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Tumor necrosis factor α-induced phosphorylation of IκBα is a signal for its degradation but not dissociation from NF-κB

(signal transduction/proteases/regulation/transcription)

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ABSTRACT **Activation of the NF-κB/Rel family of transcription factors is regulated by a cytoplasmic inhibitor, IκBα. Activity of IκBα is in turn modulated by phosphorylation and proteolysis. It has been postulated that phosphorylation of IκBα leads to its dissociation from NF-κB, and free IκBα is targeted for rapid degradation. However, this phosphorylation-mediated dissociation event has not been demonstrated *in vivo*. We demonstrate that, contrary to this hypothesis, phosphorylation of IκBα induced by tumor necrosis factor α in HeLa cells does not induce dissociation. We propose a model in which (*i*) induced phosphorylation of IκBα does not result in its dissociation from NF-κB, (*ii*) phosphorylation of IκBα serves as a signal for degradation, and (*iii*) degradation of IκBα occurs while it is still complexed with NF-κB.**

NF-κB/Rel (NF-κB) proteins regulate transcription of a variety of cellular genes including those involved in immune responses and growth control (for review, see refs. 1–3). They also regulate transcription of viral genes, such as human immunodeficiency virus, hence playing a role in the pathogenesis of AIDS (4). Unlike many other transcription factors that are localized in the nucleus, NF-κB is sequestered in the cytoplasm of most cells (5). This cytoplasmic retention of NF-κB is regulated by an inhibitor protein, IκBα, which binds to NF-κB via its ankyrin repeats (6, 7). The bound IκBα presumably masks the NF-κB nuclear localization signal and thereby inhibits its nuclear transport (8–10). IκBα is phosphorylated in response to stimulation with a variety of agents such as tumor necrosis factor α (TNF-α) or interleukin 1. After phosphorylation, IκBα is rapidly degraded (11, 12). These events lead to NF-κB translocation to the nucleus, binding to cognate DNA binding sites, and activation of transcription.

In vitro phosphorylation of partially purified IκBα seems to dissociate IκBα from NF-κB (13). The free IκBα has a short half-life *in vivo* (14, 15), supporting the idea that dissociation from NF-κB leads to rapid degradation of IκBα. In the present study, we have tested the validity of the model that *in vivo* phosphorylation of IκBα leads to dissociation from NF-κB and that free IκBα is targeted for rapid degradation. Contrary to expectations, we show that the phosphorylated form of IκBα does not dissociate from NF-κB and that phosphorylation of IκBα may be a signal for its degradation.

MATERIALS AND METHODS

Western Blot Analysis. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum and treated with recombinant human TNF-α (10 ng/ml) (Calbiochem) for the indicated periods. After rinsing with ice-cold phosphate-buffered saline, cells were scraped off the plates, centrifuged in Eppendorf tubes at 4°C, and frozen in dry ice until all the samples were ready to be processed. Cytoplasmic and nuclear extracts were prepared as described (16) in the presence of phosphatase inhibitors (50 mM NaF/0.1 mM sodium vanadate/10 mM sodium molybdate/20 mM β-glycerol phosphate/10 mM 4-nitropyrophosphate) and protease inhibitors [0.1 mM phenylmethylsulfonyl fluoride (PMSF)/aprotinin (21 μg/ml)]. For the experiment illustrated in Fig. 1*C*, cells were pretreated with calpain inhibitor I (50 μg/ml) (see below), phosphatase inhibitors were omitted during cell lysis, and cytoplasmic extracts were treated with 5 units of calf intestinal alkaline phosphatase (Boehringer Mannheim) for 20 min at 37°C prior to electrophoresis. The cytoplasmic extracts (150 μg each) were fractionated in SDS/15% polyacrylamide gel, transferred to Immobilon-P membranes (Millipore), and Western blotted using anti-MAD-3 IgG fraction (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham). The specificity of the reaction was demonstrated by competition with the epitope peptide (data not shown).

