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Inducible phosphorylation of IκBα is not sufficient for its dissociation from NF-κB and is inhibited by protease inhibitors

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ABSTRACT **The ubiquitous transcription factor NF-κB is regulated by its cytoplasmic inhibitor IκB. A variety of cellular stimuli cause the dissociation of NF-κB from IκB, allowing NF-κB to translocate to the nucleus and regulate gene expression. Although the activation of NF-κB *in vivo* is associated with the phosphorylation and degradation of IκBα, it has remained unclear how each of these events contributes to this process. Recently, studies utilizing protease inhibitors have suggested that the proteolysis of IκBα is a necessary event in the activation of NF-κB. We demonstrate in this study that these and an additional protease inhibitor also completely repress inducible phosphorylation of IκBα. This surprising result suggests a more complex role of proteases in NF-κB activation. In addition, data presented here indicate that many of these inhibitors also directly modify NF-κB and inhibit its DNA binding activity. Due to the pleiotropic effects of these protease inhibitors, it is difficult to conclude from their use how IκBα phosphorylation and degradation contribute to NF-κB activation. In the present study, a more direct approach demonstrates that phosphorylation of IκBα alone is not sufficient for NF-κB activation.**

The NF-κB/Rel transcription factor family regulates a variety of genes whose products are involved in diverse biological processes including cell growth, inflammation, and immune responses (1, 2). Members of this family, which includes the proteins NFKB1 (p105/p50), NFKB2 (p100/p52), RelA (p65), c-Rel, and RelB, are present within cells as functionally distinct homo- and heterodimers (1, 2). Inducible complexes of this family are collectively referred to as NF-κB. Typically, inactive NF-κB is found in the cytoplasm complexed to its inhibitor, IκB (3, 4). Treatment of cells with a large array of inducers, including tumor necrosis factor α (TNFα), interleukin 1β, and lipopolysaccharide (LPS), results in the dissociation of NF-κB from IκB. This allows liberated NF-κB to translocate to the nucleus and modulate target gene expression (1–4). Initial *in vitro* studies on the mechanism of NF-κB activation have suggested that phosphorylation of IκBα may be sufficient for its release from NF-κB (5, 6). Consistent with this model, a phosphorylated form of IκBα can be detected after treatment of cells with various inducers of NF-κB (7, 8). However, the role of IκBα phosphorylation *in vivo* has yet to be determined. It was also observed *in vivo* that IκBα was rapidly degraded after its phosphorylation (7–10). The ability of activated NF-κB to directly stimulate IκBα gene expression (11–13) results in the reaccumulation of inactive NF-κB–IκBα complexes in the cytoplasm.

More recent studies have addressed whether events in addition to phosphorylation may be required for NF-κB activation. In particular, the role of IκB proteolysis has been investigated (10, 11, 14–18). It has been demonstrated that IκBα is extremely unstable unless complexed with NF-κB family members (10, 15). Thus, it was proposed that the degradation of IκBα observed *in vivo* was a consequence of its dissociation from NF-κB and not a prerequisite for activation. However, other work has suggested that degradation of IκBα was necessary for NF-κB activation. It was found that pretreatment of cells with a variety of serine protease inhibitors prevented not only the degradation of IκBα but also the activation of NF-κB (11, 16–18). Here we demonstrate that many of the protease inhibitors utilized in these aforementioned studies have additional effects, including an ability to directly modify NF-κB and block its interaction with DNA. Significantly, all of the protease inhibitors effective in the repression of IκBα degradation also prevent its inducible phosphorylation. This dramatic result suggests that proteases may regulate multiple events necessary for NF-κB activation. Since these protease inhibitors interfere with both the phosphorylation and degradation of IκBα, it is difficult to ascertain from their use how each of these events contributes to NF-κB activation. As reported here, the finding that induced phosphorylation of IκBα does not cause its rapid dissociation from NF-κB is an important step in elucidating the mechanism(s) of NF-κB activation.

