Signal-dependent and -independent Degradation of Free and NF-κB-bound IκBa*

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A family of inhibitory IκB molecules regulates the activation of the transcription factor NF-κB. One member of the IκB family, IκBo, plays a major role in the rapid signal-induced activation of NF-κB. IκBo itself is transcriptionally regulated by NF-κB allowing for a tight autoregulatory loop that is both sensitive to and rapidly influenced by NF-κB activating stimuli. For this pathway to remain primed both for rapid activation of NF-κB in the presence of signal and then to suppress NF-κB activation once that signal is removed, IκBo must be exquisitely regulated. The regulation of IκBo is mainly accomplished through phosphorylation, ubiquitination, and subsequent degradation. The mechanism(s) that regulate IκBo degradation needs to be able to target IκBo for degradation in both its NF-κB bound and free states in the cell. In this study, we utilize a full-length IκBo mutant that is unable to associate with RelA/p65. We show that the signal-induced IκB kinase (IKK) phosphorylation sites on IκBo can only significantly influence the regulation of signal-dependent but not signal-independent turnover of IκBo. We also demonstrate that the constitutive carboxyl-terminal casein kinase II phosphorylation sites are necessary for the proper regulation of both signal-dependent and -independent turn-over of IκBo. These findings further elucidate how the phosphorylation of IκBo influences the complex regulatory mechanisms involved in maintaining a sensitive NF-κB pathway.

The transcription factor NF-κB is an important regulator of genes involved in immune and inflammatory responses, apoptosis, and cell proliferation (1, 2). The NF-κB/Rel family of transcription factors are a family of proteins that homo- and heterodimerize through a conserved Rel homology domain that consists of approximately 300 amino acids. The Rel homology domain of NF-κB is responsible for homo- and heterodimerization, DNA binding activity, and nuclear localization (3–5). A large number of stimuli can cause NF-κB to translocate from the cytoplasm to the nucleus, and activate target gene transcription. Stimuli that can activate NF-κB include proinflammatory cytokines, bacterial lipopolysaccharide, phorbol esters, okadaic acid, and viral infection (2, 6–8).

A group of inhibitory proteins belonging to the IκB family regulate NF-κB activation by sequestering NF-κB in the cytoplasm. IκB exerts its inhibitory effects by associating with the Rel homology domain of NF-κB proteins, effectively masking their nuclear localization signal (9–12). Although there are a number of IκB proteins, IκBo is the primary regulator of rapid signal induced activation of NF-κB. Upon stimulation by a proinflammatory cytokine such as TNFα,1 a signaling cascade is initiated that results in the activation of the IκB kinases IKK1 and IKK2 (13–17). This leads to the rapid phosphorylation of IκBo at the signal-induced phosphorylation sites, serine 32 and serine 36 (18–21). Once phosphorylated, IκBo is polyubiquitinated by the Ubc5/E3RS IκB ubiquitination enzyme pair (22, 23) on lysine 21 and lysine 22 (24–26). Polyubiquitinated IκBo is degraded by the 26 S proteasome, thus exposing NF-κB’s nuclear localization signal and allowing NF-κB to translocate to the nucleus (27–29). Once in the nucleus NF-κB activates transcription of target genes including IκBo (6, 30). When the NF-κB inducing signal is removed the newly synthesized IκBo can suppress NF-κB activity by preventing it from binding to the genomic DNA, and sequestering NF-κB in the cytoplasm (31–33).

The regulation of IκBo is carried out mainly through phosphorylation. Several phosphorylation sites have been identified on IκBo and they include the signal-induced IKK phosphorylation sites located at serine 32 and serine 36 (18–21), the constitutive CKII phosphorylation sites located in the carboxy-terminal PEST domain (34, 35), the protein kinase C site located in Ankyrin repeat 6 (ank6) (36, 37), and a tyrosine phosphorylation site that can cause the dissociation but not the degradation of IκBo from NF-κB in Jurkat T cells (38). Although extensive analysis of the signal-dependent degradation of IκBo has been done, little distinction has been made between the signal-dependent and -independent degradation of free and NF-κB-bound IκBo. Therefore, we undertook a study to examine the role of various phosphorylation sites on IκBo to determine their effects on both the signal-dependent and -independent degradation of free and NF-κB-bound IκBo.

