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# Signal-induced degradation of IκBα requires site-specific ubiquitination

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**ABSTRACT** **The inhibitor protein IκBα controls the nuclear import of the transcription factor NF-κB. The inhibitory activity of IκBα is regulated from the cytoplasmic compartment by signal-induced proteolysis. Previous studies have shown that signal-dependent phosphorylation of serine residues 32 and 36 targets IκBα to the ubiquitin–proteasome pathway. Here we provide evidence that lysine residues 21 and 22 serve as the primary sites for signal-induced ubiquitination of IκBα. Conservative Lys → Arg substitutions at both Lys-21 and Lys-22 produce dominant-negative mutants of IκBα *in vivo*. These constitutive inhibitors are appropriately phosphorylated but fail to release NF-κB in response to multiple inducers, including viral proteins, cytokines, and agents that mimic antigenic stimulation through the T-cell receptor. Moreover, these Lys → Arg mutations prevent signal-dependent degradation of IκBα *in vivo* and ubiquitin conjugation *in vitro*. We conclude that site-specific ubiquitination of phosphorylated IκBα at Lys-21 and/or Lys-22 is an obligatory step in the activation of NF-κB.**

The prototypic form of the transcription factor NF-κB is a heterodimeric complex containing p50 (NF-κB1) and p65 (RelA) (reviewed in refs. 1–4). When present in the nucleus, NF-κB stimulates the expression of numerous effector genes involved in immune, acute-phase, and inflammatory responses (1, 2). In addition, NF-κB is required for transcription of the type 1 human immunodeficiency provirus (HIV-1) (5). Normally, NF-κB is sequestered in the cytoplasm via interactions with members of the IκB family of proteins, including a 37-kDa inhibitor called IκBα (reviewed in refs. 6 and 7). In response to a broad spectrum of activation cues, IκBα is phosphorylated at Ser-32 and Ser-36 and then rapidly degraded, thus permitting translocation of NF-κB to the nucleus (6–9).

Recent *in vitro* and *in vivo* studies have shown that IκBα is degraded by the 26S proteasome following its covalent attachment to ubiquitin (Ub) (10, 11). Entry of IκBα into the Ub–proteasome pathway is dependent on signal-induced phosphorylation of this inhibitor at Ser-32 and Ser-36 (11). For most short-lived substrates, the Ub–proteasome pathway is initiated by the sequential action of at least three enzymes (termed E1, E2, and E3) that together catalyze the activation and ligation of multiple Ub molecules to susceptible lysine residues (reviewed in ref. 12). However, the mechanisms that regulate ubiquitination of conditionally labile proteins, such as IκBα, remain obscure.

In this report, we provide genetic and biochemical evidence that induced phosphorylation of IκBα at Ser-32/Ser-36 leads to ligation of Ub to Lys-21 and/or Lys-22. IκBα mutants containing simultaneous Lys → Arg replacements at these two sites act as constitutive inhibitors of NF-κB that are appropriately phosphorylated but escape from signal-dependent breakdown *in vivo* and from ubiquitination *in vitro*. These findings suggest that the Ub-conjugating machinery is recruited to the phosphorylated Ser-32/Ser-36 motif of IκBα, resulting in site-directed ubiquitination at nearby Lys-21/Lys-22.

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## MATERIALS AND METHODS

**Expression Vectors.** Complementary DNAs encoding human RelA (13), human IκBα (14), and the Tax protein of type 1 human T-cell leukemia virus (HTLV-1) (15) were inserted into the polylinker of pCMV4 (16) downstream of the cytomegalovirus immediate-early promoter. The reporter plasmid containing the chloramphenicol acetyltransferase (CAT) gene linked to the HIV-1 κB enhancer has been described (17). Site-directed mutations were introduced into the full-length IκBα cDNA by using the phosphorothioate method (18) and confirmed by DNA sequencing. Epitope-tagged derivatives of these mutants were constructed by PCR-assisted amplification with a 5′ primer that fused an epitope tag derived from the gene 10 protein of phage T7 (Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly; ref. 19) in frame with the initiator methionine of IκBα.

**Transient Transfections and CAT Assays.** Human Jurkat T lymphocytes or a derivative that stably expresses the murine interleukin 1 (IL-1) receptor (Ju.1; kindly provided by David McKean; ref. 20) were cultured and transfected by electroporation as described (8). Where indicated, cultures were treated after 48 h of growth with tumor necrosis factor α (TNF-α; 500 units/ml; Genzyme), IL-1 (1000 units/ml; Genzyme), or combinations of phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) and ionomycin (1 μM; Calbiochem) for 20 h prior to harvest. Whole-cell extracts were prepared from transfectants, normalized for protein concentration (21), and assayed for CAT activity as described (22).

