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Both Amino- and Carboxyl-Terminal Sequences within IκBα Regulate Its Inducible Degradation

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Nuclear expression and consequent biological action of the eukaryotic NF-κB transcription factor complex are tightly regulated through its cytoplasmic retention by an ankyrin-rich inhibitory protein termed IκBα. IκBα specifically binds to and masks the nuclear localization signal of the RelA subunit of NF-κB, thereby effectively sequestering this transcription factor complex in the cytoplasm. Specific cellular activation signals lead to the rapid proteolytic degradation of IκBα and the concomitant nuclear translocation of NF-κB. However, the precise biochemical mechanisms underlying the inhibitory effects of IκBα on RelA and its inducible pattern of degradation remain unclear. By using HeLa cells transfected with various cDNAs encoding epitope-tagged mutants of IκBα, our studies demonstrate the following: (i) sequences within the 72-amino-acid N-terminal region of IκBα are required for tumor necrosis factor alpha (TNF-α)-induced degradation but are fully dispensable for IκBα binding to and inhibition of RelA; (ii) serine residues located at positions 32 and 36 within the N-terminal region of IκBα represent major sites of induced phosphorylation (substitution of these serine residues with alanine abrogates TNF-α-induced degradation of IκBα); (iii) the C-terminal 40 residues of IκBα (amino acids 277 to 317), which include a PEST-like domain, are entirely dispensable for TNF-α-induced degradation and inhibition of RelA; (iv) a glutamine- and leucine-rich (QL) region of IκBα located between residues 263 and 277 and overlapping with the sixth ankyrin repeat is required for both inducible degradation and inhibition of RelA function; (v) regulation of IκBα degradation by this QL-rich region appears to occur independently of phosphorylation at serines 32 and 36. These findings thus indicate that IκBα is generally organized within distinct modular domains displaying different functional and regulatory properties. These studies have also led to the identification of a novel class of dominant-negative IκBα molecules that retain full inhibitory function on NF-κB yet fail to undergo stimulus-induced degradation. These molecules, which lack N-terminal sequences, potently inhibit TNF-α-induced activation of the human immune deficiency virus type 1 κB enhancer, thus indicating their possible use as general inhibitors of NF-κB.

Nuclear factor κB (NF-κB) is a eukaryotic transcription factor that exerts pleiotropic effects on diverse cellular genes involved in the immediate early steps of immune activation and inflammation (6, 31). In addition, NF-κB has been implicated in the transcriptional activation of several viruses, most notably human immune deficiency virus type 1 (HIV-1) (28, 33, 35, 47, 53). NF-κB corresponds to a heterodimeric complex composed of a 50-kDa (p50) and a 65-kDa (RelA [previously termed p65]) subunit. 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 (5, 7, 11, 20, 30, 32, 37, 42; for reviews, see references 21 and 46). The Rel homology domain of p50 and RelA contains sequences required for dimerization, DNA binding, and nuclear translocation (21, 46). Crystallization of the p50 subunit indicates that DNA binding is mediated through N-terminal portions of the Rel homology domain while dimerization involves more C-terminal sequences (19, 34). In addition, RelA contains a potent C-terminal transcriptional activation domain (5, 7, 11, 20, 30, 32, 37, 42–44) and serves as a physiological target for IκBα (10, 18). IκBα (previously termed MAD-3 [22]) is a cytoplasmic protein distinguished by the presence of six ankyrin-like repeats (8, 22) (for sequence alignment, see references 24 and 29). Recent studies have revealed that IκBα specifically binds to and masks the nuclear localization signal of RelA, thereby preventing the nuclear translocation of the RelA-p50 NF-κB complex (10, 18, 26, 55). Association of IκBα with NF-κB also serves to stabilize this otherwise labile cytoplasmic inhibitor (45, 50). Other IκB-like molecules have also been identified, including IκBβ, IκBγ, Bcl-3, p100, and p105, each of which contains a repeated ankyrin-like domain (for recent reviews, see references 8 and 46).

The latent cytoplasmic NF-κB complex is posttranslationally induced by a variety of extra- and intracellular signals, including mitogens such as phorbol esters, cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1, and the Tax protein from human T-cell leukemia virus type 1 (for a recent review, see reference 46). Activation of NF-κB by these and other inducers appears to involve transient phosphorylation and subsequent proteolytic degradation of IκBα, which permits nuclear translocation of the liberated NF-κB complex (9, 14, 15, 25, 39, 49, 50). Nuclear expression of the NF-κB complex leads to transcriptional activation of a broad array of cellular genes involved in immune stimulation, inflammation, and cell growth. In addition, NF-κB induces the IκBα gene itself, leading to feedback inhibition and autoregulation of NF-κB transcriptional activity (14, 45, 50).

