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Immunology

# Mutual regulation of the transcriptional activator NF-κB and its inhibitor, IκB-α

(gene regulation/cell activation/human immunodeficiency virus/transcription factor/nuclear translocation)

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ABSTRACT **The NF-κB transcription factor complex is sequestered in the cytoplasm by the inhibitory protein IκB-α (MAD-3). Various cellular stimuli relieve this inhibition by mechanisms largely unknown, leading to NF-κB nuclear localization and transactivation of its target genes. It is demonstrated here with human T lymphocytes and monocytes that different stimuli, including tumor necrosis factor α and phorbol 12-myristate 13-acetate, cause rapid degradation of IκB-α, with concomitant activation of NF-κB, followed by a dramatic increase in IκB-α mRNA and protein synthesis. Transfection studies reveal that the IκB-α mRNA and the encoded protein are potently induced by NF-κB and by homodimers of p65 and of c-Rel. We propose a model in which NF-κB and IκB-α mutually regulate each other in a cycle: saturating amounts of the inhibitory IκB-α protein are destroyed upon stimulation, allowing rapid activation of NF-κB. Subsequently, IκB-α mRNA and protein levels are quickly induced by the activated NF-κB. This resurgence of IκB-α protein acts to restore an equilibrium in which NF-κB is again inhibited.**

NF-κB is a dimeric transcription factor that binds and regulates gene expression through decameric cis-acting κB DNA motifs (reviewed in refs. 1 and 2). Although a p50/p65 heterodimer has traditionally been referred to as NF-κB and remains the prototypical and most abundant form, it has been recognized recently that several distinct but closely related homo- and heterodimeric factors are responsible for κB site-dependent DNA binding activity and regulation. The various dimeric factors are composed of members of the family of Rel-related polypeptides. One subclass of this family, distinguished by its proteolytic processing from precursor forms and lack of recognized activation domains, includes p50 (NFKB1) (3–6) and p50B (NFKB2, p52) (7–10), whereas the second subclass contains recognized activation domains and includes p65 (RelA) (11–13), RelB (14, 15), c-Rel (16), v-Rel (17, 18), and the *Drosophila* protein Dorsal (19). All Rel-related members share a 300-amino acid region of homology, responsible for DNA binding and dimerization, called the Rel homology domain.

Activation of the NF-κB transcription factor and various related forms can be initiated by a variety of agents, including tumor necrosis factor α (TNF-α) and phorbol 12-myristate 13-acetate (PMA) (1, 2). Activation proceeds through a post-translational event in which preformed cytoplasmic NF-κB is released from a cytoplasmic inhibitory protein, IκB-α (MAD-3) (20–23). IκB-α inhibits transactivation of the p50/p65 heterodimer, by binding to the p65 component, blocking the dimer's translocation to the nucleus (20, 21, 23). IκB-α also inhibits complexes containing c-Rel or RelB (24, 25). IκB-α blocks binding *in vitro* of various NF-κB dimers to κB binding sites in DNA (11, 12, 15, 22, 26, 27). Because the latter effect requires nuclear IκB-α, its relevance, *in vivo*, is unknown. Although IκB-α is generally a cytoplasmic protein (21, 23), it and its chicken homolog (pp40) have also been detected in the nucleus (refs. 28–30 and K.B., G.F., and U.S., unpublished results). In addition to the well-characterized and cloned IκB-α and its chicken and rat homologs (24, 31), another biochemically defined form, IκB-β, has been reported, but this remains to be fully characterized (26, 31, 32).

Phosphorylation of IκB-α *in vitro* by protein kinases A and C and heme-activated kinase abolished IκB-α's inhibition of DNA binding by NF-κB (24, 32, 33). Although activation of NF-κB has been conjectured to proceed through phosphorylation, no modification of IκB-α has yet been reported *in vivo*. It is also unknown how the inhibited state is maintained in unstimulated cells—that is, how the ratio of IκB-α protein to target complexes is regulated. We show here that a mutual cycle of regulation of IκB-α and NF-κB exists. Stimulation results in a rapid loss of IκB-α from cells (probably following its modification) and the rapid nuclear translocation of NF-κB. Transactivation by NF-κB, in turn, induces high levels of IκB-α synthesis, which can then restore the unstimulated inhibited state.

