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Control of NF–κB transcriptional activation by signal induced proteolysis of I κBα

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Phil. Trans. R. Soc. Lond. B 1999 **354**, 1601-1609
doi: 10.1098/rstb.1999.0504

THE ROYAL SOCIETY

Control of NF-κB transcriptional activation by signal induced proteolysis of IκBα

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In unstimulated cells the transcription factor NF-κB is held in the cytoplasm in an inactive state by IκB inhibitor proteins. Ultimately activation of NF-κB is achieved by ubiquitination and proteasome-mediated degradation of IκBα and we have therefore investigated factors which control this proteolysis. Signal-induced degradation of IκBα exposes the nuclear localization signal of NF-κB, thus allowing it to translocate into the nucleus and activate transcription from responsive genes. An autoregulatory loop is established when NF-κB induces expression of the IκBα gene and newly synthesized IκBα accumulates in the nucleus where it negatively regulates NF-κB-dependent transcription. As part of this post-induction repression, the nuclear export signal on IκBα mediates transport of NF-κB–IκBα complexes from the nucleus to the cytoplasm. As nuclear export of IκBα is blocked by leptomycin B this drug was used to examine the effect of cellular location on susceptibility of IκBα to signal-induced degradation. In the presence of leptomycin B, IκBα is accumulated in the nucleus and in this compartment is resistant to signal-induced degradation. Thus signal-induced degradation of IκBα is mainly, if not exclusively a cytoplasmic process. An efficient nuclear export of IκBα is therefore essential for maintaining a low level of IκBα in the nucleus and allowing NF-κB to be transcriptionally active upon cell stimulation. We have detected a modified form of IκBα, conjugated to the small ubiquitin-like protein SUMO-1, which is resistant to signal-induced degradation. SUMO-1 modified IκBα remains associated with NF-κB and thus overexpression of SUMO-1 inhibits the signal-induced activation of NF-κB-dependent transcription. Reconstitution of the conjugation reaction with highly purified proteins demonstrated that in the presence of a novel E1 SUMO-1 activating enzyme, Ubch9 directly conjugated SUMO-1 to IκBα on residues K21 and K22, which are also used for ubiquitin modification. Thus, while ubiquitination targets proteins for rapid degradation, SUMO-1 modification acts antagonistically to generate proteins resistant to degradation.

Keywords: IκBα modification; NF-κB activation; SUMO-1; ubiquitin

1. INTRODUCTION

To a large extent, gene expression is controlled by the frequency of transcriptional initiation at the promoter. In many cases the rate at which transcription initiates is limited by the availability or activity of DNA binding upstream activators. One such upstream activator is the transcription factor NF-κB, whose DNA-binding activity and cellular distribution are controlled by the IκB inhibitor proteins. In unstimulated cells, NF-κB is held in the cytoplasm, in a form that is unable to bind DNA, by IκB. Exposure of cells to a wide variety of stimuli results in release of the transcription factor from IκB, allowing the active DNA binding form of NF-κB to translocate to the nucleus where it binds to its recognition sites in the upstream regions of a wide variety of genes. In vertebrates, the NF-κB family of proteins is composed of transcriptionally active p65/Rel A (Nolan *et al.* 1991; Ruben *et al.* 1991), c-Rel (Wilhelmsen *et al.* 1984) and Rel B (Ryseck *et al.* 1992), and p50/NF-κB1 (Ghosh *et al.* 1990; Kieran *et al.* 1990) and p52/NF-κB2 (Bours *et al.* 1992; Neri *et al.* 1991; Schmid *et al.* 1991), which lack transcriptional activation domains. All NF-κB proteins share a conserved region known as the Rel homology domain (RHD), which contains the nuclear localization signal (NLS), as well as the dimerization and DNA-binding functions. Typically, the NF-κB form activated by extracellular signals is composed of p50 and p65. NF-κB transcriptional activity is controlled by inhibitor IκB proteins, which contain ankyrin repeat domains (ARD). The trimeric association of p50/p65 and IκB not only occludes the nuclear localization sequence of p50 and p65, leading to cytoplasmic sequestration, but also prevents NF-κB DNA-binding activity. Several IκBs have been described, including IκBα (Haskill *et al.* 1991), IκBβ (Thompson *et al.* 1995), IκBε (Whiteside *et al.* 1997) and Bcl-3 (Ohno *et al.* 1990). Additionally, the precursors of p50 (p105) and p52 (p100) possess inhibitory ARDs, which in isolation are known as IκBγ (Blank *et al.* 1991; Inoue *et al.* 1992; Liou *et al.* 1992) and IκBδ (Mercurio *et al.* 1992, 1993), respectively. Among the most important activators of NF-κB are the proinflammatory cytokines interleukin 1 (IL1) and tumour necrosis factor α (TNF) that are produced as a result of pathogenic stimulation. The role of NF-κB in transmitting signals from the extracellular environment to the cell nucleus is to initiate a new programme of gene expression in the stimulated cell. Although the list is incomplete, the genes activated by NF-κB include interferons, cytokines, acute phase proteins, cell adhesion molecules, interleukin receptors