Proteolytic degradation of IκBα has been defined as a major step in the induction of NF-κB nuclear expression. Furthermore, recent studies have indicated that degradation of IκBα is

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preceded by its phosphorylation (9, 14, 15, 49, 51). However, it appears that such phosphorylation is not sufficient to promote dissociation of IκBα from NF-κB (17, 49, 55) but rather targets IκBα for subsequent degradation by chymotrypsin-like proteases. This proteolytic reaction likely begins while the inhibitor is still associated with NF-κB, perhaps within the proteasome (38, 55). It also seems likely that phosphorylation of IκBα, which is mediated through one or more not yet identified kinases, serves to alter its conformation, thus promoting proteolysis. In the present study, we have attempted to identify sequences within IκBα that are involved in regulating its inducible degradation by TNF-α and its inhibitory interplay with RelA.

MATERIALS AND METHODS

cDNA expression vectors and reporter plasmids. pCMV4-HA was constructed by inserting a PCR-amplified DNA fragment, encoding three copies of the influenza virus hemagglutinin (HA) epitope tag (YPYDVPDYA), into the *Mlu*I and *Hin*dIII sites of the pCMV4 eukaryotic expression vector (1). C- and N-terminal truncation mutants of IκBα were generated by PCR and appropriate primers. Site-specific mutations were created by phosphorothioate oligonucleotide site-directed mutagenesis (36) (Amersham). The wild-type human IκBα cDNA and the various IκBα mutants were cloned into the *Hin*dIII and *Xba*I sites of pCMV4-HA for the generation of N-terminally HA-tagged IκBα proteins (pCMV4-HA-IκBα). The IκBα truncation mutants are designated by the specific amino acid residues retained in the mutant protein. For example, IκBα(21–317) contains the region from amino acid 21 to amino acid 317.

The κB-TATA-luciferase reporter plasmid was generated by transferring the insert, containing the HIV-1 κB enhancer and TATA box, from the κB-TATA-CAT (48) into the pGL2 plasmid 5′ of the luciferase gene (Promega).

Jurkat cell transfection and luciferase assay. Human Jurkat leukemic T cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. These cells were transfected with DEAE-dextran at a density of 3×10^5 to 6×10^5 cells per ml, as previously described (18). After 48 h of culture, the transfectants were collected and suspended in a lysis buffer (Reporter lysis buffer; Promega). Cell extracts were normalized for protein recovery (Bio-Rad), and luciferase activity was quantitated (Promega).

Immunoblotting and immunoprecipitation. HeLa cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (complete Dulbecco modified Eagle medium). The cells were seeded onto 0.1% gelatin-treated, six-well plates (1.5×10^5 cells per well) 1 day prior to transfection. Plasmid cDNA expression vectors encoding the HA tag-IκBα fusion proteins were transfected into the cells by liposome-mediated gene transfer (Lipofectamine; Gibco BRL) according to the manufacturer's instructions. A 2-μg amount of plasmid DNA and 7 μl of Lipofectamine reagent were used for each transfection. At 40 to 48 h after transfection, the recipient cells were subjected to whole-cell extract preparation by in situ lysis (18) with ELB buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.0], 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 0.5 mM dithiothreitol, 1.0 mM phenylmethanesulfonyl fluoride). The protein extracts were fractionated by sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE), electrophoretically transferred to nitrocellulose membranes, and then analyzed for immunoreactivity with a monoclonal antibody specific for the HA epitope tag (anti-HA; Babco, Berkeley, Calif.) with an enhanced chemiluminescence detection system (ECL; Amersham).

For immunoprecipitation studies, monkey kidney COS7 cells, which are maintained in Iscove's complete medium, were transfected by using DEAE-dextran as previously described (18). After 48 h, recipient cells were starved for 1 h in methionine-cysteine-deficient medium and then metabolically radiolabeled for 2 h with [^{35}S]methionine and [^{35}S]cysteine as previously described (18). Whole-cell extracts were prepared by in situ lysis in ELB buffer and subjected to immunoprecipitation analyses with a peptide-specific antiserum recognizing either the N terminus or the C terminus of IκBα (18). The immunoprecipitates were fractionated by SDS-10% PAGE and then by autoradiography.

Indirect immunofluorescence. COS7 cells were seeded on four-well chamber slides (Nunc, Naperville, Ill.) and transfected with DEAE-dextran (18). Indirect immunofluorescence was performed with anti-IκBα-specific antibodies as previously described (18).

RESULTS

Exogenous IκBα expressed by transient transfection is rapidly degraded in HeLa cells following TNF-α induction. To explore sequence elements within IκBα that regulate its inducible degradation, an in vivo system was identified that permitted detection of stimulus-coupled degradation of transiently expressed exogenous IκBα molecules. This system involved the use of HeLa cells, TNF-α as the inducing agent, and lipofectamine-mediated transfection of wild-type or mutant IκBα expression vectors (Fig. 1). To distinguish the transfected gene product from endogenous IκBα, an influenza virus HA epitope tag was incorporated into the various IκBα expression vectors. Extracts were prepared from these transiently transfected cells at various times after the addition of TNF-α (0 to 40 min) and were analyzed for IκBα expression by immunoblotting with a monoclonal antibody specific for the HA epitope tag (anti-HA; Babco). As shown in Fig. 1, the anti-HA antibody specifically reacted with a 40-kDa protein species present in cells transfected with the HA-IκBα vector (lane 2) but lacking in cells transfected with the parental pCMV4 vector (lane 1). This 40-kDa protein also reacted with an IκBα-specific antiserum (data not shown). Thus, the anti-HA antibody specifically recognized the transfected but not the endogenous IκBα species. Importantly, as observed with the endogenously synthesized IκBα (9, 14, 25, 50), TNF-α stimulation of the transfected cells led to the rapid degradation of the transfected wild-type HA-IκBα (lanes 3 to 5). Moreover, the degradation of IκBα was preceded by the appearance of a more slowly migrating IκBα species previously shown to be a phosphorylated form of this inhibitor (51).