An electrophoretic mobility-shift assay was performed with 5 μg of nuclear extracts and a palindromic κB probe as described (16). For the experiment illustrated in Fig. 2, the cells were pretreated for 1 hr before addition of TNF-α with *N*-acetylleucylleucylnorleucinal (100 μg/ml) (calpain inhibitor I; Nacalai, Teskyue, Japan), *N*-acetylleucylleucylmethional (100 μg/ml) (calpain inhibitor II; Nacalai), ethyl(+)-(2*S*,3*S*)-3-[(*S*)-methyl-1-(3-methylbutylcarbamoyl)butylcarbamoyl]-2-oxiranecarboxylate (50 μg/ml) (E-64d; a generous gift from K. Hanada, Taisho Pharmaceutical, Omiya, Japan), 50 mM NH_4Cl, 5 mM EGTA, 50 μM L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), 7-amino-1-chloro-3-tosylamido-2-heptanone (50 μg/ml) (TLCK), 0.4% dimethyl sulfoxide (DMSO), and 0.4% ethanol. In the experiment in Fig. 3*C*, stock calpain inhibitor I (25 mg/ml in DMSO) was diluted with H_2O to 250 μg/ml (lane 2), 500 μg/ml (lane 3), and 1 mg/ml (lane 4) and 1 μl was added to a final reaction volume of 10 μl.

Metabolic Labeling and Immunoprecipitation. HeLa cells (≈5 × 10^6 each) were incubated in 3 ml of DMEM without Met/Cys and supplemented with 5% dialyzed fetal calf serum for 20 min in 10-cm culture dishes. [^{35}S]Met/Cys (2.5 mCi; 1 Ci = 37 GBq) was added to each plate and incubated for an additional 4 hr. Two plates each were either treated or untreated with TNF-α (10 ng/ml) and extracts were obtained as described above. Those plates treated with TNF-α were pretreated with calpain inhibitor I (50 μg/ml) in the last 1 hr

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Abbreviations: TNF-α, tumor necrosis factor α; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TLCK, 7-amino-1-chloro-3-tosylamido-2-heptanone; DMSO, dimethyl sulfoxide.

of the labeling period. Cytoplasmic extracts were precleared with protein A-Sepharose (Pharmacia) in IP buffer (20 mM Tris·HCl, pH 7.5/150 mM NaCl/0.2% sodium deoxycholate/ 0.2% Nonidet P-40/1 mM PMSF/21 μg of aprotinin per ml) containing the phosphatase inhibitors, and equal fractions were immunoprecipitated with either anti-MAD-3 or anti-p65 IgG fraction (Santa Cruz Biotechnology). To illustrate the specificity of immunoprecipitation, equal portions from unstimulated and stimulated cells were boiled in the presence of 0.5% SDS, diluted 1:5 with IP buffer, and then incubated with anti-MAD-3 or anti-p65 IgG in the presence or absence of competing peptides (0.5 μg).

RESULTS

Phosphorylation of IκBα Precedes Its Degradation. Stimulation of HeLa cells with TNF-α causes phosphorylation and rapid degradation of IκBα with subsequent activation of NF-κB (11). Using an antibody specific to IκBα and Western blot analysis of HeLa cytoplasmic extracts treated with TNF-α, we show in Fig. 1*A* the time course of modification and degradation of IκBα in the cytoplasm. Electrophoretic mobility-shift assays by a palindromic κB site were used to show the time course of NF-κB activation in the nuclear extracts (Fig. 1*B*). The slower migrating form of IκBα (designated IκB*) appears before its complete degradation (Fig. 1*A*, lanes 4 and 5) concomitant with NF-κB DNA binding activity (Fig. 1*B*). The slower migrating IκB* is phosphorylated, since treatment with calf intestinal phosphatase leads to a loss of IκB* (Fig. 1*C*, compare lanes 2 and 4).