In this study, we characterize a full-length IκBo mutant that is unable to associate with NF-κB (designated as mutC) and exists as a free molecule in the cell. We also show that distinct phosphorylation sites can directly influence the efficiency of ubiquitination and subsequent degradation of free and NF-κB-associated IκBo in the presence or absence of stimuli. These results demonstrate the complexity of IκBo regulation necessary to ensure that NF-κB is rapidly and specifically activated by a diverse group of stimuli.

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1 The abbreviations used are: TNFα, tumor necrosis factor α; CKII, casein kinase II; MEF, mouse embryo fibroblasts; IP, immunoprecipitation.
EXPERIMENTAL PROCEDURES

MATERIALS—The proteasome inhibitor clasto-lactacystine β-lactone (β-lactone) and the ubiquitin hydrolyase inhibitor ubiquitin aldehyde (Ubal) were purchased from Boston Biochem Inc. Ubiquitin and cyclohexamide were purchased from Sigma and okadaic acid was purchased from Life Technologies, Inc. Antibodies against IκBα (c-21, sc-371), RelA/p65 (sc-109), and IKKβ (H-470) were purchased from Santa Cruz Biotechnology. Antibodies against the FLAG (MD) and HA (12CA5) tags were purchased from Eastman Kodak Co. and Roche Molecular Biochemicals, respectively. TNFα, inorganic pyrophosphatase, and creatine phosphokinase were purchased from Calbiochem.

Cell Culture—HeLa cells, human embryonic kidney 293 cells, and 1232H cells were maintained as above and selection was maintained with blasticidin (20 μg/ml). TNFα and β-lactone were used at final concentrations of 10 ng/ml and 10 μM, respectively, unless otherwise noted. Cyclohexamide was used at a final concentration of 75 μM.

Plasmids and in Vitro Translation—Mutants were generated by polymerase chain reaction-based site-directed mutagenesis and confirmed by DNA sequencing. Wild-type 1232H cells, mutant 1232Ha, and 1232Hb cells were stably engineered with Cre/lox-lox recombination. Western blot analysis of cell lysates was performed using 10% SDS-PAGE and antibodies directed against 1232Hα, 1232Hβ, and the proteasome inhibitors (0.1 mM phenylmethylsulfonyl fluoride and 10 μg/ml aprotinin). Lysates were subjected to centrifugation at 14,000 rpm, for 15 min, at 4 °C. Lysates were then concentrated and digested on 1% acrylamide gel, exposed to a PhosphorImager (Molecular Dynamics), and band intensities quantitated with ImageQuant software.

In Vitro Ubiquitination Assay—HeLa cytoplasmic extracts, used in the in vitro ubiquitination assay, were made by lysis in hypotonic buffer (buffer A) containing 10 mM HEPS (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiotreitol, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride and 10 μg/ml aprotinin). Lysates were cleared by centrifugation at 14,000 rpm, for 15 min, at 4 °C. Lysates were then concentrated and digested on 1% acrylamide gel, exposed to a PhosphorImager (Molecular Dynamics), and band intensities quantitated with ImageQuant software.

In vitro transcription—Translation was performed using the T7 promoter contained in the PCMX-PL1 vectors. Wild-type 1232H cells and the 1232Hα and 1232Hβ mutants were also ligated into the pCLBabe retroviral vectors. pcLBBabe is a derivative of the retroviral construct pcMS-1. The 1232Hα and 1232Hβ constructs and 1232Hpa were cut with HindIII and EcoRI, respectively, and then blunted with Klenow. Both were then cut with BamHI and the resulting BamHI/HindIII (blunt) 1232Hα fragment was ligated into the BamHI/EcoRI (blunt) pCBLBabe retroviral vector.

In vitro transcription—translation was performed using the T7 promoter contained in the PCMX-PL1 vectors. Wild-type and mutant IκBα were bacterially expressed in recombinant Escherichia coli. Plasmids containing the HIV-1 long terminal repeat (LTR) were driven into the pCMX-PL1 polylinker. Recombinant expression was performed using the T7 transcription-translation kit (Promega). 60 μl of the TNT reaction was used for each sample that was carried out as described (29) with the following changes. Briefly, in vitro 35S-labeled IκBα was incubated with HeLa cell extract (20 μg/ml) in 10 mM Tris (pH 7.5), 5 mM MgCl2, 2 mM ATP, 10 mM creatine phosphate, 3.5 units/ml creatine kinase, 0.6 units/ml inorganic pyrophosphatase, 1 mg/ml ubiquitin, 3 μg okadaic acid, 3 μg ubiquitin aldehyde, and 10 μg/ml β-lactone. The reaction was then quenched with H2O, rinsed with H2O, dried, and exposed to a PhosphorImager for quantification.

