase cyclins</td>
<td>(Blondel and Mann, 1996; Seufert et al., 1995)</td>
</tr>
<tr>
<td>E1A</td>
<td>Adenovirus oncprotein</td>
<td>(Hateboer et al., 1996)</td>
</tr>
<tr>
<td>E2A</td>
<td>Transcription factor</td>
<td>(Kho et al., 1997; Loveys et al., 1997)</td>
</tr>
<tr>
<td>human Rad51/Rad52</td>
<td>DNA repair</td>
<td>(Kovalenko et al., 1996; Shen et al., 1996b)</td>
</tr>
<tr>
<td>human papillomavirus Type 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>centromere DNA-binding core complex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative regulatory domain of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>the Wilm's Tumor gene product</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(WT1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fas antigen (CD95)</td>
<td>Receptor (Tumor necrosis</td>
<td>(Becker et al., 1997; Wright et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>factor receptor family</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>Transcription factor</td>
<td>(Shen et al., 1996b)</td>
</tr>
<tr>
<td>c-Jun</td>
<td>Transcription factor</td>
<td>(Gottlicher et al., 1996)</td>
</tr>
<tr>
<td>Glucocorticoid Receptor (GR)</td>
<td>Transcription factor</td>
<td>(Gottlicher et al., 1996)</td>
</tr>
<tr>
<td>TEL</td>
<td>Transcription factor (repressor of gene</td>
<td>(Chakrabarti et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>transcription)</td>
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</tr>
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</table>

112
<table>
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<tr>
<th>Protein/Complex</th>
<th>Function/Role</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETS1</td>
<td>Transcription factor (Ras and Ca2+ signalling pathways)</td>
<td>(Hahn et al., 1997)</td>
</tr>
<tr>
<td>MEKK1</td>
<td>Kinase (stress activated protein kinase pathway)</td>
<td>(Saltzman et al., 1998)</td>
</tr>
<tr>
<td>Type I TNFα receptor</td>
<td>Receptor</td>
<td>(Saltzman et al., 1998)</td>
</tr>
<tr>
<td>RanBP2</td>
<td>Nuclear transport</td>
<td>(Saitoh et al., 1997)</td>
</tr>
<tr>
<td>ATF2</td>
<td>Activating transcription factor 2 (ATF/CREB family)</td>
<td>(Firestein and Feuerstein, 1998)</td>
</tr>
<tr>
<td>mouse cRel</td>
<td>Transcription factor</td>
<td>(Tashiro et al., 1997)</td>
</tr>
<tr>
<td>IκBα</td>
<td>Inhibitor of NF-κB transcription factor</td>
<td>(Desterro et al., 1998)</td>
</tr>
<tr>
<td>human bleomycin hydrolase</td>
<td>Cysteine protease</td>
<td>(Koldamova et al., 1998)</td>
</tr>
<tr>
<td>AR, androgen receptor</td>
<td>Member of the steroid receptor family of ligand-activated transcription factors</td>
<td>(Poukka et al., 1999)</td>
</tr>
<tr>
<td>Hsp23, Hsp26, Hsp27</td>
<td>Heat shock proteins</td>
<td>(Joanisse et al., 1998)</td>
</tr>
<tr>
<td>Poly(ADP-ribose) polymerase</td>
<td>DNA replication/DNA repair</td>
<td>(Masson et al., 1997)</td>
</tr>
<tr>
<td>Cor1, Syn1</td>
<td>Synoptosomal complex components</td>
<td>(Tarsounas et al., 1997)</td>
</tr>
<tr>
<td>N-terminal transcription repression domains of Groucho</td>
<td>Corepressor</td>
<td>Ohsako, 1999, Jbioch</td>
</tr>
<tr>
<td>PML</td>
<td>Nuclear phosphoprotein component of PML nuclear bodies</td>
<td>(Boddy et al., 1996; Muller et al., 1998)</td>
</tr>
<tr>
<td>Sentrin (SUMO-1)</td>
<td>Ubiquitin-like molecule (sentrination)</td>
<td>(Gong et al., 1997)</td>
</tr>
<tr>
<td>UBL1 (SUMO-1)</td>
<td>Ubiquitin-like protein</td>
<td>(Shen et al., 1996b)</td>
</tr>
<tr>
<td>Daxx</td>
<td>Enhances Fas-mediated apoptosis</td>
<td>(Hancock et al., 1999)</td>
</tr>
</tbody>
</table>