MATERIALS AND METHODS

Cell Culture and Reagents. HeLa S3 and Jurkat T cells were grown as described (8). THP-1 cells were grown in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). LPS, 3,4-dichloroisocoumarin (DCIC), 7-amino-1-chloro-3-tosylamido-2-heptanone ("N^{α}-*p*-tosyl-L-lysine chloromethyl ketone," Sigma; TLCK), L-1-tosylamido-2-phenylethyl chloromethyl ketone ("*N*-tosyl phenylalanine chloromethyl ketone," Sigma; TPCK), and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) were obtained from Sigma; 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) was from Boehringer Mannheim; TNFα was from Promega; and okadaic acid was from LC Laboratories (Woburn, MA). TPCK was dissolved in ethanol; TLCK was dissolved in 20 mM sodium phosphate; DCIC, BTEE, and okadaic acid were dissolved in dimethyl sulfoxide; AEBSF was dissolved in H_2O; and LPS was dissolved in phosphate-buffered saline (PBS). These solvents had no detrimental effect *in vivo* or *in vitro* on NF-κB activation or its ability to bind DNA. Concentrations of protease inhibitors used and time of incubation were not toxic

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Abbreviations: LPS, lipopolysaccharide; TNFα, tumor necrosis factor α; EMSA, electrophoretic mobility shift assay; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TLCK, 7-amino-1-chloro-3-tosylamido-2-heptanone; DCIC, 3,4-dichloroisocoumarin; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; DOC, deoxycholate; DTT, dithiothreitol; NP-40, Nonidet P-40.

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to cells and are detailed in Fig. 1 unless otherwise indicated. Okadaic acid was used at 1 μM, LPS was at 10 μg/ml, and TNFα was at 10 ng/ml. Bacterially expressed RelA was a gift from C. Rosen (Human Genome Sciences, Rockville, MD).

Preparation of Extracts and Electrophoretic Mobility Shift Assays (EMSAs). Nuclear and cytoplasmic extracts were prepared as described (8). Samples standardized by protein concentration (19) were analyzed by EMSA as detailed (20). Probe sequences were as follows: NF-κB, 5′-CAGGGCTGGGGATTCCCCATCTCCACAGTTTCACTTC-3′; AP-1, 5′-TTCCGGCTGACTCATCAAGCG-3; OCT-1, 5′-TTCACGCGGTAATGAGATGGGTT-3′; SRE, 5′-CCTTTACAACAGGATGTCCATATTAGGACATCTGCGTCAGCAG-3′. Deoxycholate (DOC) activation of NF-κB *in vitro* was performed by treating cytoplasmic extracts with 0.8% DOC for 5 min followed by the addition of Nonidet P-40 (NP-40) to 1.6% (4). For *in vitro* experiments, nuclear and cytoplasmic extract or 1 μl of bacterially expressed RelA was incubated for 1 h at 4°C in binding buffer (20) lacking dithiothreitol (DTT) but containing the indicated concentrations of protease inhibitors. For some experiments DTT was also added. Cytoplasmic extracts were then incubated with DOC/NP-40. Subsequently, DTT, dI/dC, and labeled probe were added and samples were analyzed by EMSA.

Immunoprecipitation and Western Blot Analysis. Polyclonal RelA antibody (Rockland, Boyertown, PA; 2 μl of serum) was added to 400 μg of cytoplasmic extract and the volume was adjusted to 200 μl with lysis buffer (10 mM Hepes, pH 7.6/60 mM KCl/1 mM EDTA/0.3% NP-40/1 mM DTT/1 mM phenylmethylsulfonyl fluoride). The samples were incubated for 2 h at 4°C with mixing. The extract was then added to 20 μl of protein A-Sepharose previously blocked with 1% bovine serum albumin and incubated for another 2 h at 4°C with mixing. Pelleted protein A-Sepharose was washed twice with 500 μl of lysis buffer and once with lysis buffer lacking NP-40 and then resuspended in 20 μl of buffer (without NP-40). DOC was then added to 0.8% to release IκBα. Using DOC instead of boiling to dissociate IκBα from NF-κB significantly reduced the background on Western blots. The supernatant, containing IκBα previously bound to RelA, was then analyzed on Western blots. For Western blot analysis, samples were separated by SDS/PAGE, transferred to nitrocellulose, and probed with the IκBα antibody (Rockland).

