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Multiple Structural Domains within IκBα Are Required for Its Inducible Degradation by both Cytokines and Phosphatase Inhibitors

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Activation of the transcription factor NF-κB by various cellular stimuli involves phosphorylation and subsequent degradation of its inhibitor IκBα. Both the cytokine tumor necrosis factor alpha (TNF-α) and the phosphatase inhibitor calyculin A have been shown to induce rapid phosphorylation and degradation of IκBα. In the present study, we demonstrate that TNF-α and calyculin A stimulate similar although not identical pattern of IκBα phosphorylation, as demonstrated by phosphopeptide mapping. Interestingly, phosphorylation of IκBα induced by both inducers involves serine-32 and serine-36 of IκBα. Furthermore, TNF-α- and calyculin A-induced degradation of IκBα appears to require the same structural domains within IκBα. In addition to the N-terminal phosphorylation sites and the C-terminal sequences, each of the five ankyrin-like repeats of IκBα is critically required for the inducible degradation of this NF-κB inhibitor. Together, these studies suggest that degradation of IκBα by both cytokines and phosphatase inhibitors is regulated by site-specific phosphorylation and requires multiple structural domains.

Nuclear factor κB (NF-κB) is a pleiotropic eukaryotic transcription factor implicated in the transcriptional regulation of the type 1 human immune deficiency virus (HIV-1) (1–5) and various cellular genes involved in the immediate early processes of immune, acute phase, and inflammatory responses (6,7). In most cell types, the predominant inducible form of NF-κB corresponds to a heterodimeric complex composed of 50 kD (p50) and 65 kD (Rel A, previously termed p65) subunits. Both of these subunits share a region of N-terminal homology (Rel homology domain, ~300 amino acids) with the proto-oncoprotein c-Rel and other members of the NF-κB/Rel transcription factor family (reviewed in Ref. 8, 9). In resting T cells and many other non-stimulated cells, the p50/RelA heterodimer is sequestered in the cytoplasm as a latent precursor complexed with specific inhibitory proteins including IκBα and other structurally related proteins (10, 11, reviewed in Ref. 12). IκBα appears to specifically bind to and mask the nuclear localization signal of Rel A, thereby preventing the nuclear translocation of the NF-κB complex (13–16).

The latent cytoplasmic NF-κB complex is posttranslationally induced by a variety of extra- and intra-cellular signals, including phorbol esters, cytokines such as tumor necrosis factor alpha (TNF-α), phosphatase inhibitors such as okadaic acid and calyculin A, and the Tax protein from the type I human T-cell leukemia virus (HTLV-I) (9). Activation of NF-κB by these cellular stimuli involves the transient phosphorylation and subsequent proteolytic degradation of IκBα which allows nuclear translocation of the liberated NF-κB complex (17–24). Recent studies have revealed the involvement of two N-terminal serine residues, serine-32 and serine-36, in the regulation of IκBα phosphorylation and degradation (25, 26). In addition, deletion of the C-terminal sequences, especially a region rich in glutamine and leucin (QL region) also abolishes the degradation of IκBα by TNF-α (27).

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Abbreviations used: NF-κB, Nuclear factor, κB; HIV-1, type 1 human immune deficiency virus; TNF-α, tumor necrosis factor alpha; IL-1, interleukin-1; HTLV-I, type I human T-cell leukemia virus; HA, hemagglutinin; IκBα-P, phosphorylated IκBα.

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Although both TNF-α and the phosphatase inhibitor calyculin A have been shown to induce the phosphorylation and degradation of IκBα (24), it remains unclear whether different inducers eliminate IκBα with the same mechanism. Data from the present study demonstrate that although TNF-α and calyculin A stimulate different pattern of IκBα phosphorylation, degradation of IκBα by both inducers appear to be mediated by the same regulatory mechanism.

MATERIALS AND METHODS

cDNA expression vector. pCMV4HA-IκBα was constructed by inserting three copies of the influenza hemagglutinin (HA) epitope tag (YPYDVPDYA) in front of the translational initiation codon of the human IκBα cDNA (10) cloned in the pCMV4 expression vector (28). Transfection of mammalian cells with this construct leads to the expression of a 40 kD HA-IκBα fusion protein, which is about 3 kD larger than its endogenous counterpart and can be detected by both anti-IκBα and a monoclonal antibody raised against the HA epitope (anti-HA, Boehringer Mannheim). Site-directed mutations and ankyrin deletions were introduced into the full-length IκBα cDNA using a site-directed mutagenesis kit (CLONTECH Laboratories, Inc.). The IκBα mutants were analyzed by DNA sequencing.

