Signal-induced site-specific phosphorylation targets I κ B α to the ubiquitin–proteasome pathway

Zhijian Chen,¹ Jeremiah Hagler,² Vito J. Palombella,¹ Francesco Melandri,¹ David Scherer,³ Dean Ballard,³ and Tom Maniatis²

¹Myogenics, Inc., Cambridge, Massachusetts 02139 USA; ²Harvard University, Department of Molecular and Cellular Biology, Cambridge, Massachusetts 02138 USA; ³Howard Hughes Medical Institute, Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232 USA

The transcription factor NF- κ B is sequestered in the cytoplasm by the inhibitor protein I κ B α . Extracellular inducers of NF- κ B activate signal transduction pathways that result in the phosphorylation and subsequent degradation of I κ B α . At present, the link between phosphorylation of I κ B α and its degradation is not understood. In this report we provide evidence that phosphorylation of serine residues 32 and 36 of I κ B α targets the protein to the ubiquitin–proteasome pathway. I κ B α is ubiquitinated *in vivo* and *in vitro* following phosphorylation, and mutations that abolish phosphorylation and degradation of I κ B α *in vivo* prevent ubiquitination *in vitro*. Ubiquitinated I κ B α remains associated with NF- κ B, and the bound I κ B α is degraded by the 26S proteasome. Thus, ubiquitination provides a mechanistic link between phosphorylation and degradation of I κ B α .

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NF- κ B and other members of the Rel family of transcriptional activator proteins regulate the expression of the type I human immunodeficiency virus (HIV) as well as a large number of cellular genes that play essential roles in immune and inflammatory responses (for review, see Grilli et al. 1993; Baeuerle and Henkel 1994; Siebenlist et al. 1994; Thanos and Maniatis 1995). The transcriptional activities of Rel proteins are highly regulated in most cell types by a mechanism that involves specific association between heterodimeric Rel complexes and a family of monomeric inhibitor proteins designated I κ B (Baeuerle and Baltimore 1988; for review, see Beg and Baldwin 1993; Gilmore and Morin 1993). Members of this inhibitor family share a structural domain comprised of five to six ankyrin-like repeats. The best-characterized I κ B protein, I κ B α , binds to the p50 (NF- κ B1)/p65 (RelA) heterodimer of NF- κ B and masks the nuclear localization signals of these proteins (Beg et al. 1992; Ganchi et al. 1992; Henkel et al. 1992; Zabel et al. 1993). When cells are exposed to a variety of NF- κ B inducers such as lipopolysaccharide (LPS), phorbol esters, tumor necrosis factor- α (TNF α), and interleukin-1 (IL-1), I κ B α is rapidly phosphorylated and degraded, and NF- κ B translocates to the nucleus where it activates gene expression (Beg et al. 1993; Brown et al. 1993; Cordle et al. 1993; Henkel et al. 1993; Rice and Ernst 1993; Sun et al. 1993, 1994a).

An alternative pathway for regulating the activity of NF- κ B involves proteolytic processing of the p105 precursor of p50 subunit of NF- κ B (Blank et al. 1991; Fan and Maniatis 1991; Mellits et al. 1993; Mercurio et al. 1993; Donald et al. 1995). The p105 precursor contains p50 at its amino terminus and an I κ B-like sequence with ankyrin repeats at its carboxyl terminus. Unprocessed p105 can associate with p65 and other members of the Rel family to form inactive heterodimeric complexes that are sequestered in the cytoplasm (Capobianco et al. 1992; Liou et al. 1992; Neumann et al. 1992; Rice et al. 1992; Mercurio et al. 1993). Processing of p105 results in degradation of the I κ B-like carboxyl terminus and the production of the transcriptionally active p50/Rel protein heterodimer.

Recently, p105 processing was shown to require the ubiquitin–proteasome pathway (Palombella et al. 1994). This pathway requires ATP and the covalent conjugation of target proteins with multiple ubiquitin molecules (for review, see Goldberg 1992; Herskko and Ciechanover 1992; Jentsch 1992). Ubiquitination occurs in a three-step process. In the first step, ubiquitin is activated by a ubiquitin-activating enzyme (E1), and in the second step, the activated ubiquitin is transferred to a ubiquitin carrier protein (E2). In the final step, ubiquitin–protein ligase (E3) catalyzes the covalent attachment of ubiquitin

to the target protein. Additional ubiquitins are then thought to be added by a processive mechanism to form the multiubiquitin chain (for recent review, see Ciechanover 1994). The multiubiquitinated proteins are then rapidly degraded by the 26S proteasome.

The 26S proteasome consists of a 20S multicatalytic protease complex and additional regulatory subunits that are required for the recognition and degradation of multiubiquitinated proteins (for review, see Rechsteiner et al. 1993; Peters 1994). The NF κ B1 p105 protein is ubiquitinated in vitro, and ubiquitination is required for in vitro processing by purified 26S proteasome (Palombella et al. 1994). In addition, p105 processing in vitro and in vivo is blocked by peptide aldehyde inhibitors of the proteasome. The degradation of I κ B α is also blocked by such inhibitors, but this process has not yet been shown to require ubiquitination (Finco et al. 1994; Miyamoto et al. 1994; Palombella et al. 1994; Traenckner et al. 1994; Alkalay et al. 1995; DiDonato et al. 1995; Lin et al. 1995).

Although the signal transduction pathways leading to the activation of NF- κ B are not well understood, these pathways culminate in the phosphorylation of I κ B, p105, and p65 (Beg et al. 1993; Brown et al. 1993; Mellits et al. 1993; Naumann and Scheidereit 1994; Sun et al. 1994b; Donald et al. 1995). Initially, phosphorylation of I κ B α was thought to promote its dissociation from NF- κ B and its subsequent degradation (for discussion, see Beg and Baldwin 1993; Beg et al. 1993). This conclusion was consistent with the observation that tosyl-Phe-chloromethylketone (TPCK) and other alkylating agents block the degradation of I κ B α and the activation of NF- κ B (Henkel et al. 1993; Mellits et al. 1993). However, more recent studies have shown that these inhibitors actually prevent the signal-dependent phosphorylation of I κ B α and have no direct effect on proteasome function (Finco et al. 1994; Miyamoto et al. 1994; Palombella et al. 1994; Traenckner et al. 1994; Alkalay et al. 1995; DiDonato et al. 1995; Lin et al. 1995). In contrast, the presence of proteasome inhibitors leads to the accumulation of phosphorylated I κ B α bound to NF- κ B (Finco et al. 1994; Miyamoto et al. 1994; Palombella et al. 1994; Traenckner et al. 1994; Alkalay et al. 1995; DiDonato et al. 1995; Lin et al. 1995). These findings suggest that phosphorylation leads to the degradation of I κ B α by the proteasome, without inducing its dissociation from NF- κ B.