## MATERIALS AND METHODS

**Extract Preparations and Transfections.** Cell extracts were prepared by centrifugation after freeze-thawing. Nuclear extracts were prepared by the method of Dignam *et al.* (34). Transfections were performed in NTera-2 cells using the PMT2T vector plasmid as described (9). The PMT2T-IκB-α plasmid was constructed by insertion of an *Eco*RI fragment of 1550 nucleotide pairs containing full-length IκB-α/MAD-3 cDNA (22) into the *Eco*RI site of PMT2T. PMT2T-p65 and p50 plasmids were described (9). PMT2T c-Rel plasmid was constructed by cloning the *Eco*RI fragment of full-length human c-Rel cDNA (16) into the *Eco*RI site of PMT2T.

**Western Blotting.** Cell extracts were subjected to SDS/PAGE and blotted onto nitrocellulose (Schleicher & Schuell BA85), and IκB-α was detected with polyclonal rabbit antibody directed against either N- or C-terminal domains (amino acids 1–129 and 230–315, respectively) or both, followed by a second incubation with $^{125}$I-labeled protein A (New England Nuclear). Both antibodies detected the same protein in Western blots. [In addition, a peptide antibody directed against an N-terminal peptide reacted with the same protein and this could be blocked by competition with added peptide (K.B. and U.S., unpublished results).]

**Northern Blotting.** Total RNA was prepared (35), fractionated on an agarose formaldehyde gel, blotted, and hybridized

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Abbreviations: TNF-α, tumor necrosis factor α; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; EMSA, electrophoretic mobility shift assay; PD, palindromic.

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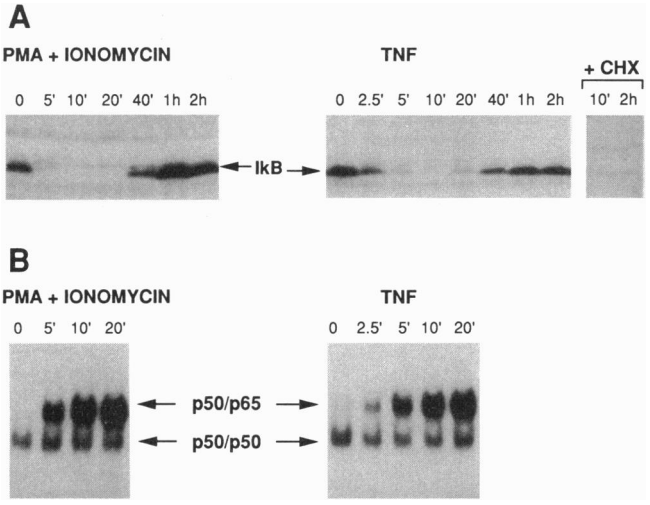


FIG. 1. Stimulation of U937 cells caused rapid degradation of IκB-α and activation of NF-κB, followed by resynthesis of IκB-α. (*A*) Western blot of IκB-α in extracts of stimulated U937 cells ($10^7$ cells per lane). PMA (20 ng/ml) and ionomycin (2 μM) or TNF-α (100 units/ml) with and without cycloheximide (CHX) (10 μg/ml) were incubated with cells ($10^6$ per ml) for times shown. (*B*) EMSA showing NF-κB binding to $^{32}$P-labeled κB DNA (PD probe) in extracts described in *A*.

(36) with a $^{32}$P-labeled 1296-bp *Xho* I–*Eco*RI restriction fragment from IκB-α/MAD-3 full-length cDNA (22).

**Electrophoretic Mobility Shift Assay (EMSA).** Whole cell and nuclear extracts were tested for binding to the palindromic (PD) NFκB binding sequence as described (9).

**Immunofluorescent Staining.** NTera-2 cells were seeded on glass coverslips, transfected, and, 36 h later washed three times in phosphate-buffered saline (PBS) and fixed for 10 min in acetone/methanol (1:1). To stain, cells were incubated 1 h at room temperature in blocking solution containing PBS plus 5% preimmune goat serum. Blocking solution was removed and replaced with a 1:50 dilution of anti-IκB-α or anti-p65 rabbit polyclonal antiserum in PBS plus 5% goat serum for 1 h at room temperature. After three washes (5 min each) fluorescein-labeled goat anti-rabbit antibody (Tago) diluted 1:50 in PBS was added and incubated for 1 h at room temperature. Slides were washed five times in PBS, air dried, and viewed under UV fluorescence (Zeiss Axioplan) with oil immersion.