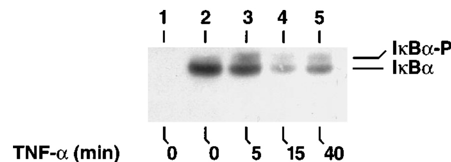

FIG. 1. TNF-α-induced degradation of exogenously expressed IκBα in HeLa cells. HeLa cells were transfected with either the parental vector pCMV4-3HA (lane 1) or a cDNA expression vector encoding the HA-IκBα fusion protein (lanes 2 to 5). These cells were subsequently stimulated with human TNF-α (20 ng/ml) for the indicated time periods, and then cellular extracts were isolated. Approximately 15 μg of each extract was then subjected to SDS-PAGE separation and immunoblotting with a monoclonal antibody specific for the HA epitope tag (anti-HA). Phosphorylated (IκBα-P) and basal forms of IκBα are indicated.

N-terminal sequences of IκBα are required for TNF-α-induced degradation. To define the IκBα sequences that are required for its inducible degradation by TNF-α, a set of N- and C-terminal deletion mutants were generated and inserted into the pCMV4-HA expression vector. The termini of these IκBα deletion mutants were selected on the basis of potentially interesting sequences or residues identified within the primary IκBα sequence (Fig. 2A).

The N-terminal sequence flanking the ankyrin-rich domain of IκBα is notable for the presence of two neighboring serines (amino acids 32 and 36), each of which is preceded by an aspartic residue (Fig. 2A; DS site [D<u>S</u>GLD<u>S</u>]), and a potential tyrosine phosphorylation site (PTK site; amino acids 39 to 47). Truncation mutants were generated to evaluate the potential function of these sites as well as more N-terminal sequences. As shown in Fig. 2B, deletion of the first 20 amino acids of IκBα [IκBα(21–317)] produced no inhibitory effect on the degradative response induced by TNF-α (upper panel, lanes 4 to 6). In contrast, deletion to residue 37 [IκBα(37–317)] or 72 [IκBα(72–317)] completely abolished TNF-α-induced degradation (upper panel, lanes 7 to 12). On the basis of homogeneous mobility, neither IκBα(37–317) nor IκBα(72–317) appeared to undergo TNF-α-induced phosphorylation (compare lanes 1 to 6 with lanes 7 to 12). Of note, under these conditions, the endogenously expressed IκBα present in the same extracts was efficiently degraded in all of the transfectants (lower panel,