and histocompatibility antigens. One of the responses to NF-κB is expression of a bank of genes that block apoptosis. As TNF initiates an apoptotic and an anti-apoptotic response via activation of NF-κB, the life or death of a cell exposed to TNF will depend on the balance between the two pathways (reviewed in Baeuerle 1998). Targeted disruption of genes coding for NFKBl, rel B and c-rel results in transgenic animals, which develop normally but have defects in immune and inflammatory responses. In contrast, p65 knockout mice are embryonic lethal due to massive liver apoptosis, while IκBα knockout mice die within eight days of birth from extensive postnatal granulopoiesis (Verma *et al*. 1995). Although an important mediator in the defence against pathogens, NF-κB also transmits signals that contribute dramatically to the progress of diseases such as acquired immune deficiency syndrome, sepsis, toxic shock and arthritis (to name but a few). NF-κB and IκB have therefore attracted considerable interest, both in academic laboratories and in the pharmaceutical industry, as potential targets for anti-inflammatory and immunosuppressive drugs.

As signals which activate NF-κB act by targeting IκBα for proteolysis, this paper will focus on the cellular mechanisms which influence the susceptibility of IκBα to signal-induced degradation.

2. SIGNAL-INDUCED DEGRADATION OF IκBα

The IκBα molecule contains a central domain of six ankyrin repeats, connected to an unstructured N-terminal extension and a complex C-terminal region (Jaffray *et al*. 1995). Following signal induction IκBα is rapidly phosphorylated and degraded (Beg *et al*. 1993; Henkel *et al*. 1993; Mellits *et al*. 1993). Sites of inducible phosphorylation are located within the N-terminal domain on residues S32 and S36 and mutation of these residues blocks signal-induced degradation (Brockman *et al*. 1995; Brown *et al*. 1995; DiDonato *et al*. 1996; Roff *et al*. 1996; Traenckner *et al*. 1995). The question of how signals, initiated by binding of TNF or IL-1 to their receptors, are transmitted from the membrane surface to IκBα has recently been answered (figure 1). Binding of TNF to the extracellular domain of the TNF receptor 1 (TNFR1) induces aggregation of the receptor via its cytoplasmic death domains (DDs), which in turn act as a target for the assembly of a large complex containing the TNFR-associated DD protein (TRADD), the TNFR-associated factor 2 (TRAF2) and the receptor-interacting protein (RIP), which is a serine–threonine kinase. Likewise, ligation of IL-1 to its receptor (IL-1R) causes receptor aggregation and recruitment of an accessory protein (AcP), TRAF6 and the IL-1R-associated kinase (IRAK) (reviewed in Baeuerle 1998). The common target for these membrane-associated protein complexes is the NF-κB-inducing kinase (NIK) (Malinin *et al*. 1997), which is directly upstream from the IκB kinase (IKK). IKK contains two subunits which are both necessary for efficient phosphorylation of IκBα and NF-κB activation (DiDonato *et al*. 1997; Mercurio *et al*. 1997; Regnier *et al*. 1997; Woronicz *et al*. 1997; Zandi *et al*. 1997). Transmission of signals to IκBα appears to be facilitated by the scaffold proteins IKAP (Cohen *et al*. 1998) and NEMO (Yamaoka *et al*. 1998) or IKKγ (Rothwarf *et al*. 1998), which assemble the various molecules into a physically associated signal-