RESULTS

Protease Inhibitors Prevent NF-κB Activation as Assayed by EMSAs. Consistent with previous results (16), pretreatment of HeLa or Jurkat T cells with the serine protease inhibitor TPCK, TLCK, BTEE, or DCIC prevented subsequent TNFα induction of NF-κB as quantitated by EMSAs of nuclear extracts (Fig. 1*A*). We demonstrate here that another serine protease inhibitor, AEBSF, had a similar effect. Other protease inhibitors, including chymostatin, leupeptin, and N^{α}-benzoyl-L-arginine ethyl ester, did not prevent NF-κB activation when tested at various concentrations (data not shown). To demonstrate that NF-κB from cells treated with protease inhibitors retains the capacity to bind DNA, EMSAs of corresponding cytoplasmic extracts were performed after treating extracts with the disrupting agent DOC. DOC activates NF-κB *in vitro* by causing its dissociation from IκB (4). Surprisingly, many of the protease inhibitors (TPCK, TLCK, and DCIC) suppressed the ability of DOC-activated NF-κB to bind DNA (Fig. 1*B*). As a control, it was confirmed that DOC can dissociate IκB from NF-κB in extracts from cells treated with any of the protease inhibitors (data not shown). BTEE and AEBSF did not negatively affect NF-κB binding (Fig. 1*B*). Pretreatment of cells with lower concentrations of protease inhibitors for shorter periods yielded similar results. However, more consistent results could be obtained at the higher concentrations. EMSAs of nuclear extracts from cells treated with only TPCK, TLCK, or DCIC indicated that these agents also suppressed basal NF-κB DNA binding activity, including that of NFKB1 homodimers (data not shown).