HeLa cell transfection and immunoblotting. HeLa cells were maintained in DMEM medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics. The cells were seeded onto 0.1% gelatin-treated, 24-well plates (5×10^4 cells/well) 14–24 hr before transfection. The pCMV4 based cDNA expression vectors encoding the wild-type or various mutant forms of the HA-tagged IκBα were transfected into the cells using liposome-mediated gene transfer according to the manufacturer's instructions (LIPOFECTAMINE™, Gibco, BRL). 0.5 μg plasmid DNA and 1.75 μl LIPOFECTAMINE™ reagent were used for each transfection. At about 48 hr posttransfection, the recipient cells were stimulated for the indicated time periods with TNF-α (80 ng/ml), and whole-cell extracts were prepared from the cells by *in situ* lysis using a detergent lysis buffer (14) supplemented with various phosphatase inhibitors (29). Human kidney 293 cells were maintained in Iscove's medium with the same supplements as for DMEM. Cells were transfected using DEAE-dextran (14) and then stimulated with calyculin A (60 ng/ml) followed by whole-cell extract preparation as described above. The extracts were fractionated by reducing 10% SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and then analyzed for immunoreactivity with anti-HA using an enhanced chemiluminescence detection system (Dupond, NEN).

Cell labeling, immunoprecipitation, and phosphopeptide mapping. Jurkat cells or transiently transfected 293 cells were incubated in a phosphate-free RPMI medium for 60 min and then radiolabeled with ^{32}P-orthophosphate (Dupont, NEN, 1 mCi/ml). After 60 min of labeling, a proteasome inhibitor, MG132 (50 μM, ProScript, Inc.) was added to the culture, and after further incubation of 60 min, the cells were treated with either TNF-α (50 ng/ml) or calyculin A (100 μM) for 15 min and then lysed in ELB buffer {50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.0, 250 mM NaCl, 0.1% Nondidet P-40, 5 mM EDTA, 1.0 mM dithiothreitol, and 1.0 mM phenylmethanesulfonyl fluoride} supplemented with various phosphatase inhibitors (29). The protein extracts were immediately supplemented with 1% SDS, heated for 5 min at 100°C, and then subjected to immunoprecipitation with a peptide-specific antiserum recognizing the C-terminus of IκBα (14). The immunoprecipitated proteins were fractionated by 10% SDS-PAGE, electrophoretically transferred to immobilon membranes (Dupond, NEN). The phosphorylated IκBα bands were identified by autoradiography, excised, and then hydrolized in 6 M HCl for phosphoamino acid analyses (30, 31). For phosphopeptide mapping, the phosphorylated IκBα bands were digested with trypsin and then fractionated in two dimensions (electrophoresis followed by chromatography) using thin-layer chromatography plates (31, 32).

RESULTS AND DISCUSSION

We have previously shown that both TNF-α and calyculin A are able to induce the phosphorylation and degradation of IκBα (24). To examine the mechanism of IκBα degradation induced by these two different inducers, phosphoamino acid and phosphopeptide analyses were performed to compare the pattern of IκBα phosphorylation in cells stimulated with TNF-α and calyculin A. These studies revealed that serine was the predominant form of phosphorylated amino acids of the phosphorylated IκBα (IκBα-P) stimulated by either of the inducers. In addition, a lower level of threonine was also detected, but no phosphorylated tyrosine was detected (data not shown). For phosphopeptide mapping, the phosphorylated IκBα was digested with trypsin followed by two-dimensional separation to distinguish the phosphopeptides. Five major phosphopeptides were detected from the IκBα-P isolated from TNF-α stimulated cells (Fig. 1, left panel, a–e). Trypsin digestion of the IκBα-P isolated from calyculin A-stimulated cells generated similar numbers of phosphopeptides (middle panel), and these peptides appeared to comigrate in the two-dimensional separation with those generated from TNF-α-induced IκBα-P, as demonstrated by phosphopeptide

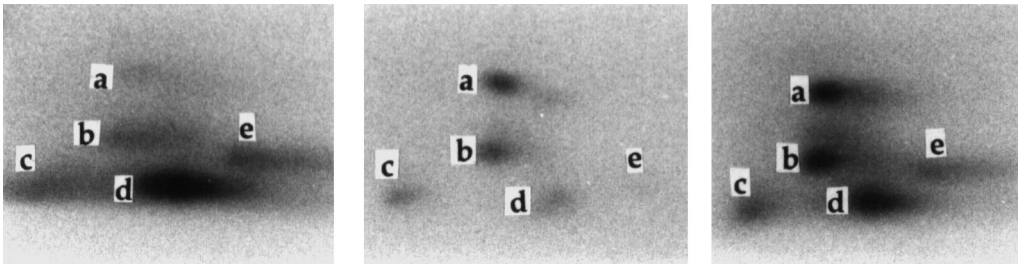

FIG. 1. Phosphopeptide analysis of TNF-α- and calyculin A-induced IκBα-P. IκBα-P isolated from human Jurkat T cells stimulated with either TNF-α (left panel) or calyculin A (middle panel) was digested with trypsin and the generated peptides were separated by two dimensions {electrophoresis (horizontal) followed by chromatography (vertical)} and then subjected to autoradiography. In the right panel, the IκBα-P isolated from the two types of cells were mixed before subjecting to the trypsin digestion and two dimensional separation. The five major phosphopeptides are named a–e.