To study the role of IκBα phosphorylation on the stability of IκBα–NF-κB interaction, stabilization of IκB* was necessary since degradation occurs rapidly, and it was difficult to distinguish the effect of phosphorylation on the IκBα–NF-κB association. Thus, we sought protease inhibitors that were capable of specifically inhibiting degradation of IκB*. Several investigators have demonstrated that serine protease inhibitors, TPCK and TLCK, inhibited degradation of IκBα and subsequent activation of NF-κB (17, 18). However, it was not demonstrated that these protease inhibitors stabilized IκB*. To examine the effects of these inhibitors on the accumulation of IκB*, HeLa cells were preincubated with 50 μM TPCK or 50 μg of TLCK per ml, which are sufficient amounts to stabilize IκBα and block NF-κB activation (refs. 17 and 18; data not shown) prior to stimulation with TNF-α. IκBα from cytoplasmic extracts of treated cells was detected by Western blot analysis. In the absence of inhibitors, TNF-α stimulation led to phosphorylation of IκBα and its degradation (Fig. 2*A*, compare lanes 1 and 2). The time course of IκBα modification varied somewhat from experiment to experiment (due to the activity of TNF-α preparations), but the phosphorylation of IκBα was consistently reproducible within an experiment. Both TPCK and TLCK blocked phosphorylation of IκBα (Fig. 2*A*, lanes 8 and 9); therefore, these protease inhibitors block the activation pathway, perhaps affecting a kinase, rather than a specific IκBα protease.

Some Calpain Inhibitors Block Degradation of IκB*. Using an *in vitro* degradation assay, we have observed that the calcium-dependent protease calpain may be involved in the degradation of IκBα (S.M., unpublished results). We thus tested inhibitors of calpain for their capacity to block degradation of IκB* *in vivo*. These included calpain inhibitors I and II, E-64d, leupeptin (19), and the calcium chelator EGTA. Fig. 2*A* demonstrates that calpain inhibitors I and II (lane 3 and 4) led to the accumulation of IκB* (lanes 3 and 4). The inhibitory effect of calpain inhibitor I was dose dependent, as demonstrated in Fig. 2*B* (compare lanes 2–5). E-64d, EGTA (Fig. 2*A*, lanes 5 and 7, respectively), and leupeptin (Fig. 2*B*, lane 7) had less effect on inhibition of IκB* degradation. The lysosomal inhibitor NH_4Cl (Fig. 2*A*, lane 6) and chloroquine (data not shown) and the solvents used for the inhibitors, DMSO, or ethanol (Fig. 2*A*, lane 10 or 11) lacked the ability to stabilize IκB*. Therefore, certain calpain inhibitors efficiently block IκB* without blocking the IκBα phosphorylation in HeLa cells.

Activation of NF-κB Is Also Inhibited by Calpain Inhibitors. We measured the time course of IκBα phosphorylation and NF-κB activation in the presence and absence of calpain inhibitor I. Fig. 3*A* shows a Western blot analysis of IκBα from HeLa cells treated with TNF-α. The phosphorylated IκB* appears by 5 min of TNF-α treatment (lane 3) and degrades by 10 min (lane 4). In the presence of calpain