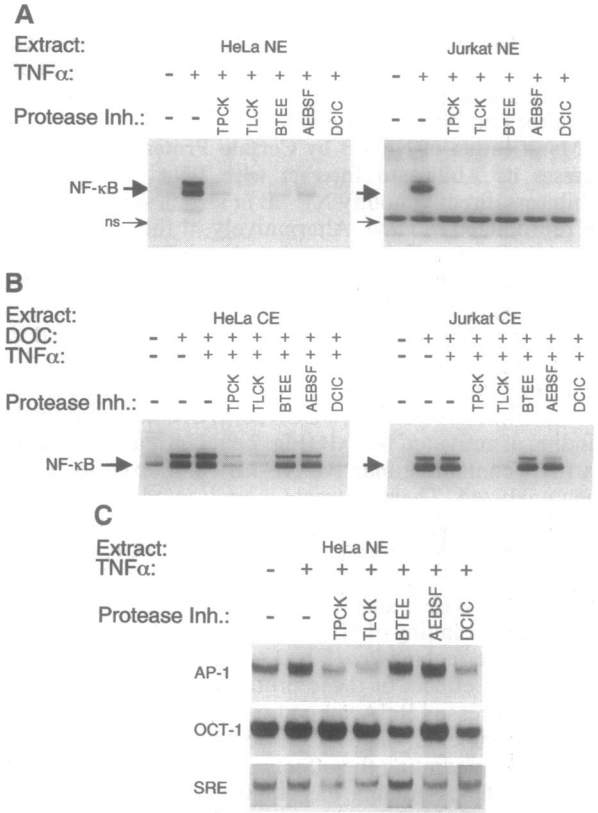

FIG. 1. *In vivo* effects of protease inhibitors on NF-κB activation and DNA binding. HeLa and Jurkat T cells were pretreated with protease inhibitors and then stimulated with TNFα for 15 min. Final concentration of inhibitors: TPCK, 50 μM; TLCK, 300 μM; BTEE, 2 mM (HeLa) or 1 mM (Jurkat); AEBSF, 1 mM (HeLa) or 2 mM (Jurkat); DCIC, 45 μM. The preincubation time prior to TNFα addition was 30 min for TPCK, TLCK, BTEE, and DCIC and was 180 (HeLa) or 90 (Jurkat) min for AEBSF. (*A*) Nuclear extracts (NEs), isolated from cells treated as indicated above each lane, were assayed by EMSA. Two major NF-κB complexes are induced by TNFα in HeLa cells but only one was induced in Jurkat cells. Inh., inhibitor. +, Added; −, not added. (*B*) Cytoplasmic extracts (CEs) corresponding to the NEs used in *A* were either directly assayed or were first treated with 0.8% DOC to dissociate IκB from NF-κB and then assayed. Two NF-κB complexes are induced by DOC. In *A* and *B*, NF-κB is indicated by a large arrow and the nonspecific band (ns) is indicated by a small arrow. Free probe is not shown. (*C*) HeLa NEs used in *A* were examined by EMSA for binding of other transcription factors. The complexes specific for each probe were competed with a 100-fold excess identical unlabeled probe but not with a 100-fold excess κB probe (data not shown). Only specific complexes are shown. The identity of each probe is indicated to the left.

We also examined the effect of these protease inhibitors on the binding of other transcription factors. AP-1 DNA binding activity in nuclear extracts from cells treated with TNFα and TPCK, TLCK, or DCIC (but not BTEE and AEBSF) was also inhibited (Fig. 1*C*). However, not all proteins were affected similarly. The binding of Oct-1 and a protein complex that specifically binds the serum response element were not significantly altered by the various protease inhibitors. Therefore, the inhibitory activities of TPCK, TLCK, and DCIC on NF-κB and AP-1 DNA binding potential appear specific. These results imply that TPCK, TLCK, and DCIC, in addition to their antagonism of protease activity, may

directly modify NF-κB and alter its ability to interact with DNA. However, it is also conceivable that these agents indirectly affect NF-κB and its DNA binding potential. To explore these possibilities, we tested the effects of these inhibitors on NF-κB binding *in vitro*.

Modification of NF-κB by Certain Protease Inhibitors Represses its Ability to Interact with DNA. If the protease inhibitors directly modify NF-κB *in vivo*, a similar effect may be reproduced *in vitro*. Alternatively, if the protease inhibitors initiate a cellular response that indirectly suppresses NF-κB DNA binding, an effect *in vitro* would not be expected since most cellular processes are inactive under these assay conditions. Nuclear extracts from TNFα-stimulated cells, containing activated NF-κB free of IκB, were investigated first. *In vitro* treatment of these extracts with increasing concentrations of TPCK, TLCK, or DCIC inhibited the DNA binding of activated NF-κB (Fig. 2*A*). Untreated cytoplasmic extracts containing inactive NF-κB complexed to IκB were then analyzed. These extracts were treated with DOC after incubation with protease inhibitors to allow analysis of DNA binding by NF-κB. As shown in Fig. 2*B*, TPCK, TLCK, and DCIC also inhibited the DNA binding potential of cytoplasmic NF-κB. BTEE and AEBSF, protease inhibitors that did not alter the binding of NF-κB when added *in vivo* (Fig. 1*B*), also had no effect *in vitro* (Fig. 2 *A* and *B* and data not shown). Finally, the effects of these protease inhibitors on the bacterially expressed RelA subunit of NF-κB were examined. In these experiments, the possible contribution of other cellular proteins in mediating the effects of TPCK, TLCK, or DCIC on NF-κB DNA binding was eliminated since only RelA and exogenously added bovine serum albumin were present. Consistent with the above results, TPCK, TLCK, and DCIC inhibited the ability of RelA to bind DNA (Fig. 2*C*). The protease inhibitors do not directly modify the κB DNA probe used in the EMSA since prolonged incubation of the probe with concentrations of TPCK, TLCK, or DCIC that completely suppressed NF-κB binding *in vivo* and *in vitro* had no inhibitory effect when the probe was subsequently diluted (to substantially lower the concentration of protease inhibitor) and used in an EMSA (data not shown). Furthermore, as determined by Western blot analysis, NF-κB is not degraded *in vivo* or *in vitro* in the presence of TPCK, TLCK, or DCIC (data not shown). Thus, these results strongly suggest that many of the protease inhibitors previously utilized in studies of NF-κB can directly interfere with the ability of this transcription factor to bind DNA (also see *Discussion*).