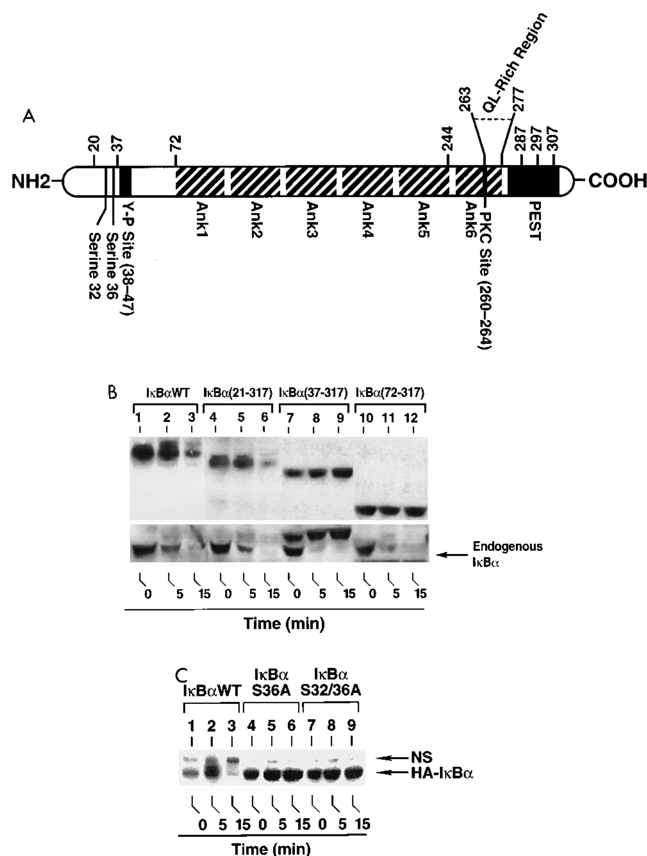

FIG. 2. Identification of N-terminal sequences required for TNF-α-induced degradation of IκBα. (A) Schematic summary of human IκBα. The N-terminal portion of IκBα flanking the ankyrin repeats contains two notable regions, including two serines at positions 32 and 36 (DSGLDS) and a consensus tyrosine phosphorylation site (Y-P site; KDEEYEQMVK) between residues 38 and 47. The C-terminal region (277 to 317) contains the PEST sequences (PEST residues) and a small adjacent region (263 to 277) that is rich in glutamine (Q) and leucine (L) (QL-rich region), which overlaps with the sixth ankyrin repeat. The six ankyrin repeats are shown in hatched boxes (Ank1 to Ank6). Amino acid numbers are indicated above the map and reflect the end points of the various truncation mutants of IκBα examined. (B) Immunoblotting analysis of IκBα N-terminal truncation mutant degradation in transfected HeLa cells stimulated with TNF-α. HeLa cells were transfected with 2 μg of the cDNA expression vectors encoding either wild-type IκBα or its various N-terminal truncation mutants as indicated. The cells were then stimulated with TNF-α (20 ng/ml) for the indicated time periods, and cell extracts were prepared and subjected to immunoblotting analysis with either anti-HA (upper panel) or anti-IκBα (lower panel). Degradation of endogenous IκBα present in the same extract is shown. The prominent nondegrading band appearing in lanes 7 to 9 of the endogenous IκBα control lane represents the epitope-tagged N-terminal deletion mutant of IκBα, which migrates in a manner similar to wild-type IκBα. This mutant, like endogenous IκBα, is immunoprecipitated by the anti-C-terminal IκBα antibody used in these studies. (C) Immunoblotting analysis of IκBα site-directed mutants S36A and S32/36A. HeLa cells were transfected with cDNA expression vectors encoding either the wild-type IκBα (WT) or its mutated versions of IκBα with an alanine-for-serine substitution at residue 36 (S36A) or residues 32 and 36 (S32/36A). After TNF-α stimulation, the cell extracts were subjected to immunoblotting with anti-HA as described in the legend to Fig. 1. NS, nonspecific binding.

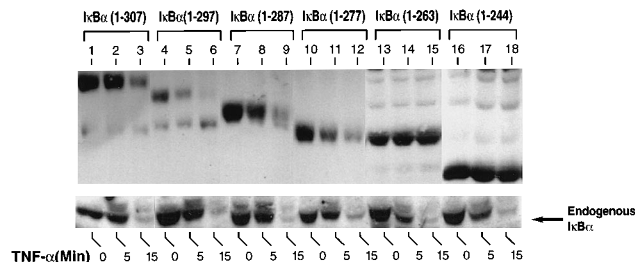

FIG. 3. A C-terminal QL-rich region (but not PEST sequences) is required for TNF-α-induced degradation of IκBα. HeLa cells were transfected with the indicated C-terminal truncation mutants of IκBα. After TNF-α stimulation, cell extracts were prepared and subjected to immunoblotting as described in the legend to Fig. 1. TNF-α-induced degradation of the endogenous IκBα present in these extracts is also shown.

lanes 1 to 12), suggesting that the failure of these transfected IκBα mutants to respond to TNF-α stimulation was due to their lack of the N-terminal sequences. Furthermore, the diminished degradation of the N-terminal deletion mutants of IκBα was not simply due to a modest change in the kinetics of breakdown, since these mutants remained fully intact after 45 min of TNF-α stimulation (data not shown). Thus, the N-terminal boundary of the domain required for IκBα degradation mapped to the 16-amino-acid region located between amino acids 21 to 36. As noted earlier, this region contains two neighboring serines located at residues 32 and 36 (Fig. 2A). To test more directly the involvement of these serine residues in the inducible phosphorylation and degradation of IκBα, site-directed mutagenesis replacing either one (serine 36) or both of these serines with alanine was performed. As shown in Fig. 3C, although the wild-type IκBα was efficiently degraded in response to TNF-α stimulation (Fig. 2C, lanes 1 to 3), mutation of either serine 36 (S36A) or both serine 32 and serine 36 (S32A/36A) to alanine abolished this response (lanes 4 to 9). Furthermore, the S32A/36A mutant failed to produce a phosphorylated form of IκBα (lanes 7 to 9). Under conditions of electrophoresis different from those presented in Fig. 2), IκBα S36A was found to yield a modified band migrating more rapidly than the band generated by TNF-α induction of wild-type IκBα. Thus, TNF-α-induced degradation of IκBα appears to be regulated by phosphorylation events occurring at these two N-terminal serine residues.

The C-terminal PEST-like sequences within IκBα are dispensable for TNF-α-induced degradation. One of the striking structural features of IκBα is the presence of a C-terminal region that is rich in the amino acids proline (P), glutamic acid (E), serine (S), and threonine (T) (22) (Fig. 2A; PEST). The presence of such PEST sequences in many other proteins predicts rapid protein turnover (41). To examine the potential role of these PEST sequences in TNF-α-induced degradation, sequential 10-amino-acid deletions were introduced at the C terminus of IκBα. Each of these C-terminal deletion mutants was then tested for TNF-α-induced degradation in HeLa cells. Sequential deletion of the final 40 amino acids of IκB which encompassed the entire PEST sequences (Fig. 2A) failed to alter TNF-α-induced degradation (Fig. 3, lanes 1 to 12). In contrast, further deletion of a 13-amino-acid sequence located at the end of the sixth ankyrin repeat (amino acids 264 to 276) and distinguished by the presence of multiple glutamine (Q) and leucine (L) residues (Fig. 2A; QL-rich region) yielded an IκBα analog [IκBα(1–263)] that failed to undergo TNF-α-induced degradation (Fig. 3, lanes 13 to 15). Despite failing to undergo such inducible proteolysis, this IκBα mutant was present in the cytoplasm normally phosphorylated, suggesting that the QL region subserves a distinct and perhaps downstream function in this degradation pathway.