Recently, serine residues 32 and 36 in I κ B α have been shown to be required for I κ B α phosphorylation and degradation in response to TNF- α , phorbol 12-myristate 13-acetate (PMA), and ionomycin (Brockman et al. 1995; Brown et al. 1995) or the Tax protein of the type 1 human T-cell leukemia virus (HTLV-1; Brockman et al. 1995). However, the mechanisms by which phosphorylation leads to the degradation of I κ B α are not understood. In this paper we show that I κ B α is ubiquitinated in vivo and in vitro and that ubiquitination is required for degradation by the 26S proteasome. In addition, we demonstrate that mutations in I κ B α that prevent its phosphorylation and degradation in vivo block ubiquitination in vitro. Together, these findings indicate that the signal-depen-

dent phosphorylation of I κ B α targets the cytoplasmic inhibitor to the ubiquitin-proteasome pathway.

Results

Inducible phosphorylation and ubiquitination of I κ B α in vivo

To determine whether I κ B α is ubiquitinated upon phosphorylation in vivo, we sought conditions that result in the stabilization of the hyperphosphorylated form of I κ B α that is rapidly degraded during cellular activation (for review, see Siebenlist et al. 1994). Previous studies demonstrated that the proteasome inhibitor MG132 (Z-Leu-Leu-Leu-H) blocks TNF α -induced degradation of I κ B α and leads to the accumulation of phosphorylated I κ B α (Palombella et al. 1994). However, only a small fraction of endogenous I κ B α remains phosphorylated under these conditions, attributable presumably to the action of endogenous phosphatases. Calyculin A and okadaic acid are phosphatase inhibitors that induce NF- κ B by maintaining phosphorylation and degradation of I κ B α (Thevenin et al. 1990; Menon et al. 1993; Lin et al. 1995). We therefore attempted to accumulate phosphorylated I κ B α in the Jurkat T-cell line using the combination of MG132 and calyculin A. Jurkat cells were treated with 40 μ M MG132 alone (Fig. 1A, lane 2), with 0.3 μ M calyculin A alone (lane 3), or with both inhibitors (lane 4), and the phosphorylation of I κ B α analyzed in a Western blot using a polyclonal antibody against the carboxyl terminus of I κ B α . Treatment with MG132 alone did not affect the level of unphosphorylated I κ B α under these conditions [although I κ B α is known to be basally phosphorylated (for example, Brown et al. 1995), we will refer to unstimulated I κ B α as unphosphorylated]. Treatment with calyculin A alone resulted in the phosphorylation and degradation of I κ B α . In contrast, phosphorylated I κ B α accumulated when cells were treated with both inhibitors. The calpain inhibitor MG102 (40 μ M), which completely inhibits calpain activity but does not inhibit the proteasome at this concentration, did not lead to accumulation of phosphorylated I κ B α in the presence of calyculin A (data not shown). These results indicate that calyculin A induces the phosphorylation-dependent degradation of I κ B α and that the proteasome is required for this process.

To determine whether ubiquitination of I κ B α occurs in vivo, we prepared cell extracts at different times after treatment of Jurkat cells with calyculin A in the presence of MG132 and then analyzed the samples by Western blotting using antibodies against I κ B α . The extracts were prepared in the presence of SDS (0.1%) and *N*-ethylmaleimide (NEM, 5 mM) to inhibit isopeptidase activities that may otherwise affect the detection of ubiquitinated proteins. As shown in Figure 1B, a ladder of high molecular mass proteins accumulated following stimulation with calyculin A/MG132 (lanes 4–9). The molecular mass increments of these ladders were $\sim$ 8.5 kD, which is the size of ubiquitin. Ubiquitination of I κ B α peaked at 5–15 min following stimulation (lanes 4–6)

Figure 1. Induced phosphorylation and ubiquitination of I κ B α in calyculin A or TNF α -treated cells. (A) Stabilization of the phosphorylated form of I κ B α by MG132. Jurkat T cells were pretreated with 40 μ M of MG132 (lanes 2,4) or DMSO (a diluent for MG132, lanes 1,3) for 30 min before incubation with 0.3 μ M of calyculin A (lanes 3,4) or DMSO (lanes 1,2) for an additional 30 min. Cytoplasmic extracts were prepared, fractionated by SDS-PAGE, and analyzed by Western blot using a rabbit polyclonal antibody against the carboxyl terminus of I κ B α (amino acids 297–317, c-21). The phosphorylated form of I κ B α is designated p-I κ B α . (B) Induced ubiquitination of I κ B α by calyculin A. Jurkat T cells were pretreated with MG132 (40 μ M, lanes 2–9) or DMSO (lane 1) for 30 min before addition of calyculin A (0.3 μ M, lanes 4–9) or DMSO (lanes 2,3). Cell extracts were prepared at 5 min (lanes 2,4), 10 min (lane 5), 15 min (lane 6), 20 min (lane 7), 40 min (lane 8), and 60 min (lanes 3,9) after adding calyculin A or DMSO. The extraction buffers contained RIPA/0.1% SDS plus 5 mM *N*-ethylmaleimide (NEM). The extracts were then subjected to Western blot analysis as described in A. (C) Jurkat T cells were treated as described in lanes 1 and 4 in A, except that cytoplasmic extracts were first immunoprecipitated with an antibody against the carboxyl terminus of I κ B α (see Materials and methods). The immunoprecipitates were then eluted with 0.1 M Capso (pH 11.2), and the eluates were separated by SDS-PAGE, followed by Western blotting with a rabbit polyclonal antibody (anti-Ub) that specifically recognizes ubiquitin conjugates. The arrow marked IgG H indicates rabbit immunoglobulin heavy chain in the immunoprecipitates that cross-reacts with the secondary antibody (alkaline phosphatase conjugated anti-rabbit Fc). (D) Induced ubiquitination of I κ B α by TNF α . Experiments were carried out as described in B, except that calyculin A was replaced with TNF α (10 ng/ml).

and then decreased by 40–60 min (lanes 8,9), possibly because of residual activities of proteasome and isopeptidases. Treatment of Jurkat cells with MG132 alone did not lead to significant accumulation of ubiquitinated I κ B α (lane 2). Longer treatment with MG132 (60 min plus 30 min of pretreatment, lane 3) led to the appearance of very faint bands corresponding to ubiquitinated I κ B α , suggesting that ubiquitination may also be involved in basal turnover of I κ B α .