Table 1. Ubc9 interacting proteins and respective function
The identified proteins that have been found to be subject to modification by SUMO-1 are shown in Table 2. Most of the proteins can be found, at least in part, inside the nucleus (Table 2). Although the cellular localisation of SUMO-1 modified IκBα has not been determined, IκBα is predominantly cytoplasmic and newly synthesised IκBα is translocated into the nucleus (Arenzana-Seisdedos et al., 1995) where is resistant to signal induced degradation (Rodriguez et al., 1999); SUMO-1 modified RanGAP1 is associated with the cytoplasmic fibbers of the NPC but it also appeared to associate with the mitotic spindle apparatus during mitosis (Mahajan et al., 1997; Matunis et al., 1996); unmodified PML and Sp100 localise to the soluble nucleoplasm and cytoplasm respectively, but SUMO-1 modified PML and Sp100 are both found in large nuclear multi-protein complexes known as PML nuclear bodies (Duprez et al., 1999; Hodges et al., 1998; Muller et al., 1998; Sternsdorf et al., 1997); p53, like IκBα, shuttles between the nucleus and the cytoplasm and blockage of nuclear export by Leptomycin B results in accumulation of p53, mdm2 complexes in subnuclear domains adjacent to the PML nuclear bodies (Lain et al., 1999). Taking this into account, one could hypothesise that SUMO-1 modification could involve nuclear import as an obligatory step. Whether the modification occurs in the nucleoplasm, during nuclear import or in the nuclear pore it remains to be shown, but recent observations on localisation of the SUMO-1 conjugation enzymes, SAE and Ubc9, support the hypothesis that the modification itself occurs in the nucleus. The mutant Sp100(447E) containing a non-functional NLS was severely compromised in SUMO-1 modification, which indicates that modification of Sp100 by SUMO-1 is tightly linked to nuclear localisation and gives strength to the above hypothesis (Sternsdorf et al., 1999).

Comparison of the sequences surrounding the acceptor lysines in proteins that have been shown to be modified by SUMO-1 reveals a striking similarity (Figure 32). Apart from the absolutely conserved SUMO-1 acceptor lysine, there appears to be an absolute requirement for a hyrophobic residue and a Glu on either site of the acceptor lysine. The consensus sequence ψKxE, may represent a
## SUMO-1 CONJUGATED PROTEINS

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ubc9 interacting protein</th>
<th>SUMO-1 interacting protein</th>
<th>Cellular localisation</th>
<th>Lysine required for modification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RanGAP-1</td>
<td>+</td>
<td>+</td>
<td>Cytoplasm</td>
<td>K526</td>
<td>(Matunis et al., 1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NPC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mitotic spindles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PML</td>
<td>-</td>
<td>+</td>
<td>Nuclear</td>
<td>K487 K490</td>
<td>(Boddy et al., 1996; Duprez et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>bodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp100</td>
<td>-</td>
<td>-</td>
<td>Nuclear</td>
<td>K297</td>
<td>(Sternsdorf et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>bodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IκBα</td>
<td>+</td>
<td>-</td>
<td>Shutting</td>
<td>K21</td>
<td>(Desterro et al., 1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cytoplasm/nucleus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>+</td>
<td>-</td>
<td>Shutting</td>
<td>K386</td>
<td>(Rodriguez et al., 1999; Shen et al., 1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cytoplasm/nucleus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV IE1</td>
<td>-</td>
<td>-</td>
<td>Nuclear</td>
<td>Unid.</td>
<td>(Muller and Dejean, 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>bodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdc3</td>
<td>-</td>
<td>-</td>
<td>Nuclear</td>
<td>Unid.</td>
<td>(Takahashi et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>bud neck</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daxx</td>
<td>+</td>
<td>+</td>
<td>Nuclear</td>
<td>Unid.</td>
<td>(Hancock et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>bodies centromeres</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. SUMO-1 conjugated proteins.**

Substrates that have been shown to be modified by SUMO-1. The ability of each substrat to interact (+) with either Ubc9 or SUMO-1 is shown. The cellular localization and the lysine required for SUMO-1 conjugation is mentioned. Unid-unidentified.
recognition site for the SUMO-1 conjugation machinery. This consensus sequence is an useful tool in the identification of additional substrates for SUMO-1 modification.

