MOLECULAR AND CELLULAR BIOLOGY, Mar. 1995, p. 1302–1311 Vol. 15, No. 3
0270-7306/95/$04.00+0

Phosphorylation of IκBα Precedes but Is Not Sufficient for Its Dissociation from NF-κB

JOSEPH A. DiDONATO,[1] FRANK MERCURIO,[2] AND MICHAEL KARIN[1]*

Department of Pharmacology, The University of California at San Diego School of Medicine, La Jolla, California 92093-0636,[1] and Signal Pharmaceuticals, San Diego, California 92121[2]

Received 20 September 1994/Returned for modification 4 November 1994/Accepted 22 November 1994

NF-κB is an important activator of immune and inflammatory response genes. NF-κB is sequestered in the cytoplasm of nonstimulated cells through interaction with the IκB inhibitors. These inactive complexes are dissociated in response to a variety of extracellular signals, thereby allowing free NF-κB dimers to translocate to the nucleus and activate transcription of specific target genes. The current dogma is that phosphorylation of the IκBs is responsible for dissociation of the inactive complexes, an event that is rendered irreversible by rapid IκB degradation. Here, we show that inducers of NF-κB activity stimulate the hyperphosphorylation of one of the IκBs, IκBα. However, contrary to the present dogma the hyperphosphorylated form of IκBα remains associated with NF-κB components such as RelA (p65). Thus, phosphorylation of IκBα is not sufficient to cause dissociation of the inactive NF-κB:IκBα complex. However, that complex is disrupted through the selective degradation of phosphorylated IκBα in response to extracellular signals. Using a variety of protease inhibitors, some of which have specificity towards the multicatalytic proteinase complex, we demonstrate that degradation of IκBα is required for NF-κB activation. The results of these experiments are more consistent with a new model according to which phosphorylation of IκBα associated with NF-κB marks it for proteolytic degradation. IκBα is degraded while bound to NF-κB. The selective degradation of IκBα releases active NF-κB dimers which can translocate to the nucleus to activate specific target genes.

NF-κB is a transcription factor (54) that plays a pivotal role in the regulation of a number of immune and inflammatory response genes and activation of various viral promoters, including the human immunodeficiency virus long terminal repeat (reviewed in references 1, 26, and 40). NF-κB is composed of a number of proteins related to the proto-oncoprotein c-Rel (reviewed in references 9 and 14). These proteins are NF-κB1 (p50), NF-κB2 (p52), c-Rel, RelA (p65), and RelB (10, 11, 25, 35, 37, 39, 41, 42, 49, 50, 52). The different NF-κB proteins associate to form a variety of homo- and heterodimers, which interact with a series of related DNA target sites, collectively known as NF-κB-binding sites (reviewed in reference 2). Most of the dimeric NF-κB complexes are stored in the cytoplasm of nonstimulated cells as inactive complexes through interactions with a group of inhibitory proteins, collectively known as IκB (reviewed in reference 4). NF-κB1 and NF-κB2 are initially synthesized as large precursors, 105 and 100 kDa in size (10, 11, 25, 35, 37, 39, 41, 52). These precursors do not bind DNA but can interact with other Rel-related proteins and function in an IκB-like fashion by sequestering various Rel-related protein homo- and heterodimers in the cytoplasm (38, 46). Proteolytic degradation of the p105 and p100 precursors leads to the production of mature DNA-binding subunits and the release of active Rel-related protein dimers, containing p50 and p52, respectively (22, 37, 38, 46). The rate of processing of both precursors appears to be regulated and can be increased in response of extracellular stimuli (38).

Central to the regulation of NF-κB activity is the movement of NF-κB dimers from the cytoplasm to the nucleus in response to appropriate extracellular stimuli (reviewed in references 1 and 2). The prototypic IκB protein involved in cytoplasmic retention of NF-κB dimers is IκBα, encoded by the *MAD-3* gene (27) and its chicken and rat homologs, pp40 and RL/IF-1, respectively (18, 59). IκBα consists primarily of a repeating ankyrin-like motif (18, 27, 28, 33). These repeats are believed to function mainly as interfaces for protein-protein interactions (60). Similar ankyrin-like repeats are present in both the p105 and p100 precursors of NF-κB1 and NF-κB2 subunits (10, 11, 25, 35, 37, 39, 41, 52) and are essential for their IκB-like activity (38, 46) and cytoplasmic localization (7, 8, 30, 38, 46).

The ankyrin-like repeats are thought to contact the Rel homology domains of the NF-κB proteins and thereby mask their nuclear transfer signals (6). Upon cell stimulation with agents such as phorbol esters, tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), hydrogen peroxide, UV light, and lipopolysaccharide, the signal is transmitted to NF-κB:IκB complexes, resulting in the dissociation of the latter and nuclear translocation of NF-κB (reviewed in references 1 and 26). As in vitro phosphorylation of IκBs by a variety of protein kinases prevented their ability to associate with NF-κB, it was proposed that the event which mediates the dissociation of inactive NF-κB:IκB complexes is the phosphorylation of IκB (24, 34, 55). Recently, it was found that NF-κB-activating stimuli cause rapid degradation of IκBα, with kinetics that parallel the activation of NF-κB (5, 13, 16, 19, 29, 36). In addition, IκBα was shown to be transiently phosphorylated after cell stimulation (5, 16, 36). Thus, the current dogma is that extracellular stimuli activate a signal transduction cascade, leading to phosphorylation of IκB proteins and dissociation of the inactive NF-κB:IκB complexes. While the liberated NF-κB dimers migrate to the nucleus, this activation process is rendered at least temporarily (until the synthesis of new IκB) irreversible through the degradation of free IκB. In the nucleus, the NF-κB dimers bind to target sequences and activate transcription of nearby genes (32), among which is the *MAD-3*

* Corresponding author. Phone: (619) 534-1361. Fax: (619) 534-8158.

gene (15, 57). This process results in synthesis of new IκBα which can then interact with NF-κB complexes to promote their dissociation from DNA and retrotransport to the cytoplasm (64).

As much of this hypothesis rests on the phosphorylation-induced dissociation of the NF-κB:IκB complex, we investigated this step in further detail. Although we find that IκBα is rapidly phosphorylated in response to cell stimulation, surprisingly, this phosphorylation does not result in the dissociation of NF-κB:IκBα complexes. A critical step in NF-κB activation however is the degradation of NF-κB-bound IκBα. That step can be inhibited by the use of peptide aldehyde protease inhibitors, which do not interfere with IκBα phosphorylation. These compounds are potent and specific inhibitors of NF-κB activation as has recently been demonstrated (43).

MATERIALS AND METHODS

Plasmids. pCMV-MAD-3 and the reporter plasmids pRSV-Luc and p2x NF-κB-Luc have been described previously (38, 48, 51). pKGMAD-3 contains the entire IκBα-coding region (amino acids 1 to 317) fused in frame to the glutathione *S*-transferase (GST) moiety of pKG (Pharmacia). pGEXmp65COOH contains the GST moiety of pGEX4T-1 (Pharmacia) fused in frame to amino acids 398 to 549 of mouse RelA (42) and was used to transform *Escherichia coli* XL-1 blue cells (Stratagene) to express the GST-RelA protein for raising polyclonal antibodies.

Antibodies. The anti-NF-κB1 and anti-NF-κB2 antisera were directed against the amino-terminal sequences of p105 and p100, respectively (37, 38). Anti-IκBα is a rabbit polyclonal antibody prepared against a histidine-tagged MAD-3 fusion protein as described previously (19). Anti-p65-C is a rabbit polyclonal antibody directed against the carboxy-terminal 151 amino acids of mouse RelA.

Cells and transfections. HeLa S3 cells were grown as adherent cells in high-glucose-containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine. HepG2 cells were grown in modified Eagle's medium (MEM) supplemented with 10% FCS, 1 mM sodium pyruvate, 1 mM nonessential amino acids, and 2 mM glutamine. To transfect HepG2 cells, 10 μg of the reporter gene was transfected by electroporation (260 V and 960 μF) into 5×10^6 cells in a total volume of 300 μl. The cells were allowed to recover on ice for 15 min before dilution into 10 ml of fresh growth medium, at which time 0.5-ml aliquots were plated into 24-well dishes. Cells were grown for 22 h after electroporation before the addition of the protease inhibitors. Cells were pretreated for 4 h with or without the inhibitors and then either were not stimulated or were stimulated with TNF-α (10 ng/ml) for 6 h. The cells were harvested at 6 h poststimulation and processed for luciferase assays (51). Transfections were performed in duplicates and repeated twice with less than 10% variation.

Cell extracts. After treatment with TNF-α or IL-1α (R & D Systems) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma Chemical Co.), the cells were harvested in ice-cold phosphate-buffered saline (PBS) and pelleted at 2,000 × *g* at room temperature for 30 s, the cell pellet was resuspended on ice in whole-cell extract (WCE) lysis buffer composed of 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.7); 0.3 M NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5% Triton X-100 (vol/vol); 3 mM dithiothreitol; the phosphatase inhibitors 30 mM β-glycerophosphate, 50 mM NaF, 1 mM sodium orthovanadate (Na_3VO_4), and 20 mM *p*-nitrophenyl phosphate (PNPP; Calbiochem, San Diego, Calif.); and the protease inhibitors aprotinin, leupeptin, bestatin, pepstatin (all at 10 μg/ml; all from Calbiochem), 100 μM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma), and 1 mM phenylmethylsulfonyl fluoride (Sigma). Lysates were rotated in the cold for 45 min and then centrifuged at 13,000 × *g* for 15 min, and the supernatants were transferred to new tubes. For calf intestinal alkaline phosphatase (Gibco-BRL) treatment of WCE, 30 μg of WCE was incubated with 50 U of calf intestinal alkaline phosphatase in phosphatase buffer supplied by the manufacturer either lacking or containing the phosphatase inhibitors described above for 60 min at 37°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer was added to the reaction mixtures, which were fractionated by SDS-PAGE, and the proteins were transferred to an Immobilon-P membrane (Millipore) for immunoblot analysis.