To further demonstrate that calyculin A induces ubiquitination of I κ B α in vivo, we immunoprecipitated I κ B α

from control and MG132/calyculin A-treated Jurkat cell extracts with an I κ B α antibody. We then performed a Western blot analysis on the immunoprecipitated proteins using a polyclonal antibody against ubiquitin (Fig. 1C). The specificity of these antibodies has been documented extensively, and the antibodies have been widely used to detect ubiquitinated proteins (Haas and Bright 1985; Lowe and Mayer 1990). The anti-ubiquitin antibody detected high molecular mass proteins in the calyculin A/MG132-treated extracts. These molecular masses ranged from 60 to 200 kD, consistent with a typ-

ical pattern seen upon multiubiquitination of proteins. Taken together, these results suggest that calyculin A induces the phosphorylation-dependent ubiquitination of I κ B α in vivo.

Because calyculin A is not a natural inducer of NF- κ B activation, it is possible that induced ubiquitination of I κ B α is an unusual effect of calyculin A. To address this possibility, we performed an experiment similar to that described in Figure 1B, except that calyculin A is replaced by TNF α (Fig. 1D), a natural inducer of NF- κ B. Strikingly, TNF α not only induces hyperphosphorylation of I κ B α in the presence of MG132 but also induces multiubiquitination of I κ B α (lanes 4–9). The kinetics of induction by TNF α is similar to the induction by calyculin A. We conclude that I κ B α is rapidly phosphorylated and ubiquitinated following stimulation by inducers of NF- κ B.

In vitro-translated I κ B α is ubiquitinated in HeLa cell extracts, and the ubiquitinated I κ B α remains associated with NF- κ B

Next, we carried out experiments to determine whether the phosphorylation and ubiquitination of I κ B α could be induced in vitro by the phosphatase inhibitor okadaic acid. Like calyculin A, okadaic acid has been shown previously to potently activate NF- κ B in vivo (Thevenin et al. 1990). Preliminary experiments revealed that incubation of HeLa cell cytoplasmic extracts in the presence of MgATP and okadaic acid led to a time-dependent phosphorylation and ubiquitination of endogenous I κ B α (data not shown). We extended this study to in vitro-translated I κ B α so that I κ B α mutants could be examined (see below). We prepared ³⁵S-labeled I κ B α by coupled in vitro transcription/translation of I κ B α mRNA in wheat germ extracts. The in vitro-translated I κ B α was then incubated in the HeLa cell cytoplasmic extract in the presence of MgATP, ubiquitin, okadaic acid, and ubiquitin aldehyde (Ubal), an isopeptidase inhibitor that prevents the breakdown of ubiquitin conjugates. As shown in Figure 2A, there was a time-dependent accumulation of high molecular mass species characteristic of multiubiquitinated I κ B α , with a concomitant decrease in unconjugated I κ B α . The molecular weights of the slowly migrating species range from 60 to >200 kD, consistent with the addition of from 10 to >20 ubiquitin molecules (8.5 kD) to each molecule of I κ B α (37–41 kD, depending on phosphorylation states). The unconjugated I κ B α present during the ubiquitination reaction is a doublet, and as described below, the upper band is likely phosphorylated I κ B α . The high molecular mass species can be immunoprecipitated by anti-ubiquitin antisera (Fig. 2B, lane 2). In addition, when glutathione S-transferase-ubiquitin (GST-Ub) was used to substitute for ubiquitin, the high molecular mass species could be precipitated by glutathione-Sepharose (data not shown). Furthermore, the high molecular mass species could be converted to lower molecular mass forms upon treatment with isopeptidase T (data not shown), which cleaves lysine-48 linked isopeptide bonds between ubiquitin molecules (Chen and Pickart 1990).

These observations, together with data shown below, clearly show that the high molecular mass species are multiubiquitinated I κ B α .

The conditions required for ubiquitination of I κ B α in vitro were examined in the experiment of Figure 2C. Both I κ B α (lanes 1–6) and FLAG epitope-tagged I κ B α (F-I κ B α , lanes 7–12) were tested in this experiment. The latter form of I κ B α was examined for comparison to a series of FLAG-tagged I κ B α mutants, which were examined previously in vivo (Brockman et al. 1995; see below and Fig. 3). With both the native and FLAG-tagged I κ B α proteins, phosphorylation and ubiquitination were dependent on the presence of okadaic acid (lanes 2 and 5, 8 and 11), and both reactions were abolished by the addition of EDTA (lanes 6,12). The upper bands labeled p-I κ B α and p-F-I κ B α are phosphorylated forms of I κ B α , as these bands could be converted to the lower bands after treatment with calf intestine alkaline phosphatase (CIAP, data not shown). The presence of Ubal was necessary for multiubiquitination of I κ B α (lanes 4,10). Without Ubal, only small amounts of low molecular mass conjugates were observed, probably because of the action of isopeptidases in the extract. Multiubiquitination of I κ B α was also inhibited by methylated ubiquitin (MeUb, lanes 3,9), which prevents the formation of multiubiquitin chains (Hershko et al. 1991). Because of the presence of competing endogenous ubiquitin in the HeLa cell extracts, MeUb functions as a ubiquitin chain terminator that leads to the generation of low levels of low molecular mass conjugates. The fact that the formation of high molecular mass conjugates is dependent on Ubal and inhibited by MeUb provides strong evidence that the high molecular mass conjugates are multiubiquitinated I κ B α . Addition of GST-c-Rel protein did not affect the in vitro ubiquitination reaction in HeLa cell extract (data not shown), presumably because the in vitro-translated I κ B α associates with excess endogenous NF- κ B proteins present in the extract (see below). Taken together, these results show that I κ B α can be ubiquitinated in vitro. Moreover, ubiquitination requires the presence of an inducing agent, such as okadaic acid.