Metabolic Labeling—6-cm plates of the 1232Hα and 1232Hβ stable cells were washed twice in phosphate-buffered saline and incubated for 1 h in 2 ml of cystine- and methionine-free Dulbecco’s modified Eagle’s medium supplemented with 10% dialyzed fetal bovine serum (Life Technologies, Inc.). Labeling was performed for 3 h with 0.5 mCi of [35S]methionine (NEN Life Science Products Inc.) per ml. Cells were then washed three times with complete medium and then chased for the appropriate time points in complete medium. After each time point the cells were washed twice with ice-cold phosphate-buffered saline and cell pellets were frozen on dry ice for later manipulation. Cell pellets were thawed on ice and whole cell lysis was performed by adding 500 μl of RIPA (20 mM Tris, pH 8.0, 100 mM NaCl, 0.2% sodium deoxycholate, 0.2% Nonidet P-40) containing 5 mM N-ethylmaleimide and the samples were subjected to immunoprecipitation (IP) by antibodies directed against IκBα or RelA/p65. IPs were carried out with 1.5 μg of the appropriate antibody for 1 h at 4 °C. Immunocomplexes were precipitated with antibody at 4 °C. The pellets were washed in RIPA buffer, SDS sample buffer was added, and the pellets were boiled for 5 min prior to SDS-polyacrylamide gel electrophoresis on 10% gels. Following electrophoresis, the gels were fixed in glacial acetic acid, amplified with 2,5-diphenyloxazole, rinsed with H2O, dried, and exposed to a PhosphorImager for quantification.
**Regulation of IkBa Degradation**

**RESULTS**

**Signal-dependent Degradation of IkBa**—To investigate the signal-dependent degradation of IkBa in *vivo*, stable pools of mouse IkBa−/− MEFs (39) were generated by injecting naive IkBa−/− MEFs with recombinant retroviral vectors containing either wild-type (wt) murine IkBa, one of the IkBa mutants (Fig. 1 schematically describes IkBa mutations), or GFP. The mutants described in Fig. 1 represent mutations of known and possible sites of serine and threonine phosphorylation. These mutants were chosen for this study because they would aid in the further elucidation of the role of serine and threonine phosphorylation in the signal-dependent and -independent degradation mechanisms of free and NF-κB-bound IkBa. The IkBa−/− MEF infections were carried out at a multiplicity of infection of much less than one to ensure that on average, after selection, each cell of a stable pool would have only a single copy of the integrated recombinant retroviral vector. This kept expression levels low and as close to endogenous expression levels as possible. Fig. 2A compares the relative expression levels of endogenous IkBa in wt MEF cells to that of the transfected IkBa−/− stable pools. It can be seen that the levels of the IkBa proteins in the transfected cells are within 1–2-fold of the endogenous IkBa (Fig. 2, lane 8, +/-).

TNFα stimulation of the IkBa−/− MEF stable pools in the presence of the translocation inhibitor cyclohexamide show that, compared with wild-type IkBa, the 3236 and M mutants were significantly more stable (Fig. 2B). The M mutant shows no change in expression over the time course while the 3236 mutant starts to show a slight reduction at the 60-min time point. The mutF mutation gives IkBa partial resistance to TNFα-dependent degradation but is less stable than the 3236 and M mutants. The mutC mutant shows no increase in stability and, in fact, is significantly less stable than wt IkBa. The 3236mutC mutation confers an increase in the stability of mutC containing IkBa, but is significantly more unstable than the 3236 mutation by itself.