EMSA and immunoblot analysis. For the electrophoretic mobility shift assay (EMSA) analysis, 10 μg of WCE was incubated with 5 μg of polyoligonucleotides (dI-dC) and 20,000 cpm (~0.2 ng) of the labeled oligonucleotide probe corresponding to the NF-κB-binding site of the mouse immunoglobulin light-chain enhancer (37). After 30 min of incubation on ice, the bound and free DNAs were fractionated on 5% native polyacrylamide gels as described previously (19, 37). Immunoblot analysis was performed essentially as described previously except that SDS–12% PAGE was used for the fractionation (19, 37).

Peptide aldehyde protease inhibitors. *N*-Acetyl-Leu-Leu-norleucinal (Ac-LLnL-CHO) and *N*-acetyl-Leu-Leu-methioninal (Ac-LLM-CHO), the calpain I and calpain II inhibitors, respectively, were purchased from Calbiochem, as was the calpain inhibitor calpeptin {benzyloxycarbonyl-Leu-norleucinal (Z-LnL-CHO [61])}. The multicatalytic protease complex (MPC) inhibitor benzyloxycarbonyl-Leu-Leu-phenylalaninal (Z-LLF-CHO [62]) was a generous gift of M. Orlowski. *N*-Acetyl-Ala-Leu-Leu-norleucinal (Ac-ALLnL-CHO) and benzyloxycarbonyl-Ile-Asp-leucinal (Z-IEL-CHO) were prepared by Signal Pharmaceuticals (San Diego, Calif.). All peptide aldehydes were dissolved in absolute ethanol and stored at −80°C.

Immunoprecipitations. Immune precipitation of endogenous proteins was done as described previously (37, 38). For immunoprecipitation of [^{35}S]methionine-cysteine-labeled proteins, HepG2 cells were metabolically labeled with [^{35}S]methionine-cysteine (Trans-label; ICN). Cells were washed two times with PBS, incubated in growth medium supplemented with 10% dialyzed FCS but lacking methionine for 45 min. Then, the medium was replaced with growth medium supplemented with 10% dialyzed FCS and [^{35}S]methionine-cysteine (100 μCi/ml) and pulse-labeled for 4 h before being chased with a 100-fold excess of cold methionine (25 mM) for 10 min prior to stimulation of the cells with IL-1α (1 ng/ml). The cells, after being treated, were washed two times with ice-cold PBS and lysed by the addition of ice-cold RIPA buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.4% deoxycholate, 1% Nonidet P-40) containing 0.1% SDS and the mixture of phosphatase and protease inhibitors described above. Cells were lysed on ice for 15 min and centrifuged for 15 min at 13,000 × *g* at 4°C. The supernatants were transferred to new tubes, 6 μl of preimmune serum was added, and the tubes were allowed to rotate for 1 h at 4°C before the addition of 50 μl of a 50/50 (wt/vol) slurry of Pansorbin (Calbiochem) to the tubes; this was followed by rotation for 90 min before centrifugation in the cold at 6,000 × *g* for 5 min. The supernatants were again transferred to new tubes, anti-IκBα antiserum (4 μl per 6×10^6 cells) was added, and the mixture was incubated at 4°C for 2 h before the addition of 30 μl of a 50/50 (vol/vol) slurry of protein A-Sepharose (Pharmacia) in RIPA buffer as described above. Immunopellets were collected as described previously (37, 38) and analyzed by SDS-PAGE as described above. The gels were then fixed, treated with Amplify (Amersham), dried, and exposed to Kodak XAR X-ray film. For immunoprecipitation of $^{32}P_i$-labeled protein, HeLa cells were washed two times with growth medium without serum and phosphate and then incubated in growth medium without phosphate but supplemented with 10% dialyzed FCS for 45 min. The medium was then replaced with fresh growth medium supplemented with 10% FCS and 2 mCi of $^{32}P_i$ per ml. The cells were incubated for 5 h before they were either not stimulated or stimulated with TNF-α (10 ng/ml) for 4 min and then harvested. Cells that were treated with protease inhibitors were administered the inhibitor 3 h prior to harvesting. Harvesting of the cells was done as described above. IκBα was immunoprecipitated as described above. The immunopellets were fractionated by SDS-PAGE and transferred to either nitrocellose or onto Immobilon-P membranes as described previously (19).

To identify the forms of IκBα which associate with RelA, WCEs from nonstimulated cells or cells pretreated with Ac-LLnL-CHO or not pretreated, as indicated, were stimulated with TNF-α (10 ng/ml) for 4 min, or for 20 min in the case of drug-treated cells, and then harvested. Washed cell pellets were lysed with a modified WCE buffer which consisted of 25 mM Tris-Cl (pH 7.9), 10 mM HEPES (pH 7.9), 100 mM NaCl, 25 mM KCl, 1 mM EDTA, 0.4% Nonidet P-40, 2 mM dithiothreitol, and the phosphatase and protease inhibitors described above. The cell extracts were then processed as described above. For immunoprecipitation, 600 μg of WCE from each treatment was diluted with an equal volume of modified WCE lysis buffer lacking NaCl and Nonidet P-40. Preimmune antiserum (2 μl) or anti-RelA-C antiserum (2 μl) was added, and the mixture was incubated in the cold between 12 and 24 h before the addition of 30 μl of a 50/50 (vol/vol) slurry of protein A-Sepharose. Immunopellets were washed three times with 1 ml of WCE lysis buffer containing Nonidet P-40 at 0.05% and 1 mM dithiothreitol. Immunopellets were fractionated by SDS–12% PAGE, and immunoblot analysis using IκBα antiserum was performed as described previously (19, 38).

Phosphopeptide mapping. IκBα was immunoprecipitated from $^{32}P_i$-labeled HeLa cells as described above, fractionated by SDS–12% PAGE, and transferred to nitrocellulose. The phosphorylated IκBα bands were identified by autoradiography and excised. The IκBα bands were digested with trypsin and analyzed by two-dimensional phosphopeptide mapping as described previously (12). Thin-layer chromatography plates were exposed to Kodak XAR film for 4 to 10 days at −80°C with an intensifying screen.

RESULTS

Signal-induced phosphorylation and degradation of IκB. Various stimuli including TNF-α, IL-1, and phorbol esters trigger the phosphorylation and eventual degradation of IκBα, resulting in activation of NF-κB DNA-binding activity (5, 36). A correlation between IκBα degradation and NF-κB activation was shown for other stimuli as well (5, 13, 19, 29, 57). To establish the order of events occurring during NF-κB activation, a detailed time course analysis of NF-κB DNA-binding activity and IκBα degradation was performed. The human

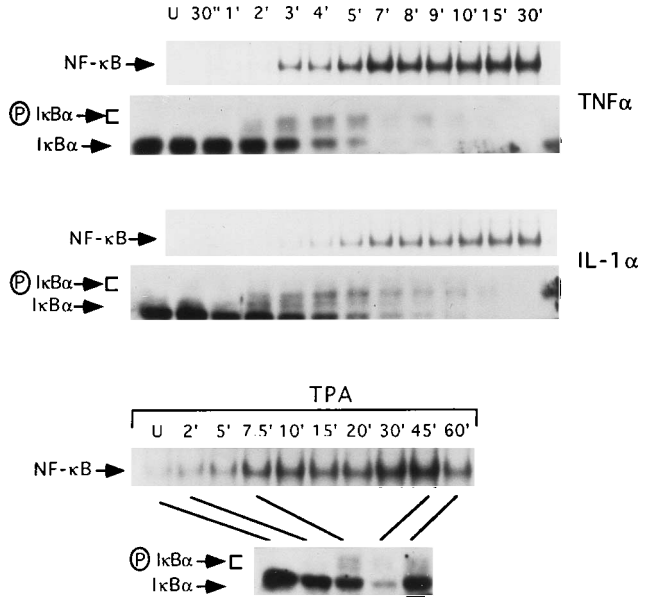

FIG. 1. Time course analysis of NF-κB DNA activation and IκBα degradation. HepG2 cells either were not stimulated (U) or were stimulated with either TNF-α (10 ng/ml), IL-1α (1 ng/ml), or TPA (100 ng/ml), as indicated. Cells were collected, and WCEs were prepared at the times (in minutes), indicated above the lanes. WCEs (10 μg) were used in EMSA analyses with a κB-binding site probe (autoradiograms of these assays are the top part of each panel, and the NF-κB protein-DNA complexes are indicated by arrows). Samples of WCEs (30 μg) were separated by SDS-PAGE, transferred to Immobilon-P membranes, and analyzed for IκBα abundance by immunoblotting with a rabbit polyclonal IκBα antiserum. These assays are displayed in the bottom half of each panel, and the hyperphosphorylated (Ⓟ IκBα) and constitutively phosphorylated (IκBα) forms are indicated.