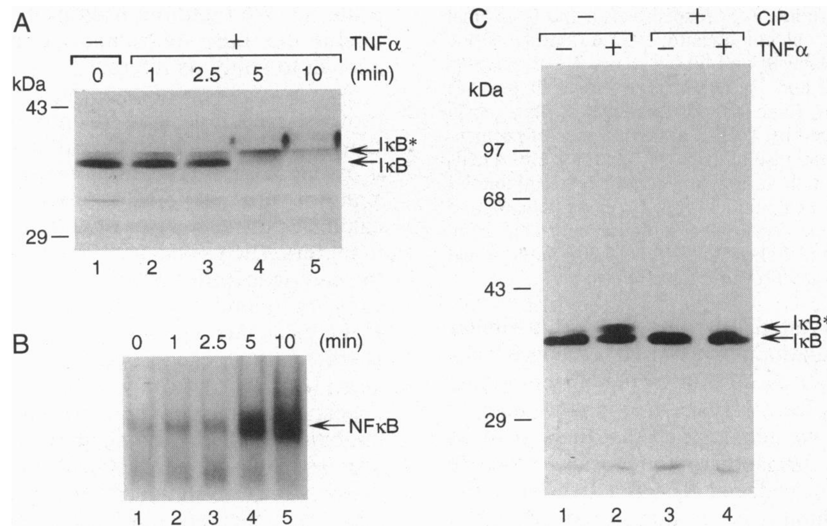

FIG. 1. NF-κB activation is accompanied by phosphorylation and degradation of IκBα. (*A*) Time course of IκBα modification induced by TNF-α. Cytoplasmic extracts isolated from cells stimulated for the indicated periods (lanes 2–5) were fractionated by SDS/PAGE, transferred to a nylon membrane, and Western blotted with the anti-MAD-3 (IκBα) IgG fraction. Arrows indicate IκBα (IκB) and phosphorylated IκBα (IκB*; see Fig. 1*C*). Other bands are nonspecific and are seen on competition with epitope peptide (data not shown). (*B*) Time course of NF-κB activation in HeLa cells stimulated by TNF-α. Nuclear extracts were prepared from cells in *A* and an electrophoretic mobility-shift assay was performed with a palindromic κB site. (*C*) Slower migrating IκBα (IκB*) is phosphorylated. Cytoplasmic extracts isolated from HeLa cells, unstimulated (lanes 1 and 3) or stimulated with TNF-α for 5 min in the presence of calpain inhibitor I (see Fig. 2*A*, lanes 2 and 4), and untreated (lanes 1 and 2) or treated (lanes 3 and 4) with calf intestinal phosphatase (CIP), were processed as in *A*.

