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Activation of NF-κB requires proteolysis of the inhibitor IκB-α: Signal-induced phosphorylation of IκB-α alone does not release active NF-κB

(transcription factor/nuclear translocation/calpain inhibitors/proteasome)

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ABSTRACT **The transcription factor NF-κB is retained in the cytoplasm by its inhibitor IκB-α. Upon cellular stimulation with a variety of pathogen- or stress-related agents, IκB-α is functionally inactivated and NF-κB translocates to the nucleus to trigger transcription of a large array of genes, many of which encode proteins critical for immune or stress responses. Here, we demonstrate that signal-induced proteolysis of IκB-α is an obligatory step for activation of NF-κB: calpain inhibitors I and II, which inhibit cysteine proteases, block activation of NF-κB by blocking degradation of IκB-α without affecting signal-induced phosphorylation of this inhibitor. This contrasts with previous models in which phosphorylation of IκB-α was postulated to be sufficient for activation. We demonstrate further that signal-induced phosphorylation of IκB-α does not by itself lead to dissociation of the inhibitor from NF-κB, providing a rationale for and confirmation of the need to proteolyze IκB-α in order to activate NF-κB. Signal-controlled, target-specific proteolysis is an unexpected, yet likely more general, mechanism for regulating transcription factors.**

The transcription factor NF-κB has been implicated as an essential component of pathogen- and stress-related responses of host organisms. Signals directly or indirectly related to pathogens or stress potently activate NF-κB, which then transcriptionally induces many genes encoding defense-related proteins (reviewed in refs. 1–3). NF-κB is a family of dimers, all of which are composed of members of the Rel/NF-κB family of polypeptides; typically, NF-κB activity is due primarily to p50/p65 (NF-κB1/RelA) heterodimers, although other dimeric combinations often coexist, such as p50/Rel or p52/p65 (NF-κB2/RelA) (3).

The mechanisms leading to activation of NF-κB are of intense interest. In unstimulated cells, NF-κB is normally held in the cytoplasm by the inhibitory protein IκB-α, which avidly binds to most dimers (in particular p50/p65), thereby shielding their nuclear translocation signals. In addition, IκB-α prevents binding of most NF-κB dimers to DNA—exceptions are homodimers of p50 and p52, which lack recognizable transactivation domains (2, 3). Appropriate cellular stimuli inactivate IκB-α, at least transiently, to allow NF-κB to translocate to the nucleus and induce gene transcription through cis-acting κB elements. The prevalent model for activation holds that phosphorylation of IκB-α in response to signals dissociates the inhibitor from the NF-κB dimer, thereby activating the transcription factor (4). The model is based on early experiments in which NF-κB was activated by kinases added to extracts *in vitro*. The activation was apparently mediated by phosphorylation of IκB-α (5–7). This hypothesis was called into question by the recent and unexpected observation that activation of NF-κB correlates with rapid proteolytic degradation of IκB-α *in vivo*, regardless of signal or cell (8–10). In addition, it was reported that inhibitors of chymotrypsin-like proteases blocked activation of NF-κB, but the mechanism by which these toxic inhibitors exerted their function was not investigated (11, 12). While the correlation between activation and degradation may suggest that degradation is necessary for activation, it does not prove this. In the view of the prevailing model, rapid degradation of IκB-α could be a consequence of phosphorylation-induced dissociation from NF-κB, conceivably reflecting IκB-α's known instability when it is a free, uncomplexed protein (8, 9, 13). In addition to observing signal-induced degradation of IκB-α, it was noted that IκB-α appeared to be phosphorylated in response to signals *in vivo* as well (8, 9); however, the phosphorylated form was transient and difficult to demonstrate due to the rapid proteolysis of IκB-α.

What then is the role of phosphorylation and degradation of IκB-α during activation of NF-κB? We now show that in contrast to established opinion, proteolysis is a required step for activation of NF-κB. Calpain inhibitors I and II, which inhibit cysteine proteases, block degradation of IκB-α and also block activation of NF-κB, but they do not block signal-induced phosphorylation of IκB-α. Furthermore, we demonstrate that the previously used inhibitors of chymotrypsin-like proteases block signal-induced phosphorylation of IκB-α; therefore, these inhibitors have unknown effects on upstream signaling events, which complicates interpretation of any experimental results obtained with them. Finally we demonstrate directly that signal-induced phosphorylation of IκB-α in cells does not dissociate it from NF-κB dimers, thus providing an answer to why IκB-α proteolysis is necessary for NF-κB activation.