## RESULTS

**Stimulation by PMA or TNF-α Causes Rapid, Transient Loss of IκB-α.** Stimulation of U937 monocytic cells or Jurkat T cells led to rapid disappearance of IκB-α as judged by Western blots of cell extracts (Figs. 1*A* and 2*A*). Fig. 1*A* shows the time course of IκB-α degradation in U937 cells following activation by PMA plus the calcium ionophore ionomycin or by TNF-α. Disappearance of IκB-α was rapid but transient. Undetectable 10 min after stimulation, IκB-α reappeared between 20 and 40 min, reaching levels equal to or greater than in unstimulated cells. The reappearance of IκB-α was inhibited by cycloheximide, demonstrating a requirement for protein synthesis (Fig. 1*A*, right). Stimulation with PMA alone gave similar results (not shown). Jurkat cells stimulated by PMA plus phytohemagglutinin (PHA) or by TNF-α (Fig. 2*A*) and human peripheral blood T cells treated with PHA plus PMA (not shown) showed modulation of IκB-α levels similar to that seen for U937 cells. The same results were obtained with different IκB-α antibodies, indicating that the disappearance of IκB-α upon stimulation was not due to shielding of a particular epitope caused by modification of the IκB-α protein.

A new, transient, activation-dependent species of IκB-α was detected in U937 cells soon after stimulation with TNF-α when cell extracts were prepared in the presence of phosphatase inhibitors (Fig. 3). This new species migrated slightly slower than the IκB-α of unstimulated cells and rapidly disappeared with further stimulation. It is presumably a phosphorylated form of IκB-α; however, this remains to be demonstrated directly. The modified form was also seen in PMA-stimulated U937 cells (not shown).

**Degradation of IκB-α Is Correlated with NF-κB Activation.** The disappearance of IκB-α from the cell following stimulation correlated closely with the activation of NF-κB. In U937

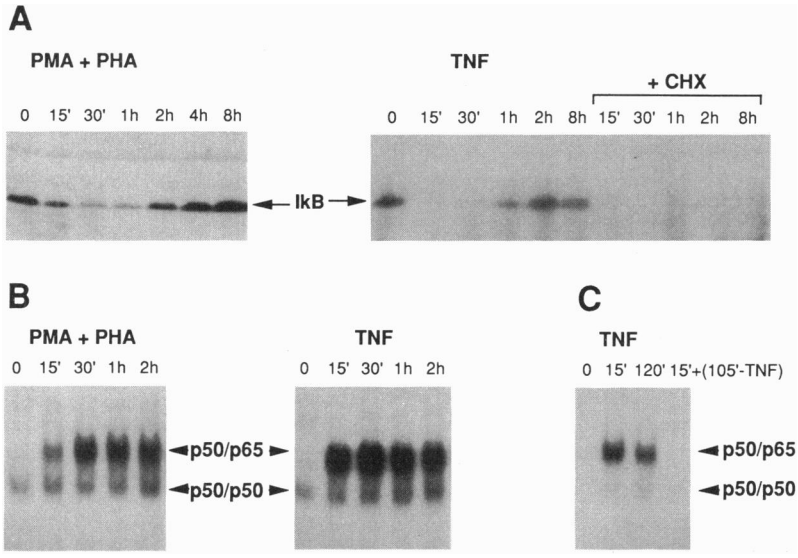


FIG. 2. Stimulation of Jurkat cells caused rapid degradation of IκB-α and activation of NF-κB followed by resynthesis of IκB-α. (*A*) Western blot of IκB-α in extracts of stimulated Jurkat cells. The experimental procedure was as in Fig. 1*A* except that in the left panel cells were stimulated with PMA (40 ng/ml) plus PHA (1 μg/ml). (*B*) EMSA showing NF-κB binding to $^{32}$P-labeled κB DNA (PD probe) in extracts described in *A*. (*C*) EMSA showing NF-κB binding to $^{32}$P-labeled κB DNA (PD probe) in nuclear extracts of Jurkat cells stimulated with TNF-α for times shown. The far right lane shows binding in nuclear extract of cells stimulated for 15 min after which cells were centrifuged, washed with warm medium, and incubated for 105 min in fresh medium with two TNF-α monoclonal antibodies (0.5 μg/ml each) (UBI, Olympus, Lake Success, NY).