The rate of IkBa degradation shown in Fig. 2B was further examined by measuring the NF-κB DNA binding activity following TNFα stimulation. The mutF mutation shows an approximate 50% reduction in DNA binding activity when compared with wt IkBa (Fig. 2C). The 3236 and M mutations reduce the gel shift activity to what is observed in unstimulated control cells. Interestingly, cell pools containing either the mutC or 3236 mutations show no significant reduction in NF-κB DNA binding activity. This is most likely due to the fact that the mutC mutation, which is a substitution of all five serine and threonine residues in the anky6 region of IkBa to alanine, disrupts the association of IkBa to NF-κB, (Fig. 3A (in vitro) and B (in vivo)), show that both mutC and 3236mutC do not associate with RelA/p65. It was recently shown that the anky6 region of IkBa makes a critical contact to NF-κB, (11, 12). Since the mutC mutation disrupts IkBs association to NF-κB and blocks any potential phosphorylation of anky6, it is possible that phosphorylation of anky6 plays a critical role in IkBa’s association to NF-κB. We conclude that (i) IKK phosphorylation is necessary for signal-dependent degradation of both free and NF-κB-associated IkBa, and (ii) that CKII phosphorylation is necessary for efficient signal-dependent degradation of NF-κB-associated IkBa.

Next, *in vitro* ubiquitination assays were performed to determine the signal-induced ubiquitination profiles of both free and NF-κB-associated IkBa mutants. A representative gel is shown for each experiment. Quantitation was done on a PhosphorImager, using ImageQuant software, and the resulting histograms display the percentage of the total labeled IkBa in the reaction that was shifted. By graphing the percentage of the total IkBa shifted we were able to correct for any loading differences between samples. Therefore, the histograms give a more accurate representation of the respective levels of ubiquitination on the various IkBa molecules. When examining a pool of both RelA/p65-associated and free IkBa it appears that IkBa containing the mutF mutation is ubiquitinated slightly, but reproducibly, more efficiently than wt IkBa (see Fig. 4A, lanes 1 and 2). The level of ubiquitination of the other IkBa mutants in Fig. 4A is consistent with the rate of degradation and the gel shift results presented in Fig. 2, B and C. The 3236 and M mutants have levels of ubiquitination just above background (Fig. 4A, lanes 3 and 4).

The data with the mutC mutants (Fig. 4A, lanes 5 and 6) represents the levels of ubiquitination on free IkBa molecules, since mutC does not associate with NF-κB (Fig. 3, A and B). MutC alone is ubiquitinated very efficiently (lane 5), even better than wt (Fig. 4A, lane 5, compared with lane 1, respectively). The mutC3236mutC mutant shows a reduction in the level of ubiquitination compared with mutC alone, but the level of ubiquitination of 3236mutC is significantly higher than IkBa containing just the 3236 mutation (Fig. 4A, lane 6, compared with lane 3, respectively). These data demonstrate that free IkBa does undergo signal-induced phosphorylation and ubiquitination and that serine 32 and serine 36 play a significant role in this process.

Fig. 4B displays the ubiquitination of the RelA/p65 associated pool of IkBa. The mutF mutation reduces the amount of signal-dependent ubiquitination below the level seen on wt IkBa when it is in association with NF-κB (Fig. 4B, lanes 1 and 2). This reduction in ubiquitination by mutF of only NF-κB-associated IkBa explains why IkBa is partially stabilized and why a reduction in the DNA binding activity of NF-κB is observed in the IkBa−/− MEF mutF stable pool (Fig. 2, B and C).

In contrast to the NF-κB-bound IkBa (Fig. 4B, lanes 1 and 2), the signal-induced ubiquitination of only the free pool of IkBa reveals that mutF is ubiquitinated slightly more efficiently than the wt (Fig. 4C, lanes 1 and 2). The 3236 and M mutants give large reductions in the amount of ubiquitination observed (Fig. 4C, lanes 4 and 5). Ubiquitination of mutC and 3236mutC, in the *in vitro* ubiquitination assay, could only be observed by direct anti-IkBa immunoprecipitation (Fig. 4A, lanes 5 and 6) because these mutants do not associate to RelA (Fig. 3A). The *in vitro* ubiquitination assay data (Fig. 4, A-C) correlates well with the stability and gel shift observations made in the IkBa−/− MEF stable pools (Fig. 2, B and C).

The *in vitro* ubiquitination results were confirmed by performing *in vivo* ubiquitination assays in the stably transfected IkBa−/− MEF stable pools. The IkBa−/− MEF stable pools were first treated with β-lactone, a potent and specific inhibitor of the proteasome, and then stimulated with TNFα (Fig. 4D). The ubiquitination patterns obtained in this experiment are similar to the corresponding in the *in vitro* assays. Wt, mutF,
3236, and M represent NF-κB associated IκBα (Fig. 4D, lanes 1–4, respectively) and give ubiquitination profiles that match those obtained for RelA/p65-associated IκBα in the in vitro assay (Fig. 4B, lanes 1–4). Wt IκBα is efficiently ubiquitinated in the presence of signal, mutF causes a decrease in ubiquitination, and 3236 and M reduce ubiquitination to almost background levels (Fig. 4D, lanes 1, 2, 3, and 4, respectively).