MATERIALS AND METHODS

Cell Culture Conditions. U937 cells were grown to a density of 10^6 cells per ml. Calpain inhibitor I (100 μM) or calpain inhibitor II (400 μM) (Boehringer) was added 1 hr prior to stimulation of cells with phorbol 12-myristate 13-acetate (PMA) (20 ng/ml; Sigma) and ionomycin (ION) (2 μM; Calbiochem) or with recombinant tumor necrosis factor α (TNF-α) (1000 units/ml; Genzyme). U937 cells were also stimulated with okadaic acid (300 nM; GIBCO) or calyculin A

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; ION, ionomycin; TNF-α, tumor necrosis factor α; DCI, 3,4-dichloroisocoumarin; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TLCK, N^{α}-(*p*-tosyl)lysine chloromethyl ketone; EMSA, electrophoretic mobility shift assay.

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(300 nM; GIBCO). The protease inhibitor 3,4-dichloroisocoumarin (DCI) (20 μM; Boehringer Mannheim), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) (25 μM; Sigma), or N^{α}-(*p*-tosyl)lysine chloromethyl ketone (TLCK) (350 μM; Sigma) was added 30 min prior to cellular stimulation.

Cell Extraction and Electrophoretic Mobility Shift Assay (EMSA). The preparation of cytoplasmic extracts (9) and whole-cell extracts (8) was as reported. EMSAs were as described also and were performed with the ^{32}P-labeled palindromic κB probe (8).

Phosphatase Treatment of Extracts. Cytoplasmic extracts (10 μl) were diluted 1:4 with 50 mM Tris (pH 8.0) solution containing 5–8 units of calf intestine alkaline phosphatase (Boehringer Mannheim) and incubated at 37°C for 5 min. The reaction was inhibited with the phosphatase inhibitors NaF (50 mM), glycerol 2-phosphate (50 mM), and sodium orthovanadate (1 mM).

Immunoblots and Immunoprecipitations. IκB-α immunoblot assays were performed as described using a polyclonal rabbit antibody directed against a C-terminal portion of IκB-α (8). The use of anti-p50 antibodies has also been described (14). Immunoprecipitations [50 mM Tris, pH 7.4/50 mM NaCl/50 mM NaF/50 mM glycerol 2-phosphate/1 mM sodium orthovanadate/100 nM okadaic acid/0.2% Nonidet P-40/protease inhibitor cocktail (8)] were performed with rabbit polyclonal antibodies directed against an N-terminal portion of IκB-α (8) and against amino acids 130–220 of p65 for 1 hr prior to addition of protein A-Sepharose. The mixture was incubated for another 1 hr at 4°C on a roller system. The immune complexes on beads were washed four times with the immunoprecipitation buffer and loaded onto SDS/PAGE gels.

RESULTS

Signal-Induced Phosphorylation of IκB-α. Activation of NF-κB occurs within minutes of appropriate cellular stimulation and temporally coincides with rapid degradation of IκB-α (8–10). Also within minutes of stimulation and just prior to a complete loss of the inhibitor protein, a signal-induced modified form of IκB-α has been detected (8, 9, 15). Because of rapid proteolysis, this modified form is detectable at low levels only; it is distinguished by a slightly slower electrophoretic mobility on SDS/PAGE. The signal-dependent modification of IκB-α is due to phosphorylation, as deduced from alkaline phosphatase treatments of modified IκB-α in extracts, which caused the collapse of the slower migrating form back to an IκB-α, which migrated at the original position present in unstimulated cells (Fig. 1*A* and refs. 8, 9, and 15). These data argue that IκB-α is phosphorylated in response to signals and further suggest that this event precedes degradation, possibly being the cause for it. In support, when NF-κB was activated by treatment of cells with okadaic acid and calyculin A, inhibitors of phosphatases 1 and 2A, a significant fraction of the IκB-α was first converted into the modified form before proteolysis finally eliminated the protein (Fig. 2*A–C*; see legend for further details). The signal-dependent phosphorylation of IκB-α may therefore tag this molecule for proteolysis.