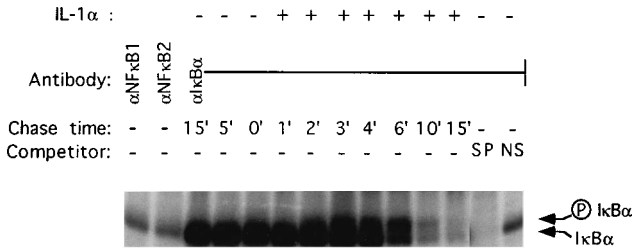

FIG. 2. Preexisting IκBα is hyperphosphorylated in response to cell stimulation. HepG2 cells were pulse-labeled with [^{35}S]methionine for 4 h and chased with growth medium supplemented with 25 mM methionine for 10 min prior to incubation in the absence or presence of IL-1α (1 ng/ml) in 25 mM methionine-containing medium. At the times (in minutes) indicated, the cells were collected and analyzed by immunoprecipitation with NF-κB1, NF-κB2, or IκBα antisera, as indicated. The first two lanes show the results of unstimulated cell lysates immunoprecipitated with either NF-κB1 or NF-κB2 antisera. IκBα coprecipitated with both of these proteins. The remaining lanes show immunoprecipitation with IκBα antiserum. The specificity of this antiserum was tested by competition with GST-IκBα (30 μg; specific competitor [SP]) or GST (80 μg; nonspecific competitor [NS]) and is shown in the last two lanes. These proteins were preincubated with the IκBα antibody for 45 min on ice prior to the addition of unstimulated cell lysates. The immunoprecipitates were collected, separated by SDS-PAGE, and visualized by autoradiography. The hyperphosphorylated and constitutively phosphorylated forms of IκBα are indicated.

hepatoblastoma cell line HepG2 was chosen because it contains receptors for both TNF-α and IL-1 (31, 56). HepG2 cells were treated with TNF-α, IL-1α, or the phorbol ester TPA for various times, and WCEs were prepared and analyzed by EMSA for NF-κB DNA-binding activity and by immunoblotting to determine IκBα levels, as described previously (19, 37). Initial immunoblot analysis with a polyclonal antibody prepared against histidine-tagged IκBα indicated that after cell stimulation with any of these inducers, a slowly migrating form of IκBα appeared (data not shown). This form of IκBα migrates as a close doublet and, as shown below, represents hyperphosphorylated IκBα. TNF-α and IL-1α caused the rapid appearance of the slowly migrating IκBα (labeled Ⓟ IκBα) within 2 min of their addition to the cells (Fig. 1). This preceded the induction of NF-κB DNA-binding activity, found detectable at 3 min poststimulation. The increase in NF-κB DNA-binding activity paralleled the disappearance of both the faster-migrating and more slowly migrating forms of IκBα, such that at 10 min poststimulation hardly any IκBα was detectable and the DNA-binding activity had reached its peak. At later time points (after 30 min), IκBα had reappeared as a slowly migrating form, presumably because of new synthesis, and its appearance preceded a decline in DNA-binding activity (data not shown). Similar findings were made with TPA as an inducer (Fig. 1), although the kinetics of the appearance of the slowly migrating form of IκBα, the disappearance of IκBα, and the induction of NF-κB DNA-binding activity were somewhat slower. These results established a temporal relationship between the appearance of modified forms of IκBα and decreased electrophoretic mobility, degradation of IκBα, and induction of NF-κB DNA-binding activity.

The slowly migrating forms of IκBα are due to phosphorylation, as we (data not shown) and others (5, 16, 36) have shown. To investigate whether the hyperphosphorylated forms of IκBα were generated through phosphorylation of preexisting IκBα, a pulse-chase analysis was performed. HepG2 cells were metabolically labeled for 4 h with [^{35}S]methionine and incubated with a 100-fold excess of unlabeled methionine for 10 min, after which the cells were either treated with IL-1α for various periods or not treated and subjected to lysis and immunoprecipitation analysis. As shown in Fig. 2, incubation with IL-1α resulted in the appearance of slowly migrating ^{35}S-labeled IκBα within 2 to 3 min. This was followed by the rapid degradation of IκBα such that after 10 min no ^{35}S-labeled IκBα was detectable. However, no loss of ^{35}S-labeled IκBα from the unstimulated cells was observed even after 25 min (10 and 15 min) of chase and no slowly migrating form was detectable. These results strongly suggest that upon cell stimulation, preexisting IκBα is rapidly hyperphosphorylated and then degraded.

Phosphorylation of IκBα does not induce its dissociation from NF-κB. Central to understanding how NF-κB is released from IκBα is the question of whether the phosphorylation of IκBα causes its dissociation from NF-κB followed by its degradation. Alternatively, phosphorylation of IκBα may mark it for degradation while associated with NF-κB. We therefore examined which forms of IκBα remain associated with RelA (p65) in the first few minutes following cell stimulation with TNF-α. Whole-cell lysates of nonstimulated and TNF-α-stimulated HepG2 cells were immunoprecipitated with either preimmune serum or an antiserum prepared against the carboxyl-terminal 151 residues of mouse RelA (42). The immune complexes were fractionated by SDS-PAGE and transferred to Immobilon-P membranes, which were then subjected to immunoblot analysis with anti-IκBα antibodies. The input lysates exhibited the characteristic patterns of the fast- and slowly migrating forms of IκBα (Fig. 3). While the preimmune serum did not precipitate any form of IκBα, both the fast- and slowly moving forms of IκBα were coprecipitated with RelA, from lysates of nonstimulated or TNF-α-stimulated cells. Most importantly, the ratio between the two forms of IκBα in TNF-α-stimulated cells associated with RelA was very similar to their

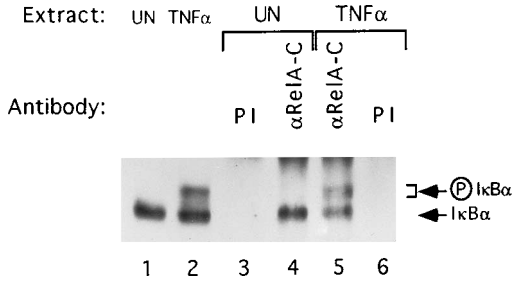

FIG. 3. Hyperphosphorylated IκBα remains associated with RelA. HeLa cells either were unstimulated or were stimulated with TNF-α for 4 min, and WCEs were prepared. Samples (600 μg) were immunoprecipitated with either preimmune serum (PI) or with rabbit polyclonal RelA antiserum (αRelA-C) as indicated. The resulting immune complexes were collected and fractionated by SDS-PAGE. Samples of WCEs from unstimulated (UN) and TNF-α-stimulated cells were loaded directly in lanes 1 and 2. Following transfer to the Immobilon-P membrane, immunoblot analysis was performed with the IκBα antiserum as described in the legend to Fig. 1.

ratio in unfractionated total cell lysates. Similar results were obtained by using anti-NF-κB1 (p50) and anti-NF-κB2 (p55) antibodies (data not shown). Since the slowly moving form of IκBα is due to signal-induced phosphorylation, it is apparent that hyperphosphorylation of IκBα is not sufficient to induce its dissociation from NF-κB.

Protease inhibitors block NF-κB activation. We investigated the possibility of using protease inhibitors to block IκBα degradation. This is critical for determining whether the degradation of IκB is an essential step in the process leading to NF-κB activation. The serine protease inhibitor and alkylating agent TPCK (53) was reported to be an effective inhibitor of NF-κB activation and IκBα degradation (29, 36). To determine the concentration of TPCK needed to block IκBα proteolysis, a dose-response experiment was performed (Fig. 4). HepG2 cells were incubated with the indicated concentration of TPCK for 45 min prior to stimulation by TPA for 30 min and the subsequent immunoblot analysis of IκBα levels. While 10 μM TPCK failed to prevent IκBα degradation, 25 to 50 μM TPCK was effective in preventing IκBα degradation. Surprisingly, TPCK not only blocked IκBα degradation but also blocked the appearance of hyperphosphorylated IκBα. Therefore, TPCK was not suitable for determining whether degradation of IκBα is necessary for NF-κB activation.