<table>
<thead>
<tr>
<th>RanGAP-1</th>
<th>523</th>
<th>L</th>
<th>K</th>
<th>S</th>
<th>E</th>
<th>D</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>IkBα</td>
<td>19</td>
<td>G</td>
<td>L</td>
<td>K</td>
<td>K</td>
<td>E</td>
<td>R</td>
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<tr>
<td>PML</td>
<td>488</td>
<td>V</td>
<td>I</td>
<td>K</td>
<td>M</td>
<td>E</td>
<td>S</td>
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<tr>
<td>PML</td>
<td>158</td>
<td>F</td>
<td>L</td>
<td>K</td>
<td>H</td>
<td>E</td>
<td>A</td>
</tr>
<tr>
<td>SP100</td>
<td>295</td>
<td>D</td>
<td>I</td>
<td>K</td>
<td>K</td>
<td>E</td>
<td>K</td>
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<td>P53</td>
<td>384</td>
<td>M</td>
<td>F</td>
<td>K</td>
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<td>Consensus</td>
<td></td>
<td>-</td>
<td>K</td>
<td>X</td>
<td>E</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Figure 32. Consensus sequence for modification by SUMO-1.**

Alignment of SUMO-1 conjugation sites known to date. An asterisk indicates the lysine residue covalently modified by SUMO-1. Identical aminoacids are in the dark shading and similarities are in the light shading.

SUMO-1 modifications have been implicated in the targeting of proteins to a particular subcellular compartment and in converting proteins into a form that is resistant to ubiquitin-mediated degradation. In yeast the enzymes required for SUMO-1 conjugation and deconjugation are both essential and their inactivation leads to a G2/M block (Li and Hochstrasser, 1999; Seufert et al., 1995). Since SUMO-1 conjugation of PML and SplO0 is regulated in a cell cycle dependent manner (Roger Everrett, personal communication) it is likely that SUMO-1 modification is a dynamic mechanism by which the cell controls diverse processes, including cell cycle, differentiation and virus infection.

Evidence that the SUMO-1 modification state of target proteins may change during certain cell cycle stages was demonstrated with PML. During mitosis PML and Sp100 become deconjugated and, by this mechanism might regulate PML nuclear bodies disassembly, which occurs during this phase of the cell cycle (Sternsdorf et al., 1997). SUMO-1 plays an important role in the biological activity of nuclear body associated proteins and/or may promote the assembly of these and maybe other nuclear structures. These nuclear structures may represent storage sites.
for proteins and nuclear factors, which can be liberated in the nucleoplasm for cellular processes such as transcription or cell division.

Several viral proteins associate with PML bodies and interfere with the organisation of these structures. Infection with herpes simplex virus 1 (HSV-1 Vmw110 or ICP0), adenovirus (E4ORF3) and human cytomegalovirus (IE1) disrupts PML bodies (Carvalho et al., 1995; Everett and Maul, 1994; Everett et al., 1997; Kelly et al., 1995; Maul et al., 1993). Although the biological significance of the destruction of PML nuclear bodies upon viral infection is still unclear, an attractive but speculative hypothesis is that viral proteins interfere with the cellular SUMO-1 conjugation/deconjugation pathway. CMV IE1 can specifically abrogate the SUMO-1 modification of PML and Sp100. It has been shown that IE1 protein itself is a substrate for SUMO-1 modification (Muller and Dejean, 1999). Although it represents the first viral protein to undergo this posttranslational modification, a large number of other viral proteins are potential substrates and one could speculate that viral and cellular proteins may compete for the same pool of SUMO-1 during virus infection and thus interfere with cellular processes.