MutC and 3236mutC represent free IκBα in the IκBα2/2 MEF stable pools (Fig. 3B), and they also give ubiquitination patterns in vivo (Fig. 4D, lanes 5 and 6) that correlate with...
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FIG. 4. In vitro and in vivo signal-dependent ubiquitination of the IkB\(\alpha\) mutants. All experiments in this figure were done under stimulated conditions. The HeLa cytoplasmic extracts used in the in vitro ubiquitination assays were activated with a reaction mixture that contained 3 \(\mu\)M okadaic acid. Control samples, designated by “C,” were performed exactly like the wt sample except the reaction mixture was replaced by 50 mM Tris (pH 7.5). A, the in vitro signal-dependent ubiquitination assays were carried out as described under “Experimental Procedures.” The IkB\(\alpha\) IP was carried out to examine total ubiquitination of both free and NF-κB-associated IkB\(\alpha\). Quantitation was done on the PhosphorImager, using ImageQuant software. The percentage of labeled IkB\(\alpha\) shifted by ubiquitination was calculated by dividing the shifted counts by the total count. This calculation corrects for any differences in the input amounts of labeled IkB\(\alpha\). Three independent samplings were performed and the average of the three values obtained is depicted graphically. B, the RelA IP was done to isolate the labeled IkB\(\alpha\) that had become associated to NF-κB present in the activated HeLa extract. Everything else as was done for A. C, the supernatant from the RelA IP done in B was subjected to a second round of IP with anti-IkB\(\alpha\) sera. This isolated the free IkB\(\alpha\) in the in vitro ubiquitination assay. D, in vivo signal-dependent ubiquitination. The IkB\(\alpha\)^{−/−} MEF stable pools were treated with \(\beta\)-lactone for 1 h and then stimulated with TNF\(\alpha\) for 15 min. Cytoplasmic extracts were made and an IkB\(\alpha\) immunoblot was performed. Ub, ubiquitin.

those obtained in vitro (Fig. 4A, lanes 3 and 6). MutC alone is ubiquitinated very efficiently and the 3236mutC mutation gives a reduction in the amount of ubiquitination but the level of ubiquitination seen is significantly higher than that seen with just the 3236 mutant (Fig. 4D, compare lanes 3 and 6). These data show that free IkB\(\alpha\) is ubiquitinated in the presence of signal even when it is lacking its signal-dependent phosphorylation sites. It is likely that signal-independent degradation mechanisms that are involved in removing free IkB\(\alpha\) from the cell are contributing to the ubiquitination of the mutC containing mutants in these experiments.

From the in vitro and in vivo ubiquitination data we conclude that phosphorylation of serine 32 and serine 36 by the IKK is necessary for efficient signal-dependent ubiquitination and degradation of free and NF-κB-associated IkB\(\alpha\). We also conclude that the constitutive CKII phosphorylation of the carboxyl terminus of IkB\(\alpha\) enhances the extent of ubiquitination of free IkB\(\alpha\) (Fig. 4C, lane 2), but reduces the ubiquitination of NF-κB-associated IkB\(\alpha\) (Fig. 4, B and D, lane 2).

Signal-independent Turnover of IkB\(\alpha\).—To investigate whether or not the constitutive or signal-dependent phosphorylation sites are involved in the signal-independent turnover of NF-κB-associated IkB\(\alpha\), pulse-chase experiments were performed on the wt, mutF, and 3236 IkB\(\alpha^{−/−}\) MEF stable pools (Fig. 5, A-C). Compared with the half-life of wt IkB\(\alpha\), the mutF mutation gives a 2-fold increase in IkB\(\alpha\) half-life. The 3236 mutation has no effect on basal turnover, resulting in a half-life very similar to that of wt IkB\(\alpha\) (compare A and C). These results demonstrate that only the carboxyl-terminal CKII phosphorylation sites play a significant role in the basal turnover of NF-κB-associated IkB\(\alpha\). The inducible phosphorylation sites (serine 32 and 36) have no effect on the basal turnover of NF-κB-bound IkB\(\alpha\).