Calpain Inhibitors Block Activation of NF-κB and Block Degradation but Not Phosphorylation of IκB-α. Given the proven signal-induced phosphorylation of IκB-α *in vivo* and given the prevailing model whereby phosphorylation of IκB-α dissociates it from NF-κB (4–7), what significance does IκB-α degradation have for activation of NF-κB, if any? Contradicting a simple dissociation model, inhibitors of chymotrypsin-like protease activity blocked degradation of IκB-α and, apparently in consequence, blocked activation of NF-κB (11, 12). We have made similar observations (Fig. 3*A Top* and *Middle*) but discovered that experiments utilizing these protease inhibitors cannot provide conclusive evidence on the critical role of IκB-α proteolysis. This is because the inhibitors DCI, TPCK, and TLCK [as well as *N*-acetyl-DL-Phe-β-naphthyl ester (data not shown)] prevent the formation of the slower migrating phosphorylated form of IκB-α (Fig. 3*A*, *Middle*). This phosphorylated form would be expected to accumulate under conditions that block only proteolysis. Inhibitors of chymotrypsin-like protease activity somehow block a signaling step upstream of IκB-α phosphorylation, and therefore their use cannot distinguish whether phosphorylation or proteolysis of IκB-α (or both) is required for activation of NF-κB. These inhibitors thus appear to have unappreciated activities (see also legend to Fig. 3), which precludes their use in determining this question.

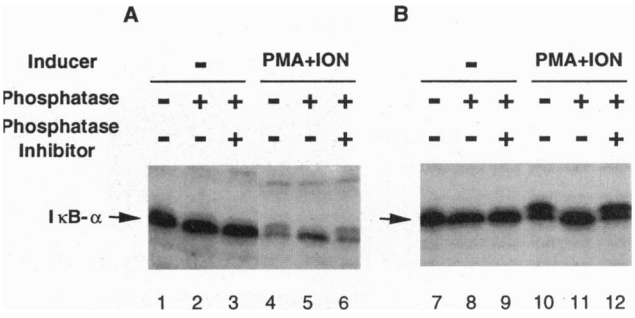

FIG. 1. Activation of NF-κB proceeds via phosphorylation of IκB-α. (*A*) U937 cells were either left unstimulated or were stimulated with PMA and ION for 4 min, as indicated. Cell extracts were digested with alkaline phosphatase in the presence or absence of phosphatase inhibitors, as indicated. IκB-α was visualized by immunoblotting with anti-IκB-α antibodies. (*B*) Same as for *A*, except that cells were pretreated with calpain inhibitor I prior to stimulation with PMA/ION for 4 min.

While searching for more specific protease inhibitors that do not also block IκB-α phosphorylation, we discovered that calpain inhibitor I and, at higher concentrations, calpain inhibitor II efficiently blocked NF-κB activation and IκB-α degradation. Unlike chymotrypsin-like inhibitors, these cysteine protease inhibitors did allow the signal-phosphorylated form of IκB-α to accumulate and thus to be easily detected (Fig. 3*B*). This slower migrating form of IκB-α obtained in the presence of calpain inhibitors I and II appeared to be identical to that transiently seen during stimulation without inhibitors, since it could also be converted back to the faster migrating species when treated with phosphatases *in vitro* (Fig. 1*B*). The calpain inhibitors I and II functioned similarly regardless of the extracellular agent employed, including PMA/ION, TNF-α (Fig. 3*C*), PMA (data not shown), okadaic acid, and calyculin A (Fig. 2*D*). In addition to these experiments, which were performed with U937 cells, calpain inhibitor I also blocked activation of NF-κB and degradation of IκB-α in Jurkat T cells stimulated with phytohemagglutinin/PMA and in 70Z/3 pre-B cells stimulated with PMA, PMA/ION, lipopolysaccharide, or interleukin 1β (data not shown). These experiments provide evidence for a critical role of IκB-α proteolysis in the activation of NF-κB, although this remains to be demonstrated more directly (see below). Other inhibitors of cysteine and serine protease activity, such as antipain, chymostatin, leupeptin, and phenylmethylsulfonyl fluoride, had no effect on NF-κB activation (data not shown).