In contrast to the results presented here, other investigators (17, 21) have reported that TPCK does not inhibit the DNA binding activity of NF-κB *in vitro*. This discrepancy may be a result of the assay conditions in which the experiments were performed. We have found that DTT suppresses the *in vitro* inhibitory activity of TPCK toward NF-κB DNA binding (Fig. 2*D*). It is possible that other investigators did not detect the inhibitory effects of TPCK *in vitro* because DTT was present. As our *in vitro* results are identical to those observed *in vivo*, we believe that the *in vitro* conditions used in this study more closely reflect protease inhibitor activity within cells.

Protease Inhibitors Block Both Phosphorylation and Degradation of IκBα *in Vivo*. Pretreatment of cells with TPCK suppresses degradation of IκBα by inducers of NF-κB including phorbol 12-myristate 13-acetate, interleukin 1β, and LPS (11, 15–17). We demonstrate here that the other tested serine protease inhibitors, in addition to TPCK, also repressed TNFα-induced degradation of IκBα in HeLa cells (Fig. 3*A*). It is well established that TNFα-induced degradation of IκBα is preceded by its phosphorylation (8, 22). Therefore, in the absence of degradation, phosphorylated IκBα would be expected to accumulate in cells. However, newly phosphorylated IκBα, as detected on a Western blot and based on its reduced mobility during SDS/PAGE (7, 8, 22), was not observed in samples stimulated with TNFα for 15 min in the presence of protease inhibitors (Fig. 3*A*). This result indicates that protease inhibitors may prevent the phosphorylation of IκBα in addition to its degradation. Another explanation is that IκBα is rapidly dephosphorylated by a cytoplasmic phosphatase in the absence of degradation. To distinguish between these possibilities, cytoplasmic extracts from HeLa cells treated with TNFα for shorter periods of time were examined. Stable phosphorylated IκBα can be detected by Western blot analysis at these earlier time points