Several studies have been performed to evaluate the role of phosphorylation in the degradation of free IkB\(\alpha\) in the absence of stimulation (35, 45, 46). It has been shown that the mutF mutation or the removal of the carboxyl terminus of IkB\(\alpha\) will increase the stability of free IkB\(\alpha\) by approximately 2-fold (35, 46). Here we look at the effects of the 3236 mutation on the basal turnover of free IkB\(\alpha\). Unstimulated IkB\(\alpha^{−/−}\) MEF stable pools expressing either the mutC mutant or the 3236mutC mutant (Fig. 6, A and B) were analyzed. As has been shown (Fig. 3B) mutC and 3236mutC do not associate to RelA/p65, thus all the IkB\(\alpha\) is in a free state in the IkB\(\alpha^{−/−}\) MEF stable pools. Free IkB\(\alpha\) is at least 5 times more rapidly degraded in the absence of signal when compared with NF-κB-bound IkB\(\alpha\) (compare Fig. 5A and 6A). Interestingly, the 3236 mutation in the mutC background increases the rate of signal-independent turnover of free IkB\(\alpha\) by about 3-fold (Fig. 6, A and B).

To determine whether or not ubiquitination plays a role in the basal turnover of NF-κB-associated IkB\(\alpha\), the IkB\(\alpha^{−/−}\) MEF stable pools were treated with the proteasome inhibitor \(\beta\)-lactone in the absence of signal. Unfortunately, the prolonged treatments with \(\beta\)-lactone necessary to see the accumulation of ubiquitinated IkB\(\alpha\) bound to NF-κB in the absence of signal seems to activate the NF-κB pathway (data not shown). This is
most likely caused by the tremendous stress that the cell experiences when its proteasomes are inactivated for prolonged periods of time. Consequently, no concrete conclusions could be made about the role of ubiquitination in the basal turnover of NF-κB-associated IκBα.

The signal-independent ubiquitination of free IκBα is quite robust (Fig. 7, lanes 2–4) which may explain the rapid basal turnover of free IκBα when compared to NF-κB-associated IκBα (110 and 550 min, respectively). Free IκBα is very unstable in the absence of signal (Fig. 6A) and a detectable pool of ubiquitinated mutC and 3236mutC is observed in the absence of any stimulation and in the absence of a proteasome inhibitor (Fig. 7). The 3236 mutation in the mutC background does not reduce signal-independent ubiquitination of free IκBα (Fig. 7, lane 4). Therefore, phosphorylation of serine 32 and serine 36 is not necessary for efficient signal-independent ubiquitination and degradation of free IκBα.

We have previously shown that the carboxyl-terminal CKII sites are necessary for the efficient basal turnover of free IκBα (35, 46). Here we conclude (i) that efficient basal turnover of NF-κB-associated IκBα requires the carboxyl-terminal CKII phosphorylation and (ii) that IKK phosphorylation (serine 32 and 36) plays no role in the basal turnover of free or NF-κB associated IκBα.

**DISCUSSION**

IκBα functions as the primary regulator of NF-κB in both stimulated and unstimulated cells. To accomplish this, IκBα...
itself must be a tightly regulated protein. NF-κB activity is positively and negatively regulated through IkB phosphorylation and NF-κB-dependent synthesis of IkBα, respectively. Mechanisms that ensure proper NF-κB activity must exist to regulate signal-dependent degradation of free and NF-κB-associated IkBα as well as the signal-independent turnover of free and NF-κB-associated IkBα. These mechanisms also must allow newly synthesized IkBα the opportunity to enter the nucleus in order to remove NF-κB from the DNA and/or inhibit further activation upon the removal of signal (33). One way that the cell can easily and efficiently regulate the multiple states and fates of IkBα is through phosphorylation. Phosphorylation seems to be involved in almost all aspects of IkB regulation. We demonstrate when and how some of the different phosphorylation sites on IkBα can influence ubiquitination, degradation, and the overall stability of free and NF-κB-associated IkBα in the presence or absence of NF-κB inducing stimuli. Our conclusions about the role of phosphorylation and ubiquitination in the regulation of signal-dependent and -independent degradation of free and NF-κB-associated IkBα are summarized in Fig. 8.