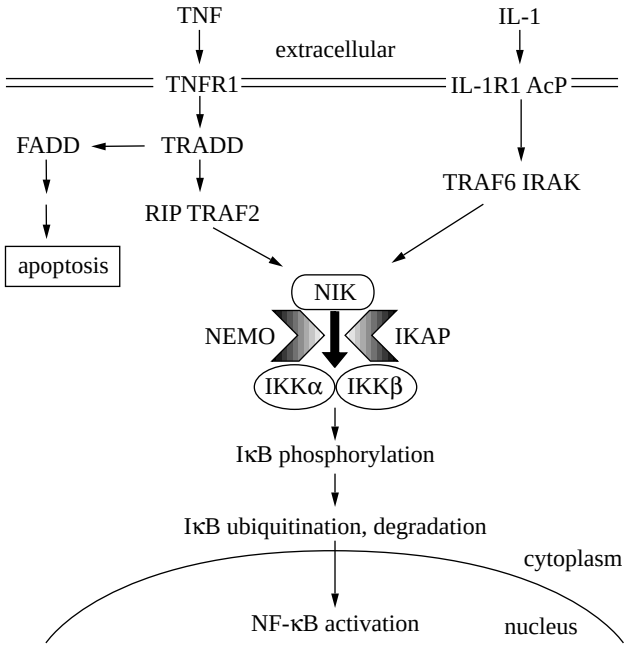

Figure 1. Pathway by which signals from TNFα and IL-1β induce IκBα degradation and NF-κB activation.

ling module. 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 (Alkalay *et al*. 1995; Chen *et al*. 1995; Li *et al*. 1995; Roff *et al*. 1996). Mutational analysis has indicated that K21 and K22 are the primary sites for addition of multiubiquitination chains with K38 and K47 as secondary sites (Baldi *et al*. 1996; Rodriguez *et al*. 1996; Scherer *et al*. 1995). Although signal-induced phosphorylation and ubiquitination of IκBα take place on the N-terminus of the protein, deletion of the C-terminus of IκBα renders the protein resistant to signal-induced degradation (Brown *et al*. 1995; Rodriguez *et al*. 1995; Sun *et al*. 1996; Whiteside *et al*. 1995). It is thought that the C-terminus of IκBα functions post-ubiquitination via interactions with the catalytic core of the proteasome (Kroll *et al*. 1997).

Ubiquitin addition is accomplished via a thioester cascade, with ubiquitin first being activated by a unique E1 enzyme which uses ATP to adenylate the C-terminal glycine of ubiquitin. Release of AMP accompanies the formation of a thioester bond between the C-terminus of ubiquitin and a cysteine residue in the E1 protein. In a transesterification reaction the ubiquitin is transferred from the ubiquitin-activating enzyme to an E2 ubiquitin-conjugating enzyme, which may, in turn, transfer the ubiquitin to an E3 ubiquitin protein ligase. In many cases it is this enzyme that recognizes the protein substrate and catalyses formation of an isopeptide bond between the C-terminus of ubiquitin and the ε-amino group of lysine in the target protein. Proteins destined for degradation via the proteasome are coupled to multiple copies of ubiquitin by formation of further isopeptide bonds between additional ubiquitin molecules and lysine residues in the bound ubiquitin (Hershko & Ciechanover 1998). In the case of IκBα, Ubch5 acts as the E2 (Chen *et al*. 1996), but ubiquitination requires the presence of an additional