**Subcellular Fractionation and Extract Analyses.** Cytosolic and nuclear fractions were prepared from T-cell transfectants as described (8). Gel-retardation assays were performed under published reaction conditions (8) and a $^{32}$P-labeled palindromic κB enhancer probe (κB-pd; 5′-CAACGGCAGGGGAATTCCCCTCTCCTT-3′) (23). Epitope-tagged IκBα was isolated from cytosolic extracts by incubation with monoclonal anti-T7 antibody (T7-Tag; Novagen) and protein A-agarose beads (Boehringer Mannheim). Immunoprecipitates were washed in low-stringency buffer, fractionated by SDS/PAGE, and transferred to polyvinylidene difluoride membranes (DuPont) as described (8). Membranes were incubated with anti-peptide antisera directed against C-terminal sequences of either IκBα (aa 229–317) or RelA (aa 529–551). Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham).

Abbreviations: CAT, chloramphenicol acetyltransferase; HIV-1, type 1 human immunodeficiency virus; HTLV-1, type 1 human T-cell leukemia virus; Ub, ubiquitin; TNF-α, tumor necrosis factor α; IL-1, interleukin 1; PMA, phorbol 12-myristate 13-acetate; CHX, cycloheximide.

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***In Vitro* Ubiquitination Assay.** Epitope-tagged derivatives of each IκBα cDNA were subcloned into pBluescript SK (+) (Stratagene), linearized with *Not* I, and transcribed *in vitro*. Radiolabeled IκBα substrates for ubiquitination reactions were synthesized from these transcripts by using wheat germ extracts (TNT; Promega) supplemented with [$^{35}$S]methionine (11). Preparation of active HeLa cell cytoplasmic extracts (S100 fraction) and reaction parameters for *in vitro* Ub conjugation have been described in detail (11). Under these conditions, efficient phosphorylation and ubiquitination of IκBα are dependent on the presence of okadaic acid, a potent inducer of NF-κB *in vivo* (24–26).

## RESULTS

Phosphorylation of IκBα at Ser-32 and Ser-36 is required for Ub conjugation (11); however, the specific lysine residues in IκBα that serve as Ub acceptors are not known. Inspection of the deduced N-terminal sequences for the human (14), rat (27), and porcine (28) homologs of IκBα revealed that Ser-32 and Ser-36 are flanked by four lysine residues at positions 21, 22, 38, and 47. To determine whether these invariant lysines play a role in the regulation of IκBα, we used site-directed mutagenesis to introduce conservative Lys → Arg substitutions at each of these positions in the full-length inhibitor. Considering the potential for functional redundancy, two other mutants of IκBα were constructed containing simultaneous replacements at either Lys-21/Lys-22 or Lys-38/Lys-47. All of these Lys → Arg mutants were expressed at comparable levels *in vivo* (data not shown).

**Lys → Arg Mutants of IκBα Inhibit NF-κB-Directed Transcription in Tax-Expressing T Cells.** Prior studies have shown that the Tax protein of HTLV-1, a potent viral inducer of NF-κB, targets IκBα for degradation by stimulating its phosphorylation at Ser-32/Ser-36 (8). To determine whether the lysine residues flanking these phosphoacceptors are required for Tax-dependent degradation of IκBα, we examined the effects of Lys → Arg mutations on IκBα activity *in vivo*. Human Jurkat T lymphocytes were cotransfected with a Tax expression vector and a CAT expression plasmid under the control of two NF-κB binding sites from the HIV enhancer (HIV-κB-CAT) (Fig. 1*A*). Consistent with previous findings (8), Tax stimulated transcription from the NF-κB reporter at least 30-fold in the presence of wild-type IκBα (WT). This activity was not significantly affected by point mutations at Lys-21, Lys-22, Lys-38, or Lys-47 (mutants K21R, K22R, K38R, and K47R, respectively). In contrast, replacement of both Lys-21 and Lys-22 with arginine (mutant K21/22R) in IκBα markedly attenuated Tax-mediated transactivation of HIV-κB-CAT. However, IκBα protein containing simultaneous mutations at both Lys-38 and Lys-47 (mutant K38/47R) failed to inhibit the Tax response, indicating that neither of these downstream lysines is essential for signal-dependent inactivation of IκBα.

**Lys → Arg Mutants of IκBα Inhibit NF-κB Function During T-Cell Activation.** In addition to HTLV-1 Tax, NF-κB activity is stimulated by specific receptor-mediated pathways that lead to the rapid degradation of IκBα, including those initiated by peptide antigens (29), TNF-α (30–34), and IL-1 (20, 32, 33). To examine these Tax-independent signaling cascades, Jurkat T cells were transfected with HIV-κB-CAT and cDNA expression vectors encoding either wild-type IκBα (WT) or mutant K21/22R. Transfected cells were then treated with TNF-α, IL-1, or PMA and ionomycin (PMA/IONO), a combination which mimics activation through the T-cell receptor (35, 36). As shown in Fig. 1*B*, each of these NF-κB inducers stimulated transcription from the HIV-κB enhancer in the presence of the wild-type inhibitor. In contrast, induction of HIV-κB-CAT by these agents was largely prevented in T cells expressing the K21/22R mutant of IκBα. These results confirm that Lys-21 and Lys-22 of IκBα serve an essential function in both viral and immune pathways for NF-κB activation.