Signal-dependent Degradation of NF-κB-associated IkBα—Several groups have shown that Ser-32 and Ser-36 undergo signal-dependent phosphorylation and that mutating these sites stabilizes IkBα in the presence of stimuli (18–21). There are many different views on the role of the carboxy terminus and the CKII phosphorylation sites in signal-dependent degradation of IkBα (19, 21, 29, 35, 46–49). Using a variety of phosphorylation site mutations stably expressed in an IkBα−/− MEF cell line, we have confirmed that by mutating serine 32 and serine 36 to alanine, IkBα is stabilized in the presence of TNFα stimulation (18–21). We have also shown that the mutation of the carboxy-terminal CKII sites (mutF) causes a significant stabilization of IkBα during TNFα stimulation but to a lesser extent than the 3236 mutation. Although work shown here was carried out using transduced IkBα−/− MEF cells, similar results were also obtained using transduced HeLa cell lines (data not shown). From these results we conclude that both IKK and CKII phosphorylation play an important role in the signal-dependent degradation of NF-κB-associated IkBα.

Experiments were also carried out to study the critical step of IkBα polyubiquitination during signal-dependent degradation of NF-κB-associated IkBα. In vitro and in vivo systems clearly showed that IkBα stability was increased in the 3236, M, and mutF mutants due to decreased levels of ubiquitination. The 3236 mutant simply blocks the signaling cascade from continuing to the ubiquitination step, while the mutF mutation is only able to decrease the efficiency of the reactions that lead to IkBα degradation. Thus, in agreement with others we conclude that both the amino-terminal IKK sites and the carboxyl-terminal CKII sites play significant roles in the signal-dependent degradation of NF-κB-associated IkBα. We also conclude that both amino-terminal IKK and carboxyl-terminal CKII phosphorylation is necessary for efficient signal-induced ubiquitination of NF-κB associated IkBα.

Signal-dependent Degradation of Free IkBα—To maintain a sensitive and rapidly responding NF-κB pathway the cell must be relatively free of unbound IkBα. If newly synthesized IkBα does not associate with NF-κB, the cell must clear this free population of IkBα before proper activation of NF-κB can take place. There is evidence that free IkBα is degraded in a signal-dependent manner and that this event is mainly regulated by the amino terminus of IkBα (45, 46). There are also reports that large carboxyl-terminal deletions that stretch into the sixth ankyrin repeat, and are believed to disrupt NF-κB association, can stabilize IkBα in the presence of stimuli (21, 47, 48, 50).

Here we investigated the role of the amino- and carboxyl-terminal phosphorylation sites in the context of a full-length IkBα molecule using both in vitro and in vivo systems. We were able to identify a mutant, mutC, that is degraded upon stimulation but does not associate to NF-κB. The mutC mutant contains five serine and threonine to alanine mutations in the ankyr6 region. The 3236 mutation in the mutC background is able to partially stabilize IkBα in the presence of TNFα without...
having any effect on NF-κB DNA binding activity. The 3236mutC mutant also shows reduced signal-induced ubiquitination in vitro compared with the wild type levels seen in the presence of mutC alone (Fig. 4). A reduction in signal-dependent ubiquitination of free IkBα containing the 3236 mutation was also observed in vitro. Therefore the amino-terminal IKK phosphorylation sites play a critical role in signal-induced ubiquitination and degradation of free IkBα.

Several groups have reported on the effects of deleting or mutating the carboxyl-terminal PEST domain. Five groups report that the PEST domain does play a role in signal-dependent degradation (19, 21, 29, 45, 48), while four other groups report that it does not (46, 47, 49, 50). Here we examine free full-length IkBα containing five serine and threonine to alanine mutations in its PEST domain (mutF). In our in vitro ubiquitination system the mutF mutation caused a slight increase in the extent of signal-induced ubiquitination of free IkBα when compared with wild type IkBα. This is in contrast to the decrease in ubiquitination that was seen for mutF while in association with NF-κB. In the presence of NF-κB inducing stimuli, the differential regulation of IkBα stability by the carboxyterminal CKII sites indicates that a very interesting and sophisticated mechanism is utilized to regulate the different populations of IkBα during signaling. We conclude that CKII phosphorylation is necessary for efficient signal-dependent degradation of NF-κB-associated IkBα, but it is not necessary for efficient signal-dependent degradation of free IkBα (46). In fact, the absence of CKII phosphorylation on free IkBα, under stimulated conditions, may potentiate the rate at which it is ubiquitinated and subsequently degraded by the 26 S proteasome. This ensures that NF-κB activation is maintained until stimuli is removed and IKK activation is down-regulated.