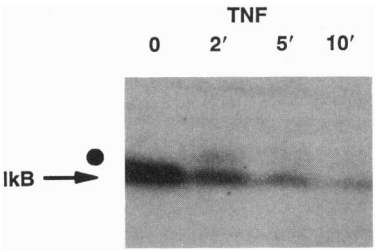


FIG. 3. Stimulated U937 cells produced a modified, short-lived form of IκB-α. Western blot of IκB-α in cell extracts of U937 stimulated with TNF-α for the times shown. The modified form of IκB-α is indicated by a dot. The experimental procedure was as in Fig. 1*A* except that following stimulation, cells were washed in PBS containing 1 mM sodium orthovanadate and extracts were prepared by lysis on ice in Tris·HCl buffer (50 mM, pH 7.4) containing NaCl (100 mM), sodium pyrophosphate (30 mM), NaF (50 mM), sodium orthovanadate (1 mM), 0.5% Nonidet P-40, phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (10 μg/ml), and aprotinin (10 μg/ml).

cells NF-κB binding to κB sites (i.e., p50/p65) was easily detected 2.5–5 min after stimulation with TNF-α (Fig. 1*B*). The induced binding activity was composed largely of p50/p65 heterodimers as determined by supershifting with specific antibodies. These experiments also confirmed that the faster migrating species present in unstimulated and stimulated cells was composed of p50 homodimers [not shown; the band assignments concur with earlier reports (22, 37–39)]. NF-κB binding activity continued to increase in concert with the loss of IκB-α for up to 10 min of stimulation with PMA plus ionomycin or with TNF-α. Similar results were obtained with stimulated Jurkat T cells (Fig. 2*B*).

Analysis of nuclear and cytosolic extracts of resting and stimulated Jurkat and U937 cells revealed that the loss of IκB-α correlated with translocation of NF-κB from the cytoplasm into the nucleus (Fig. 2*C* shows nuclear extracts of Jurkat cells; U937 data not shown). Provided that the external stimulus was maintained, nuclear p50/p65 levels remained high despite the appearance of newly synthesized IκB-α (a 2-h stimulation is shown in Fig. 2*C*). The mechanism by which NF-κB persists in the nucleus with continued stimulation is unclear. When TNF-α was removed after 15 min by washing and addition of TNF-α antibodies, nuclear p50/p65 binding activity declined rapidly, in accord with a prior report (40) (a 105-min time point following removal of TNF-α is shown in Fig. 2*C*). Thus, brief stimulation by TNF-α produced a transient loss of IκB-α and only a transient increase of nuclear NF-κB (p50/p65).

**Kinetics of IκB-α mRNA Induction Following Stimulation.** Stimulation of U937 by PMA or TNF-α for short periods, up to 10 min, had little effect on the level of IκB-α mRNA (Fig. 4). By this time IκB-α protein had disappeared and NF-κB

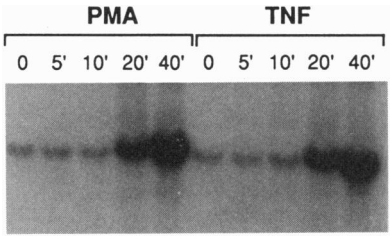


FIG. 4. Stimulation of U937 cells caused induction of IκB-α mRNA. Northern blot of IκB-α RNA extracted from U937 cells ($10^6$ per ml) stimulated with PMA (20 ng/ml) or TNF-α (100 units/ml) for times shown. Each lane contained 20 μg of total RNA and the blot was probed with $^{32}$P-labeled IκB-α cDNA. As a control the blot shown was reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Clontech). No change in the level of GAPDH mRNA with stimulation was seen (not shown).

was activated. IκB-α mRNA levels surged, however, by 20 min. Again, Jurkat cells behaved similarly (not shown). Thus, although the loss of IκB-α that follows quickly after stimulation was not associated with a loss of mRNA, the new IκB-α protein synthesis observed subsequently was associated with a massive increase in IκB-α mRNA, which accords with the cycloheximide sensitivity of this IκB-α protein induction.

**Induction of IκB-α by p65 and c-Rel.** The induction of IκB-α mRNA and protein shortly after activation of NF-κB suggested that NF-κB was directly responsible. Undifferentiated NTera-2 human embryonic carcinoma cells were transfected with various plasmid constructs directing the synthesis of components of NF-κB. These cells are devoid of NF-κB activity (9) and contained no detectable IκB-α protein or mRNA (Fig. 5 *A* and *B*, respectively). Transfection of NTera-2 cells with p65, known to form potently transactivating homodimers (7, 13, 41), generated high levels of endogenous IκB-α mRNA and protein expression (Fig. 5 *B* and *A*, respectively). The simplest explanation for the increase in IκB-α mRNA is direct transactivation of this gene,

