Critical Role for Lysines 21 and 22 in Signal-induced, Ubiquitin-mediated Proteolysis of IκB-α*

Lucia Baldi, Keith Brown, Guido Franzoso, and Ulrich Siebenlist‡

Laboratory of Immunoregulation, NIAID, National Institutes of Health, Bethesda, Maryland 20892-1876

The NF-κB transcription factor induces rapid transcription of many genes in response to a variety of extracellular signals. NF-κB is readily activated from normally inhibited cytoplasmic stores by induced proteolytic degradation of IκB-α, a principal inhibitor of this transcription factor. Following the inhibitor's degradation, NF-κB is free to translocate to the nucleus and induce gene transcription. The IκB-α inhibitor is targeted for degradation by signal-induced phosphorylation of two closely spaced serines in its NH2 terminus (Ser32 and Ser36). Proteolytic degradation appears to be carried out by proteasomes which can recognize ubiquitinated intermediates of the IκB-α inhibitor. We provide evidence which supports a ubiquitin-mediated mechanism. Amino acid substitutions of two adjacent potential ubiquitination sites in the NH2 terminus of IκB-α (Lys21 and Lys22) almost completely block the rapid, signal-induced degradation of the mutant protein, while they do not interfere with induced phosphorylation. The mutant IκB-α also does not permit signal-induced activation of NF-κB bound to it. The data suggest that ubiquitination at either of the two adjacent lysines (21 and 22) is required for degradation following induced phosphorylation at nearby serines 32 and 36. Such dependence on ubiquitination of specific sites for protein degradation is unusual. This mechanism of degradation may also apply to IκB-β, an inhibitor related to and functionally overlapping with IκB-α, as well as to cactus, an IκB homolog of Drosophila.

The transcription factor complexes known collectively as NF-κB function primarily as mediators of inducible transcription in response to a variety of environmental signals (for recent reviews, see Refs. 1–5). Stress- and pathogen-related signals in particular are known to activate NF-κB, leading to induced expression of a large number of genes, including many genes which encode functions relevant to immune responses. In most cell types, p50-p65 heterodimers represent the vast majority of the rapidly inducible NF-κB complexes, although several other NF-κB dimers may coexist and may become activated also. All NF-κB dimers are composed of members of the Rel/NF-κB family of polypeptides, and in vertebrates this family is comprised of p50 (NF-κB1), p65 (RelA), c-Rel, p52 (NF-κB2), and RelB. The various NF-κB dimers usually lie dormant in the cytoplasm of cells, kept there by inhibitory ankyrin-containing members of the IκB family of proteins, in particular IκB-α. IκB-α strongly associates with p50/p65 heterodimers and appears to shield the nuclear localization sequences contained in both subunits; this is presumed to be the mechanism by which this protein retains the heterodimers in the cytoplasm (6–9). Activation of NF-κB proceeds via rapid, signal-induced proteolytic degradation of the inhibitor, liberating the transcription factor which is now free to translocate to the nucleus (10–17). Degradation is carried out by proteasomes and is preceded by signal-induced phosphorylation of IκB-α itself (18–24). Induced phosphorylation occurs on two closely spaced serines in the NH2 terminus of the protein (amino acids 32 and 36), mediated by an as yet unknown kinase(s) (25–28). It has recently been shown that signal-induced phosphorylation can lead to ubiquitination of IκB-α (29). Since ubiquitin-tagged proteins are generally subject to proteasome-mediated proteolysis (30), these observations suggest that degradation of IκB-α is triggered by ubiquitination. However, a ubiquitin-independent mechanism of degradation is not necessarily excluded, since only a fraction of the total pool of IκB-α could be shown to be ubiquitinated (under conditions of proteolysis inhibition). Furthermore, precedents exist for a ubiquitin-independent, but proteasome-dependent degradation mechanism (31). Therefore, we sought to demonstrate ubiquitin-dependence by investigating the requirement for lysines in IκB-α degradation, because lysines are the sites at which ubiquitin is ligated (30). Here we provide evidence which strongly suggests that rapid, signal-regulated degradation of IκB-α proceeds primarily via a ubiquitin-independent mechanism. An IκB-α mutant bearing conservative substitutions at two potential ubiquitination sites is remarkably resistant to signal-regulated degradation. The results imply that two adjacent NH2-terminal lysines (Lys21 and Lys22) are the primary targets of signal-induced ubiquitination. That specific lysines play such an important role in ubiquitin-mediated protein degradation is uncommon (30).