To determine whether the I κ B α ubiquitinated in HeLa cell extracts is associated with NF- κ B, the reaction mixture (Fig. 3, lane 1) was precipitated with an antibody against RelA (lane 2). Both ubiquitinated and unconjugated I κ B α were present in the anti-RelA precipitates, indicating that both are associated with NF- κ B. The ratio of ubiquitinated I κ B α to unconjugated I κ B α in the immunoprecipitates is similar to that in the reaction mixture prior to immunoprecipitation, suggesting that both forms of I κ B α associate with NF- κ B with similar affinity.

Serine residues 32 and 36 are necessary for ubiquitination of I κ B α in vitro

Serine residues 32 and 36 of I κ B α are required for the phosphorylation and degradation of I κ B α in vivo (Brockman et al. 1995; Brown et al. 1995). However, a mechanistic link between phosphorylation and degradation had

Figure 2. Induced phosphorylation and ubiquitination of I κ B α in vitro. (A) Time course of ubiquitination. I κ B α was in vitro-translated in wheat germ extract (Promega TNT system) in the presence of [35 S]methionine. The labeled I κ B α was then incubated in HeLa extracts at 37°C in the presence of Mg-ATP (2 mM), ubiquitin (1 mg/ml), okadaic acid (3.3 μ M), and Ubal (3 μ M). An aliquot of the reaction was then quenched at the indicated times in SDS sample buffer, followed by SDS-PAGE and fluorography. (B) Double immunoprecipitation with I κ B α and ubiquitin antibodies. Ubiquitinated I κ B α was synthesized as described in A, and the reaction mixture was precipitated with I κ B α antibody (lane 1). An aliquot of the immune complex was then boiled for 5 min in the presence of 0.5% SDS, and the liberated ubiquitinated I κ B α was then precipitated again with anti-ubiquitin antibody (lane 2). The samples were analyzed by SDS-PAGE followed by fluorography. (C) Conditions required for phosphorylation and ubiquitination of I κ B α in vitro. Both I κ B α (lanes 1–6) and FLAG-epitope-tagged I κ B α (F-I κ B α , lanes 7–12) were 35 S-labeled by in vitro translation and used as substrates for ubiquitination. The reaction conditions were as described in A, except for the following: Lanes 3 and 9 received MeUb (1.3 mg/ml) instead of Ub; lanes 4 and 10 lacked Ubal in the reaction; lanes 5 and 11 lacked okadaic acid in the reaction; lanes 6 and 12 received EDTA (40 mM) instead of Mg-ATP. After incubation at 37°C for 90 min, I κ B α and F-I κ B α were immunoprecipitated with I κ B α antisera (c-21) and then analyzed by SDS-PAGE. In A–C, the bands below I κ B α are probably nonspecific partial proteolytic cleavage products of I κ B α , as the generation of these bands is not affected by EDTA, MeUb, okadaic acid, or Ubal (see C).

not been established. We therefore tested a series of phosphorylation-defective mutants of I κ B α in the in vitro ubiquitination assay. Wild-type and mutant I κ B α proteins tagged at their amino termini by the FLAG epitope (Brockman et al. 1995) were produced by in vitro translation (Fig. 4A). These same mutants were analyzed previously for their effects on the inducible degradation of I κ B α in vivo (Brockman et al. 1995).

The first 36 amino-terminal amino acids have been deleted in the Δ N mutant, whereas the S32A and S36A mutants are serine to alanine substitutions at positions 32 and 36, respectively. The S32A/S36A mutant is a double serine to alanine substitution at positions 32 and 36. All of these mutant proteins are stable when expressed in cells treated with TNF α , PMA/ionomycin, or in the presence of the HTLV Tax protein. Moreover, they retain their ability to associate with RelA, and are dominant negative inhibitors of NF- κ B activation (Brockman et al.

1995). The S32E and S36E mutants are serine to glutamic acid substitutions, which are designed to mimic the negative charge of phosphorylation (note mobility differences on SDS-polyacrylamide gel; Fig. 4B). These substitutions restore the ability of the mutant I κ B α to be degraded in response to inducing agents in vivo (Brockman et al. 1995). The Δ C mutant lacks 75 amino acids at the carboxyl terminus of I κ B α . This mutation removes a carboxy-terminal PEST sequence, as well as a putative sixth ankyrin repeat. The sixth ankyrin repeat has been shown to be required to bind NF- κ B (Ernst et al. 1995). In addition, deletion of the PEST sequence has been shown to stabilize I κ B α in vivo (Miyamoto et al. 1994; Brockman et al., 1995; Brown et al. 1995). PEST sequences in other proteins have been implicated in protein degradation (Rogers et al. 1986). (Note that this Δ C mutant is different from another recently described Δ C mutant, which is missing only 41 residues at the carboxyl termi-

Figure 3. Association of ubiquitinated I κ B α with NF κ B. Conditions for the synthesis of ubiquitinated I κ B α were as described in Fig. 2A except that the reaction was carried out at 37°C for 2 hr. Ten percent of the reaction mixture was saved for SDS-PAGE analysis (lane 1), and I κ B α in the remaining mixture was coimmunoprecipitated with RelA using antisera against RelA/p65 (see Materials and methods). The precipitates were boiled in SDS sample buffer, followed by SDS-PAGE and fluorography (lane 2). An amount equivalent to ~50% of the initial reaction volume was loaded in lane 2, which is fivefold greater than that loaded in lane 1. The bulk of the high molecular mass conjugates in lane 2 is approximately 100 kD, as opposed to 200 kD seen in lane 1, and this may be attributable to “trimming” by isopeptidases during the immunoprecipitation.

nus, and can associate with RelA/p65; Brown et al. 1995).