The nonspecific effect of TPCK on IκBα phosphorylation could be due to it being an alkylating agent. We therefore

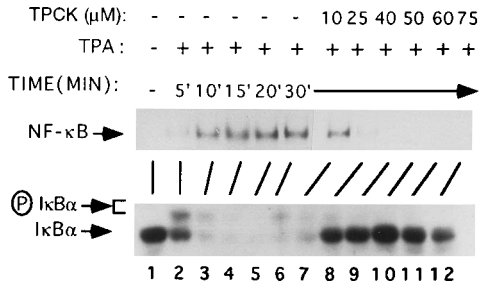

FIG. 4. TPCK interferes with IκBα hyperphosphorylation. HepG2 cells were incubated in the absence or presence of the indicated micromolar concentrations of TPCK for 45 min prior to incubation in the absence or presence of TPA (100 ng/ml) for 0 to 30 min as indicated. The top panel shows the results of EMSA analysis of 10 μg of WCEs, and the bottom panel shows the results of immunoblot analysis of 30-μg WCE samples with anti-IκBα antibodies. The locations of the NF-κB protein-DNA complexes and the two forms of IκBα are indicated.

examined the effect of a different class of protease inhibitor, peptide aldehydes, on IκBα degradation and NF-κB activation. Peptide aldehydes are thought to function as competitive inhibitors of various proteases through binding to their catalytic pockets (44, 47). The peptide aldehyde inhibitors used included the calpain inhibitors Ac-LLnL-CHO, Ac-LLM-CHO, and Ac-LnL-CHO (calpeptin [61]); an inhibitor of the MPC, Z-LLF-CHO (62); and two novel compounds, Ac-ALLnL-CHO and Z-IEL-CHO. The calpain inhibitors were chosen because calpains are cytosolic Ca^{2+}-activated proteases that could conceivably be involved in stimulus-induced IκB proteolysis (reviewed in references 17 and 58). The MPC inhibitor was chosen because this complex was initially suggested to be involved in the processing of the p105 and p100 precursors (22), and this has recently been affirmed (43). In addition, certain MPC inhibitors were found to inhibit NF-κB activation and IκBα degradation (43). HepG2 (data not shown) or HeLa cells were pretreated with the various peptide aldehydes at 1 to 200 μM or with ethanol as a control for 5 h prior to stimulation with TNF-α. After 20 min, WCEs were prepared and analyzed by EMSA for NF-κB-binding activity and by immunoblotting for IκBα levels. Three of the inhibitors, Z-LLF-CHO, Ac-LLnL-CHO, and Ac-ALLnL-CHO, blocked IκBα degradation but did not inhibit its hyperphosphorylation (Fig. 5A). The same three compounds inhibited the induction of NF-κB DNA-binding activity. Most importantly, the inhibition of NF-κB induction was directly proportional to the extent of inhibition of IκBα degradation. Z-LnL-CHO, Z-IEL-CHO, and Ac-LLM-CHO failed to block IκBα degradation and NF-κB DNA-binding activity. To further examine the relationship between IκBα degradation and NF-κB activation, a detailed dose-response experiment using the two most potent inhibitors was performed (Fig. 5B). The 50% inhibitory concentrations for IκBα degradation and NF-κB activation were essentially identical and were 3.4 μM for Z-LLF-CHO and 12.1 μM for Ac-LLnL-CHO. Z-LLF-CHO, however, at concentrations of 50 μM or greater, caused an apparent decrease in IκBα hyperphosphorylation. This could be the result of cytotoxic effects of this peptide aldehyde caused by a large accumulation of ubiquinated protein in the cell (42a), which was not observed with Ac-LLnL-CHO at the various concentrations used (19a).

The different peptide aldehyde inhibitors were also examined as to their effects on IκB degradation and NF-κB activation following stimulation of HeLa cells with IL-1α or TPA. As observed for TNF-α-stimulated cells, only Z-LLF-CHO, Ac-LLnL-CHO, and Ac-ALLnL-CHO inhibited IκBα degradation and NF-κB DNA binding in IL-1α- or TPA-treated cells (Fig. 5C). Ac-LLM-CHO partially inhibited IκBα degradation and NF-κB activation only in TPA-stimulated cells and not in IL-1α-treated cells. A time course analysis of TNF-α-stimulated HeLa cells preincubated with Ac-LLnL-CHO revealed that 5 min after TNF-α stimulation, most of the IκBα was converted to the hyperphosphorylated form. After that, the amount of the hyperphosphorylated IκBα decreased while the amount of the unmodified IκBα remained more or less constant for about 20 min (Fig. 5D). These results suggest that Ac-LLnL-CHO did not completely block IκBα degradation but slowed it considerably. In agreement with this assertion, NF-κB DNA-binding activity was induced with very slow kinetics and was detectable only after 20 min compared with the usual 2 to 3 min (Fig. 5D). Under these conditions, it therefore appears that the hyperphosphorylated form of IκBα is the preferred target for proteolytic degradation.

Peptide aldehyde inhibitors do not alter IκBα phosphorylation. The slowly migrating form of IκBα observed in cellular

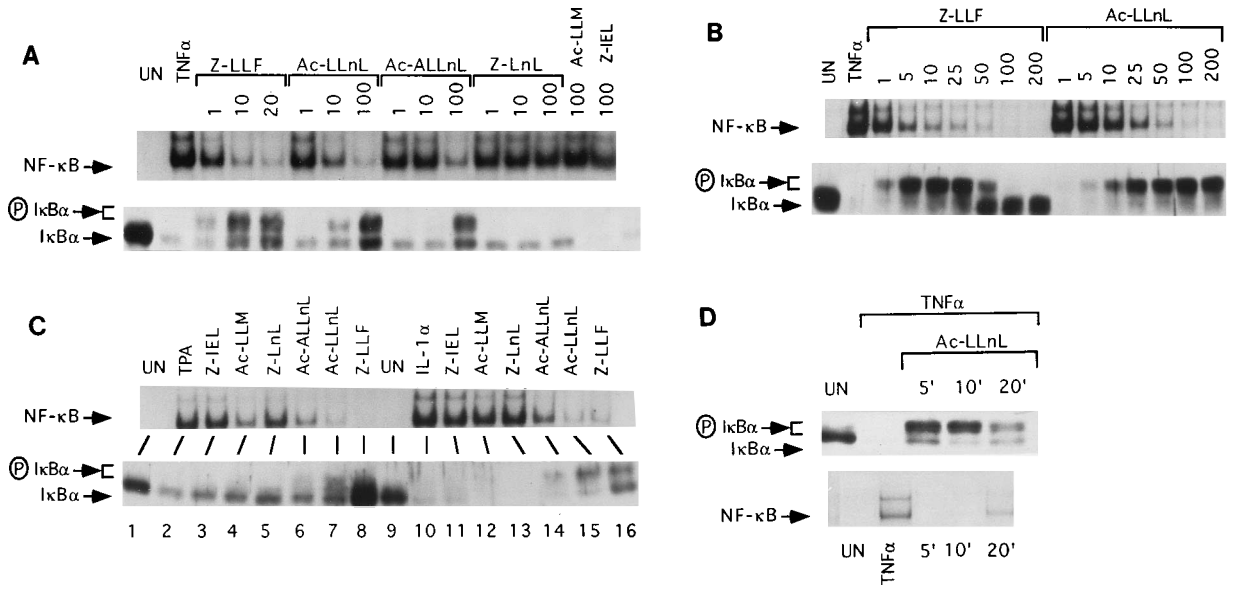

FIG. 5. Effects of peptide aldehyde protease inhibitors on activation of NF-κB DNA-binding activity and IκBα degradation. The indicated peptide aldehydes were added at 1 to 200 μM, as indicated, to HeLa cells for 5 h prior to their incubation in the absence (UN) or presence of TNF-α (10 ng/ml) (A, B, and D) and IL-1α (1 ng/ml) or TPA (100 ng/ml) (C). WCEs were prepared after 20 min of incubation with TNF-α or 30 min after incubation with TPA or IL-1α. EMSA and immunoblot analyses were performed as described in the text. (A to C) The top halves of the panels show the results of the EMSA analyses, while the bottom halves show the immunoblotting results. This order is reversed in panel D. Panels A and B show the results of dose-response analyses with the indicated inhibitors. (C) All the inhibitors were tested at 100 μM, except for Z-LLF-CHO, which was tested at 20 μM. (D) The cells were treated with 100 μM Ac-LLnL-CHO prior to the addition of TNF-α (10 ng/ml) as indicated. In this case, extracts were prepared at 5, 10, and 20 min after the addition of TNF-α.

extracts of peptide aldehyde-treated cells was converted to the fast-migrating form by phosphatase treatment, and this conversion could be blocked by phosphatase inhibitors (data not shown). These results confirm that the slowly migrating form of IκBα detected after peptide aldehyde treatment is indeed due to its phosphorylation.

To ascertain whether Ac-LLnL-CHO blocked IκBα proteolysis by actually inhibiting the IκBα protease or by altering the IκBα phosphorylation pattern, HeLa cells metabolically labeled with $^{32}P_i$ were incubated in the absence or presence of Ac-LLnL-CHO for 3 h. IκBα was immunoprecipitated from total lysates of unstimulated or TNF-α-stimulated cells, and samples were fractionated by SDS-PAGE and transferred to nitrocellulose membranes for tryptic digestion. IκBα isolated from cells not treated with Ac-LLnL-CHO was transferred onto an Immobilon-P membrane and was initially autoradiographed (Fig. 6). This revealed that even the fast-migrating form of IκBα was ^{32}P labeled and that, following TNF-α stimulation, the slowly migrating form, which also is ^{32}P labeled, appeared (lanes 1 and 2). Immunoblot analysis of the same blot revealed the relative amounts of the fast- and slowly migrating forms of IκBα (lanes 3 and 4). On the basis of densitometric and phosphoimaging analyses, we found an almost threefold increase in the specific radioactivity of the slowly migrating form of IκBα compared with IκBα isolated from unstimulated cells. A similar increase in specific radioactivity was found in Ac-LLnL-CHO-pretreated cells (data not shown). Phosphoamino acid analysis of both forms of IκBα revealed the presence of phosphoserine and phosphothreonine but not phosphotyrosine (data not shown).