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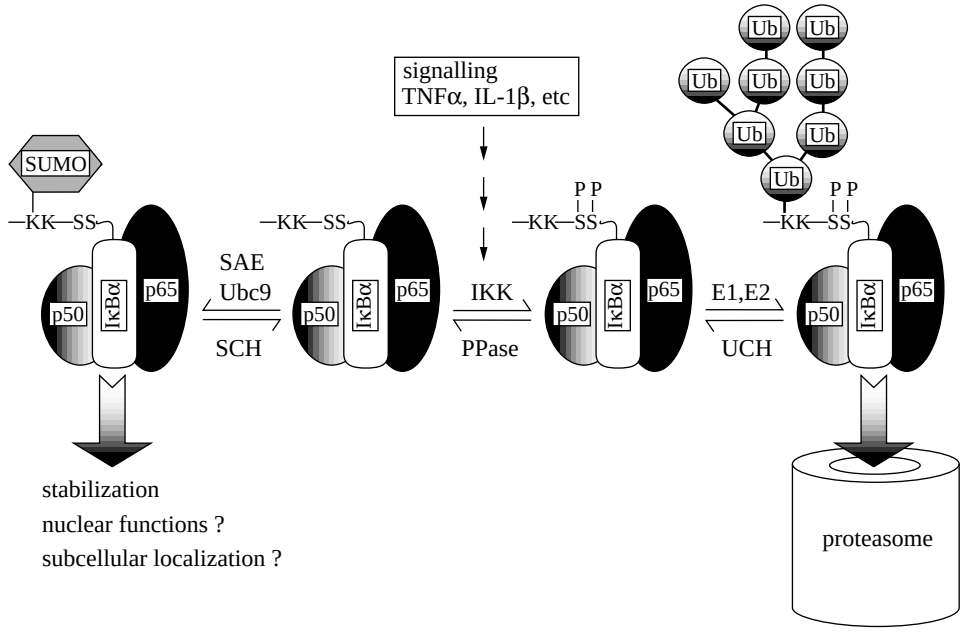

Figure 2. Fates of ubiquitin and SUMO-1 modified IκBα. See text for details. Abbreviations: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; UCH, ubiquitin C-terminal hydrolase; IKK, IκB B kinase; PPase, phosphatase; SAE, SUMO-activating enzyme; Ubc9, SUMO-1-conjugating enzyme; SCH, SUMO C-terminal hydrolase.

ubiquitin protein ligase activity which recognizes IκBα phosphorylated on residues S32 and S36 (Chen *et al.* 1996; Yaron *et al.* 1997).

3. SUMO-1 MODIFICATION OF IκBα

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 shown to encode the human protein Ubc9, which is homologous to ubiquitin-conjugating enzymes. In yeast, Ubc9 is essential for cell cycle progression (Seufert *et al.* 1995) and mammalian homologues have been isolated repeatedly from yeast two-hybrid screens in association with a wide variety of proteins. As IκBα undergoes signal-induced ubiquitination within the N-terminal domain, our expectation was that Ubc9 could participate in the ubiquitination of IκBα. However, 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 Ubc9. Furthermore, Ubc9 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 Ubc5. Although covalent modification of proteins by ubiquitin is now well documented, it is also clear that a number of other small protein molecules can be linked to target proteins in a similar fashion to ubiquitin. Whereas addition of multiple copies of ubiquitin targets proteins for degradation, it is now widely recognized that covalent attachment of other ubiquitin-related molecules does not result in degradation of the modified protein. Recently a small ubiquitin-like protein variously known as SUMO-1, sentrin, GMP1, 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.* 1997; Mahajan *et al.* 1997; Matunis *et al.* 1996; Shen *et al.* 1996). As it was reported that the ubiquitin-like protein SUMO was present in complexes containing Ubc9, it had been suggested that Ubc9 might be involved in SUMO-1 conjugation rather then ubiquitination (Saitoh *et al.* 1997). We demonstrated that this was indeed the case as Ubc9 could form a thioester with SUMO-1 provided that a SUMO-1-activating activity was present (Desterro *et al.* 1997).

As Ubc9 could interact with IκBα we were prompted to search for physiological situations in which IκBα is modified by SUMO-1. When special precautions were taken to avoid deconjugation, SUMO-1-modified IκBα was detected in many cell lines, although the extent of modification varies from low levels to almost 50% of the total (Desterro *et al.* 1998). While umodified IκBα was susceptible to signal-induced degradation, SUMO-1-modified IκBα was completely resistant to degradation (figure 2). To investigate the biochemical requirements for SUMO-1 modification of IκBα, an *in vitro* system was developed that could accurately mimic the situation *in vivo*. In the presence of recombinant SUMO-1, recombinant Ubch9 and a fraction from HeLa cells containing E1 activity (Desterro *et al.* 1997), SUMO-1 was conjugated to IκBα in an ATP-dependent fashion. 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 recognizes the substrate and brings it in to intimate contact with the E2, which catalyses the 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 coexists with a number of ubiquitin-like molecules and the E1 enzymes must distinguish between