Inhibition of RelA by IκBα does not require the N-terminal region or the C-terminal PEST-like sequences of IκBα but is critically influenced by the carboxyl QL-rich element. Studies were then performed to examine whether the sequences regulating the inducible degradation of IκBα were also required

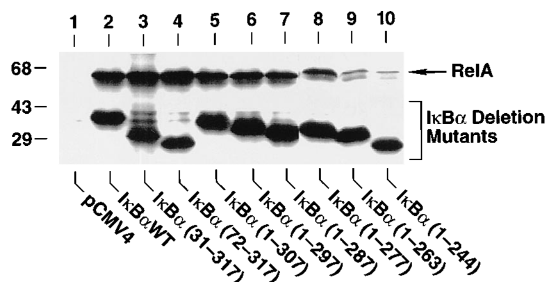

FIG. 4. Analyses of N- and C-terminal sequences needed for the physical association of IκBα with RelA. COS7 cells were transfected with the RelA cDNA expression vector together with the indicated IκBα expression vectors or the parental pCMV4 vector. The transfected cells were metabolically radiolabeled with [^{35}S]methionine and [^{35}S]cysteine, and then cell extracts were prepared. These extracts were then subjected to coimmunoprecipitation analysis with peptide-specific antibodies specific for either the C terminus (lanes 1 to 4) or the N terminus (lanes 5 to 10) of IκBα. The migrations of IκBα and its mutants as well as the coimmunoprecipitated RelA are indicated to the left.

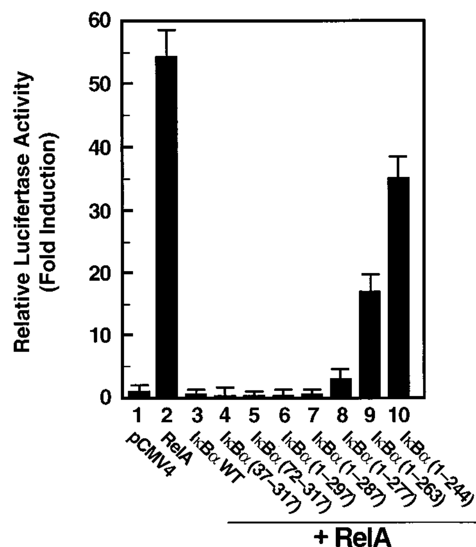

FIG. 5. Mapping of IκBα sequences required for inhibition of RelA-mediated transcriptional activation. Human Jurkat T cells were transfected with either the parental pCMV4 vector or cDNA expression vectors encoding either RelA alone or RelA and the indicated IκBα wild-type or deletion mutants. These cells were also transfected with a luciferase reporter plasmid containing the HIV-1 κB enhancer (κB-TATA-luc). Luciferase activities were measured after 48 h of transfection and are presented as relative fold induction over basal levels obtained with cells transfected with pCMV4 alone (column 1). The values shown are the means ± standard errors of the means obtained in three independent experiments.

for IκBα inhibition of NF-κB. First, the capacities of the various IκBα mutants to bind to and coimmunoprecipitate with RelA were analyzed. The expression vectors encoding each of the IκBα mutants were cotransfected into COS7 cells with a cDNA expression vector encoding RelA (Fig. 4, lanes 2 to 10). As a control, RelA was cotransfected with the parental vector ($pCMV4_1$), which lacks a cDNA insert (lane 1). After transfection, the cells were metabolically radiolabeled with [^{35}S]methionine and [^{35}S]cysteine and subjected to immunoprecipitation analysis with peptide-specific antisera reactive with either the C terminus (lanes 1 to 4) or the N terminus (lanes 5 to 10) of IκBα. As shown in Fig. 4, these antisera specifically immunoprecipitated both the 37-kDa wild-type IκBα (lane 2) and all of the various IκBα deletion mutants (25 to 37 kDa; lanes 3 to 10) but failed to react with RelA alone (lane 1). Furthermore, as expected, RelA was coimmunoprecipitated with wild-type IκBα (lane 2), suggesting the formation of stable complex between IκBα and this NF-κB subunit. Coimmunoprecipitation experiments also revealed that deletion of the entire N-terminal region (amino acids 1 to 71) or the C-terminal PEST-like sequences (amino acids 278 to 317) of IκBα had no marked effect on its physical association with RelA (lanes 3 to 8). However, further deletion of a 13-amino-acid element between residues 277 and 263 corresponding to the QL-rich region (Fig. 2A) or beyond to the beginning of the sixth ankyrin repeat (1 to 244) significantly diminished the binding of IκBα to RelA (lanes 9 and 10). Thus, although the N-terminal region and the C-terminal PEST sequences appear largely dispensable for the binding of IκBα to RelA, the QL-rich region appears critically required for association with RelA as well as inducible degradation of IκBα.