MATERIALS AND METHODS

Site-directed Mutagenesis—Mutations in full-length, human IκB-α cDNA (32) were generated essentially as described previously (25, 33). In each case, lysine codons were substituted with arginine codons (for positions 21, 22, 47 and 67: AAG was changed to AGG; for 38: AAA was changed to CGA; for 87: AAG was changed to CGG). The mutated IκB-α cDNAs were excised from a Bluescript vector (Stratagene, La Jolla, CA) by EcoRI (1500 base pairs) and subcloned into the PMT2T mammalian expression vector (34). Mutations were confirmed by subsequent DNA sequence analysis.

Transient Transfections—Ntera-2, human embryonal carcinoma cells were transfected via calcium phosphate-mediated transfer as described elsewhere (35) with the following vectors: a CAT* reporter plasmid containing the tandemly repeated κB sites of human immunodeficiency virus (5 μg) (35); the p65-PMT2T expression vector (0.2 μg) (35); PMT2T vectors carrying the wild-type or mutant IκB-αs (see above) depending on the experiment; between 0.15 and 0.6 μg were found to be required for near-maximal inhibition, which was the point used in the experiments). Cells were stimulated with PMA (10 ng/ml)

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†To whom correspondence should be addressed: Dr. Ulrich Siebenlist, NIH, Bldg. 10, Rm. 11816, Bethesda, MD 20892-1876. Tel.: 301-496-7662; Fax: 301-402-0070; E-mail: us3n@nih.gov.

†The abbreviations used are: CAT, chloramphenicol acetyltransferase; PMA, phorbol 12-myristate 13-acetate.
for 6 h prior to harvesting, stimulation starting at about 36 h posttransfection, and CAT activity was measured as previously described (25, 35–38).

Permanent Transfections—EL-4 murine T lymphoma cells were maintained in RPMI medium in the presence of 10% FCS (Life Technologies, Inc.) and were stably transfected with the various PMT2T-IκBα vectors, together with a plasmid conferring neomycin resistance, as described elsewhere (25). Briefly, 20 μg of PMT2T-IκBα DNA and 2 μg of neomycin-resistant plasmid DNA were electroporated into 10^7 EL-4 cells using the Bio-Rad Gene Pulser, set at 250 V, 960 microfarads. Stable neomycin-resistant transfectants were selected after 3–4 weeks of incubation with genetin (G418, Life Technologies, Inc.), used at 400 μg/ml during the first week and at 200 μg/ml thereafter. The resulting cell lines were screened for expression of human IκBα using a rabbit polyclonal antibody directed against full-length IκBα (10, 25) and ECL technology (Amersham Corp.). Cells were stimulated with PMA (40 ng/ml) and ionomycin (2 μM), and calpain inhibitor I was used at 100 μM, starting 30 min prior to stimulation.

RESULTS

Since ubiquitin is ligated to proteins through lysine residues, we substituted the lysines in IκBα by site-directed mutagenesis and tested the resulting mutant proteins for defects in signal-dependent degradation. Human IκBα (Mad-3) contains lysine at positions 21, 22, 38, 47, 67, 87, 98, 177, and 238 (32). Lysines 22, 38, 87, and 238 are perfectly conserved in pig, rat, and chicken (pp40) IκBα; lysines 21, 47, 67, and 98 are absent in chicken; and lysine 177 is not conserved at all (39). We substituted each of the NH2-terminal lysines (21, 22, 38, 47, 67, and 87) and the pair, 21 + 22, with arginine residues to block ubiquitination of these sites which all lie near the inducibly phosphorylated serines 32 and 36. Arginine was chosen so as not to change the charge of the protein. As an initial test of the mutant proteins, we transiently transfected expression constructs for the various IκBα mutants into NTera-2 embryo-stimulated carcinoma cells, together with an expression construct for NF-kB/p65. Undifferentiated NTera-2 cells serve as an internal control. 