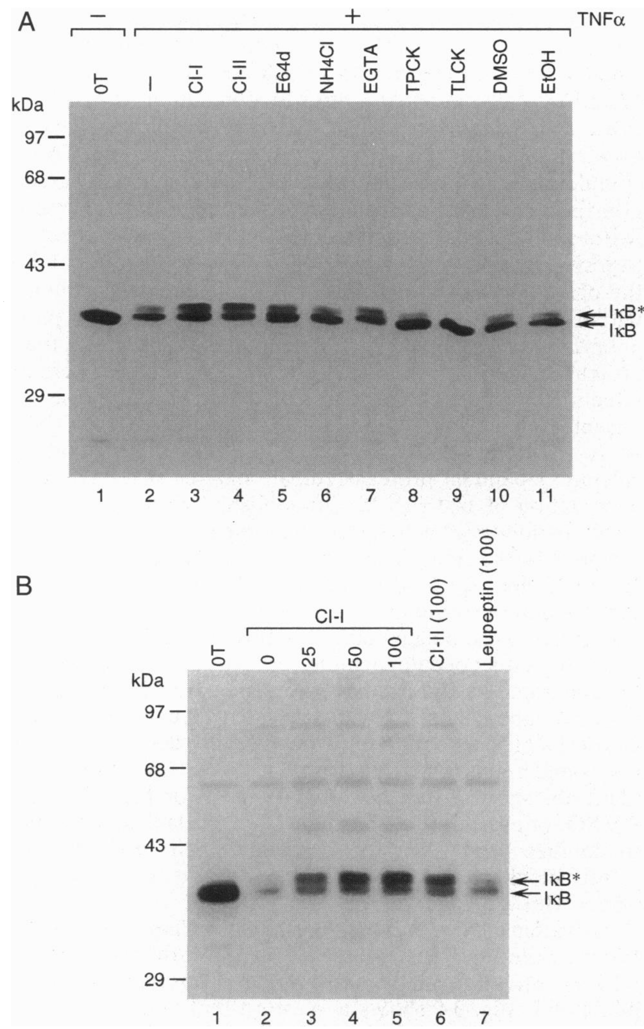

FIG. 2. Some calpain inhibitors stabilize the phosphorylated form of IκBα (IκB). (*A*) Inhibition of IκB* degradation by some calpain inhibitors. HeLa cells were pretreated with calpain inhibitors I and II (Cl-I and Cl-II) and E-64d (lanes 3–5, respectively), the lysosomal inhibitor NH_4Cl (lane 6), the calcium chelator EGTA (lane 7), serine protease inhibitors TPCK (lane 8) and TLCK (lane 9), or solvents DMSO and EtOH (lanes 10 and 11, respectively) for 1 hr prior to addition of TNF-α for 7 min. Lane 2 shows the level of phosphorylation and degradation induced by TNF-α in the absence of preincubated inhibitors. (*B*) Dose–response of calpain inhibitor I. HeLa cells were preincubated for 1 hr with various doses of calpain inhibitor I (μg/ml) (lanes 3–5), 100 μg of calpain inhibitor II per ml (lane 6), and 100 μg of leupeptin per ml (lane 7) prior to stimulation with TNF-α (10 ng/ml) for 10 min. Lanes 1 and 2 show the level of IκBα without and with 10 min of stimulation with TNF-α, respectively.

inhibitor I, the phosphorylated IκB* appears with a similar time course but its degradation is prevented (compare lanes 3 and 4 with lanes 6 and 7). According to the current model (1, 13), IκB* dissociates from NF-κB, allowing its nuclear translocation. However, we demonstrate that the activation of NF-κB is minimal (Fig. 3*B*, lane 7) in the presence of stable IκB* (Fig. 3*A*, lane 7). The reduced NF-κB activation in the presence of calpain I inhibitor is not due to reduced DNA binding activity since this inhibitor does not directly inhibit DNA binding in electrophoretic mobility-shift assays (Fig. 2*C*, compare lane 1 with lanes 2–4). DNA binding activity of NF-κB was also undetected in the cytoplasm but was inducible by treatment with deoxycholate, suggesting that NF-κB was still associated with IκBα in the cytoplasm (data not shown). Therefore, the phosphorylation of IκBα induced by TNF-α does not directly lead to dissociation from NF-κB.

Phosphorylated IκBα Remains Associated with NF-κB. To directly demonstrate the lack of dissociation of NF-κB–IκBα upon phosphorylation or the presence of a stable NF-κB–IκB* complex, coimmunoprecipitation was performed with antisera to either IκBα or to the p65 subunit of NF-κB. Extracts from *in vivo* [^{35}S]Met/Cys-labeled HeLa cells, either unstimulated or stimulated with TNF-α in the presence of calpain inhibitor I, were used for coimmunoprecipitation. When equal fractions were immunoprecipitated with antiserum to IκBα, both IκBα and IκB* could be immunoprecipitated (Fig. 4, lanes 1 and 2). p65 was also coimmunoprecipitated by the IκBα antiserum. When antiserum to p65 was used, IκBα as well as IκB* was immunoprecipitated (Fig. 4, lanes 3 and 4), directly demonstrating that IκB* is still complexed with NF-κB. Specificity of p65–IκBα complex formation and immunoprecipitation was demonstrated by boiling to disrupt protein complexes (lanes 5 and 6 for IκBα and lanes 8 and 9 for p65) and competition with corresponding epitope peptides (lanes 7 and 10 for IκBα and p65, respectively). These data demonstrate that TNF-α-induced phosphorylation of IκBα does not result in its dissociation from NF-κB.