The effects of the calpain inhibitors I and II do not necessarily imply an involvement of calpain in activation of NF-κB, although calpain is a major source of extralysosomal proteolytic activity in cells. Calpain could be demonstrated to cleave IκB-α *in vitro* in a manner dependent on Ca^{2+} and inhibitable by calpain inhibitors I and II as well as by leupeptin; however, degradation of IκB-α was not inhibited by leupeptin when added to cells (data not shown). This argues against calpain involvement. While the identity of the protease remains to be determined directly, a protease inhibitable by calpain inhibi-

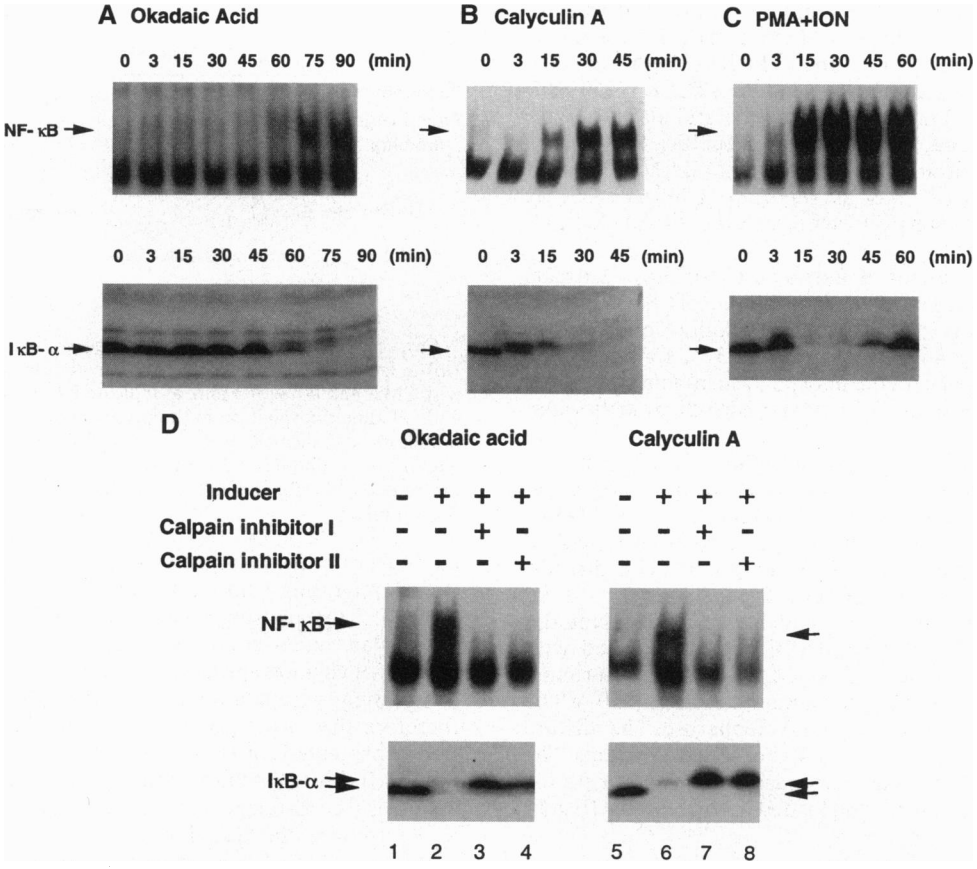

FIG. 2. (*A–C*) Okadaic acid and calyculin A both activate IκB-α via phosphorylation and degradation of IκB-α. U937 cells were stimulated with okadaic acid (*A*), calyculin A (*B*), or PMA/ION (*C*) for the times indicated. Cell extracts were prepared and EMSAs were performed to detect activated NF-κB (*Upper*; marked by arrow), and immunoblot assays were carried out to detect IκB-α (*Lower*; both forms of IκB-α are marked by a single arrow). Okadaic acid requires ≈1 hr to activate NF-κB. Calyculin A activates with faster kinetics, though somewhat more slowly than PMA/ION. With both okadaic acid and calyculin A, the upper, modified form accumulates to easily detectable levels before final proteolysis. Since activated NF-κB potently induces its own inhibitor (8), the IκB-α protein was resynthesized already within an hour of stimulation with the fast-acting agents PMA/ION. (*D*), Analysis for NF-κB and IκB-α as in *A–C*. Cells were stimulated with okadaic acid and calyculin A for 90 min and 30 min, respectively. Calpain inhibitors I and II block activation of NF-κB by okadaic acid and by calyculin A (*Upper*), as shown. Arrows in *Lower* specifically indicate slower and faster migrating forms of IκB-α (the slower form results from signal-induced phosphorylation). The arrow in *Upper* in *A–D* indicates activated NF-κB, which is almost entirely due to p50/p65 heterodimers; the constitutive, faster migrating bandshift is due to p50 homodimers (8).

tors I and II appears critical for IκB-α proteolysis (see *Discussion*).