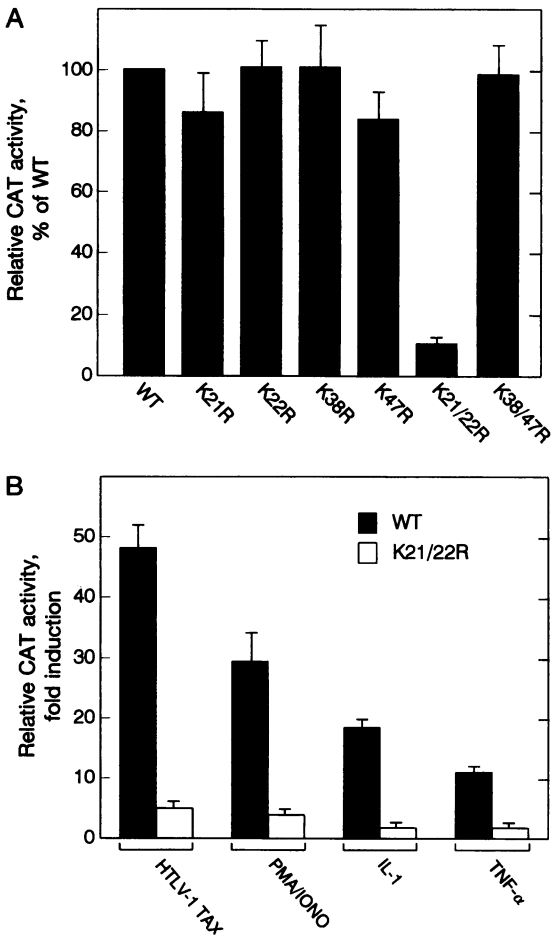


FIG. 1. Effect of Lys → Arg mutations in IκBα on the activation of NF-κB. (*A*) Jurkat T cells were cotransfected with the indicated IκBα effector plasmids (2.5 μg each) and a CAT reporter construct containing the HIV κB enhancer (HIV-κB-CAT; 5 μg), in the presence or absence of a Tax expression vector (5 μg). Tax-dependent increases in reporter gene activity (mean ± SEM, from at least three independent transfections) are expressed as a percentage of the activity induced in cells transfected with wild-type IκBα (WT; mean fold induction = 35). (*B*) Jurkat T cells were transfected with HIV-κB-CAT (5 μg) and the indicated IκBα expression vectors (2.5 μg). After 48 h of growth, half of each culture was stimulated for 20 h with TNF-α, IL-1, or combinations of PMA and ionomycin (PMA/IONO). Transfectants stimulated with IL-1 correspond to a Jurkat derivative that stably expresses the IL-1 receptor (Ju.1; ref. 19). Results are reported as the mean fold induction ± SEM in CAT activity measured in extracts from stimulated versus unstimulated cells. Results obtained in control experiments with HTLV-1 Tax-expressing Jurkat cells (see *A*) are shown at left.

**Functional Interactions of Lys → Arg Mutants with RelA.** The RelA transactivator subunit of NF-κB serves as a high-affinity receptor for IκBα (37, 38). To examine whether the K21/22R mutant of IκBα is competent to regulate the nuclear translocation of RelA, Jurkat T cells were cotransfected with expression vectors encoding RelA and selected Lys → Arg mutants in the presence or absence of a Tax expression vector. Nuclear extracts were then prepared from recipient cells and analyzed for κB-specific DNA-binding activity in gel retardation assays. As shown in Fig. 2*A*, two nucleoprotein complexes were detected in extracts from control cells expressing ectopic RelA alone (lane 1), which correspond to RelA homodimers (upper) and NF-κB (lower) (8). Consistent with their ability to