DISCUSSION

We have shown that upon signal induction, IκBα, associated with NF-κB protein in the cytoplasm, is phosphorylated and targeted for proteolytic degradation without dissociating from NF-κB. In light of these results, we propose a model for NF-κB activation (Fig. 5). A central feature of our model is the demonstration that induced phosphorylation of IκBα prior to NF-κB activation does not result in its dissociation from NF-κB but rather targets IκBα for degradation. Support for our model comes from the data showing that inhibition of degradation of the phosphorylated form of IκBα (IκB*) leads to negligible NF-κB activity in the nucleus (Fig. 3). Although IκBα degradation appears to be specifically blocked by the serine protease inhibitors TPCK and TLCK (16–18), it is now clear that they also prevent IκBα phosphorylation (Fig. 2*A*). Therefore, the inhibition of NF-κB activation by these inhibitors (16–18) is most likely due to inhibition of phosphorylation events rather than specific inhibition of an IκBα protease. We therefore used protease inhibitors that do not inhibit the IκBα phosphorylation to stabilize IκB* long enough to study its interactions with NF-κB.

We have observed that the calcium-dependent cysteine protease calpain I can efficiently degrade IκBα *in vitro* and that the presence of the C-terminal PEST-like sequence (20) is essential for degradation *in vitro* and *in vivo* (S.M. and D. Van Antwerp, unpublished observation). Since the activity of calpain I can be inhibited by cell-permeable cysteine protease inhibitors, such as calpain inhibitors I and II, E-64d, calpeptin, and leupeptin (19), we tested these inhibitors for their capacity to inhibit degradation of IκB* induced by TNF-α in HeLa cells. Among these inhibitors, calpain inhibitors I and II efficiently blocked degradation of IκB* (Fig. 2). However, when IκB* degradation was inhibited, NF-κB activity did not appear in the nucleus (Fig. 3*B*). This was not due to the direct inhibition of DNA binding activity of nuclear NF-κB by the protease inhibitor (Fig. 3*C*). If phosphorylation led to IκBα dissociation (1, 13), inhibition of IκB* degradation would still allow the appearance of nuclear NF-κB. Thus, we conclude that IκBα does not dissociate from NF-κB upon phosphorylation induced by TNF-α in HeLa cells. We directly demonstrated by coimmunoprecipitation of the NF-κB–IκB* complex (Fig. 4) that IκB* remains associated with the complex. Furthermore, we argue that the substrate for protease in this case is not free IκB* but rather IκB* associated with NF-κB (Fig. 5). It is important to emphasize that the lack of a dissociation event following induced phosphorylation of