Signal-Induced Phosphorylation of IκB-α Does Not Dissociate the Inhibitor from NF-κB. While the results with the calpain inhibitors I and II clearly indicate a need for IκB-α proteolysis to activate NF-κB, they do not indicate why this need exists. The role of proteolysis is most easily understood if phosphorylation of IκB-α fails to cause its dissociation from NF-κB and that IκB-α must therefore be degraded to liberate the transcription factor. When IκB-α was coimmunoprecipitated from cells with anti-NF-κB/p65 antibodies, both the normal and the slower migrating phosphorylated form of IκB-α could be detected (Fig. 4, lane 8) despite ongoing proteolysis of IκB-α, which leads to its rapid loss. When calpain inhibitor I was employed to block signal-induced degradation and thus increase the amount of IκB-α, the signal-modified, slower migrating form was clearly seen to coimmunoprecipitate with p65 (Fig. 4, lane 4). Importantly, the ratio of the amounts for the two forms precipitated with anti-p65 antibodies was similar to the ratio obtained with anti-IκB-α antibody precipitations (compare lanes 2 and 4). This indicates that both forms associate with p65 and do so equally well. Furthermore, cellular stimulation did not result in a specific decrease in the total amount of IκB-α coimmunoprecipitated with anti-p65 relative to that immunoprecipitated with anti-IκB-α (compare lanes 1 and 2 with lanes 3 and 4). These data strongly argue that the phosphorylation-modified form of IκB-α remains associated with p65 (as does the resting form).

DISCUSSION

We demonstrate here that activation of NF-κB requires the proteolysis of IκB-α, which follows signal-induced phosphorylation of the inhibitor. Phosphorylation by itself is insufficient to activate NF-κB. Calpain inhibitors I and II can block proteolysis of IκB-α and thereby the activation of NF-κB, while having no effect on the signal-induced phosphorylation of the inhibitor. The distinct newly phosphorylated form of IκB-α remains tightly bound to p65/NF-κB and continues to inhibit the complex in the absence of proteolysis. These data suggest a model for activation in which signal-dependent phosphorylation of IκB-α merely tags the inhibitor for proteolysis but does not dissociate it from NF-κB. To activate NF-κB, proteolysis is absolutely required, because the inhibitor IκB-α remains bound to NF-κB even though it is phosphorylated in response to signals. This model for NF-κB activation refutes the currently held view in which activation of NF-κB is effected via dissociation of IκB-α in response to phosphorylation.

The signal-induced phosphorylation of IκB-α appears to occur just prior to its degradation, consistent with a causal