Signal-independent Turnover of NF-κB-associated IkBα—IkBα is a more stable molecule while associated to NF-κB than in its free state (45, 51, 52). However, in the associated state, IkBα is not totally stabilized because NF-κB activation and IkBα degradation can be observed within a couple of hours after treating cells with cyclohexamide to block new protein synthesis (53, 54). This activation is due to the signal-independent turnover of the NF-κB-associated IkBα. We show that mutating the IKK phosphorylation sites in IkBα has no effect on the basal degradation rate of NF-κB-associated IkBα (Fig. 5, A and C). On the other hand, mutating the CKII sites caused IkBα half-life to double (Fig. 5B). We were unable to determine if ubiquitination plays a significant role in the basal turnover of NF-κB-associated IkBα because long term treatment (2–4 h) of the cells with β-lactone seems to activate the NF-κB pathway. A role for ubiquitination in the basal turnover of NF-κB-associated IkBα may be observed in Fig. 7 (comparing lanes 1 and 2). It appears that there may be a detectable pool of ubiquitinated IkBα even in the absence of an NF-κB inducing signal. Therefore, we conclude that only the constitutive CKII phosphorylation sites are important for signal-independent degradation of NF-κB-associated IkBα.

Basal Turnover of Free IkBα—We have previously shown that both a carboxyl-terminal deletion of 39 amino acids and that the mutF mutation can increase the stability of free IkBα in the absence of signal (35, 46, 49). In this report we go on to show that free IkBα is very efficiently ubiquitinated in the absence of signal, and that polyubiquitinated IkBα can be detected even in the absence of a proteasome inhibitor (Fig. 7, lane 3). We also demonstrate that the 3236 mutation is unable to block ubiquitination of free IkBα in the absence of signal (Fig. 7, lane 4). We propose that only the CKII sites are significant regulators of the signal-independent degradation of free IkBα, and that this degradation is mediated by ubiquitination and the 26 S proteasome.

An important component of IkBα regulation/degradation that still needs to be identified is the signal-independent ubiquitin-conjugation-ligase complex. We have shown that free and possibly NF-κB-bound IkBα undergo signal-independent ubiquitination. It has also been shown that the IkBα-ligase complex, that was recently identified (22, 23, 55, 56), only binds to and ubiquatinates IkBα when it is phosphorylated at its signal-induced phosphorylation sites (22, 23, 55–57). This suggests that a signal-independent IkBα ubiquitin-ligase complex must exist. It is possible that this signal-independent ubiquitin ligase recognizes the same residues as the signal-dependent ligase but only when serine 32 and serine 36 are unphosphorylated. When IkBα is bound to NF-κB the signal-independent ligase may only be able to associate to IkBα with a weak affinity due to steric hindrance caused by IkBα association to NF-κB. When IkBα is free in the cell the steric hindrance may be relieved allowing the signal-independent ubiquitin ligase to bind to IkBα with a much higher affinity. This would explain the differences in the rate of ubiquitination and basal turnover between free and NF-κB-bound IkBα. This could also explain why we observed an increase in the basal turnover of free IkBα containing the 3236 mutation (Fig. 6B). By changing serine 32 and serine 36 to alanine we may have created a molecule with an even higher affinity for the signal-independent ubiquitin-ligase complex. An increase in the affinity of this ligase for its target would most likely result in an increase in the rate of its ubiquitination and subsequent degradation. Identification of the signal-independent ubiquitin ligase would allow further dissection of the mechanisms involved in IkBα regulation.

Although our results are internally consistent and supported by previous biochemical and structural studies it is always necessary to consider the possibility that introducing mutations into any given protein may have unintended effects. It is for this reason that we utilized point mutations rather than gross deletions as has been done in other studies. It was our hope that by minimizing the changes made to IkBα and by utilizing full-length proteins that we would be able to obtain the most meaningful results.

In summary, we have investigated the role of phosphorylation in the regulation of IkBα stability. We have demonstrated that specific phosphorylation events can have very different implications depending on the state of the cell and of IkBα. The complexity of the regulation of IkBα is a reflection of its importance to the cell. It will be interesting and of much importance to further explore the effects that other components of the IkB kinase complex and degradation machinery have on the phosphorylation, ubiquitination and degradation of IkBα.

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