Figure 1: Inducibility of a NF-kB-dependent CAT reporter in the presence of wild-type (wt) or mutant IκBαs in transiently transfected NTera-2 cells. NTera-2 cells were transfected with PMT2T-IκBα/p65, wild-type or mutant PMT2T-IκBα expression vectors and the NF-kB-dependent CAT reporter plasmid (see “Materials and Methods”). (p65, transfected alone, potently stimulated CAT activity, and cotransfection of the IκBα vectors inhibited this transactivation to near-background levels (25).) The p65/IκBα cotransfected cells were stimulated with PMA, and inducibility was calculated as the ratio of PMA-stimulated CAT activity to unstimulated activity. The inducibilities are shown as a percent of that seen with matched, wild-type IκBα, which represents an at least 10-fold stimulation. In several independent experiments, only the K21R/K22R (R22R22) mutant blocked PMA induction of CAT activity.

To confirm these interpretations and to rule out a potential defect in phosphorylation of the K21R/K22R mutant, we directly evaluated mutants for phosphorylation and degradation. Murine EL-4 T cells were permanently transfected with the various IκBα mutants, and then the cells were stimulated with PMA and ionomycin. We showed previously that exogenously derived human IκBα is subject to the same signal-induced phosphorylation and degradation as the endogenous murine IκBα (25). Endogenous murine IκBα serves as an internal positive control in these experiments. Since it migrates slightly faster than the transfected human protein, both proteins could be simultaneously visualized (25). Among the mutants tested, only the K21R/K22R mutant was resistant to signal-induced degradation in the EL-4 cells (Fig. 2), while all other transfected human (h) mutant proteins bearing individual lysine substitutions appeared to be degraded as efficiently as the endogenous murine IκBα (25). All mutant proteins, including the double mutant K21R/K22R, were rapidly phosphorylated in response to signals, as indicated by the shift in mobility of IκBα in the presence of calpain inhibitor I, which inhibits proteasomes (20–24) (Fig. 2, data not shown for K38R, K47R, K67R, and K87R). This is as expected for the rapidly degraded IκBα proteins. In the case of the double mutant (K21R/K22R), the proteasome inhibitors were not needed to see the phosphorylation, since this mutant was...

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not efficiently degraded. The presence of the proteasome inhibitor did, however, increase the amount of the K21R/K22R IκB-α mutant observed, suggesting that these mutations may not completely block induced degradation. Nonetheless, the K21R/K22R mutation afforded this IκB-α significant protection from degradation, and the results are consistent with the Ntera-2 experiments shown in Fig. 1.

DISCUSSION

We have demonstrated a critical requirement for the presence of either of two lysines at positions 21 and 22 in signal-induced degradation of IκB-α and, as a consequence, in signal-induced transactivation by NF-κB/p65. The substitution of both lysines 21 and 22 with arginines in IκB-α (K21R/K22R) caused a severe block to rapid signal-induced degradation of that IκB-α in stably transfected EL-4 cells. The mutant protein was, however, still phosphorylated at nearby serine sites (S32 and S36), indicating that the defect lies downstream of the phosphorylation step; it also suggests that the protein was not grossly altered by these conservative substitutions, since the kinase(s) activity on this substrate appears unaffected. The K21R/K22R mutant prevented the signal-induced, p65-mediated transactivation of a κB-dependent reporter in transient transfection experiments using Ntera-2 cells. In contrast to the K21R/K22R mutant, mutants bearing substitutions of individual lysines, including those at residue 21 or 22, had no measurable effect and behaved like wild type. Taken together these data demonstrate that either of the two lysines at positions 21 and 22 is necessary for rapid degradation (but not for phosphorylation) and they provide a compelling argument for obligatory ubiquitination prior to degradation of IκB-α: at least one of the two potential ubiquitination sites must be present for rapid signal-induced degradation to proceed. Although degradation is dramatically inhibited, it appears not to be absolutely blocked (see Fig. 2); this may indicate that ubiquitination can also occur at other sites, albeit less efficiently, or that another mechanism allows for a slower degradation. Finally, the data do not tell us if Lys21 or Lys22 are sufficient for ubiquitin-mediated degradation. It is possible, for example, that some other, not necessarily specific, lysine is necessary also. To formally test this less likely possibility would require a mutant IκB-α bearing substitutions of all lysines other than 21 and 22.