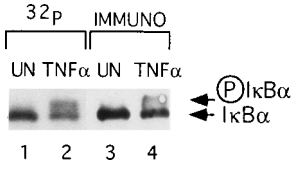

FIG. 6. IκBα is a phosphoprotein which becomes hyperphosphorylated upon cell stimulation. HeLa cells were metabolically labeled with $^{32}P_i$ for 5 h prior to incubation in the absence or presence of TNF-α (10 ng/ml). Cell lysates were immunoprecipitated with IκBα antiserum and separated by SDS-PAGE. Lanes 1 and 2 are autoradiographic images of IκBα isolated from unstimulated (UN) and TNF-α-stimulated cells, respectively. Lanes 3 and 4 show the results of the immunoblot analysis of the identical samples with IκBα antiserum.

To confirm that Ac-LLnL-CHO did not affect the phosphorylation pattern of IκBα, ^{32}P-labeled fast- and slowly migrating forms of IκBα isolated from either nonstimulated or TNF-α-treated cells as described above were subjected to phosphopeptide mapping (Fig. 7). TNF-α stimulation resulted in the appearance of three new tryptic phosphopeptides, labeled a, b, and c (compare panel B to panel A). The same phosphopeptide pattern was detected upon analysis of IκBα isolated from TNF-α-stimulated cells that were pretreated with Ac-LLnL-CHO (panel C). Mixing equal amounts of slowly migrating IκBα isolated from cells that were either pretreated with Ac-LLnL-CHO or untreated confirmed that the peptide aldehyde inhibitor had no effect on the pattern of IκBα phosphorylation (panel D). These results provide the first demonstration that cell stimulation does indeed alter the phosphorylation pattern of IκBα, as previously suspected.

Peptide aldehyde inhibitors block reporter gene activation. We examined the effect of peptide aldehyde protease inhibitors on activation of NF-κB-dependent reporter gene expression. HepG2 cells were transiently transfected with either the pRSV-Luc or p2x NF-κB-Luc reporter plasmid (48, 51) and either were untreated or were incubated with various peptide aldehyde protease inhibitors for 4 h prior to stimulation with TNF-α. Cells were then harvested 6 h postinduction, and luciferase expression was determined. Expression of the pRSV-Luc reporter was not induced by TNF-α and was not affected

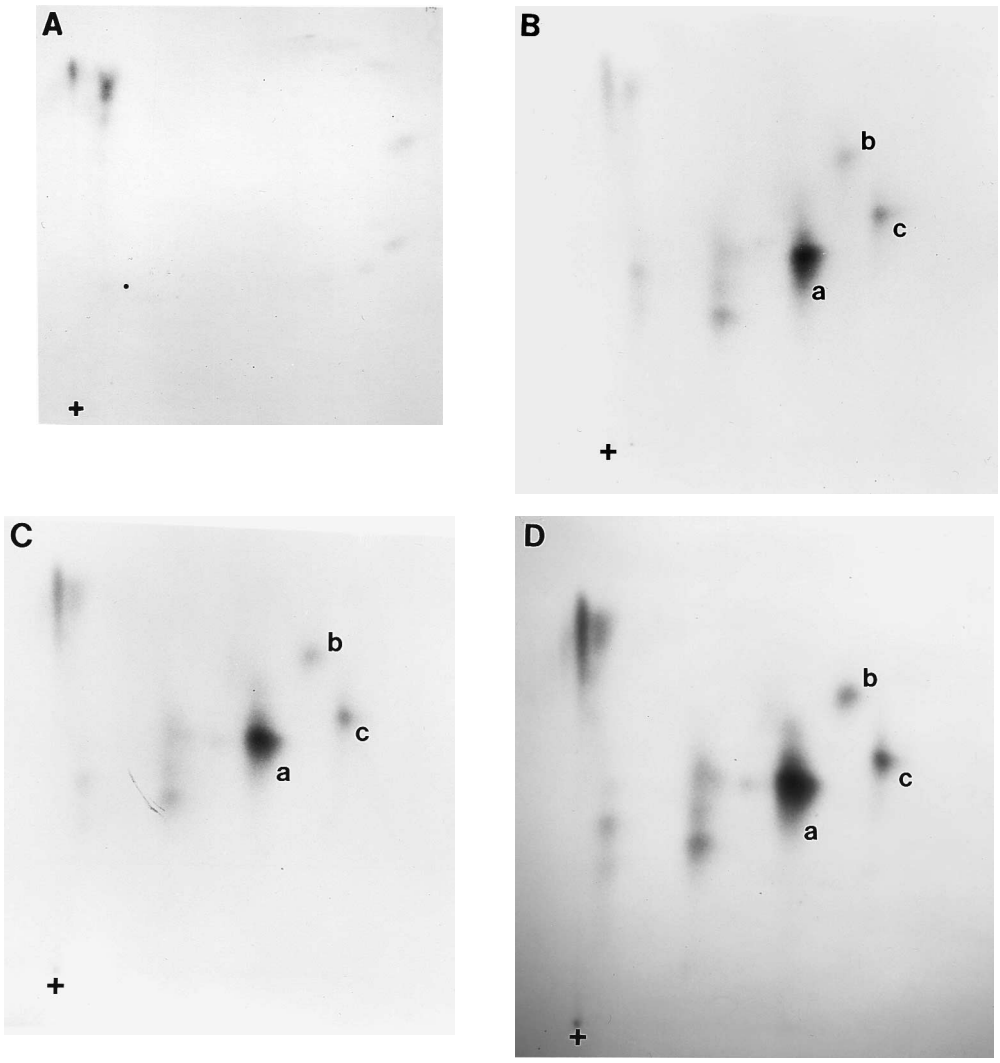

FIG. 7. Phosphopeptide maps of IκBα. HeLa cells were metabolically labeled with $^{32}P_i$, and IκBα was isolated by immunoprecipitation as described in the legend to Fig. 6. Phosphopeptide maps of IκBα isolated from unstimulated cells (A) and hyperphosphorylated IκBα isolated from cells stimulated for 4 min with TNF-α (10 ng/ml) (B). The newly appearing phosphopeptides are labeled a, b, and c. (C) Phosphopeptide map of hyperphosphorylated IκBα isolated from cells pretreated with Ac-LLnL-CHO (100 μM) for 3 h prior to stimulation with TNF-α (10 ng/ml) for 20 min. (D) Phosphopeptide map of mixture of equal amounts (in counts per minute) of IκBα isolated from TNF-α-stimulated cells that were either pretreated with Ac-LLnL-CHO or untreated. A total of 1,000 cpm (A and B), 500 cpm (C), or 250 cpm (D) of each IκBα sample was used. The origin is marked with a plus sign. The direction of electrophoresis was from left to right, and that of chromatography was from bottom to top.

by any of the drug treatments (Fig. 8). Expression of the p2x NF-κB-Luc reporter, however, was induced six- to sevenfold by TNF-α and was inhibited by cotransfection with an IκBα (*MAD-3*) expression vector. Induction of p2x NF-κB-Luc expression was also inhibited by various peptide aldehydes, with Z-LLF-CHO and Ac-LLnL-CHO being the most effective. While 1 μM Z-LLF-CHO inhibited expression of p2x NF-κB-Luc by 77%, 1 μM Ac-LLnL-CHO resulted in a 44% inhibition of TNF-α-stimulated p2x NF-κB-Luc expression. Good correlation was observed between the effects of the various inhibitors on induction of p2x NF-κB-Luc (Fig. 8) and their effects on induction of NF-κB DNA-binding activity and IκBα degradation (Fig. 5A to C).

DISCUSSION

Hyperphosphorylation of IκBα does not cause dissociation from NF-κB and is required for proteolysis. It was previously shown that treatment of cells with potent inducers of NF-κB activity, such as TNF-α and IL-1α results in the appearance of slowly migrating, presumably hyperphosphorylated, forms of IκBα (5, 36). Weak inducers of NF-κB did not seem to induce the appearance of this form (5, 13, 45, 57). Here we show not only that potent NF-κB inducers, such as TNF-α and IL-1α, induce IκBα hyperphosphorylation but also that a weaker inducer, TPA, causes IκBα hyperphosphorylation (Fig. 1 and 4). The appearance of hyperphosphorylated IκBα in TPA-treated cells was comparable to that observed with TNF-α and IL-1α, although it occurred with slightly slower kinetics (Fig. 1 and 4, lane 2). Thus, the only difference between strong and weak NF-κB inducers is probably the extent to which they activate the putative IκBα-kinase: the more potent the activation of the kinase is, the faster the hyperphosphorylated form of IκBα appears and can be detected prior to its degradation. With potent inducers such as TNF-α and IL-1α, the hyperphospho-