Analysis of these mutants in the *in vitro* ubiquitination assay revealed an excellent correlation between their ability to be degraded *in vivo* in response to inducers and their ability to be ubiquitinated *in vitro* (Fig. 4C). Specifically, Δ N (lane 12), S32A (lane 14), S36A (lane 16), and S32A/S36A (lane 18), which escape signal-dependent degradation *in vivo* (Brockman et al. 1995), are also not ubiquitinated *in vitro*. In contrast, wild-type I κ B α (lane 11), S32E (lane 15), and S36E (lane 17) are all ubiquitinated in this assay, consistent with their functional phenotype *in vivo* (Brockman et al. 1995). The Δ C mutant protein was ubiquitinated *in vitro* (lane 13), but its degradation is not enhanced *in vivo* following stimulation of cells. However, the basal turnover of this mutant is indistinguishable from that of the wild type (data not shown). Given that this Δ C mutant does not associate with RelA, it is likely that its behavior reflects signal-independent (basal) turnover of free I κ B α (albeit at low level) in the cell. It is possible that ubiquitination is also involved in the basal turnover of free I κ B α and that the carboxy-terminal sequence including the PEST region is not required for ubiquitination of free I κ B α .

A shorter exposure of the film shown in Figure 4, A

and C, showed a remarkable correlation between phosphorylation and ubiquitination of these mutants in HeLa extracts (Fig. 4B,D). A clear difference in electrophoretic mobility of the unconjugated I κ B α mutants following incubation in the HeLa cell extracts can be observed. For example, after incubation in the okadaic acid-supplemented extracts, the unconjugated wild-type I κ B α exhibits a slightly slower migrating band in addition to the band observed prior to incubation (cf. Fig. 4B, lane 1, and 4D, lane 11). This “mobility shift” phenomenon is not observed in Δ N (lanes 2,12) and S32A/S36A mutants (lanes 8,18). This slower migrating band is attributable to phosphorylation of I κ B α . The S32A and S36A mutant proteins were also phosphorylated during the reaction (lanes 4 and 14, 6 and 16), probably because the adjacent unaltered serine residues (S36 and S32, respectively) can serve as phosphoryl group acceptors. The S32E and S36E mutants both migrate more slowly than S32A, S36A, or S32A/S36A even before the ubiquitin conjugation reaction (lanes 5 and 15, 7 and 17), an indication that the negative charges on S32E and S36E decrease the mobility of both proteins. The mobility of the S32E and S36E mutants did not change appreciably after the reaction, suggesting that additional phosphorylation at the alternate serine residue S36 and S32, respectively, did not change significantly the electrophoretic mobility of I κ B α . These results demonstrate that phosphorylation of the I κ B α mutants in HeLa cell extracts correlates with their ability to be ubiquitinated.

The alkylating agent TPCK and the antioxidant pyridinedithiocarbamate (PDTC) have been widely used to inhibit the induced degradation of I κ B α (Beg et al. 1993; Henkel et al. 1993; Sun et al. 1993). These agents act by inhibiting the phosphorylation of I κ B α . As shown in Figure 4, C and D, TPCK (50 μ M) also inhibited phosphorylation and ubiquitination of I κ B α in HeLa cell extracts (lane 9). In contrast, PDTC (50 μ M) did not inhibit either phosphorylation or ubiquitination of I κ B α in this assay (lane 10), probably because PDTC acts upstream of the okadaic acid activation step or, alternatively, generation of reactive oxygen intermediates (ROI) was not faithfully reproduced in this system. Taken together, there was an excellent correlation between phosphorylation and ubiquitination of I κ B α *in vitro*. We conclude that both serine residues 32 and 36 are required for ubiquitination of I κ B α *in vitro*, most likely through direct phosphorylation of these sites (see discussion).

Ubiquitinated I κ B α bound to NF- κ B is degraded by the 26S proteasome

To determine whether ubiquitination of I κ B α is required for degradation by the 26S proteasome *in vitro*, we compared the fate of conjugated and unconjugated I κ B α when incubated with purified 26S proteasome. I κ B α labeled with [35 S]methionine was produced by *in vitro* translation and incubated in HeLa cell extracts in the presence or absence of EDTA. (EDTA blocks ubiquitination and therefore serves as a control; see Fig. 2C). The I κ B α /NF- κ B complex formed *in vitro* was then immunopre-

Figure 4. Mutations that prevent the phosphorylation of I κ B α in vivo also abolish ubiquitination in vitro. (A) In vitro-translated I κ B α mutants. I κ B α mutants were translated in wheat germ extracts (Promega TNT system) in the presence of [35 S]methionine. The translation products were analyzed by SDS-PAGE and fluorography. (B) A shorter exposure of A showing the I κ B α mutants before ubiquitination reactions. (C) Ubiquitination assays. I κ B α mutants shown in A were incubated in HeLa cell extracts at 37°C for 1 hr under conditions described in Fig. 2A. The reaction mixtures were then subjected to SDS-PAGE and fluorography. In lanes 9 and 10, TPCK (50 μ M) and PDTC (50 μ M) were added to the reactions containing wild-type I κ B α , respectively. (D) A shorter exposure of C, showing the phosphorylation properties of the remaining unconjugated I κ B α mutants. p-I κ B α is the phosphorylated form of I κ B α or I κ B α bearing glutamic acid substitutions at position 32 or 36.

cipitated with an anti-RelA antibody (see above and Fig. 3). Both unconjugated and ubiquitinated I κ B α were isolated as a complex with NF- κ B. The immunoprecipitated I κ B α proteins were then separated by SDS-PAGE as shown in the fluorograph of Figure 5A (lanes 1,2). Quantitation of the data by PhosphorImager analysis showed that ubiquitin-conjugated I κ B α contained 67% of the total radioactivity, and the remaining 33% was unconjugated I κ B α . When both conjugated and unconjugated I κ B α were incubated with purified 26S proteasomes (23 nM) in the presence of Mg and ATP, a significant (47%) reduction in the level of conjugated I κ B α was observed (cf. lanes 2 and 4), whereas the amount of unconjugated I κ B α did not significantly change (cf. lanes 1 and 3). The level of unconjugated I κ B α in lane 4 is slightly higher than that in lane 2, probably because of isopeptidase activities associated with the 26S proteasome (Eytan et al. 1993). To directly measure the degradation of ubiquitinated I κ B α by the 26S proteasomes, the degradation products were separated from undegraded I κ B α by trichloroacetic acid (TCA) precipitation, and the TCA-soluble radioactivity was determined. The percentage of conjugate degradation, taking into account the unconjugated I κ B α present in the conjugate sample, is plotted in Figure 5B. Ubiquitinated I κ B α is efficiently degraded by the 26S proteasome (17 nM, open square). Approximately 19% of the substrate was degraded within 5 min, and by 1 hr, 51% of the conjugates was degraded. Inclusion of EDTA in the degradation reaction abolished degradation of the conjugates (solid square), indicating that the 26S proteasome-catalyzed degradation is Mg-ATP dependent. The 20S proteasome, which functions as the proteolytic core of the 26S complex, did not degrade ubiquitinated I κ B α (open triangle). This is consistent with the role of the 26S proteasome in degrading ubiquiti-