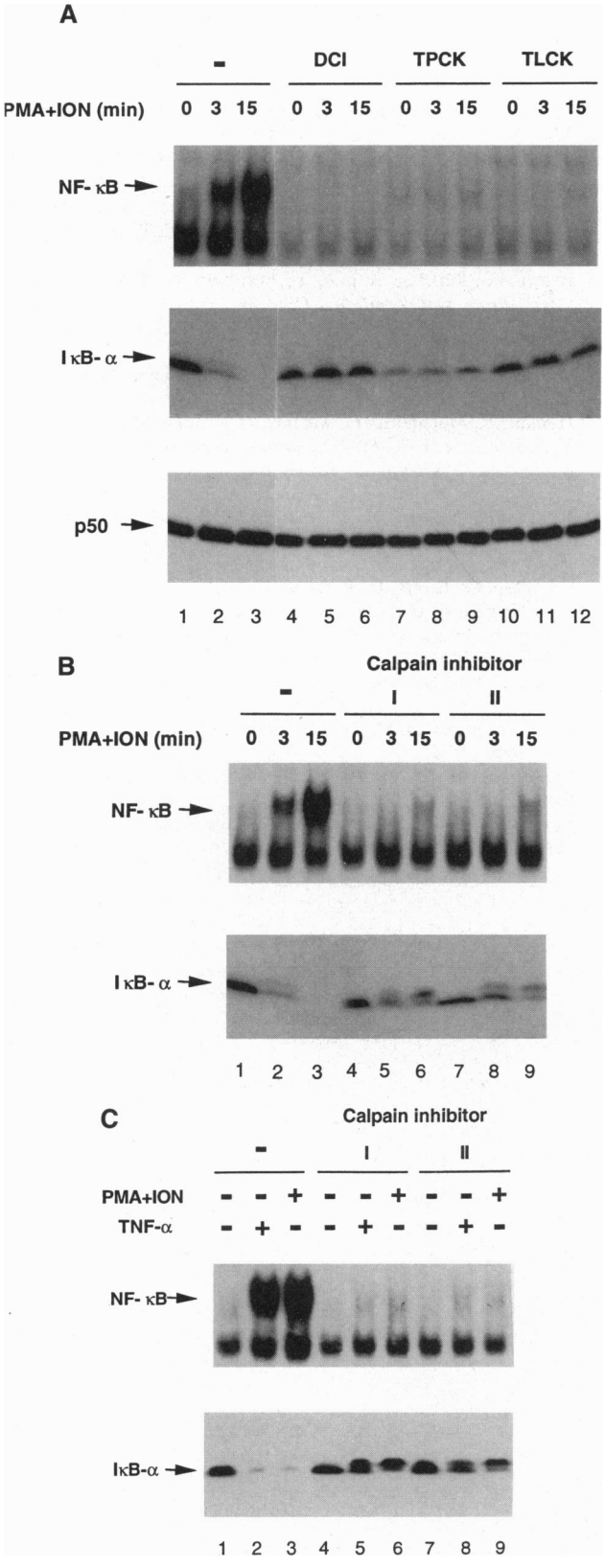

FIG. 3. Effect of various protease inhibitors on NF-κB activation: only calpain inhibitors I and II block NF-κB activation and IκB-α degradation without inhibiting the signal-induced phosphorylation of IκB-α. (*A*) U937 cells were stimulated with PMA and ION for the times indicated, and NF-κB activity was measured (EMSA analysis; *Top*); IκB-α (*Middle*) and p50 (*Bottom*) protein were detected by immunoblot. The protease inhibitors DCI, TPCK, and TLCK were tested as indicated. The slower migrating, modified form of IκB-α was not detected after treatment with these protease inhibitors, but this form was detected transiently during the course of stimulation without protease inhibitors (lane 2). These inhibitors of chymotrypsin-like proteases appear to have several nonspecific effects: while the amount of p50 protein was unaffected by the inhibitors (*Bottom*), the DNA-binding ability of p50 homodimers was significantly decreased (*Top*; faster migrating, constitutive κB-binding activity). Activated NF-κB activity is marked by an arrow (see Fig. 2). (*B*) Cells were stimulated for the times indicated in the presence or absence of calpain inhibitor I or II, as indicated. The signal-induced phosphorylated form of IκB-α accumulates with time. (*C*) Cells were stimulated for 15 min with PMA/ION or TNF-α, with or without calpain inhibitor I or II, as indicated. The extent to which the upper form of IκB-α accumulated varied slightly between experiments, probably due to the variable degree of activation and/or variable dephosphorylation of IκB-α after cell lysis. For activated (arrow) and constitutive κB-binding activities shown in the upper panels see Fig. 2.