To explore the biological function of each of the various IκBα mutants, human Jurkat T cells were cotransfected with an expression vector encoding RelA and an HIV-1 κB-luciferase reporter plasmid (κB-TATA-luc). As shown in Fig. 5, in the absence of IκBα, RelA potently stimulated the κB-directed transcription (~55-fold stimulation; Fig. 5, column 2) relative to the background luciferase level obtained with the parental pCMV4 vector (column 1). As expected, RelA-mediated activation of the κB enhancer was completely inhibited when cells were cotransfected with wild-type IκBα expression vectors (column 3). In keeping with the coimmunoprecipitation studies, the N-terminal deletion mutants, which retained the ability to associate with RelA, exhibited full inhibitory function (columns 4 and 5). Deletional analyses within the C-terminal portion of IκBα revealed that removal of the final 30 amino acids [IκBα(1–287)] had no effect on IκBα inhibitory function (column 7). Further deletion of 10 additional amino acids [IκBα(1–277)], which removed the entire PEST or acidic region, modestly compromised IκBα inhibition of RelA (column 8). However, deletion to residue 263, which removes the QL-rich region or beyond [IκBα(1–244)], produced a marked loss of IκBα inhibitory function (columns 9 and 10). These findings were consistent with the inefficient physical association of these mutants with RelA (Fig. 4, lanes 9 and 10). Taken together, the QL-rich region appears to be importantly involved in the regulation of RelA functional activity as well as the inducible degradation of this inhibitor. In contrast, the N-terminal domain 21 to 36, which is required for inducible degradation because of serine phosphorylation at positions 32 and/or 36, is fully dispensable for IκBα inhibition of RelA function. The PEST sequences are not required for physical association; however, their removal very modestly inhibits IκBα regulation of RelA.

Parallel immunofluorescence assays were performed to localize RelA expression in the presence of the various IκBα mutants (Fig. 6). These studies indicated that inhibition of RelA-mediated transactivation by the various functional IκBα mutants precisely correlated with the ability of these mutants to block nuclear translocation of RelA (Fig. 6A to G). In brief, deletion of N terminal sequences of IκBα (72 to 317 [Fig. 6C]) or removal of the C-terminal PEST sequences (1 to 277 [panel E]) did not alter the largely cytoplasmic pattern of RelA localization. In sharp contrast, deletion of the QL region (1 to 263 [panel F]) or all of the sixth ankyrin repeat (1 to 244 [panel G]) resulted in a predominantly nuclear pattern of RelA expression. Together, these deletional analyses suggest that a 13-amino-acid sequence element, which is located at the C terminus of the sixth ankyrin-like repeat and rich in glutamines

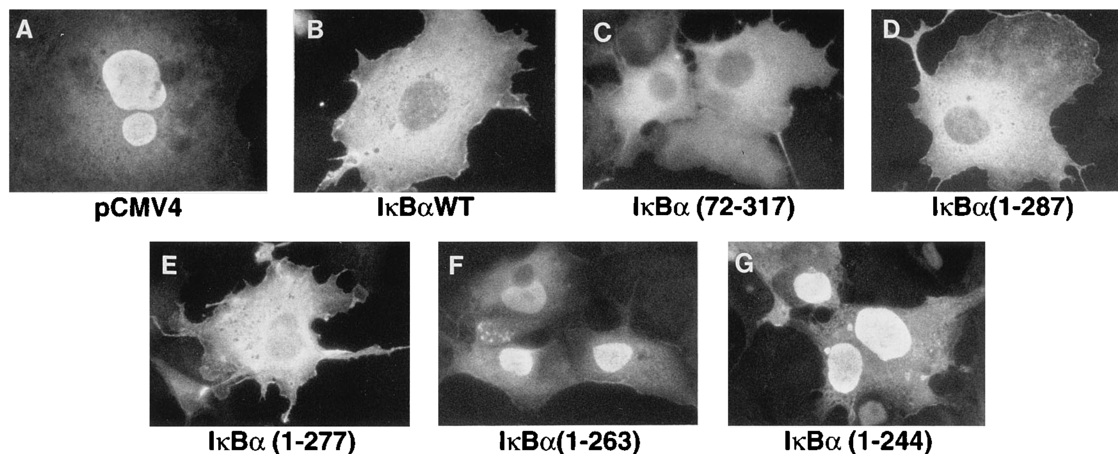

FIG. 6. Inhibitory activities of the various IκBα mutants correlated with their abilities to inhibit the nuclear translocation of RelA. COS7 cells were cotransfected with a RelA C-terminal truncation mutant, p65(1–312), which assembles normally with IκBα (18), and either the parental pCMV4 vector or the indicated wild-type (WT) or mutant IκBα expression vectors. After 48 h, the transfected cells were subjected to indirect immunofluorescence assaying with RelA-specific antisera and Texas red-conjugated anti-rabbit immunoglobulin secondary antibody. Principally cytoplasmic patterns of RelA staining are seen in panels B, C, and D, while nuclear staining is seen in panels A, F, and G. A whole-cell pattern of staining is seen in panel E.

and leucines, is critical for IκBα assembly with RelA and for inhibition of RelA action. In contrast, the entire N-terminal sequences and the C-terminal PEST sequences flanking the ankyrin repeats appear dispensable for these inhibitory functions of IκBα on RelA.