nated substrates. Similarly, no degradation of the conjugates occurred in the absence of the 26S proteasome (solid triangle). Importantly, unconjugated I κ B α was not degraded by the 26S proteasome (open diamond). These results clearly demonstrate that ubiquitination of I κ B α targets the protein for degradation by the 26S proteasome. Thus, it appears that I κ B α is not only phosphorylated and ubiquitinated while associated with NF- κ B, but ubiquitinated I κ B α also serves as a substrate for the 26S proteasome when complexed to NF- κ B.

Discussion

The transcription factor NF- κ B is activated in response to a large number of distinct extracellular signals, all of which result in the phosphorylation of I κ B proteins (for review, see Siebenlist et al. 1994). Recently, serine residues 32 and 36 were shown to be required for phosphorylation and degradation of I κ B α , and activation of NF- κ B (Brockman et al. 1995; Brown et al. 1995). In this paper we have shown that I κ B α is ubiquitinated in response to stimulation both in vivo and in vitro, and that phosphorylation of I κ B α is required for ubiquitination. In addition, we show that ubiquitinated I κ B α is degraded by the 26S proteasome in vitro. The latter observation is consistent with previous observations showing that proteasome inhibitors block the degradation of I κ B α in vivo (Finco et al. 1994; Miyamoto et al. 1994; Palombella et al. 1994; Traenckner et al. 1994; Alkalay et al. 1995; DiDonato et al. 1995; Lin et al. 1995). Although these inhibitors were shown to act on purified proteasomes, they can also inhibit other proteases, such as calpains and cathepsins. A series of peptide-aldehyde inhibitors with different potencies (IC $_{50}$ s) against the purified proteasome in vitro (Rock et al. 1994) were tested for their

Figure 5. Degradation of ubiquitinated I κ B α by the 26S proteasome. (A) In vitro-translated 35 S-labeled I κ B α was incubated in HeLa extracts at 37°C for 2 hr under ubiquitination conditions (see Materials and methods), except that either Mg-ATP (lanes 2,4) or EDTA (lanes 1,3) was added to the reaction. The reaction mixtures were then immunoprecipitated by an antibody against RelA under conditions that allow coprecipitation of conjugated (lanes 2,4) or unconjugated (lanes 1,3) I κ B α . The immunoprecipitates were then used directly for the degradation assay by the 26S proteasome. Lanes 1 and 2 are minus (–) 26S; lanes 3 and 4 are plus (+) 26S (23 nM). The degradation reactions were carried out at 37°C for 1 hr in the presence of Mg-ATP, and the reaction mixtures were separated by SDS-PAGE, followed by fluorography. (B) Ubiquitinated I κ B α in the immunoprecipitates described in A (lane 2) was incubated with 26S proteasome (17 nM) at 37°C in the presence of Mg-ATP (□). At indicated time points, an aliquot of the reaction was precipitated by 10% TCA. The TCA-soluble radioactivity was then determined by liquid scintillation counting. Similarly, degradation of unconjugated (free) I κ B α was also determined (◇). In other reactions, 40 mM EDTA was added (■), 26S proteasome was omitted (▲), and 20S proteasome (49 nM) was added (△) instead of the 26S proteasome.

ability to inhibit NF- κ B in vivo (Palombella et al. 1994; Read et al. 1995). The rank order potencies of these compounds in vitro and in vivo were in excellent agreement. In contrast, other calpain and cathepsin inhibitors, even at high concentrations, did not block NF- κ B activation. Thus, it seems likely that the antagonistic effects of these agents on NF- κ B activation derive from their inhibitory activity on the proteasome. Moreover, in related studies it was found that lactacystin, a highly specific inhibitor of the proteasome (Fenteany et al. 1995), also prevents the processing of p105, the degradation of I κ B α , and the activation of NF- κ B in vivo (J. Hagler, O.J. Rando, G. Fenteany, S.L. Schreiber, and T. Maniatis, unpubl.). In addition, a new class of synthetic proteasome inhibitors, which do not affect any other known cellular proteases, also blocks I κ B α degradation and NF- κ B activation (V. Palombella and Z. Chen, unpubl.).

In this paper we show that deletion of the amino-terminal 36 amino acids of I κ B α , or serine to alanine substitutions at either position 32 or 36, block in vitro ubiquitination. Although direct biochemical proof for phosphorylation of I κ B α at serine residues 32 and 36 is still lacking, many independent lines of evidence indicate that these two residues are most likely the sites of phosphorylation. First, peptide mapping localizes inducible phosphorylation to the amino terminus of I κ B α (Brown et al. 1995). Second, mutants of I κ B α containing phos-

phoserine mimetics (but not alanine) at serine 32 or 36 are competent for degradation in vivo (Brockman et al. 1995). Third, the electrophoretic mobility of mutants containing mimetics at these serine sites coincides with that of the hyperphosphorylated form of endogenous I κ B α in activated cells (Fig. 4; data not shown). Fourth, disruption of all other potential phosphorylation sites in the amino terminus of I κ B α has no effect on the function of I κ B α (Brockman et al. 1995; Brown et al. 1995). Fifth, removal of the carboxy-terminal PEST domain of I κ B α fails to prevent inducible hyperphosphorylation in vivo (Brown et al. 1995). Taken together, we propose that phosphorylation of serine residues 32 and 36 targets I κ B α to the ubiquitin–proteasome pathway.

The amino terminus of I κ B α , which is not required for its association with NF- κ B, is highly susceptible to protease cleavage, and this susceptibility is unaffected by binding to the p65 subunit of NF- κ B (Jaffray et al. 1995). Thus, the amino terminus of I κ B α appears to be exposed in the NF- κ B complex and can therefore be recognized by an I κ B kinase and presumably ubiquitination enzymes. In contrast, the central region of I κ B α , which contains a tandem array of ankyrin repeats, is protease resistant and connected to the acidic carboxy-terminal domain containing a PEST sequence (Jaffray et al. 1995). Recent mutational studies have suggested that this PEST sequence may be required for signal-induced degradation of I κ B α .