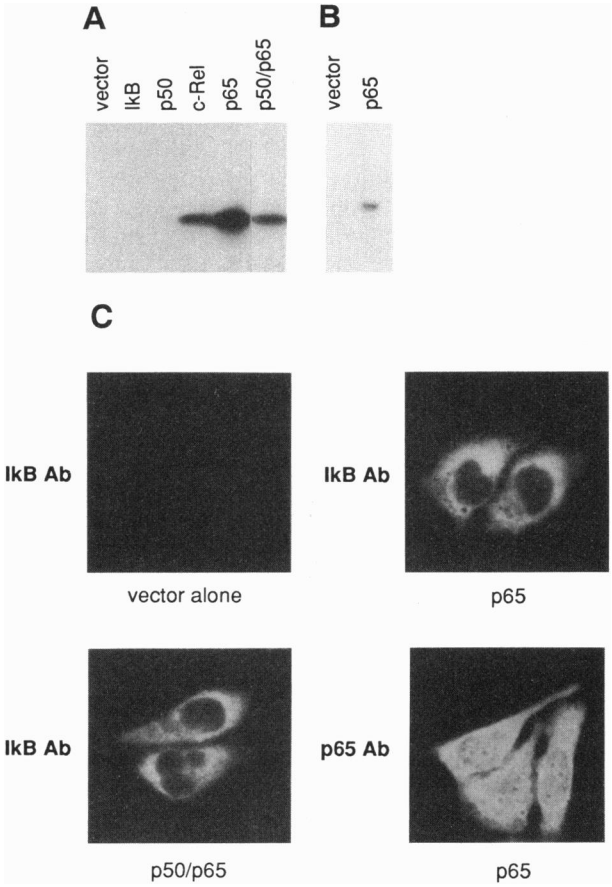


FIG. 5. p65, p50/p65, and c-Rel induced IκB-α in transfected NTera-2 cells. (*A*) Western blot of IκB-α protein produced in cell extracts of NTera-2 transiently transfected with PMT2T (vector)-bearing cDNAs as indicated. The Western blotting procedure was as in Fig. 1*A*. Cells ($5 \times 10^6$) in 100-mm Petri dishes were transfected with 4 μg of plasmid DNA and harvested 48 h after transfection. Each lane was loaded with the extract of $1.5 \times 10^6$ cells. (*B*) Northern blot of IκB-α mRNA produced in NTera-2 cells transfected with 4 μg of PMT2T (vector) or PMT2T-p65. Each lane was loaded with 20 μg of total RNA and the blot was probed with $^{32}$P-labeled IκB-α cDNA. (*C*) Immunofluorescent stained transfected NTera-2 cells. The upper left panel shows cells transiently transfected with the PMT2T vector plasmid (4 μg) stained for IκB-α. The upper right and lower left panels show cells transfected with p65 (4 μg) and p65 plus p50 (2 μg each) cDNAs, respectively, and stained for IκB-α. The lower right panel shows cells transfected with p65 stained for p65. Ab, antibody.

presumably through a κB element. It is formally possible, however, that the increase is mediated indirectly or is due to mRNA stabilization. In contrast to p65, transfection with p50, which generates non-transactivating p50 homodimers (7, 9, 13, 14, 41), did not induce IκB-α synthesis. It is curious that transfection with the IκB-α expression construct did not result in detectable levels of the encoded protein (Fig. 5*A*), despite the fact that high, functional levels of IκB-α can be expressed from this plasmid when cotransfected with potential targets for inhibition and binding, such as p65 (39). Binding to target proteins may therefore stabilize IκB-α, allowing its detection in Western blots. We speculate further that the inherent instability of free IκB-α is the basis for its rapid removal from the cell upon activation-induced dissociation from NF-κB (see *Discussion*). Consistent with this notion, the transfection of p50 plus p65 and of c-Rel also induced considerable levels of IκB-α (Fig. 5*A*), the respective dimers being capable of transactivation and being targets for binding and inhibition by IκB-α (11, 20–24, 31, 42–45).

Immunofluorescent staining of IκB-α confirmed the induction and cytoplasmic location of this protein in NTera-2 cells transfected with p65 or with p50/p65 (Fig. 5*C*). Staining for p65 revealed high levels of this protein in p65 transfected cells. The distribution of p65 in nuclear and cytoplasmic compartments suggested that the highly abundant exogenously introduced p65 is partially retained in the cytoplasm by the induced endogenous IκB-α.

## DISCUSSION

We show here that IκB-α is physiologically regulated by rapid removal from the cell in response to stimulation. In three cell types tested—the monocytic cell line U937, Jurkat T lymphocytes, and peripheral blood T cells—IκB-α was present in unstimulated cells but disappeared within a few minutes of stimulation by PMA or TNF-α. The removal of IκB-α is coupled to a concomitant translocation of NF-κB (predominantly p50/p65) from the cytoplasm into the nucleus.