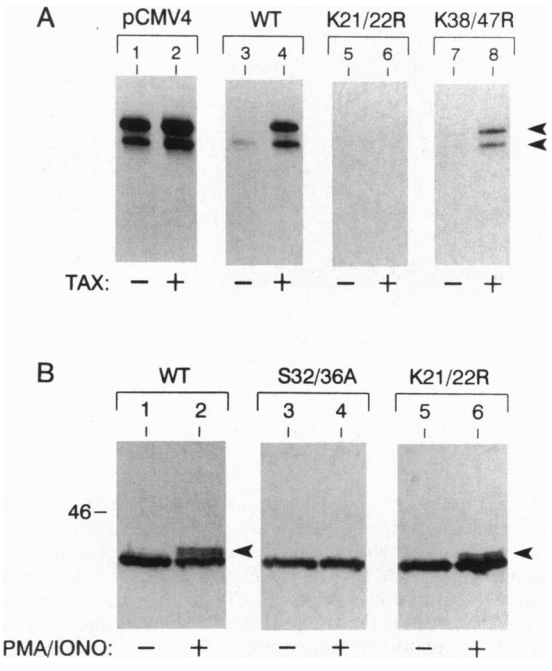


FIG. 2. Effects of Lys → Arg mutations in IκBα on NF-κB DNA binding and IκBα phosphorylation. (*A*) Jurkat cells were cotransfected with pCMV4-based expression vectors for RelA (10 μg), Tax (5 μg), and the indicated IκBα mutants (5 μg). After 48 h of growth, cultures were treated with cycloheximide (CHX) (50 μg/ml) for 2 h. Nuclear extracts were prepared and analyzed in gel retardation assays as described (8). Arrows indicate positions of nucleoprotein complexes containing RelA homodimers (upper) and NF-κB (lower). (*B*) Jurkat cells were cotransfected with an expression vector encoding RelA (10 μg) and the indicated T7-tagged IκBα constructs (10 μg). After 48 h of growth, half of the cultures were pretreated with MG132 (100 μM; 30 min) and then stimulated with PMA and ionomycin (PMA/IONO) for 15 min. Tagged proteins were immunoprecipitated with monoclonal anti-T7 antibodies and analyzed by immunoblotting with IκBα-specific antisera. The positions of hyperphosphorylated IκBα (arrow) and molecular mass standards (in kDa) are indicated.

bind RelA (data not shown), formation of both complexes was efficiently blocked by cotransfection with wild-type IκBα (WT; Fig. 2*A*, lane 3), K21/22R (Fig. 2*A*, lane 5), or K38/47R (Fig. 2*A*, lane 7). Moreover, coexpression with Tax led to inactivation of wild-type IκBα and K38/47R, as evidenced by the accumulation of functional RelA and NF-κB in the nuclear compartment (Fig. 2*A*, lanes 4 and 8). In contrast, Tax failed to activate latent complexes containing the K21/22R mutant of IκBα (Fig. 2*A*, lane 6). This observation is fully consistent with the dominant-negative effects of K21/22R on NF-κB-directed transcription *in vivo* (Fig. 1).

**Signal-Dependent Phosphorylation of Lys → Arg Mutants *in Vivo*.** Recent studies have shown that IκBα is phosphorylated at Ser-32 and Ser-36 prior to its proteolysis (8, 9). Hyperphosphorylated IκBα is readily detected as a more slowly migrating electrophoretic species that accumulates in cells treated with the proteasome inhibitor MG132 (10). To determine whether K21/22R is a substrate for induced phosphorylation *in vivo*, Jurkat cells were cotransfected with expression vectors encoding RelA and wild-type IκBα, K21/22R, or a mutant containing Ser → Ala replacements at Ser-32 and Ser-36 (denoted S32/36A). In the absence of an NF-κB-inducing signal, each of these proteins migrated as a single species during SDS/PAGE (Fig. 2*B*; lanes 1, 3, and 5). However, stimulation with PMA and ionomycin led to the rapid induction of hyperphosphorylated IκBα (Fig. 2*B*, lane 2) and K21/22R (Fig 2*B*, lane 6) in cells pretreated with MG132. In contrast, this hyperphosphorylated species was not detected in control cells expressing the S32/36A mutant (Fig. 2*B*, lane 4). Taken together, these *in vivo* results indicate that K21/22R is appropriately phosphorylated at Ser-32/Ser-36 but fails to release NF-κB in response to multiple inducers.

**Mutant K21/22R Escapes from Signal-Induced Breakdown *in Vivo*.** To examine whether Lys-21 and Lys-22 of IκBα are required for proteasome-mediated degradation, we compared the stabilities of wild-type IκBα and K21/22R in CHX-arrested cells following treatment with either TNF-α or PMA and ionomycin. As shown in Fig. 3, IκBα remained stable in control cells treated with translation inhibitor alone (CHX; *Top*) but was rapidly degraded in transfectants exposed to either of these NF-κB-inducing agents (Fig. 3 *Middle* and *Bottom*, lanes 1–5). In contrast, the K21/22R derivative of IκBα was relatively resistant to induced proteolysis under both stimulatory conditions (Fig. 3 *Middle* and *Bottom*, lanes 6–10). These findings suggest that the K21/22R mutation prevents phosphorylated IκBα from acquiring a requisite degradation signal recognized by the 26S proteasome.