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these molecules. As distinct E1 activities have been described for ubiquitin (Handley *et al*. 1991), Smt3p (Johnson *et al*. 1997) and Rublp (Lammer *et al*. 1998; Liakopoulos *et al*. 1998), we undertook the isolation and characterization 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 (Desterro *et al*. 1999). This enzyme could also transfer activated SUMO-1 to Ubc9, the conjugating enzyme involved in this process (Desterro *et al*. 1997; Johnson & Blobel 1997; Saitoh *et al*. 1998; Schwarz *et al*. 1998). While the E1 activity for ubiquitin is contained within a single large polypeptide, the E1 activity of SUMO-1, like that of Smt3p and Rublp, is partitioned between two smaller polypeptides, SAE1 and SAE2. Sequence comparisons between the E1 enzymes indicate that SAE1 is homologous to Aoslp, Ulalp and the N-terminus of the ubiquitin-activating enzymes, while SAE2 is homologous to Uba2p, Uba3p and the C-terminus of the ubiquitin-activating enzymes. The association between SAE1 and SAE2 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. Each SAE subunit contains a conserved nucleotide binding motif, GXGXXG, while the putative cysteine (Cys173) which forms a thioester bond with the C-terminal glycine of SUMO-1 is in an active site consensus sequence (KXXPZCTXXXXP) found in 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 localization signals, which are also present in the C-terminal region of Uba2p (Dohmen *et al*. 1995).

To precisely define the requirements for SUMO-1 conjugation, recombinant SAE protein was tested in a purified system containing recombinant Ubc9, a recombinant IκBα substrate and an ATP-regenerating system. Under these conditions SUMO-1 was efficiently conjugated to IκBα, indicating that conjugation does not require the presence of an E3-like protein ligase activity (Desterro *et al*. 1999). However, we cannot rule out the possibility that *in vivo* such proteins could increase the efficiency of conjugation. As our initial yeast two-hybrid screen demonstrated an interaction between Ubc9 and IκBα (Desterro *et al*. 1997), it is likely that substrate specificity is achieved by Ubc9. 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.

It was further demonstrated that IκBα was modified by SUMO-1 on the same lysine residues that are used for ubiquitin conjugation. This provides an explanation for the stability of SUMO-1-modified IκBα, in that SUMO-1-modified IκBα cannot be ubiquitinated and is therefore resistant to proteasome-mediated degradation. SUMO-1-modified IκBα remains bound to NF-κB, thus creating a 'privileged' pool of NF-κB–IκBα–SUMO-1 complexes that do not respond to signal induction. 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 reporters containing other promoters are not affected. Immediately following 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. 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. 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. 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, has been reported (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. Whether these SUMO-1-deconjugating enzymes are identical to the enzymes that proteolytically process the C-terminus of SUMO-1 remains to be established. Removal of the four C-terminal amino acids of SUMO-1 is required to expose G97, the carboxyl terminus of which is directly coupled to the ε-amino group of lysine in the target protein (Mahajan *et al*. 1998). Western blotting with an antibody that detects 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 the endogenous SUMO-1 is conjugated to proteins (Matunis *et al*. 1996). This is also the case in yeast, where virtually all the SUMO-1 homologue Smt 3p is present in conjugates (Johnson & Blobel 1997). These data indicate that endogenous SUMO-1 is limiting and suggest that SUMO-1 deconjugation is required to release free SUMO-1 for further modification.

The existence of IκBα–SUMO-1 conjugates that are resistant to signal-induced degradation may explain why, in many reports, only a fraction of IκBα is degraded in response to inducers such as TNFα or IL1. It is likely that IκBα–SUMO-1 conjugates remain after exposure of the cells to inducers, but when the cell extracts are prepared without special precautions, SUMO-1 is removed from the IκBα and the unmodified protein appears to be resistant to degradation.

While SUMO-1 modification of IκBα can serve to block signal-induced ubiquitination and thus degradation of IκBα, SUMO-1 modification of RanGAP1 serves to direct the modified protein to the nuclear pore complex (Mahajan *et al*. 1997, 1998; Matunis *et al*. 1996, 1998). 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 (Duprez *et al*. 1999; Muller *et al*. 1998; Sternsdorf *et al*. 1997).