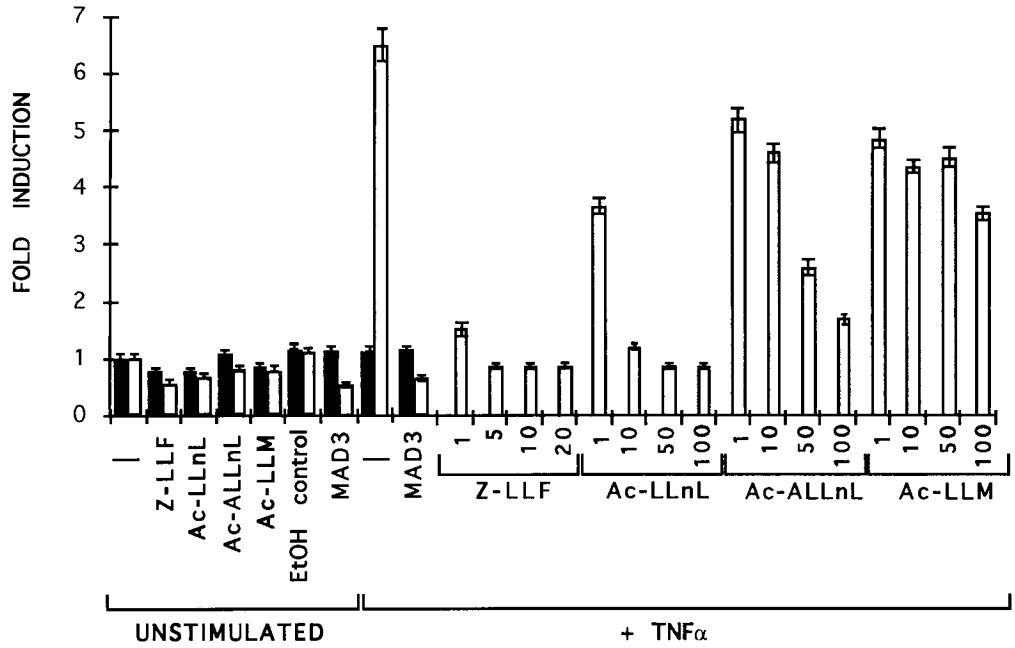

FIG. 8. Effects of peptide aldehyde protease inhibitors on NF-κB-driven reporter gene expression. HepG2 cells were transfected with either the pRSV-Luc (■) or p2x NF-κB-Luc (□) reporter plasmid (10 μg per 5×10^6 cells) without or with the IκBα expression vector (pCMV-MAD-3) by electroporation. Duplicate transfections were pooled, in order to normalize for transfection efficiency, and the cells (2.5×10^5) were plated into 24-well dishes. Transfections which included the IκBα expression plasmid contained 6 μg of pCMV-MAD-3 and 4 μg of reporter plasmid. Transfected cells were either untreated or were treated with the inhibitors listed at the indicated micromolar concentrations. For unstimulated cells, the concentration of the peptide aldehyde inhibitors was 100 μM, except for Z-LLF-CHO, which was used at 20 μM. Activity was defined as the relative units of light per microgram of protein. The fold induction was calculated by comparison of the activity of each sample with that of the unstimulated, non-inhibitor-treated sample, which served as the standard. Transfections were performed twice in duplicate samples, and the average fold induction for each condition in the two experiments is shown. The fold induction values for each treatment varied less than 10% in both experiments and are indicated by error bars.

rylated form of IκBα appears within 2 to 3 min of cytokine addition, indicating very rapid signal transduction from the activated membrane receptors to the cytoplasmic NF-κB:IκBα complexes. As previously shown, nonstimulated cells contain very little free IκBα (45). Most importantly, we demonstrate for the first time that NF-κB inducers not only stimulate phosphorylation of IκBα but also actually alter the pattern of its phosphorylation (Fig. 7). Although not conclusively proven, the appearance of three new phosphopeptides following cell activation suggests that TNF-α may stimulate the phophorylation of IκBα on several new sites. We show that the signal-induced phosphorylation, consistent with its putative regulatory role, affects previously synthesized IκBα molecules present in nonstimulated cells (Fig. 2).

In vitro phosphorylation studies suggested that phosphorylation of IκB resulted in its dissociation from NF-κB and was therefore the necessary step for NF-κB activation (24, 34, 55). Other studies have supported this idea (5, 13, 36, 57). We have directly examined whether phosphorylation of IκBα in intact cells results in its dissociation from NF-κB. As clearly shown, both the basal form and the hyperphosphorylated forms of IκBα can be coprecipitated with RelA (p65) following cell stimulation (Fig. 3). This interaction was quite stable since the duration of the immunoprecipitation step ranged from 12 h (as in Fig. 3) to as long as 24 h (data not shown). Furthermore, both IκBα forms present after stimulation were also immunoprecipitated by anti-NF-κB1 (p50) and anti-NF-κB2 (p52) antibodies (unpublished results), suggesting that hyperphosphorylated IκBα remains associated with most, if not all, types of NF-κB dimers and not only with RelA-containing complexes. Additionally, the enriched hyperphosphorylated form of IκBα following treatment with peptide aldehyde protease inhibitors was associated with RelA (data not shown), and, most importantly, the ratio between the basal and hyperphosphorylated forms of IκBα associated with RelA was the same as the ratio between them in total cell lysates (Fig. 3). These results indicate that hyperphosphorylation of IκBα is not sufficient to induce its dissociation from NF-κB. We suggest an alternative model, consistent with all of the present as well as previously published results, according to which phosphorylation of IκBα associated with NF-κB dimers precedes its proteolytic degradation. We suggest that phosphorylation of IκBα marks it for proteolytic degradation and that the selective degradation of NF-κB-associated phosphorylated IκBα results in the release of active NF-κB dimers (Fig. 9).

That hyperphosphorylation of IκBα marks it for degradation is supported by the results with TPCK (Fig. 4) and pyrrolidinedithiocarbamate (PDTC) (data not shown), which block NF-κB activation (29, 36). These agents differ in their modes of action. While TPCK is an alkylating agent known to inhibit serine proteases (53), PDTC is a metal chelator and antioxidant (3). We find that contrary to a previous publication (29), these compounds block the appearance of hyperphosphorylated IκBα (Fig. 4). Since the appearance of this form precedes the degradation of IκBα, these compounds somehow block the activation or the action of the IκB-kinase rather than the IκB-protease as previously suggested (29, 36). The failure to detect hyperphosphorylated IκBα in that earlier study was due to insufficient electrophoretic separation and the use of an antiserum that poorly reacts with hyperphosphorylated IκBα (6a).

Inhibitors of IκBα degradation block NF-κB activation but not IκBα phosphorylation. Because of the problem associated

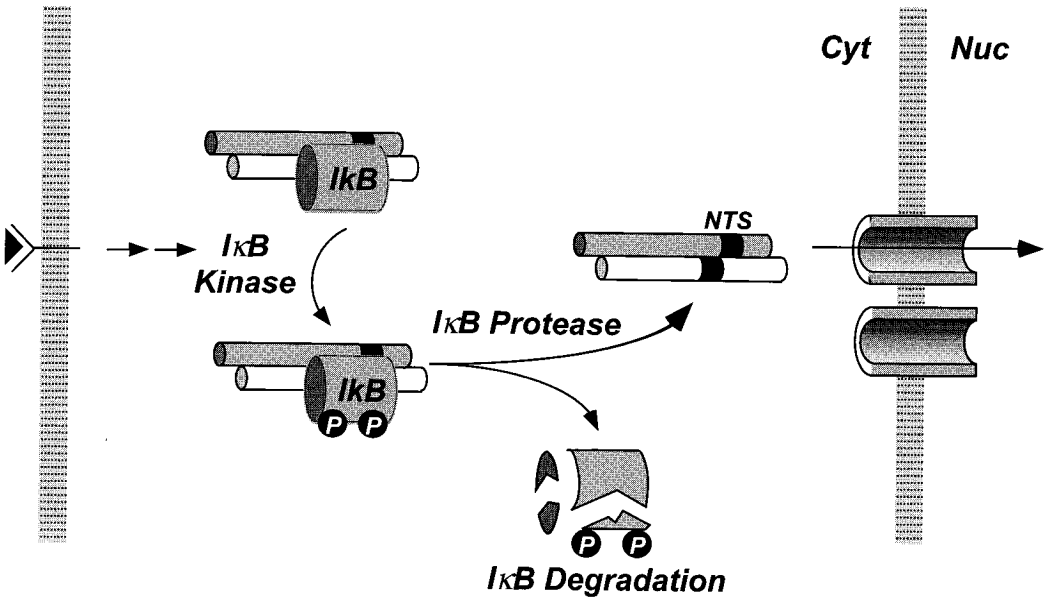

FIG. 9. Mechanism for IκBα degradation and NF-κB activation. Extracellular stimuli initiate a signaling cascade which results in the activation of the IκBα-kinase. The IκBα-kinase phosphorylates IκBα associated with NF-κB in the cytoplasm, marking it for proteolytic degradation. The hyperphosphorylated IκBα in the inactive NF-κB:IκBα complex is then selectively degraded, freeing the NF-κB dimers, which are then able to translocate to the nucleus and bind to their target genes (see the text for details).

with the use of TPCK and PDTC (see above), we searched for other types of protease inhibitors that would have no effect on IκBα phosphorylation and yet would inhibit its degradation. We chose to use peptide aldehyde inhibitors that target two different types of proteases, calpain and the MPC. The calpains were chosen as possible candidates for the IκB-protease because of their activation by extracellular stimuli that cause Ca^{2+} mobilization (reviewed in references 17 and 58). Such stimuli may therefore induce a protease activity that activates NF-κB. The MPC was chosen as a candidate for the IκB-protease on the basis of the previous suggestion by Fan and Maniatis (22) that processing of the NF-κB1 precursor may involve this proteolytic complex, otherwise known as the proteasome (20, 21). Furthermore, our own results have shown certain functional similarities between NF-κB1 and NF-κB2 precursor processing and the process leading to NF-κB activation (38). While this work was being prepared for publication, Palombella et al. (43) provided additional evidence that the MPC is involved in NF-κB1 precursor processing and that the same proteolytic system is also involved in IκBα degradation. As we have shown, two peptide aldehyde inhibitors, Z-LLF-CHO and Ac-LLnL-CHO, were found to be particularly useful and potent inhibitors of IκBα degradation and induction of NF-κB DNA-binding and transcriptional activities. Most importantly, these inhibitors had no effect on the stimulation of IκBα phosphorylation by TNF-α (Fig. 5A, B, and D). By slowing down the proteolysis of IκBα, these inhibitors also provide evidence that it is the hyperphosphorylated form that is preferentially degraded (Fig. 5D). Hyperphosphorylation could therefore serve as a tag to mark IκBα for proteolytic degradation.