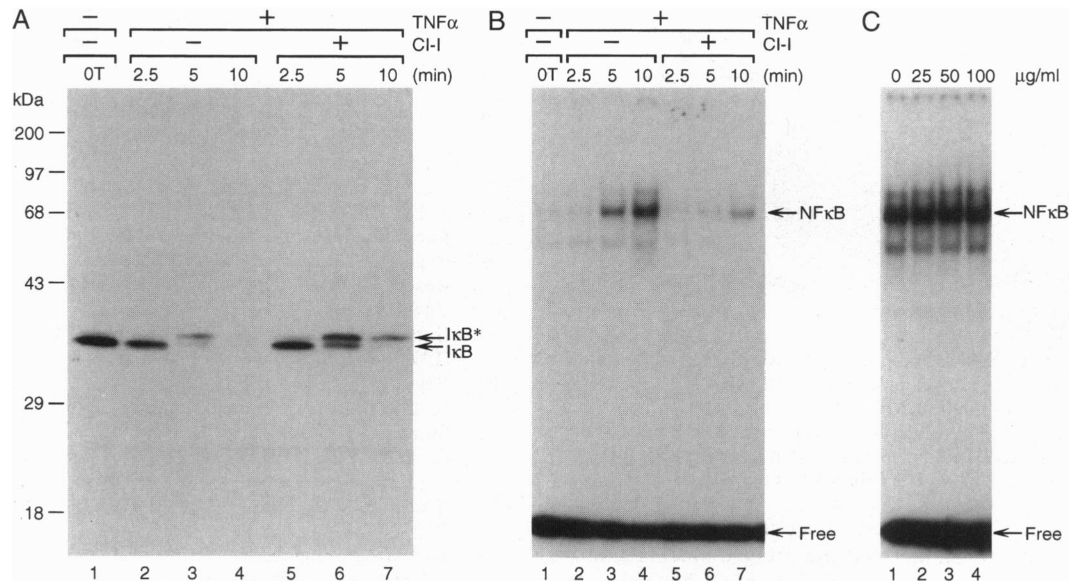

FIG. 3. Phosphorylation of IκBα (IκB) does not lead to nuclear translocation of NF-κB. (A) Time course of calpain inhibitor I (CI-I) inhibition of IκBα degradation. HeLa cells were pretreated for 1 hr with (lanes 5–7) or without (lanes 2–4) calpain inhibitor I (50 μg/ml) and treated with TNF-α (10 ng/ml) for the indicated periods. Cytoplasmic extracts were Western blotted with anti-MAD-3 IgG as in Fig. 1. (B) Nuclear extracts isolated from cells in A were assayed for NF-κB activity by electrophoretic mobility-shift assay as in Fig. 1B. (C) Calpain inhibitor I does not directly inhibit DNA binding activity of NF-κB. Calpain inhibitor I was added directly to a nuclear extract isolated from TNF-α-stimulated HeLa cells with the indicated concentrations (μg/ml) (lanes 2–4) and the NF-κB–DNA complex was resolved by electrophoretic mobility-shift assay.

IκBα is tested only for the TNF-α-induced pathway in HeLa cells. It is still possible that other NF-κB activating agents may induce phosphorylation of IκBα that results in its dissociation as implicated from the *in vitro* experiments (13). Such a hypothesis predicts the presence of different inactivating IκBα phosphorylation sites depending on the activating signals that may or may not lead to IκBα dissociation events. Thus, further studies are necessary to determine a potential involvement of IκBα dissociation in other NF-κB activation pathways.

Although an *in vivo* IκBα protease is not known, a criterion for its identification should include the specificity for the phosphorylated form of IκBα, which can be complexed with NF-κB. Recently, it was shown that p105, the precursor of the p50 subunit of NF-κB, is processed by a ubiquitin-dependent pathway involving the proteasome (22). It was also suggested that IκBα degradation involves the proteasome. Since calpain inhibitors I and II can also inhibit the proteasome activity (21, 22), the role of calpain in IκBα degradation is tentative at present. However, it is worth noting that cysteine protease inhibitor E64-d and leupeptin efficiently block IκB* degradation induced by lipopolysaccharide in murine pre-B-cell line 70Z/3 (S.M., unpublished observation). Since the distributions of calpain and IκBα are similar (both cytoplasmic and ubiquitously expressed; ref. 23), calpain may be involved in IκBα degradation under