**Rescue of IκBα Mutants Containing Multiple Lys → Arg Substitutions.** To extend these findings, Jurkat T cells expressing HTLV-1 Tax and the HIV-κB-CAT reporter (see Fig. 1*A*) were cotransfected with a mutant of IκBα containing simultaneous Lys → Arg substitutions at positions 21, 22, 38, and 47. As expected, this IκBα mutant functioned as a strong constitutive inhibitor of NF-κB in Tax-expressing cells (Fig. 4; mutant KO) and was hyperphosphorylated in a signal-dependent manner (data not shown). This functional defect in IκBα could be rectified by converting either Arg-21 or Arg-22 back to Lys (mutants R21K and R22K, respectively) or by introducing an Arg → Lys substitution at Arg-17 (mutant R17K) (Fig. 4). In contrast, Arg → Lys reversions at positions

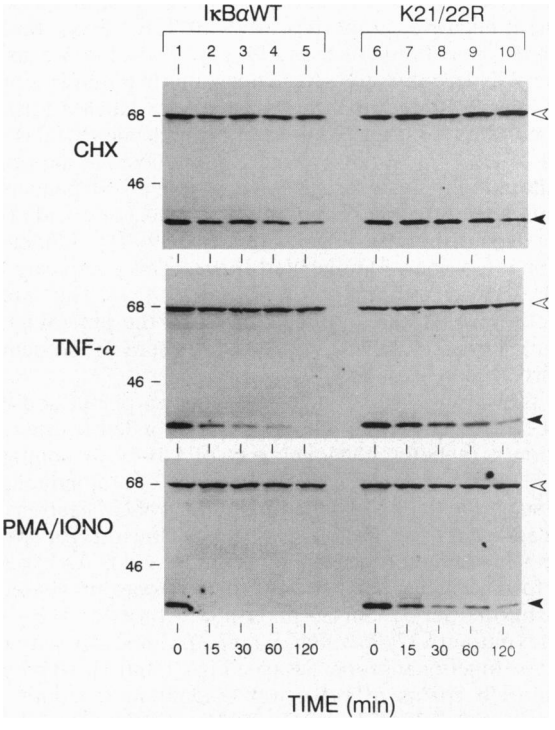


FIG. 3. Effect of Lys → Arg mutations in IκBα on signal-induced degradation. Jurkat T cells were transfected with 10 μg of expression vectors encoding either wild-type IκBα (IκBαWT) or mutant K21/22R. After 48 h of growth, cells were translationally arrested with CHX (50 μg/ml) for 1 h and then treated with medium (*Top*), TNF-α (*Middle*), or PMA and ionomycin (PMA/IONO; *Bottom*) for the indicated times. Cytoplasmic extracts were prepared and analyzed by immunoblotting with IκBα-specific antisera. The positions of IκBα (closed arrowheads), a nonspecific immunoreactive species (internal control; open arrowheads), and molecular mass markers (in kDa) are indicated.

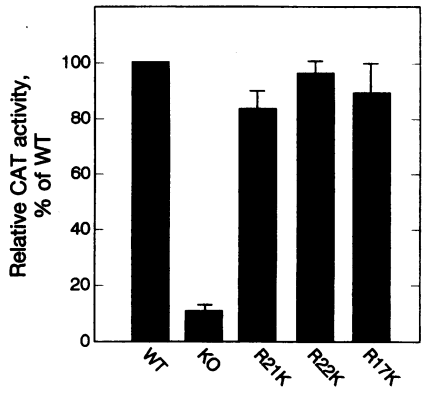


FIG. 4. Functional rescue of multiple Lys → Arg mutations in IκBα. Jurkat T cells were cotransfected with HIV-κB-CAT (5 μg) and equivalent doses (2.5 μg) of the indicated IκBα expression vector in the presence or absence of a Tax expression vector (5 μg). After 48 h of culture, whole-cell extracts were normalized for protein content and assayed for CAT activity. Tax-dependent increases in reporter gene activity (mean ± SEM from at least three independent transfections) are expressed as a percentage of the activity induced in cells transfected with wild-type IκBα (WT; mean fold induction = 24).

38 and 47 failed to reconstitute the Tax response (Fig. 1*A*; mutant K21/22R). We conclude that a single lysine at a position N-terminal but not C-terminal to the Ser-32/Ser-36 motif is sufficient to restore the signaling activity of IκBα, even if an artificial lysine site is created in this subregion.