Peptide aldehydes, because of their low intrinsic toxicity, are increasingly being used to identify and study protease activities involved in important physiological and cellular events. One major drawback to their use however is their lack of specificity. The peptide aldehydes used in this study have previously been characterized by their ability to inhibit calpain (63) and various proteolytic activities of the MPC (23, 62). In in vitro studies, both Ac-LLnL-CHO and Ac-LLM-CHO were found to inhibit calpain I (the low-Ca^{2+}-requiring isozyme) and calpain II (the highest-Ca^{2+}-requiring isozyme) with 50% inhibitory concentrations of 0.4 to 0.6 μM (63). Calpeptin, Z-LnL-CHO, is the most potent inhibitor of calpain, capable of complete inhibition at 0.5 to 1 μM, while Ac-LLnL-CHO and Ac-LLM-CHO have to be used at nearly 10 μM to fully inhibit calpain II activity (23). Ac-LLnL-CHO and Ac-LLM-CHO have also been demonstrated to inhibit the chymotrypsin-like activity of the MPC (23, 62). However, in contrast to their inhibitory properties on calpains I and II where they display similar 50% inhibitory concentrations (63), Ac-LLnL-CHO is a much more potent inhibitor of the MPC chymotrypsin-like activity (six-fold) than is Ac-LLM-CHO (62). Calpeptin, on the other hand, was found to be ineffective against any of the MPC's proteolytic activities (23). Z-LLF-CHO has not been analyzed as a calpain inhibitor, but when used as an MPC inhibitor, it shows a marked selectivity toward its chymotrypsin-like activity and can inhibit this activity nearly 10-fold more effectively than Ac-LLnL-CHO and 100-fold more effectively than Ac-LLM-CHO (62). Comparison of the results of these previous studies with the results shown in Fig. 5A to C suggests that the cellular target most likely involved in IκBα degradation and NF-κB activation is the MPC rather than the calpains. However, more experiments will be required to establish the role of the MPC in IκBα degradation and NF-κB activation.

Model for IκBα degradation and NF-κB activation. On the basis of the results of previous studies and those presented here, we suggest the following model (Fig. 9) to explain IκBα degradation and NF-κB activation. Initially, an extracellular stimulus initiates a signaling cascade leading to activation of the IκBα kinase, which then specifically phosphorylates IκBα associated with NF-κB in the cytoplasm. Once hyperphosphorylated, the IκBα is tagged for proteolysis as it remains complexed to NF-κB. The tagged IκB–NF-κB complex is then selectively proteolyzed so that only IκBα is degraded, freeing

the NF-κB dimer to translocate to the nucleus where it binds its target genes (32). NF-κB target gene activity quickly results in the resynthesis of IκBα (15, 57) which is made in excess of Rel-related proteins and results in its reassociation with free NF-κB dimers, thereby dampening and eventually turning off NF-κB activity in the absence of a new signal.

ACKNOWLEDGMENTS

We thank M. Orlowski for the gift of Z-LLF-CHO, T. Hunter for helpful tips on phosphopeptide mapping, and A. Baldwin and Y. Ben-Neriah for useful discussions. We thank A. Lewis and D. Anderson of Signal Pharmaceuticals for support and encouragement, J. W. Li for technical assistance, S. Garvin for help with the artwork, and C. Rosette for help with the manuscript. J.A.D. also is indebted to M.A.D., A.N.D., and A.J.D. for their understanding.

J.A.D. was supported by National Institutes of Health postdoctoral grant DK-0858401. This work was supported by NIH grants CA50528 and HL35018 to M.K.

REFERENCES

1. **Baeuerle, P. A.** 1991. The inducible transcription activator NF-κB: regulation by distinct protein subunits. Biochim. Biophys. Acta **1072:**63–80.
2. **Baeuerle, P. A.** 1991. The physiology of the NF-κB transcription factor, p. 423–446. *In* P. Cohen and J. G. Foulkes (ed.), The hormonal control regulation of gene transcription. Elsevier Science Publishers, Amsterdam.
3. **Bartoli, G. M., A. Muller, E. Cadenas, and H. Sies.** 1983. Antioxidant effect of diethyldithiocarbamate on microsomal lipid peroxidation assessed by low-level chemiluminescence and alkane production. FEBS Lett. **164:**371–3744.
4. **Beg, A. A., and A. Baldwin, Jr.** 1993. The IκB proteins: multifunctional regulators of Rel/NF-κB transcription factors. Genes Dev. **7:**2064–2070.
5. **Beg, A. A., T. S. Finco, P. V. Nantermet, and A. Baldwin, Jr.** 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Mol. Cell. Biol. **13:**3301–3310.
6. **Beg, A. A., S. M. Ruben, R. I. Scheinman, S. Haskill, C. A. Rosen, and A. Baldwin, Jr.** 1992. IκB interacts with the nuclear localization sequences of the subunits of NF-κB: a mechanism for cytoplasmic retention. Genes Dev. **6:**1899–1913.

6a. **Ben-Neriah, Y.** Personal communication.

7. **Blank, V., P. Kourilsky, and A. Israel.** 1991. Cytoplasmic retention, DNA binding and processing of the NF-κB p50 precursor are controlled by a small region in its C-terminus. EMBO J. **10:**4159–4167.
8. **Blank, V., P. Kourilsky, and A. Israel.** 1992. NF-κB and related proteins: Rel/dorsal homologies meet ankyrin-like repeats. Trends Biochem. Sci. **17:** 135–140.
9. **Bose, H., Jr.** 1992. The Rel family: models for transcriptional regulation and oncogenic transformation. Biochim. Biophys. Acta **1114:**1–17.
10. **Bours, V., P. R. Burd, K. Brown, J. Villalobos, S. Park, R. P. Ryseck, R. Bravo, K. Kelly, and U. Siebenlist.** 1992. A novel mitogen-inducible gene product related to p50/p105-NF-κB participates in transactivation through a κB site. Mol. Cell. Biol. **12:**685–695.
11. **Bours, V., J. Villalobos, P. R. Burd, K. Kelly, and U. Siebenlist.** 1990. Cloning of a mitogen-inducible gene encoding a κB DNA-binding protein with homology to the rel oncogene and to cell-cycle motifs. Nature (London) **348:**76–80.
12. **Boyle, W. J., P. van der Geer, and T. Hunter.** 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol. **201:**110–149.
13. **Brown, K., S. Park, T. Kanno, G. Franzoso, and U. Siebenlist.** 1993. Mutual regulation of the transcriptional activator NF-κB and its inhibitor, IκBα. Proc. Natl. Acad. Sci. USA **90:**2532–2536.
14. **Brownell, E., N. Mittereder, and N. R. Rice.** 1989. A human rel proto-oncogene cDNA containing an Alu fragment as a potential coding exon. Oncogene **4:**935–942.
15. **Chiao, P. J., S. Miyamoto, and I. M. Verma.** 1994. Autoregulation of IκBα activity. Proc. Natl. Acad. Sci. USA **91:**28–32.
16. **Cordle, S. R., R. Donald, M. A. Read, and J. Hawiger.** 1993. Lipopolysaccharide induces phosphorylation of MAD-3 and activation of c-Rel and related NF-κB proteins in human monocytic THP-1 cells. J. Biol. Chem. **268:**11803–11810.
17. **Croall, D. E., and G. N. DeMartino.** 1991. Calcium-activated neutral protease (calpain) system: structure, function, and regulation. Physiol. Rev. **71:**813–847.
18. **Davis, N., S. Ghosh, D. L. Simmons, P. Tempst, H. C. Liou, D. Baltimore, and H. Bose, Jr.** 1991. Rel-associated pp40: an inhibitor of the rel family of transcription factors. Science **253:**1268–71.
19. **Devary, Y., C. Rosette, J. A. DiDonato, and M. Karin.** 1993. NF-κB activation by ultraviolet light not dependent on a nuclear signal. Science **261:**1442–1445.

19a. **DiDonato, J. A.** Unpublished observations.