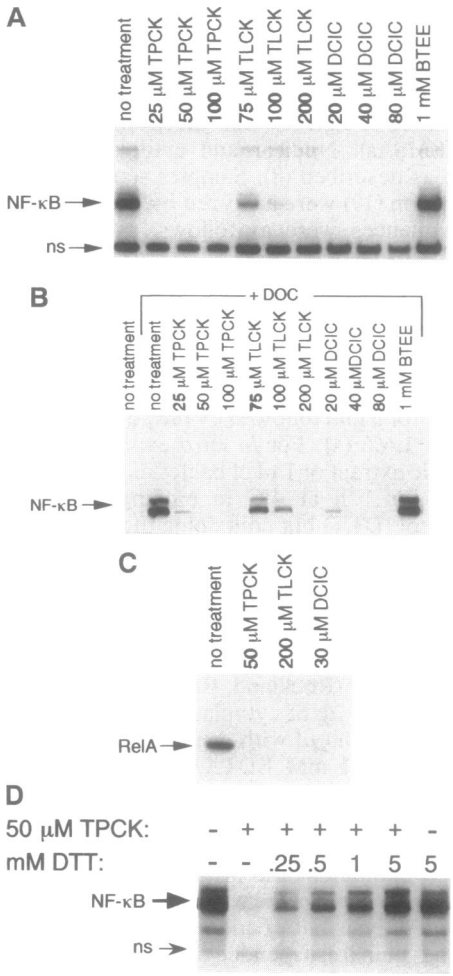

FIG. 2. *In vitro* effects of protease inhibitors on NF-κB DNA binding. (*A*) Protease inhibitors prevent DNA binding of activated nuclear NF-κB *in vitro*. Nuclear extracts from Jurkat cells stimulated with TNFα for 15 min were incubated at 4°C for 1 h alone or with TPCK, TLCK, BTEE, or DCIC in the absence of DTT. DTT was then added and samples were analyzed by EMSA. The final concentration of protease inhibitors is indicated. ns, Nonspecific band. (*B*) Protease inhibitors prevent DOC-induced binding of cytoplasmic NF-κB *in vitro*. Experiments using untreated Jurkat cytoplasmic extracts were performed as described in *A* except for the *in vitro* activation of NF-κB by DOC after the 1-h incubation at 4°C with protease inhibitors. The final concentration of protease inhibitor for each sample is shown. (*C*) Protease inhibitors prevent DNA binding of the bacterially expressed RelA subunit of NF-κB. Experiments were performed as in *A* by using 1 μl of RelA and the indicated concentrations of protease inhibitors. (*D*) DTT suppresses the inhibitory effect of TPCK on NF-κB binding. As indicated, HeLa nuclear extracts from cells treated with TNFα for 15 min were incubated for 1 h at 4°C alone, with 50 μM TPCK, or with 50 μM TPCK and the concentrations of DTT indicated. Afterwards, DTT was added to 5 mM in all samples, which were then analyzed by EMSA. NF-κB and the nonspecific (ns) band are labeled to the left; free probe is not shown.

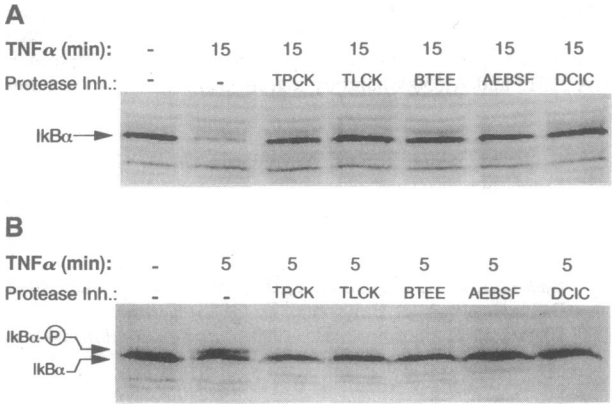

FIG. 3. Protease inhibitors prevent TNFα-induced phosphorylation and degradation of IκBα. (*A*) Protease inhibitors block IκBα degradation. HeLa cells, untreated or preincubated with the indicated protease inhibitors, were induced with TNFα for 15 min. Cytoplasmic extracts were then isolated and analyzed on a Western blot by using a polyclonal IκBα antibody. Protease inhibitor concentrations and time of preincubation are as in Fig. 1. IκBα is indicated at the left and the addition of TNFα (for 15 min) and protease inhibitors is also indicated. No other bands were specifically recognized by the antibody. (*B*) Protease inhibitors block IκBα phosphorylation. Experiments were performed as in *A* except TNFα induction was for 5 min. The phosphorylated form of IκBα induced by TNFα, which has a reduced mobility on SDS/PAGE, is indicated to the left. Samples were treated as indicated above the blot.