**Mutations at Lys-21 and Lys-22 of IκBα Prevent Ub Conjugation *in Vitro*.** Recent studies have indicated that IκBα is ubiquitinated both *in vivo* and *in vitro* (11). Coupled with the dominant-negative properties of K21/22R, these findings raised the possibility that Lys-21 and Lys-22 serve as the primary Ub acceptors of IκBα. To test this hypothesis directly, we performed ubiquitination assays on $^{35}$S-labeled forms of these mutants by using HeLa cell cytoplasmic extracts as a source of Ub-conjugating enzymes (11). Phosphorylation and ubiquitination of IκBα in this *in vitro* system is dependent on the presence of the phosphatase inhibitor okadaic acid (11), a known NF-κB-inducing agent (24, 26, 39–41). Under our reaction conditions, radiolabeled IκBα forms complexes with NF-κB derived from the HeLa cell extract (11). This interaction thus permits immunoprecipitation of the physiologically relevant substrate with RelA-specific antisera for subsequent analysis by SDS/PAGE.

As shown in Fig. 5, control reactions performed with $^{35}$S-labeled forms of the wild-type inhibitor led to the accumulation of a heterogeneous array of Ub–IκBα conjugates (lane 1), which likely reflects the capacity of a monoubiquitinated substrate to undergo multiple rounds of Ub ligation (12). These adducts reacted with either Ub-specific or IκBα-specific antisera in immunoprecipitation analyses (11). As expected (11), formation of Ub–IκBα conjugates was prevented by replacing the Ser-32/Ser-36 phosphoacceptors of IκBα with alanine (mutant S32/36A; Fig. 5, lane 2). Consistent with their wild-type functional properties (see Figs. 1 and 4), attachment of multi-Ub chains to IκBα was evident in reactions programmed with K38/47R, K21R, K22R, R21K, or R17K (Fig. 5, lanes 3, 5, 6, 8, and 9). In contrast, the efficiency of formation of high molecular weight conjugates was significantly reduced (70–90% of wild type) in reaction mixtures containing dominant-negative mutants of IκBα that lacked Lys-21 and Lys-22, including K21/22R and KO (Fig. 5, lanes 4 and 7). The residual level of ubiquitination observed with these mutants resulted in the generation of relatively low molecular weight adducts, which may be degraded less efficiently than the large Ub conjugates (42). These species were not detected in okadaic acid-depleted reactions and presumably derive from Ub attachment to secondary sites in IκBα. Taken together, these *in vitro* findings correlated strongly with the functional phenotype of each IκBα mutant when expressed *in vivo*.

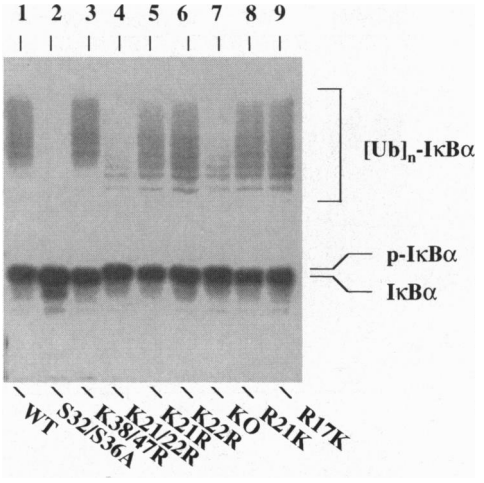


FIG. 5. Effect of Lys → Arg mutations in IκBα on ubiquitination *in vitro*. $^{35}$S-labeled IκBα mutants were preincubated with HeLa cell extracts supplemented with leupeptin (10 μg/ml), aprotinin (10 μg/ml), and MG132 (40 μM) at 4°C for 15 min. Ubiquitination reactions were initiated by addition of MgATP, Ub, okadaic acid, and ubiquitin aldehyde as described (11). After 1 h at 37°C, NF-κB-bound IκBα was isolated by immunoprecipitation with RelA-specific antisera and analyzed by SDS/PAGE and fluorography. The positions of unmodified (IκBα), hyperphosphorylated (p-IκBα), and ubiquitinated (Ub-IκBα) forms of IκBα are indicated on the right. Shorter exposures revealed hyperphosphorylated intermediates for all IκBα mutants except S32/36A (ref. 11 and data not shown).

## DISCUSSION

The Ub–proteasome pathway governs the biologic activity of at least two ankyrin-rich cytoplasmic inhibitors of the NF-κB/Rel family of transcription factors (4). One of these inhibitors, termed p105, is constitutively processed by the 26S proteasome to the functional p50 subunit of NF-κB (10, 43). Fully processed forms of NF-κB are retained in the cytoplasm by other inhibitors, including IκBα (6, 7). Within minutes after cellular stimulation, IκBα is completely degraded, thus permitting the rapid nuclear import of NF-κB (6, 7). Destabilization of IκBα appears to involve signal-dependent phosphorylation at Ser-32 and Ser-36 (8, 9). More recent studies have shown that induced phosphorylation of IκBα at these sites leads to its subsequent ubiquitination, which renders IκBα susceptible to breakdown by the 26S proteasome (11). However, the biochemical mechanisms for coupling these three modification steps remain unclear.