The HSV-1 Vmw110 immediate-early regulatory protein induces the proteasome-dependent degradation of several cellular proteins. Vmw110 associates with PML bodies, which are disrupted within a few hours as a consequence of degradation of several PML forms in a proteasome dependent fashion (Chelbi-Alix and de The, 1999; Everett et al., 1998; Everett and Maul, 1994; Maul and Everett, 1994; Maul et al., 1993). Vmw110 also binds to centromeres and induces proteasome dependent degradation of CENP-C, a centromeric protein. CENP-C degradation disrupts kinetochore structure and induces profound defects in mitotic events (Everett et al., 1999). There are striking similarities between the interactions of Vmw110 with the PML bodies and centromeres and one could speculate that SUMO-1 conjugation or deconjugation might have a role in both processes, creating a possible connection between these nuclear structures. It would be interesting to determine if CENP-C is modified by SUMO-1. The destruction of CENP-C may be
important for the virus to prevent the cell from aborting the viral infection by inducing an apoptotic response.

Centromere assembly, like PML bodies, may be regulated by a mechanism involving SUMO-1 or a similar ubiquitin-like protein modification. Smt3p, the yeast homologue of SUMO-1, also becomes conjugated to several proteins posttranslationally (Johnson et al., 1997). It was originally isolated as high copy suppressor of mutations in the MIF2 gene (Meluh and Koshland, 1995) which encodes a CENP-C like centromere-binding protein, required for normal DNA replication and mitotic spindle integrity in yeast (Brown et al., 1993; Meluh and Koshland, 1995). Whether Mif2p is a target for Smt3p has not yet been determined. The first identified target for this modification in yeast is Cdc3, a component of septin rings at the mother-bud neck (Takahashi et al., 1999). Although, the biological significance of Smt3p-conjugation is not well understood a function similar to SUMO-1 conjugation is expected as SUMO-1 can complement yeast smt3 null mutants (Johnson et al., 1997). The most plausible function for Smt3p conjugated Cdc3 involves its stability. Cdc3 is conjugated to Smt3p during the cell cycle but is unstable during cytokinesis and this instability may be related with disassembly of the septin structure. The possible involvement of SUMO-1 pathway in centromere assembly is also demonstrated by the specific interaction of Ubc9 with all three subunits of the Saccharomyces cerevisiae centromere DNA-binding core complex, CBF3 (Jiang and Koltin, 1996). Thus, it appears very likely that the function of Ubc9p in the G2/M phase of S. cerevisiae could be related to regulation of centromere proteins in chromosome segregation during mitosis. In S. pombe, pmt3p, homologue of Smt3p is required for a number of nuclear events including the control of telomere length and kinetochore functions involved in chromosome segregation (Katsunori et al., 1999). hDaxx can localise with CENP-C during interphase (Pluta et al., 1998). hDaxx is a human homologue of a murine protein originally identified as a mediator of the Fas/CD95 apoptotic pathway is a substrate for SUMO-1 modification (Hancock et al., 1999).
Recent studies describing a SUMO-1/Smt3 deconjugating enzyme in yeast, Ulp1, suggest the presence of SUMO-1 deconjugating homologues in mammalian cells and it appears that SUMO-1 conjugation and deconjugation of target proteins is a dynamic cell cycle regulated process. Deconjugation from one or more proteins by Ulp1 is required for normal cell cycle progression but conjugation must also positively regulate the G2/M transition, as Ubc9 mutants show cell cycle arrest. Different targets and timings for SUMO-1/Smt3p conjugation may explain these negative and positive cell-cycle regulatory effects. Although human HAUSP (herpes associated ubiquitin-specific protease) has been localised in the nuclear bodies (Everett et al., 1997), that contain SUMO-1 modified proteins, the involvement of this enzyme in the deconjugation of SUMO-1 has not been described and human SUMO-1 deconjugating enzymes have yet to be characterised.