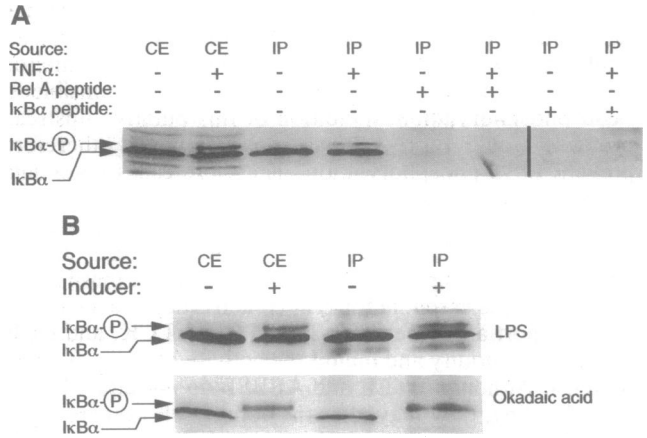

FIG. 4. Phosphorylation of IκBα is not sufficient for its dissociation from NF-κB. (*A*) TNFα-induced phosphorylation of IκBα does not cause its dissociation from NF-κB. NF-κB complexes from cytoplasmic extracts of either untreated cells or cells treated with TNFα for 5 min were immunoprecipitated with a RelA polyclonal antibody. Washed complexes, containing NF-κB and associated proteins, were then treated with 0.8% DOC to release IκBα bound to NF-κB. Liberated IκBα was subsequently analyzed on a Western blot with an IκBα polyclonal antibody. Also shown is 50 μg of cytoplasmic extract from untreated cells or cells stimulated with TNFα for 5 min, neither of which underwent immunoprecipitation. Sample treatment, including controls for specificity, was as indicated above the blot. (*B*) Phosphorylated IκBα, induced by LPS or okadaic acid, remains associated with NF-κB. Experiments were performed as in *A* except cytoplasmic extracts from THP-1 cells treated 1 hr with LPS or Jurkat T cells treated 1 h with okadaic acid were used. As indicated, both cytoplasmic extracts and immunoprecipitations using the RelA antibody were analyzed on a Western blot with the IκBα antibody. The various forms of IκBα are indicated to the left and the inducer is indicated to the right. CE, cytoplasmic extract; IP, immunoprecipitation.

(8). Treatment of cells with TNFα alone for 5 min resulted in the appearance of phosphorylated IκBα. However, this form of IκBα was not present when cells were first preincubated with any of the protease inhibitors and then stimulated with TNFα (Fig. 3*B*). Essentially identical results were obtained in Jurkat cells (data not shown). Thus, these serine protease inhibitors repress both the phosphorylation and degradation of IκBα. Because the inhibitors block both phosphorylation and degradation, it is difficult to assess from their use how each event contributes to the activation of NF-κB.

Phosphorylated IκBα Remains Associated with NF-κB. To better understand the mechanism(s) responsible for NF-κB activation, we determined *in vivo* whether induced phosphorylation of IκBα was sufficient for its dissociation from NF-κB. Specifically, we ascertained whether the phosphorylated form of IκBα observed after stimulation of HeLa cells with TNFα for 5 min could be coimmunoprecipitated with an antibody to the RelA subunit of NF-κB. The results, shown in Fig. 4*A*, indicate that phosphorylated IκBα remains associated with NF-κB. Neither unphosphorylated nor phosphorylated IκBα was detected when the RelA antibody used in the immunoprecipitation or the IκBα antibody used in the Western blot was preincubated with its corresponding peptide, demonstrating the specificity of the assay.

We also investigated whether similar results could be observed for other inducers of NF-κB. Studies have observed that LPS activation of NF-κB in the monocytic cell line THP-1 involves the phosphorylation and degradation of IκBα (ref. 7 and Fig. 4*B*). As shown in Fig. 4*B*, the phosphorylated form of IκBα induced by LPS also retains association with NF-κB. Okadaic acid, a specific inhibitor of the serine/threonine phosphatases 1 and 2A, induces NF-κB DNA binding activity in Jurkat T cells (23). This activation is mediated primarily through the phosphorylation and degradation of IκBα (T.S.F. and A.S.B., unpublished data). Consistent with results obtained using TNFα and LPS, the phosphorylated form of IκBα induced by okadaic acid also remains complexed to NF-κB (Fig. 4*B*). It is evident from these results that under a variety of conditions the inducible phosphorylation of IκBα does not cause its dissociation from NF-κB. These data strongly suggest that other events significantly contribute to the activation of NF-κB.

DISCUSSION

In this report we demonstrate that protease inhibitors used thus far in studies of NF-κB (11, 16–18) have previously uncharacterized properties that may complicate conclusions based on their use. For example, TPCK, TLCK, and DCIC interfere with NF-κB DNA binding potential. It is known that these inhibitors block protease activity by direct alkylation or acylation of the protease active site (24–26). Moreover, TPCK, TLCK, and DCIC alter the activity of proteins in addition to proteases such as glycogen phosphorylase *b*, TFIIIC, and Ets-1 through direct modifications (27–29). Furthermore, NF-κB DNA binding activity is redox-sensitive and can be inhibited by alkylation within its DNA binding domain (30, 31). Based on these observations and the data in this report, it is highly likely that the effects elicited *in vivo* and *in vitro* by these agents on NF-κB DNA binding occur through a direct modification of NF-κB and not by an indirect effect. It is also apparent that NF-κB can be modified when free or complexed to IκBα. An awareness of these effects on NF-κB DNA binding will be beneficial during relevant experiments and in the interpretation of results. BTEE and AEBSF, protease inhibitors that do not appear to interfere with NF-κB DNA binding activity, may be better suited for studies of NF-κB.