The present study provides further evidence that the Ub–proteasome pathway plays a critical role in the regulated turnover of IκBα (11). Specifically, we have identified a dominant-negative mutant of IκBα, designated K21/22R, which contains conservative Lys → Arg substitutions exclusively at positions 21 and 22. This mutant undergoes induced phosphorylation but not degradation, thus revealing a defect in Ub conjugation. Consistent with this interpretation, mutations at Lys-21 and Lys-22 prevented efficient ubiquitination of IκBα *in vitro*, despite the presence of two other potential sites for Ub attachment in the N-terminal region of IκBα (Lys-38 and Lys-47). Furthermore, replacement of either Arg-21 or Arg-22 with lysine in a mutant of IκBα lacking all four of these potential Ub attachment sites rectified the signaling function of IκBα *in vivo* (Fig. 4). Taken together, we conclude that Lys-21 and Lys-22 serve as the primary Ub acceptors in wild-type IκBα.

Although our data imply that ubiquitination of IκBα is site specific, previous studies with other substrates have suggested that the Ub-conjugating apparatus has the capacity to utilize alternative lysines when the primary acceptors are disrupted (44, 45). In this regard, recent studies have demonstrated that hyperphosphorylated (10, 26, 40, 41, 46) and ubiquitinated (11) forms of IκBα remain bound to NF-κB. Therefore, Lys-21 and Lys-22 may be uniquely positioned in a region of NF-κB-bound IκBα that is sterically accessible to the ubiquitination machinery. This proposed default mechanism for Ub attachment is supported by several observations. First, the N-terminal region of IκBα is dispensable for association with NF-κB *in vivo* (8, 9). Second, this domain is highly susceptible to protease cleavage when IκBα is complexed with RelA, whereas all distal regions of IκBα are relatively resistant (47). Third, the C-terminal PEST domain of IκBα is devoid of lysine residues (14). Fourth, dominant-negative mutants of IκBα that lack Lys-21 and Lys-22 are efficiently rescued by Arg → Lys mutations at a nearby site (Arg-17) or by fusion of a lysine-rich peptide to the N terminus of IκBα (Fig. 4 and data not shown).

How does the ubiquitination machinery target these particular lysines for modification in a signal-dependent manner? One intriguing possibility is that induced phosphorylation at Ser-32 and Ser-36, which is a requisite step for both degradation *in vivo* (8, 9) and ubiquitination *in vitro* (11), regulates the interaction of IκBα with a specific Ub-protein ligase (E3). Indeed, recent studies suggest that E3 activity actually corresponds to a family of ligases with distinct substrate specificities (12). Consistent with their spatial proximity in the N terminus of IκBα, a specific E3 may be recruited to the phosphorylated Ser-32/Ser-36 motif, which in turn facilitates Ub attachment to Lys-21 and/or Lys-22. Further resolution of this signal-dependent coupling model for the regulated turnover of IκBα awaits identification of the relevant Ub-conjugating enzymes.