Identification of select N-terminal IκBα deletion mutants as dominant negative repressors of NF-κB. The results from the studies described above demonstrated that the N-terminal IκBα deletion mutants IκBα(37–317) and IκBα(72–317) retained full inhibitory function but failed to undergo degradation in response to TNF-α stimulation. These results raised the intriguing question as to whether such IκBα mutants might function as dominant negative repressors of NF-κB. To test this possibility, Jurkat T cells were transfected with the HIV κB luciferase reporter plasmid either alone or together with IκBα or the N-terminal IκBα deletion mutants 37 to 317 or 72 to 317. The recipient cells were then either untreated or incubated for 5 h with TNF-α, and then cell extracts were isolated for luciferase assay (Fig. 7). As expected, in the absence of IκBα, TNF-α potently stimulated (~35-fold) κB-directed luciferase gene expression (Fig. 7, column 1, open bar). The transfection of wild-type IκBα moderately inhibited this response (column 2); however, the stimulatory effects of TNF-α were completely abrogated in the presence of either of the two N-terminal truncation mutants of IκBα (columns 3 and 4, open bars). Apparently, by virtue of their ability to bind to and sequester RelA in the cytoplasm but not to undergo TNF-α-induced degradation (Fig. 2B, 4, 5, and 6), both of these N-terminal deletion mutants of IκBα functioned as potent dominant negative repressors of NF-κB.

DISCUSSION

NF-κB is a potent transcriptional activator of many growth-related cellular genes as well as of certain viruses, notably HIV-1 (6, 31). The transparent activity of NF-κB is tightly controlled through its cytoplasmic retention by a family of inhibitors including IκBα (2–4, 8, 56). IκBα specifically recognizes RelA and is believed to mask its nuclear localization signal (10, 18, 26, 57). IκBα is distinguished by the presence of six ankyrin domain repeats. Similar repeats are present in the other members of the IκB protein family, including IκBβ (52), IκBγ, p105, p100, and Bcl-3 (8). Recent in vitro studies have suggested that the ankyrin-rich domain and a C-terminal acidic region (also referred to as the PEST sequence) of IκBα are both necessary and sufficient for the association of IκBα with RelA and for the inhibition of RelA DNA-binding activity (23). In the present study, we have analyzed the sequences within IκBα that are required for its physical association with RelA and inhibition of RelA nuclear translocation and transcriptional function in vivo. In keeping with the prior in vitro study (23), we have found that deletion of the entire N-terminal region (71 amino acids) located upstream of the ankyrin-rich domain has no effect on the physical binding of IκBα to RelA or on IκBα inhibition of RelA nuclear translocation or transcriptional activity (see schematic summary in Fig. 8).

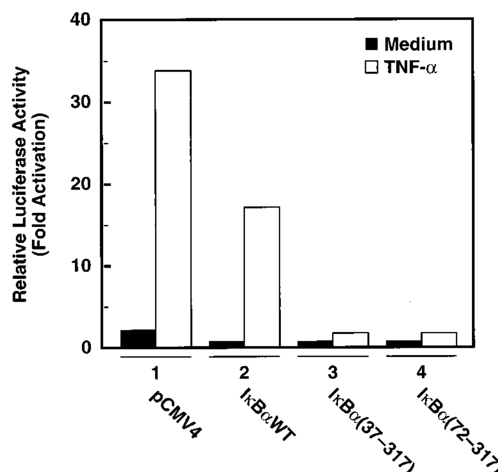

FIG. 7. Nondegradable N-terminal deletion mutants of IκBα function as dominant negative inhibitors of NF-κB. Human Jurkat cells were transfected with either the parental vector pCMV4 or the indicated wild-type (WT) and N-terminally truncated IκBα cDNA expression vectors and a luciferase reporter plasmid containing the HIV-1 κB enhancer. After 48 h, the cells were stimulated with TNF-α (10 ng/ml) for 5 h, and the cells were then collected for luciferase assaying. Luciferase activity is presented as relative fold induction over the basal level obtained for cells transfected with pCMV4 alone (not shown). Similar results were obtained in two additional experiments.

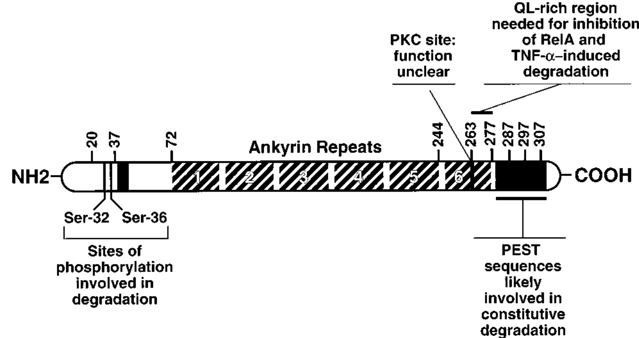

FIG. 8. Schematic summary of various functional domains identified within IκBα.