As virtually all SUMO-1 in the cell is conjugated to target proteins, changes in the SUMO-1 modification state of individual proteins must involve not only the conjugation enzymes SAE and Ubc9 but also the deconjugating enzymes (SUMO-1 specific proteases, SSPs). Thus, it is likely that these enzymes are subject to highly specific regulation. These control mechanisms are likely to operate during the cell cycle and be responsive to signals from the extracellular environment. As the nuclear DNA viruses all disrupt the normal cell cycle it is perhaps not surprising that they have also targeted the SUMO-1 modification pathway. Using the tools described in this thesis it is hoped that the next few years will reveal the details of how proteins are selected, in time and at specific subcellular locations, for SUMO-1 modification.
5. Appendix I  Vector Maps
Comments for pcDNA3 (5446 nucleotides)
Supplier: Invitrogen
Accession number: IG1046
CMV promoter: bases 209-863
T7 promoter: bases 864-882
Polylinker: bases 889-994
Sp6 promoter: bases 999-1016
BGH poly A: 1018-1249
SV40 promoter: bases 1790-2115
SV40 origin of replication: bases 1984-2069
NeoR ORF: bases 2151-2932
SV40 poly A: bases 3120-3250
pUC19 backbone: bases 3272-5446
AmpR ORF: bases 4450-5310
Cloning primers:
Upstream (BamHI): 5'-ACA AAC CAT GGA TCC ATG* TCG GGG ATC GCC CTC AGY
Downstream (Xhol): 5'-TCC GAG CTC GAG TTA* GTC CAA TCC CAG CAA AGG GTT TGG GAT TGA GGG CGC CGC AAA CTT CTT GGC-3'

Ubch9:
Organism: Homo sapiens
Coding region: 474 bp
GB accession number: NM_003345, U29092, U31882, AJ002385, U31933, X96427.

Antibodies:
Mouse 336 monoclonal antibody against PK-SV5 peptide (IPNPLLGLG)
Sheep anti-Ubch9 polyclonal antibody against Ubch9 c-terminal peptide (CEYEKRVRAQAKKFAPS)
Cloning primers:
Upstream (BamHI): 5'-AAC GGA TCC ATG* GTG GAG AAG GAG GAG GCT GGC-3'
Downstream (EcoRI): 5'-G CGG GAA TTC TCA*CTT GGG GCC AAG GCA CTC CAC AA-3'

SAE1:
Subunit of SUMO-1 activating enzyme
Organism: Homo sapiens
Coding region: 1041bp
GB accession number: NP_005491, AAD24433

Antibodies:
Sheep anti-SAE1 polyclonal antibody against recombinant SAE1
**Cloning primers**
Upstream (EcoRI): 5'-GAG GAA TTC ATG* GCA CTG TCG CGG GGG CTG-3'
Downstream (EcoRI): 5'-GAG GAA TTC TCA* ATC TAA TGC TAT GAC ATC-3'

**SAE2:**
Subunit of SUMO-1 activating enzyme
Coding region: 1923 bp
GB accession number: NP_005490, AAD24434
Cloning primers:
Upstream (KpnI): 5'-CAT GGG AAA GCC GAT CCC AAA CCC TTT GCT GGG ATT GGA CTC CAC CG-3'

Downstream (BamHI): 5'-GAT CCG GTG GAG TCC AAT CCC AGC AAA GGG TTT GGG ATC GGC TTT CCC ATG GTA C-3'

PK-SV5 tag:
Peptide: IPNPLLGLE
The tag linker codes amino acids 95 to 108 derived from SV5 P and V proteins.

Antibodies:
Mouse 336 monoclonal antibody against PK-SV5 peptide obtained from R.E. Randall, University of St Andrews (Hanke et al., 1992).
Cloning primers:
Upstream (BamHI): 5'-ACA AAC GGA TCC ATG* TCT GAC CAG GAG GCC AAA-3'
Downstream (BamHI): 5'-GCC GAG GGA TCC CTA* ACC CCC CGT TTG TTC CTG-3'

SUMO-1 (1-97 a.a.):
C-terminus cleaved form of SUMO-1, which allows it to be conjugated to other proteins via the conserved C-terminal Gly residue
Organism: Homo sapiens
Coding region: 291 bp,
GB accession number: NP_003343, Q93068, AAC50996.

Antibodies:
Mouse 336 monoclonal antibody against PK-SV5 peptide obtained from R.E. Randall, University of St Andrews (Hanke et al., 1992).
Mouse 21C7 monoclonal antibody against full-length recombinant GMP1 protein obtained from Zymed (Cambridge Bioscience).
**Cloning primers:**
Upstream (BamHI): 5'-AAC GGA TCC ATG* GTG GAG AAG GAG GAG GCT GGCG-3'

Downstream (EcoRI): 5'-G CGG GAA TTC TCA*CTT GGG GCC AAG GCA CTC CAC AA-3'

**SAE1:**
Subunit of SUMO-1 activating enzyme
Organism: Homo sapiens
Coding region: 1041 bp
GB accession number: NP_005491, AAD24433