Although we have not shown that SUMO-1 modification alters the cellular location of IκBα, one feature that the known SUMO-1 substrates (RanGAP1, PML, Sp100 and IκBα) have in common is that they undergo regulated transport between the cytoplasm and the nucleus. Nuclear transport is required for IκBα to mediate post-induction repression of NF-κB-dependent transcription (Arenzana-Seisdedos *et al*. 1995; Beg *et al*. 1995; Cressman & Taub 1993; Zabel *et al*. 1993). However, mutation of K21 and K22 does not appear to affect the ability of IκBα 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 IκBα. IκBα also possesses a leucine-rich nuclear export signal (NES), which allows the protein to export NF-κB–IκBα complexes out of the nucleus (Arenzana-Seisdedos *et al*. 1997; Ossareh-Nazari *et al*. 1997). RanGAP1 may shuttle between nucleus and cytoplasm in a similar fashion as it contains a number of NES-like sequences, although they have yet to be functionally defined (Matunis *et al*. 1998).

Signal-induced ubiquitination of IκBα takes place primarily on residues K21 and K22 (Baldi *et al*. 1996; Rodriguez *et al*. 1996; Scherer *et al*. 1995), with K38 and K47 as secondary sites (Rodriguez *et al*. 1996). 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 (Ciechanover 1994; Hou *et al*. 1994; King *et al*. 1996). In contrast, SUMO-1 modification appears to be highly specific. IκBα appears to be modified by SUMO-1 predominantly on K21, while RanGAP1 is conjugated to SUMO-1 solely via K526 (Mahajan *et al*. 1998). Comparison of the sequences surrounding the acceptor lysines in IκBα, RanGAP1 and the recently identified sites in PML (Duprez *et al*. 1999) reveals a striking similarity, which suggests that the sequence—LKxE—may represent a recognition site for the SUMO-1 conjugation machinery. It is likely that this recognition is achieved by Ubch9, as an interaction between Ubch9 and the N-terminus of IκBα was detected in a yeast two-hybrid screen (Desterro *et al*. 1997). Protein affinity chromatography experiments using immobilized GST–Ubch9 have demonstrated a direct protein–protein interaction between Ubch9 and recombinant human IκBα.

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, is a poor substrate for SUMO-1 conjugation. It thus appears that SUMO-1 acts antagonistically to ubiquitination: while multi-ubiquitination of IκBα targets the protein for destruction SUMO-1 modification creates a pool of IκBα that is resistant to degradation (figure 2). This new function of SUMO-1 is rather similar to that observed when mutations are introduced into ubiquitin in the lysine residues that are used for multi-ubiquitination. K29R and K48R mutants in ubiquitin generate proteins that can be conjugated to substrates but which cannot form multi-ubiquitin chains. As such, the modified proteins are resisitant 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.

4. COMPARTMENTALIZED DEGRADATION OF IκBα

After signal-induced degradation of IκBα, NF-κB translocates from the cytoplasm to the nucleus where it activates responsive genes. As the promoter for IκBα is NF-κB dependent, IκBα mRNA levels rise and IκBα protein is rapidly resynthesized. At this stage cytoplasmic levels of NF-κB are low and free IκBα translocates to the nucleus where it terminates NF-κB-dependent transcription. This is accomplished by inhibition of the NF-κB–DNA interaction and export of NF-κB back to the cytoplasm (Arenzana-Seisdedos *et al*. 1995). IκBα does not contain a recognizable basic type nuclear localization signal (NLS), but it has been demonstrated that nuclear import of free IκBα is mediated by sequences present in the ankyrin repeats (Sachdev *et al*. 1998; Turpin *et al*. 1999). However, as nuclear import of IκBα is blocked by an excess of peptide containing a basic-type NLS, it is likely that IκBα is imported into the nucleus via a 'piggy back' mechanism. Once bound to NF-κB in the nucleus, the nuclear localization signals in NF-κB and IκBα are mutually occluded. The NF-κB–IκBα complex is transported from the nucleus to the cytoplasm by virtue of a leucine-rich nuclear export sequence (NES) present in the C-terminal region of IκBα (Arenzana-Seisdedos *et al*. 1997). Sequences homologous to the IκBα NES are found in many proteins, including the human immunodeficiency virus-type 1 Rev protein and the protein kinase A inhibitor (Fischer *et al*. 1995; Fritz & Green 1996; Wen *et al*. 1995). Such NESs constitute transferable transport signals, which are necessary for rapid and active export from the nucleus to the cytoplasm. The nuclear protein CRM1 (also known as Exportin 1) has been recently identified as the NES receptor (Fornerod *et al*. 1997*a*; Fukuda *et al*. 1997; Ossareh-Nazari *et al*. 1997; Stade *et al*. 1997). CRM1 belongs to the karyopherin β family, which contains a homologous Ran-GTP binding domain (Fornerod *et al*. 1997*b*). Formation of CRM1–NES complex is facilitated by the presence of Ran in its GTP-bound form and it has been suggested that this is transported through the nuclear pore complex and dissociated in the cytoplasm as a result of GTP hydrolysis by Ran-GAP (Fornerod *et al*. 1997*b*). In addition, CRM1 has been shown to be the cellular target of the drug leptomycin B (LMB), which inhibits NES-mediated protein export both *in vivo* and *in vitro* (Fornerod *et al*. 1997*a*; Fukuda *et al*. 1997; Ossareh-Nazari *et al*. 1997; Wolff *et al*. 1997).