However, in contrast to the prior in vitro study, our C-terminal deletion analyses have revealed that removal of the C-terminal region (amino acids 278 to 317) containing the entire PEST element does not significantly impair the physical association of IκBα with RelA in vivo. The prior study suggested that the entire ankyrin-rich domain and a C-terminal acidic region (or PEST sequence) were essential for this function of IκBα. These differences could be due to the fact that the IκBα construct used in the in vitro studies did not contain the N-terminal sequences or to methodological differences in the assay of the IκBα-RelA association (23). Nevertheless, our results are consistent with a recent report (16) which demonstrated that deletion of the acidic C-terminal region (amino acids 279 to 317) of IκBα had no effect on the binding of this inhibitor to RelA. While the PEST element was not important in vivo, our studies revealed that the adjacent glutamine- and leucine-rich region (amino acids 264 to 276), located at the C-terminal end of the sixth ankyrin repeat of IκBα, was critical both for IκBα association with RelA and for inhibition of RelA transcriptional activity. These results are supported by protease protection analyses of IκBα domain structure reported by Jaffray et al. (27). These investigators have found that the QL region is protected from proteolytic cleavage in the presence of RelA, suggesting a contact of these proteins in this region.

Recent studies have revealed that the induction of NF-κB nuclear expression by various stimuli is associated with rapid proteolytic degradation of this inhibitor (9, 14, 15, 25, 39, 49, 50). Although the precise biochemical mechanism underlying this inducible degradation of IκBα remains unclear, its proteolysis appears to be preceded by phosphorylation (9, 14, 15, 49, 51). However, in contrast to prior in vitro studies, phosphorylation of IκBα in vitro does not cause its immediate dissociation from the NF-κB complex. Rather, this inducible posttranslational modification likely serves to target the inhibitor for subsequent degradation by chymotrypsin-like proteases apparently associated with the proteasome (17, 38, 49, 55). Therefore, phosphorylation of IκBα probably causes a conformational change in the transcription factor-inhibitor complex, which in turn allows for protease attack while the inhibitor is still associated with NF-κB. Recent evidence further suggests that IκBα undergoes ubiquitination following phosphorylation, presumably further targeting degradation within the proteasome (14a). Our studies have revealed that an N-terminal 16-amino-acid sequence (amino acids 21 to 36) containing two closely spaced serine residues (D<u>S</u>GLD<u>S</u>) at positions 32 and 36 is critically required for TNF-α-induced degradation. In keeping with several recent studies (12, 13, 54), we have found that mutation of either one or both of these serine residues abolishes TNF-α-induced degradation of IκBα. Thus, degradation of IκBα appears to be triggered by its site-specific phosphorylation. However, additional sequences are required for degradation of this inhibitor.

Previous studies have demonstrated that IκBα contains a region (the C-terminal 40 amino acids) rich in PEST residues near its C terminus (22). These PEST sequences are frequently associated with rapid protein turnover (41). The PEST (or acidic) sequences in IκBα have been proposed to be involved in TNF-α-induced degradation of IκBα, since deletion of the C-terminal 61 amino acids containing these sequences abolishes the degradation of this inhibitor (40). We have examined the role of the PEST sequences in the inducible degradation of IκBα in vivo by performing more detailed deletional analyses. Our studies have demonstrated that deletion of the C-terminal 40 amino acids that span this PEST domain had no effect on TNF-α-induced IκBα degradation. However, further deletion of 13 amino acids from the C terminus, which removes a glutamine- and leucine-rich segment (Fig. 8; QL-rich region), completely abolished TNF-α-induced degradation. Thus, the QL-rich region, rather than the PEST (or acidic) sequences, which is located at the C-terminal part of IκBα appears to be involved in the inducible degradation of this inhibitor. However, this nondegraded IκBα mutant lacking the QL-rich region is normally phosphorylated, suggesting that this segment likely subserves a function downstream of serines 32 and 36 phosphorylation that is required for induced degradation in the proteasome. Of note, the QL-rich region is further distinguished by its high sequence divergence from other members of the IκB family. This finding may be relevant to the observation that IκBα is the only mammalian IκB species currently shown to undergo rapid degradation in response to cellular activation. We suspect that the PEST sequences, which are common to all IκB family members (8), may be involved in constitutive rather than stimulus-coupled turnover of IκBα. In fact, our pulse-chase experiments have revealed that the C-terminal truncation mutants lacking these PEST sequences exhibit somewhat longer half-lives than the wild-type IκBα in unstimulated COS7 cells (data not shown).

Delineation of the structural domains of IκBα that are required for its inducible degradation and inhibitory function may facilitate the design of inhibitors of the NF-κB transcription factor. In this regard, our studies have revealed the function of the N-terminal mutants of IκBα lacking serines 32 and 36 as dominant negative repressors of NF-κB. These IκB analogs retain full inhibitory effects on RelA because of their ability to assemble with RelA and their subsequent failure to undergo TNF-α degradation. As such, it is possible that these or related IκB mutants could be used in the future to negatively modulate NF-κB action in vivo.

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