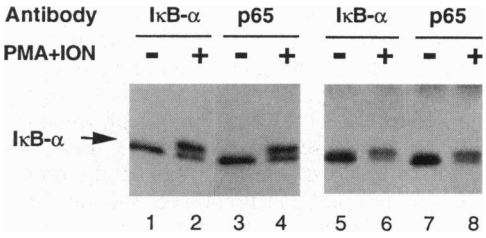

FIG. 4. Signal-induced phosphorylation of IκB-α does not dissociate the NF-κB–IκB-α complex. anti-IκB-α and anti-p65 antibodies were used to immunoprecipitate IκB-α from extracts of U937 cells stimulated with PMA/ION for 15 min (lanes 2 and 4) or for 4 min (lanes 6 and 8) or of unstimulated cells, as indicated. The immune complex was resolved by SDS/PAGE, and immunoblot assays were performed with anti-IκB-α antibodies. The phosphorylated IκB-α (upper band) was complexed with p65 in extracts of cells treated with calpain inhibitor I (*Left*) or untreated (*Right*). These experiments were controlled with preimmune sera; in additional control experiments, use of peptide antibodies to both IκB-α and p65 yielded similar results, and in both cases immunoprecipitation of IκB-α was blocked by addition of the appropriate peptides (data not shown).

relationship. That phosphorylation precedes degradation is most easily visualized by stimulation of cells with the phosphatase inhibitors calyculin A and okadaic acid, which activate NF-κB more slowly than stimuli such as PMA/ION. It is not known if the targets of these NF-κB activators (i.e., phosphatases 1 and 2A) are normally regulated in their activity during extracellular signaling. Perhaps a more likely possibility is that these phosphatases counteract basal, unstimulated activities of kinases that normally function in signaling to IκB-α. If so, inhibition of these phosphatases may indirectly lead to activation. Simian virus 40 small tumor antigen has been reported to activate the Raf–mitogen-activated protein (MAP)/extracellular signal-regulated protein kinase (MEK)-MAP kinase pathway by blocking phosphatase 2A from counteracting the phosphorylation and activation of MEK, implying that there exists a basal level of kinase activity that targets MEK in unstimulated cells (16). Interestingly, the Raf pathway has been implicated in activation of NF-κB (17, 18).

The discovery of protease inhibitors that block degradation but not phosphorylation of IκB-α permits the easy detection of the phosphorylated form of IκB-α, which accumulates in their presence. This contrasts with inhibitors of chymotrypsin-like proteases, which interfere by unknown mechanisms with signaling to phosphorylate IκB-α. The inhibition of signaling in turn may account for the observed absence of proteolysis of IκB-α and activation of NF-κB.

Our data imply that after stimulation, IκB-α is either degraded within the complex or that if dissociation of IκB-α occurs at all, it must be mechanistically linked with its degradation. It is possible, for example, that the initial signal-dependent recognition of IκB-α by a protease may cause its

removal from NF-κB followed by degradation; alternatively, an initial proteolytic cleavage of IκB-α may liberate NF-κB, again prior to complete degradation of the inhibitor. It remains to be determined how proteolysis is initiated. Possible mechanisms include the direct recognition of a phosphorylated site on IκB-α by a protease complex or the recognition of a phosphorylation-induced change, either in the conformation of IκB-α or in its interaction with NF-κB. Finally, the existence of a distinct, signal-induced activation of the IκB-α protease cannot be ruled out. As for the proteolytic activity responsible for IκB-α degradation, calpains are probably not involved since leupeptin can inhibit these proteases while it does not block the degradation of the inhibitor or NF-κB activation. Multicatalytic proteasomes, however, seem likely candidates. Recently proteasomes have been reported to be efficiently inhibited by calpain inhibitors (19); in addition, proteasomes are abundant and regulated in their activity and they can completely digest proteins, as appears to be the case for IκB-α.

Signal-regulated proteolysis may be a newly emerging mechanism for activating mammalian transcription factors. SREBP-1, the sterol-regulated factor controlling expression of the low density lipoprotein receptor and the hydroxymethylglutaryl-CoA synthase genes, is liberated from cytoplasmic retention by sterol-regulated proteolytic cleavage, allowing SREBP-1 to translocate to the nucleus (20). Although details of the two model systems differ, the NF-κB and SREBP-1 examples could indicate a more widespread role for regulated proteolysis in the activation of transcription factors.

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