Individual ubiquitination sites do not usually play a dominant role in protein degradation, where often multiple functional ubiquitination sites exist and targeted mutations have little effect (30), although the degradation of Mos may be another exception to this rule (44). It is possible that ubiquitination of IκB-α may be specifically directed to lysines 21 and 22, or, alternatively, that these lysines are the only ones accessible for ligation (no other lysines exist NH2-terminal to the phosphorylation sites). This latter possibility may be supported by the observation that ubiquitination occurs with IκB-α still bound to NF-κB (29), which should partially shield the inhibitor. The central part of IκB-α consists of 6 ankyrin repeats whose primary function is to interact with NF-κB; this part may be largely buried in the cleft between the two NF-κB subunits, as suggested by x-ray crystallographic data of p50 homodimers (45, 46). The fairly short COOH-terminal region of IκB-α is required for inhibition of DNA binding by NF-κB, implying that it too may interact with NF-κB proteins (25, 43). By contrast, the NH2-terminal part of the protein is not required for these functions, rather, it must be accessible to a kinase(s) to allow inducible phosphorylation. An as yet undetermined protein may then recognize the phosphorylated protein, presumably by recognizing the phosphorylated serines or local changes induced in the protein as a consequence of phosphorylation (no major conformational changes are expected, since the phosphorylated species remains tightly bound to NF-κB and continues to inhibit) (18–24). Thus, the two lysines NH2-terminal to the two serines may present the only obvious targets. It remains to be shown whether highly ubiquitinated IκB-α is removed from the complex just prior to degradation, or if degradation is initiated while ubiquitinated IκB-α is still in the complex. In contrast to bound IκB-α, the free unbound form may present additional sites for ubiquitination.

Chicken IκB-α (pp40) contains only one of the two lysine residues important for degradation (the Lys equivalent to that at position 22 in the human protein), suggesting that a single substitution of that lysine may be sufficient to block rapidly inducible degradation of pp40. Recently IκB-β was cloned and shown to be inducibly degraded in response to certain signals, such as interleukin-1 and lipopolysaccharide (47). While IκB-α and IκB-β share high overall similarity, their NH2-terminal

![Fig. 2. Signal-induced degradation and phosphorylation of wild-type and mutant IκB-α expressed in stably transfected EL-4 cells.](http://www.jbc.org/)

![Fig. 3. Sequence comparisons of IκB-α, IκB-β, and cactus.](http://www.jbc.org/)
regions are surprisingly different, save for a few conserved amino acids; however, these few amino acids appear to be highly significant in that they suggest shared regulatory features of these proteins (Fig. 3). Both inducibly phosphorylated serines and a few surrounding residues are conserved as is the lysine equivalent to that at position 22 in IκB-α. (This is the only lysine in the entire NH2-terminal part of the IκB-β protein, which may suggest that it is absolutely required for signaling in that protein). This limited but significant conservation of functional sites can also be found in cactus, the Drosophila homolog of IκB proteins (48, 49) (see Fig. 3), suggesting that all three proteins may be regulated in a similar fashion. Cactus, which contains a much larger NH2-terminal domain than either IκB-α or IκB-β, may offer additional sites for regulation.

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