We also show that all protease inhibitors that repress IκBα degradation also completely prevent its phosphorylation. Therefore, it is difficult to conclude whether the suppressive effects of these inhibitors on NF-κB activation are due to

their inhibition of IκBα phosphorylation or instead its degradation. Several models exist to explain how these protease inhibitors prevent IκBα phosphorylation. (*i*) The inhibitors may target a protease that regulates a kinase responsible for IκBα phosphorylation; inhibition of this putative upstream protease would repress kinase activation and, therefore, prevent IκBα phosphorylation. Thus, proteases potentially exist at two steps in the NF-κB signal transduction pathway, one upstream of the kinase and one that acts directly on IκBα. (*ii*) It is conceivable that the protease inhibitors directly interfere with a signal transduction component that is not a protease but is essential for NF-κB activation. As described in this report and others (27–29), TPCK, TLCK, and DCIC can directly modify and inhibit the activity of nonproteolytic proteins. Although BTEE and AEBSF do not effect NF-κB DNA binding, they may alter the activity of proteins within the cell besides proteases. This second model includes the possibility that direct modification of NF-κB and/or IκBα by certain protease inhibitors may prevent their phosphorylation and/or activation. (*iii*) Phosphorylation and proteolysis are coupled events. This model would be relevant in situations where the kinase and protease directly interact, for example, as part of a multiprotein complex. Interaction of the protease inhibitors with the protease could cause a conformational change in the complex that then alters kinase activity. (*iv*) A combination of the above models may also explain how all of these mechanistically distinct protease inhibitors prevent IκBα phosphorylation; although the inhibitors are targeting different proteins in the signal transduction pathway, the outcome is identical.

The identification of inhibitors that block IκBα degradation but allow phosphorylation may clarify the role of proteolysis in NF-κB activation. Recently, an inhibitor of NF-κB activation that targets the proteasome has been shown to prevent degradation of IκBα without altering its phosphorylation (32). Intriguingly, two of the protease inhibitors used in the present study, DCIC and AEBSF, repress proteolytic activities within the proteasome (33, 34). However, they must be altering additional cellular processes due to their ability to inhibit IκBα phosphorylation. DCIC and AEBSF may interact with the proteasome in a manner that also inhibits IκBα phosphorylation or an additional target of DCIC and AEBSF within the cell may be the proposed upstream protease.

Previous studies have suggested that the phosphorylation of IκBα is responsible for its dissociation from NF-κB, after which free IκBα is rapidly degraded (5, 6, 10, 15). However, we show in this report that *in vivo* phosphorylation of IκBα alone is not sufficient for its dissociation from NF-κB. This important result indicates that additional cellular events are required for NF-κB activation. Perhaps other phosphorylation events, either on IκBα or NF-κB, are necessary. Consistent with this idea, we have observed that the RelA subunit of NF-κB is also phosphorylated after treatment of cells with inducers of NF-κB (unpublished observation). Thus, within an NF-κB–IκBα complex, phosphorylation of NF-κB or IκBα alone may have no consequence, but both phosphorylation events together would cause the dissociation of IκBα. Another possible role for inducible phosphorylation of IκBα is that it serves as a signal for a subsequent essential event. For example, phosphorylated IκBα may be a better substrate for a cytoplasmic protease. The proteolysis of phosphorylated IκBα complexed to NF-κB would then result in the activation of NF-κB.

Elucidation of the signals required for NF-κB activation is important in deciphering how this family of transcription factors is regulated. An understanding of this complex process may lead to the discovery of pharmacologic modulators of NF-κB activity that are beneficial in the treatment of immune and inflammation disorders, in the regulation of tumorigenesis, and in the treatment of AIDS.

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