**Antibodies:**
Mouse 336 monoclonal antibody against PK-SV5 peptide obtained from R.E. Randall, University of St Andrews (Hanke et al., 1992)

Sheep anti-SAE1 polyclonal antibody against recombinant SAE1
**Cloning primers:**
Upstream (Kpnl): 5'-C ATG GCT TCA TAT CCT TAC GAT GTT CCA GAC TAC GCT TCC CTT G -3'

Downstream (BamHI): 5'-GA TCC AAG GGA AGC GTA GTC TGG AAC ATC GTA AGG ATA TGA AGC CAT GGT AC-3'

**HA tag:**
Peptide: YPYDVPDYA
The tag linker codes for a 9 aminoacid peptide from influenza hemagglutinin protein.

**Antibodies:**
Mouse 12CA5 monoclonal antibody against HA peptide obtained from Babco.
Cloning primers:
Upstream (BamHI): 5'-ACA AAC GGA TCC ATG* TCT GAC CAG GAG GCC AAA-3'
Downstream (BamHI): 5'-GCC GAG GGA TCC CTA* ACC CCC CGT TTG TTC CTG-3'

SUMO-1 (1-97 a.a.):
C-terminus cleaved form of SUMO-1, which allows it to be conjugated to other proteins via the conserved C-terminal Gly residue
Organism: Homo sapiens
Coding region: 291 bp,
GB accession number: NP_003343, Q93068, AAC50996.

Antibodies:
Mouse 12CA5 monoclonal antibody against HA peptide obtained from Babco.
Mouse 21C7 monoclonal antibody against full-length recombinant GMPI protein obtained from Zymed (Cambridge Bioscience).
**Cloning primers:**
Upstream (BamH1): 5'-G ATC CAT GGC TTC ATA TCC TTA CGA TGT TCC AGA CTA CGC TTC CCT TG-3'

Downstream (EcoRI): 5'-AAT TCA AGG GAA GCG TAG TCT GGA ACA TCG TAA GGA TAT GAA GCC ATG-3'

**HA tag:**
Peptide: YPYDVPDYA
The tag linker codes for a 9 aminoacid peptide from influenza hemagglutinin protein.

**Antibodies:**
Mouse 12CA5 monoclonal antibody against HA peptide obtained from Babco.
Cloning primers
Upstream (EcoRI): 5'-GAG GAA TTC ATG* GCA CTG TCG CGG GGG CTG-3'
Downstream (EcoRI): 5'-GAG GAA TTC TCA* ATC TAA TGC TAT GAC ATC-3'

SAE2:
Subunit of SUMO-1 activating enzyme
Coding region: 1923 bp
GB accession number: NP_005490, AAD24434

Antibodies:
Mouse 12CA5 monoclonal antibody against HA peptide obtained from Babco.
Cloning primers:
Upstream (KpnI): 5’-CAT GGC TCA TCA TCA TCA TCA TCA TGG TG-3’

Downstream (BamHI): 5’-GAT CCA CCA TGA TGA TGA TGA TGA GCC ATG GTA C-3’

6XHis tag:
The tag linker codes for 6 histidines which enables affinity purification of 6xHis-tagged proteins on Ni-NTA-agarose beads.
**Cloning primers:**
Upstream (BamHI): 5'-ACA AAC **GGA** TCC ATG* TCT GAC CAG GAG GCC AAA-3'

Downstream (BamHI): 5'-GCC GAG **GGA** TCC CTA* ACC CCC CGT TTG TTC CTG-3'

**SUMO-1 (1-97 a.a.):**
C-terminus cleaved form of SUMO-1, which allows it to be conjugated to other proteins via the conserved C-terminal Gly residue

**Organism:** Homo sapiens
**Coding region:** 291 bp,
**GB accession number:** NP_003343, Q93068, AAC50996.

The expression system provides possible affinity purification on Ni-agarose beads of proteins conjugated to 6xHis-SUMO-1
Comments for pGEX-2T (4948 nucleotides)