(Miyamoto et al. 1994; Brockman et al. 1995; Brown et al. 1995). The PEST sequences on the cyclin CLN3 protein contain phosphorylation sites, and these sites have been shown to be required for CLN3 degradation during the cell cycle (Yaglom et al. 1995). It should be noted that the ΔC mutant tested in this study lacks the PEST sequence as well as additional carboxy-terminal sequences that are required for binding to NF- κ B (Brockman et al. 1995). Although the in vitro-translated ΔC protein is ubiquitinated in vitro, the significance of this observation with respect to the regulated degradation of NF- κ B-bound I κ B α remains unclear. Notwithstanding this uncertainty, these findings suggest that the PEST sequences of I κ B α are not required for ubiquitination of free I κ B α .

Investigation of the amino acid sequence requirements for the ubiquitin-proteasome-dependent degradation of other proteins has not revealed a common recognition element (for review, see Ciechanover 1994). For example, a "destruction box" sequence has been shown to be required for the ubiquitin-mediated degradation of mitotic cyclins (Glutzer et al. 1991; Luca et al. 1991). In contrast, a region containing PEST sequences and multiple phosphorylation sites is required for the degradation of CLN3 (Yaglom et al. 1995). In another example, degradation of the transcription factor MAT α 2 requires two different regions of the protein, and these sequences have not been found in other proteins (Hochstrasser et al. 1991). These and other examples strongly suggest that different protein substrates are recognized for degradation by the ubiquitin-proteasome pathway by distinct mechanisms (Ciechanover 1994). The recognition of specific substrates may involve the use of specific ubiquitin protein ligases (Hershko et al. 1994).

Remarkably, neither phosphorylation nor ubiquitination results in the dissociation of I κ B α and NF- κ B, and we have shown that the 26S proteasome recognizes and degrades ubiquitinated I κ B α in the ternary NF- κ B complex. The three-dimensional structure of an NF- κ B p50 homodimer bound to DNA has been determined recently (Ghosh et al. 1995; Muller et al. 1995). In addition, specific amino acids in the highly conserved Rel homology domain have been shown to be required for interactions between the *Drosophila* Dorsal and Cactus proteins, homologs of NF- κ B and I κ B, respectively (Lehming et al. 1995). The location of these amino acids in the three-dimensional structure of the Rel homology domain suggests that I κ B may fit within a deep groove formed between the dimerization domain of the two subunits (Ghosh et al. 1995; Lehming et al. 1995; Muller et al. 1995). Thus, I κ B α must be phosphorylated and ubiquitinated on the surface away from the dimerization domain, as the modified I κ B α remains bound to NF- κ B. How, then, is the I κ B α degraded as part of the NF- κ B complex? An interesting possibility is suggested by the recent observations that molecular chaperones are required for the degradation of certain ubiquitinated substrates by the proteasome (D.H. Lee, M. Sherman, and A.L. Goldberg, pers. comm.). Perhaps, the 26S proteasome binds to the ubiquitin chains on I κ B α and, in con-

junction with chaperones, strips I κ B α away from NF- κ B and then unfolds and degrades free I κ B α .

We have shown that in vitro-translated I κ B α is phosphorylated and ubiquitinated in HeLa cell extracts in the presence of the phosphatase inhibitor okadaic acid. Previous studies have shown that I κ B α can be inactivated in vitro by sphingomyelinase or ceramide (Machleidt et al. 1994), LPS (Ishikawa et al. 1995), or ζ protein kinase C (ζ PKC) (Diaz-Meco et al. 1994). In contrast, the behavior of the in vitro system described here suggests a low level of constitutive I κ B α phosphorylation that would ordinarily not be detected because of the presence of endogenous phosphatases. However, in the presence of okadaic acid, the constitutively phosphorylated I κ B α accumulates.

Phosphorylation-dependent ubiquitination of I κ B α could occur via two mechanisms, which are not mutually exclusive. First, the phosphorylation of I κ B α may enhance its affinity for constitutive ubiquitination enzymes (E2s and E3s). Alternatively, one or more of the enzymes involved in ubiquitination may be activated by the same signal transduction cascade. An example of such regulation is the activation of a cyclin ubiquitin protein ligase (E3) by cdc2 (Hershko et al. 1994).

There are now several examples in which the ubiquitin-proteasome pathway plays an essential role in the regulation of transcription factor levels. These examples include the degradation of yeast MAT α 2 (Hochstrasser et al. 1991) and GCN4 (Kornitzer et al. 1994) proteins, and the mammalian c-Jun (Treier et al. 1994) and p53 proteins (Scheffner et al. 1993). The example of NF κ B1/p105 is exceptional in that the proteasome selectively degrades the carboxyl terminus of an inactive precursor protein, leaving the amino terminus intact (Palombella et al. 1994). The complete degradation of I κ B α leads to a rapid and transient activation of NF- κ B. The transient nature of the activation is a consequence of the positive autoregulation of the I κ B α gene by the activated NF- κ B and the subsequent restoration of the cytoplasmic I κ B α pool (Sun et al. 1993). In contrast, when the degradation of another I κ B protein, I κ B β , is induced by LPS and IL-1, the activation of NF- κ B persists (Thompson et al. 1995). The degradation of I κ B β , like that of I κ B α , is inhibited by TPCK, which seems to block the activities of one or more I κ B kinases. I κ B β contains an amino-terminal sequence strikingly similar to the Ser-32/Ser-36-like region of I κ B α (Thompson et al. 1995). Thus, it seems likely that the degradation of I κ B β also involves the ubiquitin-proteasome pathway.

Because of the central role played by NF- κ B and other Rel family members in the immune and inflammatory responses, their activation would be an attractive target for the development of pharmacological inhibitors. For example, the genes encoding the cell adhesion molecules expressed on the surface of the vascular endothelium require NF- κ B for their induced expression by TNF α and other inflammatory cytokines (for review, see Collins et al. 1995). Recent studies have shown that the proteasome inhibitor MG132 blocks the induction of the leukocyte adhesion molecules E-selectin, VCAM-1, and

ICAM-1 (Read et al. 1995). The functional consequence of this inhibition was the prevention of lymphocyte attachment to TNF α -treated endothelial monolayers. The finding that ubiquitination is required for the proteasome-dependent degradation of I κ B α provides additional, and possibly more specific, targets for inhibition of the inflammatory response.