To examine the role of nuclear export in NF-κB metabolism, nuclear export of IκBα was inhibited by the drug

LMB. Inhibition of IκBα nuclear export not only prevents the post-induction repression of NF-κB-dependent transcription but also strongly represses the initial activation of NF-κB upon cell stimulation. Indeed, nuclear IκBα appears to be resistant to signal-induced phosphorylation and degradation and this results in nuclear accumulation of transcriptionally inactive IκBα–NF-κB complexes. Although the inhibition of CRM1 by LMB is highly specific, it was important to rule out the possibility that LMB might be interfering with the signal transduction pathways that lead to NF-κB activation. To address this point we employed a lacZ fusion protein containing the N- and C-termini of IκBα. When this protein is expressed in cells it is unable to translocate to the nucleus, but undergoes signal-induced degradation in response to agents such as TNFα and IL-1β (Kroll *et al.* 1997). Signal-induced degradation of the fusion protein was unaffected by LMB, thus indicating that LMB does not inhibit the signal transduction pathway that leads to IκBα degradation. It is also clear that the LMB does not inhibit transcription in a non-specific fashion as activity of an integrated RSV-driven lacZ reporter was unaffected by the presence of LMB. The conclusion from these experiments is that, in HeLa cells, signal-induced phosphorylation and degradation of IκBα occurs exclusively in the cytoplasm (Rodriguez *et al.* 1999). One possibility to explain this restriction is that an essential component of the signal transduction pathway which leads to IκBα phosphorylation cannot gain access to the nucleus. IκB kinases (IKK α and β) are present in a large signalling complex (figure 1) containing upstream kinases such as NF-κB-inducing kinase (Baeuerle 1998; Karin & Delhase 1998) and scaffolding proteins such as NEMO (Yamaoka *et al.* 1998). It has yet to be determined if this large complex can be imported into the nucleus. The alternative argument would be that the signal modification machinery has access to the nucleus, but that the nuclear IκBα is in some way refractile to modification. Mechanisms to achieve this could include prior covalent modification of IκBα to a form that is no longer recognized by the IKK signalling complex. As mentioned previously, IκBα is modified by SUMO-1 to a form that is resistant to signal-induced degradation. Although the known proteins that are substrates for SUMO-1 modification have been detected in the nucleus, or are involved in nuclear transport (Hodges *et al.* 1998), we have no evidence to support the notion that nuclear IκBα detected in the presence of LMB is resistant to signal-induced phosphorylation because it is modified by SUMO-1. It is also possible that IκBα could interact with a nuclear protein which occludes the region in IκBα-containing residues S32 and S36, thus protecting it from signal-induced phosphorylation.