mapping using a mixture containing equal amounts of radiolabeled IκBα-P isolated from TNF-α and calyculin A-stimulated cells (Fig. 1, right panel). These results suggested that the TNF-α- and calyculin A-induced IκBα phosphorylation might share certain phosphorylation sites. However, the relative intensity of the phosphopeptides generated from calyculin A-induced IκBα-P differed markedly from that observed with TNF-α-induced IκBα-P (compare the left and middle panels). While phosphopeptide d represented the predominant form in TNF-α-induced IκBα-P, phosphopeptides a and b were much more abundant than phosphopeptide d in calyculin A-induced IκBα-P. Thus, the cytokine TNF-α and the phosphatase inhibitor calyculin induced similar but not identical patterns of IκBα phosphorylation.

Prior studies have shown that mutation of serine-32 or serine-36 abolished both the phosphorylation and subsequent degradation of IκBα in cells stimulated with TNF-α or T-cell mitogens (25, 26). Since the TNF-α- and calyculin A-induced IκBα-P share certain phosphopeptides, we examined whether serine-32 and serine-36 were also involved in calyculin A induced IκBα phosphorylation and degradation. For these studies, HA-tagged IκBα was transiently transfected into human kidney 293 cells (Fig. 2). Degradation of these exogenously transfected IκBα molecules were examined by stimulating the transfectants with the phosphatase inhibitor calyculin A followed by immunoblotting analysis of the exogenously transfected IκBα proteins using a monoclonal antibody against the HA epitope tag. As shown in Fig. 2, stimulation of the cells transfected with the wildtype IκBα for 30 min with calyculin A led to the appearance of more slowly migrating IκBα species (lane 2, HA-IκBα-P) that had previously been shown to be the inducibly phosphorylated IκBα (24, 27). Further stimulation of the cells till 60 min resulted in the degradation of IκBα (lane 3). More importantly, the IκBα mutants bearing a mutation (serine to alanine) at either serine-32 (S-32A) or serine-36 (S-36A) was resistant to the inducible degradation, although both mutants

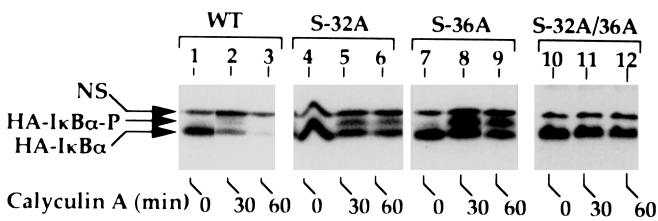

FIG. 2. Serine-32 or serine-36 are required for calyculin A-induced IκBα degradation. Human 293 cells were transfected with cDNA expression vectors encoding either the wild-type (WT) or indicated mutant forms of IκBα and then stimulated with calyculin A (60 ng/ml) for the indicated time periods. Whole-cell extracts were subjected to immunoblotting using anti-HA antibody. Both the basal (HA-IκBα) and phosphorylated (HA-IκBα-P) IκBα are indicated. NS is a protein species nonspecifically reacting with anti-HA.

were still phosphorylated (lanes 4–9). Simultaneous mutation of serine-32 and serine-36 to alanines (S-32/S36) abolished both the phosphorylation and degradation of IκBα (lanes 10–12). Thus, like seen with TNF-α, calyculin A-induced phosphorylation and degradation of IκBα also required the two N-terminal serine residues.

To further explore the biochemical mechanism underlying the degradation of IκBα, each of the five ankyrin-like repeats of IκBα was selectively deleted, and the generated IκBα mutants were subjected to degradation analysis. For these studies, the wildtype IκBα and its mutants were transfected into HeLa cells followed by stimulating the transfectants with TNF-α. Immunoblotting analyses revealed that, following TNF-α stimulation, the wildtype IκBα was rapidly phosphorylated and then degraded (Fig. 3, lanes 1–3). Remarkably, deletion of each of the ankyrin repeats of IκBα either partially (△Ank 2 and 5) or completely(△Ank 1, 3, and 4) inhibited the degradation of IκBα, although these mutants were still phosphorylated (lanes 4–18). Parallel studies performed with 293 cells that were stimulated with calyculin A revealed similar pattern of IκBα phosphorylation and degradation (data not shown). Furthermore, phosphorylation analysis by 32p metabolic labeling revealed that deletion of the ankyrin repeats had little or no effect on the constitutive phosphorylation of IκBα (Fig. 3B). Thus, the ankyrin-like repeats are required for the degradation but not phosphorylation of IκBα. These studies also suggest that TNF-α- and calyculin A-induced degradation of IκBα may be regulated by a similar mechanism, requiring the same sequence elements and structural domains. In further support of this notion, deletion of N- and C-terminal sequences of IκBα abolished its degradation by both calyculin A (Fig. 4, lanes 5–12) and TNF-α (27).