Materials and methods

Materials

The proteasome inhibitor MG132 (Z-Leu-Leu-Leu-H) has been described before (Palombella et al. 1994; Rock et al. 1994). Calyculin A and okadaic acid were purchased from GIBCO BRL. Antibodies against I κ B α (c-21, sc-371) and RelA/p65 (sc-109), as well as the agarose conjugates of the RelA antibody (sc-109AC) were purchased from Santa Cruz Biotechnology. Affinity-purified antibody specific for conjugated ubiquitin was provided by Dr. Cecile Pickart (State University of New York, Buffalo). Ubiquitin was purchased from Sigma, and MeUb was prepared according to Hershko and Heller (1985). Fluorescamine analysis showed that >95% of the lysine residues on MeUb was blocked. Ubal was prepared according to Mayer and Wilkinson (1989). 20S and 26S proteasomes were purified according to published methods (Hough et al. 1987; Ganoth et al. 1988).

Plasmids, in vitro translation, and cell culture

The I κ B α mutants are described by Brockman et al. (1995). These mutants were subcloned into pBluescript [SK(+), Stratagene] or pSP72 (Promega) for in vitro translation. Wild-type and mutant I κ B α proteins were produced and labeled with [³⁵S]methionine by in vitro translation in TNT wheat germ extracts (Promega) using RNA transcribed from *NotI* linearized plasmids. The translation products were used directly in ubiquitination assays (see below). Jurkat cells (ATCC) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. For metabolic labeling with [³⁵S]methionine/cysteine, 200 μ Ci/ml of EXPRE³⁵S (Dupont NEN) was used in the labeling media lacking methionine and cysteine.

Preparation of cell extracts

Preparation of HeLa cytoplasmic extracts (S100) was described earlier (Fan and Maniatis 1991). These extracts were further concentrated by ammonium sulfate (80%) precipitation, followed by extensive dialysis in 20 mM Tris (pH 7.6), 0.5 mM DTT. The extracts were stored in aliquots at -80°C .

Jurkat cell cytoplasmic extracts were prepared by lysing the cells in a hypotonic buffer (buffer A) containing 10 mM HEPES (pH 7.4), 1 mM EDTA, 10 mM KCl, 1 mM DTT, phosphatase inhibitors (50 mM NaF, 50 mM glycerol-2-phosphate, 1 mM sodium orthovanadate, 0.1 μ M okadaic acid), and protease inhibitors (0.1 mg/ml of PMSF, 10 μ g/ml of leupeptin, 10 μ g/ml of aprotinin). Following incubation on ice for 15 min, 0.2% NP-40 was added to the lysate, and the mixture was placed on ice for another 5 min. After centrifugation at 16,000g for 5 min at 4°C , the supernatant (cytoplasmic extract) was stored at -80°C .

Immunoprecipitation and Western blot analysis

Immunoprecipitation was carried out in RIPA buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate) plus 0.1% SDS. Antibodies were incubated with cytoplasmic extracts at 4°C for 1 hr. Protein A–trisacryl (Pierce) equilibrated in the same buffer was then added to the mixture, and the incubation was continued for another hour. When anti-

RelA–agarose was used for immunoprecipitation, the step involving addition of protein A–trisacryl was omitted. After a brief centrifugation, the resin was washed four times with RIPA/0.1% SDS and then boiled in SDS sample buffer.

Coimmunoprecipitations were carried out similar to the immunoprecipitation described above, except that buffer A/0.2% NP-40 (see above) instead of RIPA/0.1% SDS was used for antibody incubations. The resin was then washed with buffer B (10 mM HEPES at pH 7.4, 1 mM EDTA, 10 mM KCl, 50 mM NaF, 50 mM glycerol-2-phosphate, 1 mM sodium orthovanadate, 0.1 mg/ml of PMSF, 0.2% NP-40, and 90 mM NaCl). The washed resin was either boiled in SDS sample buffer, eluted with 0.1 M Capso (pH 11.2), or used directly for assays (see below). Western blot analysis was performed according to Fan and Maniatis (1991).

Ubiquitination assay

In vitro-translated ³⁵S-labeled I κ B α was incubated with HeLa extract (4.5 mg/ml) in the presence of an ATP regenerating system (50 mM Tris at pH 7.6, 5 mM MgCl₂, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/ml of creatine kinase, 0.6 U/ml inorganic pyrophosphatase), together with ubiquitin (1 mg/ml), okadaic acid (3 μ M), and Ubal (3 μ M). The reactions were incubated at 37°C for 1 hr unless otherwise indicated. After terminating the reaction with SDS sample buffer, the reaction mixture was subjected to SDS-PAGE (9%) and fluorography.

Isolation of ubiquitin-I κ B α conjugates

Ubiquitinated I κ B α was synthesized in a 300- μ l reaction mixture containing 60 μ l of in vitro-translated ³⁵S-labeled I κ B α , 1.5 mg of HeLa extract, and other components of the ubiquitination reaction. The control reaction contained 40 mM EDTA instead of Mg-ATP in the mixture. After 2 hr of incubation at 37°C , ubiquitinated I κ B α and unconjugated I κ B α were coimmunoprecipitated with RelA using 30 μ l of anti-RelA agarose conjugates (0.25 μ g/ μ l of antibody). Following incubation at 4°C for 1 hr, the resin was washed three times with buffer B and once with buffer D (50 mM Tris at pH 7.6, 0.5 mM DTT). The resin was then resuspended in buffer D and used directly in the conjugate degradation assay.

Conjugate degradation assay

Ubiquitinated I κ B α suspension (~ 2000 cpm) was incubated with 17 nM of 26S proteasome in an ATP-regenerating system (see above). At the desired time points, the reaction was quenched by addition of 125 μ l of 4% BSA and 575 μ l of 12% TCA. After removal of the TCA precipitates by centrifugation, 600 μ l of the supernatants was counted in a scintillation counter. The results are expressed as percentage of the conjugates that are degraded to TCA-soluble counts.

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Z Chen, J Hagler, V J Palombella, et al.

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