In unstimulated cells there is clearly a requirement for the transcription of essential NF-κB-dependent genes. Low-level transcription of these genes does not take place simply as a consequence of NF-κB-independent transcription, as IκBα overexpression effectively abolishes the activity of an NF-κB-dependent reporter in unstimulated cells. Thus it appears that the cell has evolved a highly dynamic system to provide for continued low-level transcription of NF-κB-dependent genes. This homeostatic mechanism requires the continuous proteasome-mediated breakdown of IκBα, which generates a stream of free NF-κB which can translocate to the nucleus. Once in the nucleus, NF-κB activates responsive genes including that of IκBα. IκBα mRNAs, after transport to the cytoplasm, are translated and the free IκBα is directed to the nucleus where it interacts with DNA-bound NF-κB and dissociates the DNA–protein complex. By virtue of the presence of an NES in IκBα (Arenzana-Seisdedos *et al.* 1997), NF-κB–IκBα complexes are recognized by CRM1, which mediates nuclear export (Ossareh-Nazari *et al.* 1997). At this point equilibrium is re-established. Thus, rather than having a simple on–off switch, the cell can delicately alter the NF-κB transcriptional response by varying the rate at which IκBα is turned over. The most extreme perturbation of this equilibrium comes after exposure of the cells to agents such as TNFα or IL-1β. In this situation cytoplasmic IκBα is completely degraded and a massive pulse of NF-κB is released into the nucleus to initiate high-level transcription of NF-κB-dependent genes. However, the same mechanism is used to bring the system back into homeostasis (Arenzana-Seisdedos *et al.* 1995, 1997). A remarkably similar homeostatic mechanism seems to operate to control the level of p53 within the cell. In this case the product of the hdm2 gene targets p53 for ubiquitin-mediated proteasomal degradation, and disruption of this interaction during the damage response leads to the accumulation of p53. Nuclear translocation of p53 activates transcription of the hdm2 gene and the newly synthesized protein enters the nucleus where it terminates p53-dependent transcriptional activation. Hdm2 also contains an NES and this is employed to export the p53–hdm2 complex to the cytoplasm using the same pathway that is used for nuclear export of IκBα. Inhibition of hdm2-mediated export revealed that nuclear export of hdm2 is required to accelerate the degradation of p53 (Roth *et al.* 1998). In the case of both IκBα and p53, ubiquitin-mediated proteasomal degradation occurs in the cytoplasm, even though proteasomes are found in both compartments. However, proteasomal components are distributed differentially between the nucleus and the cytoplasm (Palmer *et al.* 1996; Wojcik *et al.* 1995), suggesting that nuclear and cytoplasmic proteasomes may have unique properties. The advantage to the cell of these homeostatic mechanisms to control NF-κB- and p53-dependent transcription is that they are both highly sensitive to perturbation and they can provide a finely tuned response to external signals.

This work was supported by the MRC, BBSRC and the EU.

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Discussion

A. Hershko (*Technion—Israel Institute of Technology, Haifa, Israel*). Is the SUMO conjugation to IκB regulated?

R. T. Hay. We haven't seen situations where we see a massive increase or disappearance of SUMO modification.

M. Hochstrasser (*University of Chicago, Illinois, USA*). Is the SUMO-modified IκB still associated with NF-κB?

R. T. Hay. Yes it is.

M. Hochstrasser. Only a fraction of the protein is modified by SUMO. What fraction of NF-κB is normally activated when you treat cells with TNF? Is this enough to explain inhibition?

R. T. Hay. It's a good point. It is difficult to say, though, because we can't really measure the amount of SUMO-conjugated IκB. Even with the rapid assay we may be losing significant amounts of the conjugated form. There could be other effects of the SUMO-modified protein as well. Perhaps it has some trans-dominant effect for example.

T. Toda (*Imperial Cancer Research Fund, London, UK*). What is the ubiquitin conjugating enzyme for IκB?

R. T. Hay. It has been published that UBC5 is involved in IκB ubiquitination.

M. Tyers (*Mount Sinai Hospital, Toronto, Canada*). Is there an E3 involved in IκB ubiquitination?

R. T. Hay. It's too early to talk about that just now.

K. A. Nasmyth (*Research Institute for Molecular Pathology, Vienna, Austria*). If you put SUMO-conjugated IκB into a ubiquitination assay, does this block ubiquitination? For example, if just one of the lysine residues is attached to SUMO, does this block ubiquitination at the other lysine?

R. T. Hay. That would be an interesting experiment to try.

R. T. Hunt (*ICRF Clare Hall Laboratories, Hertfordshire, UK*). I'm still a little unclear about what you think the role of SUMO conjugation is.

R. T. Hay. Well, I still think it could block degradation. If there are conditions which raise the levels of SUMO conjugation, this would stabilize IκB.

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