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1. Grilli, M., Chiu, J. J.-S. & Lenardo, M. J. (1993) *Int. Rev. Cytol.* **143,** 1–62.
2. Baeuerle, P. A. & Henkel, T. (1994) *Annu. Rev. Immunol.* **12,** 141–179.
3. Siebenlist, U., Franzoso, G. & Brown, K. (1994) *Annu. Rev. Cell Biol.* **10,** 405–455.
4. Thanos, D. & Maniatis, T. (1995) *Cell* **80,** 529–532.
5. Nabel, G. & Baltimore, D. (1987) *Nature (London)* **326,** 711–713.
6. Beg, A. A. & Baldwin, A. S., Jr. (1993) *Genes Dev.* **7,** 2064–2070.
7. Gilmore, T. D. & Morin, P. J. (1993) *Trends Genet.* **9,** 427–433.
8. Brockman, J. A., Scherer, D. C., McKinsey, T. A., Hall, S. M., Qi, X., Lee, W. Y. & Ballard, D. W. (1995) *Mol. Cell. Biol.* **15,** 2809–2818.
9. Brown, K., Gerstberger, S., Carlson, L., Franzoso, G. & Siebenlist, U. (1995) *Science* **267,** 1485–1491.
10. Palombella, V. J., Rando, O. J., Goldberg, A. L. & Maniatis, T. (1994) *Cell* **78,** 773–785.
11. Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D. & Maniatis, T. (1995) *Genes Dev.* **9,** 1586–1597.
12. Ciechanover, A. (1994) *Cell* **79,** 13–21.
13. Ruben, S. M., Dillon, P. J., Schreck, R., Henkel, T., Chen, C.-H., Maher, M., Baeuerle, P. A. & Rosen, C. A. (1991) *Science* **251,** 1490–1493.
14. Haskill, S., Beg, A. A., Tompkins, S. M., Morris, J. S., Yurochko, A. D., Sampson-Johannes, A., Mondal, K., Ralph, P. & Baldwin, A. S., Jr. (1991) *Cell* **65,** 1281–1289.
15. Smith, M. R. & Greene, W. C. (1990) *Genes Dev.* **4,** 1875–1885.
16. Andersson, S., Davis, D. L., Dahlback, H., Jörnvall, H. & Russell, D. W. (1989) *J. Biol. Chem.* **264,** 8222–8229.
17. Stein, B., Rahmsdorf, H., Steffen, A., Liffin, M. & Herrlich, P. (1989) *Mol. Cell. Biol.* **9,** 5169–5181.
18. Nakamaye, K. & Eckstein, F. (1986) *Nucleic Acids Res.* **14,** 9679–9698.
19. Rosenberg, A. H., Lade, B. N., Chui, D., Lin, S.-W., Dunn, J. J. & Studier, F. W. (1987) *Gene* **56,** 125–135.
20. McKean, D. J., Huntoon, C. & Bell, M. (1994) *J. Exp. Med.* **180,** 1321–1328.
21. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
22. Neumann, J., Morency, C. & Russian, K. (1987) *BioTechniques* **5,** 444–447.
23. Ballard, D. W., Walker, W. H., Doerre, S., Sista, P., Molitor, J. A., Dixon, E. P., Peffer, N. J., Hannink, M. & Greene, W. C. (1990) *Cell* **63,** 803–814.
24. Thevenin, C., Kim, S.-J., Rieckmann, P., Fujiki, H., Norcross, M. A., Sporn, M. B., Fauci, A. S. & Kehrl, J. H. (1990) *New Biol.* **2,** 793–800.
25. Menon, S. D., Qin, S., Guy, G. R. & Tan, Y. H. (1993) *J. Biol. Chem.* **268,** 26805–26812.
26. Lin, Y.-C., Brown, K. & Siebenlist, U. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 552–556.
27. Tewari, M., Dobrzanski, P., Mohn, K. L., Cressman, D. E., Hsu, J. C., Bravo, R. & Taub, R. (1992) *Mol. Cell. Biol.* **12,** 2898–2908.
28. deMartin, R., Vanhove, B., Cheng, Q., Hofer, E., Csizmadia, V., Winkler, H. & Bach, F. H. (1993) *EMBO J.* **12,** 2773–2779.
29. Kang, S.-M., Tran, A.-C., Grilli, M. & Lenardo, M. (1992) *Science* **256,** 1452–1456.
30. Brown, K., Park, S., Kanno, T., Franzoso, G. & Siebenlist, U. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2532–2536.
31. Sun, S.-C., Ganchi, P., Ballard, D. W. & Greene, W. C. (1993) *Science* **259,** 1912–1915.
32. Osborn, L., Kunkel, S. & Nabel, G. J. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2336–2340.
33. Beg, A. A., Finco, T. S., Nantermet, P. V. & Baldwin, A. S., Jr. (1993) *Mol. Cell. Biol.* **13,** 3301–3310.
34. Donald, R., Ballard, D. W. & Hawiger, J. (1995) *J. Biol. Chem.* **270,** 9–12.
35. Manger, B., Weiss, A., Weyand, C., Goronzy, J. & Stobo, J. (1985) *J. Immunol.* **135,** 3669–3673.
36. Weiss, A. & Littman, D. (1994) *Cell* **76,** 263–274.
37. Baeuerle, P. & Baltimore, D. (1989) *Genes Dev.* **3,** 1689–1698.
38. Urban, M. & Baeuerle, P. (1990) *Genes Dev.* **4,** 1975–1984.
39. Sun, S.-C., Ganchi, P., Béraud, C., Ballard, D. W. & Greene, W. C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1346–1350.
40. Finco, T. S., Beg, A. A. & Baldwin, A. S., Jr. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11884–11888.
41. Traenckner, E. B.-M., Wilk, S. & Baeuerle, P. A. (1994) *EMBO J.* **13,** 5433–5441.
42. Hershko, A. & Heller, H. (1985) *Biochem. Biophys. Res. Commun.* **128,** 1079–1086.
43. Fan, C.-M. & Maniatis, T. (1991) *Nature (London)* **354,** 395–398.
44. Bachmair, A. & Varshavsky, A. (1989) *Cell* **56,** 1019–1032.
45. Treier, M., Staszewski, L. M. & Bohmann, D. (1994) *Cell* **78,** 787–798.
46. Miyamoto, S., Maki, M., Schmitt, M. J., Hatanaka, M. & Verma, I. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12740–12744.
47. Jaffrey, E., Wood, K. M. & Hay, R. T. (1995) *Mol. Cell. Biol.* **15,** 2166–2172.