In summary, the current study demonstrates that the cytokine TNF-α and the phosphatase inhibitor calyculin A induces similar although not identical pattern of IκBα phosphorylation. Apparently, serine-32 and serine-36 are involved in the phosphorylation of IκBα induced by both inducers. Our results also demonstrate that, in addition to the N- and C-terminal sequences, each of the ankyrin-like repeats of IκBα is also required for the degradation of IκBα induced by both

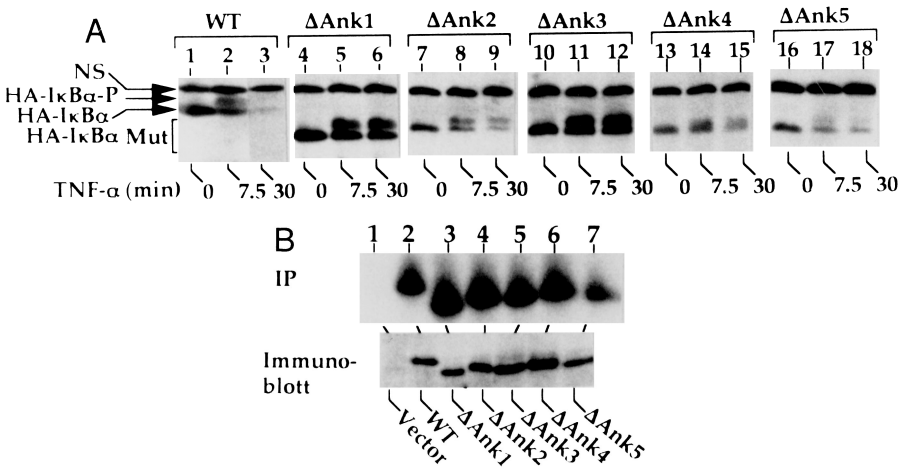

FIG. 3. The ankyrin-like repeats of IκBα are required for the degradation but not for the phosphorylation of IκBα. (A) Phosphorylation and degradation of IκBα by TNF-α. HeLa cells were transfected with either the wildtype IκBα (WT) or IκBα mutants lacking each of the ankyrin-like repeats (ΔAnk1-ΔAnk5) and stimulated with TNF-α for the indicated time periods. Whole-cell extracts were subjected to immunoblotting using anti-HA. The basal and phosphorylated forms of HA-tagged IκBα are indicated. (B) Constitutive phosphorylation of IκBα and its mutants. 293 cells were transfected with the wildtype and mutant forms of IκBα and then metabolically labeled with 32P-orthophosphate. Whole-cell extracts were subjected to immunoprecipitation (upper panel) or immunoblotting (lower panel) using anti-HA. The immunoblotting shown in the lower panel shows that the expression level of the various IκBα constructs are similar.

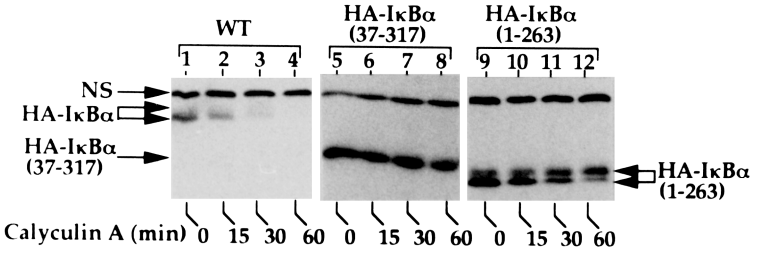

FIG. 4. Both the N- and C-terminal sequences are required for calyculin A-induced IκBα degradation. 293 cells were transfected with either the wildtype IκBα (WT) or its deletion mutants lacking either the N-terminal 36 {IκBα(37–317)} or the C-terminal 54 {IκBα(1–263)} amino acids. The transfectants were stimulated with calyculin A (60 ng/ml) for the indicated time periods, and the cell extracts were subjected to immunoblotting using anti-HA. The upper bands of the wildtype IκBα (WT) and IκBα(1–263) are the phosphorylated forms these IκBα molecules. Due to the lack of serine-32 and serine-36, IκBα(37–317) did not generate the more slowly migrating phosphorylated form. NS represents a nonspecific band.

TNF-α and calyculin A. Together, these results suggest that degradation of IκBα induced by various agents is regulated by phosphorylation and requires multiple structural domains.

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