20. **Driscoll, J., and A. L. Goldberg.** 1990. The proteasome (multicatalytic protease) is a component of the 1500-kDa proteolytic complex which degrades ubiquitin-conjugated proteins. J. Biol. Chem. **265:**4789–4792.
21. **Eytan, E., D. Ganoth, T. Armon, and A. Hershko.** 1989. ATP-dependent incorporation of 20S protease into the 26S complex that degrades proteins conjugated to ubiquitin. Proc. Natl. Acad. Sci. USA **86:**7751–7755.
22. **Fan, C. M., and T. Maniatis.** 1991. Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway. Nature (London) **354:**395–398.
23. **Figueiredo-Pereira, M. E., N. Banik, and S. Wilk.** 1994. Comparison of the effect of calpain inhibitors on two extralysosomal proteinases: the multicatalytic proteinase complex and m-calpain. J. Neurochem. **62:**1989–1994.
24. **Ghosh, S., and D. Baltimore.** 1990. Activation *in vitro* of NF-κB by phosphorylation of its inhibitor IκB. Nature (London) **344:**678–682.
25. **Ghosh, S., A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolan, and D. Baltimore.** 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to rel and dorsal. Cell **62:**1019–1029.
26. **Grilli, M., J. J. Chiu, and M. J. Lenardo.** 1993. NF-κB and Rel: participants in a multiform transcriptional regulatory system. Int. Rev. Cytol. **143:**1–62.
27. **Haskill, S., A. A. Beg, S. M. Tompkins, J. S. Morris, A. D. Yurochko, A. Sampson-Johannes, K. Mondal, P. Ralph, and A. Baldwin, Jr.** 1991. Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. Cell **65:**1281–1289.
28. **Hatada, E. N., A. Nieters, F. G. Wulczyn, M. Naumann, R. Meyer, G. Nucifora, T. W. McKeithan, and C. Scheidereit.** 1992. The ankyrin repeat domains of the NF-κB precursor p105 and the protooncogene bcl-3 act as specific inhibitors of NF-κB DNA binding. Proc. Natl. Acad. Sci. USA **89:**2489–2493.
29. **Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, and P. A. Baeuerle.** 1993. Rapid proteolysis of IκBα is necessary for activation of transcription factor NF-κB. Nature (London) **365:**182–185.
30. **Henkel, T., U. Zabel, K. van Zee, J. M. Muller, E. Fanning, and P. A. Baeuerle.** 1992. Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-κB subunit. Cell **68:**1121–1133.
31. **Hirayama, M., Y. Kohgo, H. Kondo, N. Shintani, K. Fujikawa, K. Sasaki, J. Kato, and Y. Niitsu.** 1993. Regulation of iron metabolism in HepG2 cells: a possible role for cytokines in the hepatic deposition of iron. Hepatology **18:**874–880.
32. **Hunter, T., and M. Karin.** 1992. The regulation of transcription by phosphorylation. Cell **70:**375–387.
33. **Inoue, J., L. D. Kerr, D. Rashid, N. Davis, H. Bose, Jr., and I. M. Verma.** 1992. Direct association of pp40/IκBβ with rel/NF-κB transcription factors: role of ankyrin repeats in the inhibition of DNA binding activity. Proc. Natl. Acad. Sci. USA **89:**4333–4337.
34. **Kerr, L. D., J. Inoue, N. Davis, E. Link, P. A. Baeuerle, H. Bose, Jr., and I. M. Verma.** 1991. The rel-associated pp40 protein prevents DNA binding of Rel and NF-κB: relationship with IκBβ and regulation by phosphorylation. Genes Dev. **5:**1464–1476.
35. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israel.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the rel oncogene product. Cell **62:**1007–1018.
36. **Mellits, K. H., R. T. Hay, and S. Goodbourn.** 1993. Proteolytic degradation of MAD-3 (IκBα) and enhanced processing of the NF-κB precursor p105 are obligatory steps in the activation of NF-κB. Nucleic Acids Res. **21:**5059–5066.
37. **Mercurio, F., J. DiDonato, C. Rosette, and M. Karin.** 1992. Molecular cloning and characterization of a novel Rel/NF-κB family member displaying structural and functional homology to NF-κB p50/p105. DNA Cell Biol. **11:**523–537.
38. **Mercurio, F., J. A. DiDonato, C. Rosette, and M. Karin.** 1993. p105 and p98 precursor proteins play an active role in NF-κB-mediated signal transduction. Genes Dev. **7:**705–718.
39. **Meyer, R., E. N. Hatada, H. P. Hohmann, M. Haiker, C. Bartsch, U. Rothlisberger, H. W. Lahm, E. J. Schlaeger, A. P. van Loon, and C. Scheidereit.** 1991. Cloning of the DNA-binding subunit of human nuclear factor κ B: the level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor α. Proc. Natl. Acad. Sci. USA **88:**966–970.
40. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326:**711–713.
41. **Neri, A., C. C. Chang, L. Lombardi, M. Salina, P. Corradini, A. T. Maiolo, R. S. Chaganti, and R. Dalla-Favera.** 1991. B cell lymphoma-associated chromosomal translocation involves candidate oncogene lyt-10, homologous to NF-κB p50. Cell **67:**1075–1087.
42. **Nolan, G. P., S. Ghosh, H. C. Liou, P. Tempst, and D. Baltimore.** 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a rel-related polypeptide. Cell **64:**961–969.

42a. **Orlowski, M.** Personal communication.

43. **Palombella, V. J., O. J. Rando, A. L. Goldberg, and T. Maniatis.** 1994. The ubiquitin-proteasome pathway is required for processing the NF-κB1 pre-

cursor and the activation of NF-κB. Cell **78:**773–785.
44. **Powers, J., and J. Harper.** 1986. Inhibitors of serine proteinases, p. 55–152. *In* A. J. Barrett and G. Salvensen (ed.), Proteinase inhibitors. Elsevier Science Publishers, Amsterdam.
45. **Rice, N. R., and M. K. Ernst.** 1993. In vivo control of NF-κB activation by IκBα. EMBO J. **12:**4685–4695.
46. **Rice, N. R., M. L. MacKichan, and A. Israel.** 1992. The precursor of NF-κB p50 has IκB-like functions. Cell **71:**243–253.
47. **Rich, D. H.** 1986. Inhibitors of cysteine proteinases, p. 153–178. *In* A. J. Barrett and G. Salvensen (ed.), Proteinase inhibitors. Elsevier Science Publishers, Amsterdam.
48. **Rosette, C., and M. Karin.** Cytoskeletal control of gene expression: depolymerization of microtubules activates NF-κB. J. Cell Biol., in press.
49. **Ruben, S. M., P. J. Dillon, R. Schreck, T. Henkel, C. H. Chen, M. Maher, P. A. Baeuerle, and C. A. Rosen.** 1991. Isolation of a rel-related human cDNA that potentially encodes the 65-kD subunit of NF-κB. Science **251:** 1490–1493.
50. **Ryseck, R. P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobrzanski, and R. Bravo.** 1992. RelB, a new Rel family transcription activator that can interact with p50-NF-κB. Mol. Cell. Biol. **12:**674–84.
51. **Saatcioglu, F., T. Deng, and M. Karin.** 1993. A novel cis element mediating ligand-independent activation by c-ErbA: implications for hormonal regulation. Cell **75:**1095–1105.
52. **Schmid, R. M., N. D. Perkins, C. S. Duckett, P. C. Andrews, and G. J. Nabel.** 1991. Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. Nature (London) **352:**733–736.
53. **Schoellmann, G., and E. Shaw.** 1963. Direct evidence for the presence of histidine in the active center of chymotrypsin. Biochemistry **2:**252–255.
54. **Sen, R., and D. Baltimore.** 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell **47:**921–928.
55. **Shirakawa, F., and S. B. Mizel.** 1989. In vitro activation and nuclear translocation of NF-κB catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. Mol. Cell. Biol. **9:**2424–2430.
56. **Stopeck, A. T., A. C. Nicholson, F. P. Mancini, and D. P. Hajjar.** 1993. Cytokine regulation of low density lipoprotein receptor gene transcription in HepG2 cells. J. Biol. Chem. **268:**17489–17494.
57. **Sun, S. C., P. A. Ganchi, D. W. Ballard, and W. C. Greene.** 1993. NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. Science **259:**1912–1915.
58. **Suzuki, K., T. C. Saido, and S. Hirai.** 1992. Modulation of cellular signals by calpain. Ann. N. Y. Acad. Sci. **674:**218–227.
59. **Tewari, M., P. Dobrzanski, K. L. Mohn, D. E. Cressman, J. C. Hsu, R. Bravo, and R. Taub.** 1992. Rapid induction in regenerating liver of RL/IF-1 (an IκB that inhibits NF-κB, RelB-p50, and c-Rel-p50) and PHF, a novel κB site-binding complex. Mol. Cell. Biol. **12:**2898–2908.
60. **Thompson, C. C., T. A. Brown, and S. L. McKnight.** 1991. Convergence of Ets- and notch-related structural motifs in a heteromeric DNA binding complex. Science **253:**762–768.
61. **Tsujinaka, T., Y. Kajiwara, J. Kambayashi, M. Sakon, N. Higuchi, T. Tanaka, and T. Mori.** 1988. Synthesis of a new cell penetrating calpain inhibitor (calpeptin). Biochem. Biophys. Res. Commun. **153:**1201–1208.
62. **Vinitsky, A., C. Michaud, J. C. Powers, and M. Orlowski.** 1992. Inhibition of the chymotrypsin-like activity of the pituitary multicatalytic proteinase complex. Biochemistry **31:**9421–9428.
63. **Wang, K. K.** 1990. Developing selective inhibitors of calpain. Trends Pharmacol. Sci. **11:**139–142.
64. **Zabel, U., T. Henkel, M. S. Silva, and P. A. Baeuerle.** 1993. Nuclear uptake control of NF-κB by MAD-3, an IκB protein present in the nucleus. EMBO J. **12:**201–211.