**Supplier:** Pharmacia

Accession number: U13850

tac promoter-10: bases 205-211

tac promoter-35: bases 183-188

lac operator: bases 217-237

Start codon for GST: base 258

Ampr ORF: bases 1356-2214

lacIq ORF: bases 3297-4377

Site of replication initiation: base 2974

Multiple Cloning Site: bases 930-945

For inducible, high level expression of genes as fusions with glutathione S-transferase (GST) gene from *Schistosoma japonicum*, which forms an affinity tail on the protein products of genes inserted into the multiple cloning site (MCS). Expression is under the control of a tac promoter which enables inducible, high-level production of fusion proteins.
**Cloning primers:**

**Upstream (BamHI):** 5'-ACA AAC GGA TCC ATG* TCT GAC CAG GAG GCC AAA-3'

**Downstream (BamHI):** 5'-GCC GCG GGA TCC CTA* AAC TGT TGA ATG ACC-3'

**SUMO-1:**
- **Organism:** Homo sapiens
- **Coding region:** 303 bp
- **GB accession number:** NP_003343, Q93068, AAC50996

**Antibodies:**
- Mouse 21C7 monoclonal antibody against full-length recombinant GMP-1 protein obtained from Zymed (Cambridge Bioscience)

**Bacterial Expression:**
- Expressed as a GST fusion protein in *E. coli* B834
Cloning primers:
Upstream (BamHI): 5'-ACA AAC GGA TCC ATG* TCT GAC CAG GAG GCC AAA-3'
Downstream (BamHI): 5'-GCC GAG GGA TCC CTA* ACC CCC CGT TTG TTC CTG-3'

SUMO-1 (1-97 a.a.):
C-terminus cleaved form of SUMO-1, which allows it to be conjugated to other proteins via the conserved C-terminal Gly residue
Organism: Homo sapiens
Coding region: 291 bp,
GB accession number: NP_003343, Q93068, AAC50996.

Antibodies:
Mouse 21C7 monoclonal antibody against full-length recombinant GMP1 protein obtained from Zymed (Cambridge Bioscience).

Bacterial expression:
Expressed as a GST fusion protein in E. coli B834.
Cloning primers:
Upstream (BamHI): 5'-ACA AAC GGA TCC ATG* TCG GGG ATC GCC CTC AGC-3'

Downstream (EcoRI): 5'-GCC GCG GAA TTC TTA* TGA GGG CGC AAA CTT CTT GGC-3'

Ubch9:
Organism: Homo sapiens
Coding region: 474 bp
GB accession number: NM_003345, U29092, U31882, AJ002385, U31933, X96427.

Antibodies:
Sheep anti-Ubch9 polyclonal antibody against Ubch9 C-terminal peptide (CEYEKRVRAQAKKFAPS)

Bacterial Expression:
Expressed as a GST fusion protein in E. coli DH5α
Cloning primers:
Upstream (BamHI): 5'-ACA AAC GGA TCC ATG* GCG CTG AAG AGG ATT CAG-3'
Downstream (EcoRI): 5'-GCC GCG GAA TTC TTA* CAT TGC ATA TTT CTG AGT CC-3'

Ubch5:
Ubiquitin conjugating enzyme
Organism: Homo sapiens
Coding region: 459 bp
GB accession number: X78140

Bacterial Expression:
Expressed as a GST fusion protein in *E. coli* DH5α. The recombinant GST-Ubch5 is not digested by Thrombin
Cloning primers:
Upstream (BamHI): 5'-AAC GGA TCC ATG* GTG GAG AAG GAG GAG GCT GGC-3'

Downstream (EcoRI): 5'-G CGG GAA TTC TCA* CTT GGG GCC AAG GCA CTC CAC AA-3'

SAE1:
Subunit of SUMO-1 activating enzyme
Organism: Homo sapiens
Coding Region: 1041 bp
GB accession number: NP_005491, AAD24433

Antibodies:
Sheep anti-SAE1 polyclonal antibody against recombinant SAE1

Bacterial expression:
Expressed as a GST fusion protein in E.coli B834
**Linker primers:**
Upstream: 5'-GAT CCG CAG GTT CCG-3'
Downstream: 5'-GAT CCG GAA CCT GCG-3'

**Linker:**
The linker codes for a 5 aminoacid peptide (GSAGS) between GST and Ubch5, to facilitate a better access to the Thrombin cleavage site.

**Bacterial expression:**
Expressed as a GST fusion protein in *E.coli* B834.
6. Bibliography


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7. Publications