How does activation increase the lability of IκB-α protein? There are at least two possible explanations: (*i*) modification of IκB-α and/or its bound partners (e.g., p50/p65), rendering it susceptible to hydrolysis by a cytoplasmic protease; this increased susceptibility could result simply from the release of the modified IκB-α as a free, labile molecule; (*ii*) activation of a cytoplasmic protease with specificity for IκB-α. Though not excluding the latter explanation, our data support the former. The observation of a short-lived, more slowly migrating species of IκB-α in stimulated U937 cells suggests that modification of IκB-α occurs and may precede dissociation from NF-κB and its degradation. Secondly, because no IκB-α protein could be demonstrated even upon transfection with IκB-α expression vectors in NTera-2 cells that lack NF-κB, IκB-α appears to be inherently unstable in the absence of NF-κB binding partners. IκB-α is stabilized and readily detected in NTera-2 cells transfected with p65, p50/p65, or c-Rel. Our observations are consistent with the hypothesis that activation-induced modification of IκB-α and/or its bound NF-κB partner leads to dissociation of IκB-α, rendering it susceptible to immediate protease attack.

The loss of IκB-α and the activation of NF-κB upon stimulation are transient phenomena. IκB-α mRNA transcription is quickly induced, leading to replenishment of the IκB-α protein level. The induction of endogenous IκB-α mRNA upon transfection of NTera-2 cells with transactivators such as p65 suggests direct transactivation of the IκB-α gene by NF-κB, possibly through a κB element. Thus, p65 positively regulates IκB-α, probably by transcriptional activation, and may also regulate IκB-α turnover by post-translational stabilization. In addition to p65 homodimers, p50/p65 and c-Rel have similar effects on the induction of IκB-α, as may other Rel-related, transactivating combinations that can associate with IκB-α.

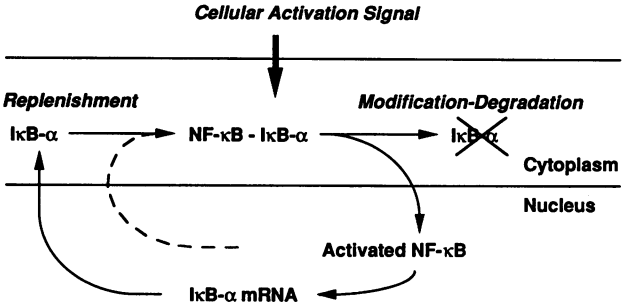


FIG. 6. Model depicting the mutual regulation of IκB-α and NF-κB. Activation-induced IκB-α degradation is coupled to NF-κB nuclear translocation, which in turn leads to new IκB-α synthesis. The broken line indicates possible recycling of nuclear NF-κB (see text).

Our working model for NF-κB activation is shown in Fig. 6. Stimulation triggers signal transduction, which may lead to modification of IκB-α, probably by phosphorylation, and dissociation from cytoplasmic NF-κB. Components of the NF-κB factor may also undergo modification assisting the release of IκB-α. The resulting free, modified IκB-α is rapidly degraded by a protease. NF-κB translocates to the nucleus, causing a pulse of κB-dependent transcription that is prolonged only if external stimulation continues. IκB-α is likely to be one of the genes targeted by NF-κB, resulting in a surge of IκB-α mRNA synthesis, restoring its protein levels in the cell. In the absence of further stimulation, the newly formed IκB-α may assist in removing nuclear NF-κB, since no NF-κB binding remains soon after elimination of the signal (Fig. 2*C*). This action of IκB-α could be the physiological role for the inhibitor's ability to remove NF-κB from DNA *in vitro*. Newly formed IκB-α may also sequester *de novo* synthesized NF-κB factors in the cytoplasm. Activation-induced synthesis of components of NF-κB factors does not commence until well after 2 h of stimulation (5, 9, 44, 45).

The mutual regulation of IκB-α and NF-κB ensures that activation of NF-κB by environmental stimuli is limited and transient. Activation of this transcription factor is subject to a negative feedback regulation through strong induction of IκB-α synthesis. IκB-α production is geared to saturate its free NF-κB partners, acting to restore an equilibrium of inhibition following stimulation. Any excess of free IκB-α is quickly degraded. NF-κB and IκB-α are thus locked in a tightly controlled circuit of mutual regulation.

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