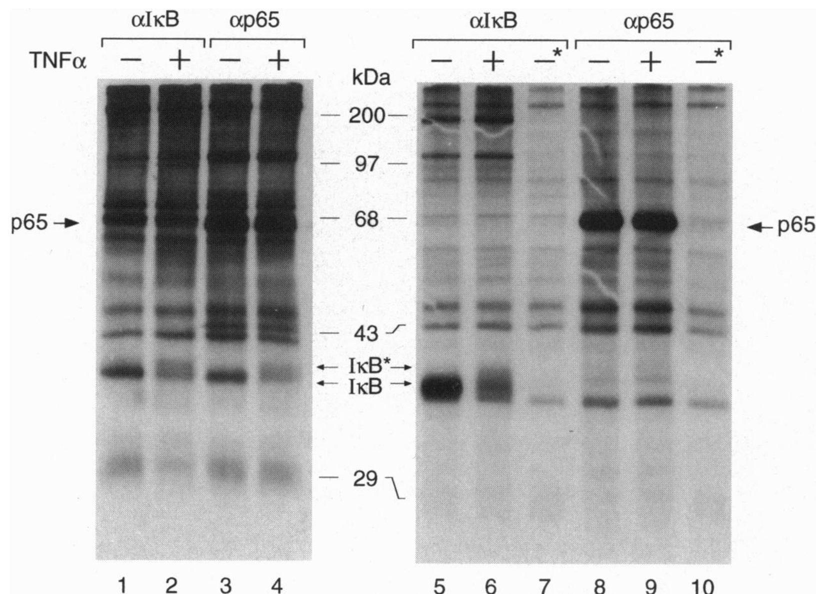

FIG. 4. Phosphorylated IκBα (IκB) remains associated with NF-κB in TNF-α treated HeLa cells. HeLa cells were metabolically labeled with [^{35}S]Met/Cys and treated with TNF-α for 7 min. The cell lysate was prepared and immunoprecipitated with anti-MAD-3 (lanes 1, 2, and 5–7) or anti-p65 IgG fraction (lanes 3, 4, and 8–10). Lanes 5–10, cell extracts were boiled in 0.5% SDS to disrupt the NF-κB–IκBα complex before immunoprecipitation. Lanes 7 and 10, immunoprecipitations were carried out in the presence of epitope for MAD-3 (lane 7) and p65 (lane 10).

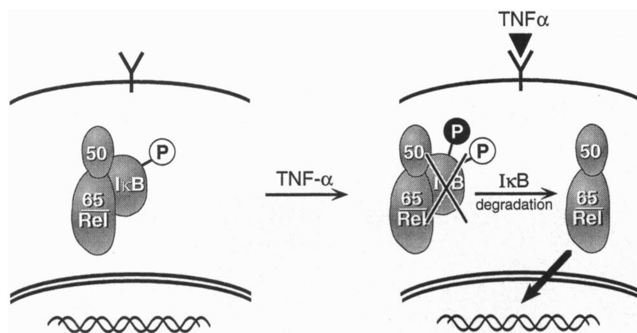

FIG. 5. Model for NF-κB activation. p50/p65(or Rel) proteins are sequestered in the cytoplasm complexed with IκBα (IκB) (a phosphoprotein). Upon stimulation with TNF-α, IκBα protein is further phosphorylated. The hyperphosphorylated form of IκBα (IκB*) is then targeted for degradation, releasing p50/p65(or Rel) complex to translocate to the nucleus where it binds to cognate κB sites.

certain *in vivo* conditions. Thus, it would be of interest to focus subsequent experiments on the precise mechanism of phosphorylation-dependent degradation of IκBα prior to NF-κB activation in various cell types.

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1. Baeuerle, P. A. (1991) *Biochim. Biophys. Acta* **1072,** 63–80.
2. Grilli, M., Chiu, J.-S. & Lenardo, M. J. (1993) *Int. Rev. Cytol.* **143,** 1–62.
3. Miyamoto, S. & Verma, I. M., *Adv. Cancer Res.* in press.
4. Nabel, G. & Baltimore, D. (1987) *Nature (London)* **326,** 711–713.
5. Baeuerle, P. A. & Baltimore, D. (1988) *Cell* **53,** 211–217.
6. Inoue, J.-I., Kerr, L. D., Rashid, D., Davis, N., Bose, H. R., Jr., & Verma, I. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4333–4337.
7. Hatada, E. N., Naumann, M. & Scheidereit, S. (1993) *EMBO J.* **12,** 2781–2788.
8. Baeuerle, P. A. & Baltimore, D. (1988) *Science* **242,** 540–546.
9. Beg, A. A., Ruben, S. M., Scehinman, R. I., Haskill, S., Rosen, C. A. & Baldwin, A. S., Jr. (1992) *Genes Dev.* **6,** 1899–1913.
10. Ganchi, P. A., Sun, S.-C., Greene, W. C. & Ballard, D. W. (1992) *Mol. Biol. Cell* **3,** 1339–1352.
11. Beg, A. A., Finco, T. S., Nantermet, P. V. & Baldwin, A. S., Jr. (1993) *Mol. Cell. Biol.* **13,** 3301–3310.
12. Sun, S.-C., Ganchi, P. A., Beraud, C., Ballard, D. W. & Greene, W. C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1346–1350.
13. Ghosh, S. & Baltimore, D. (1990) *Nature (London)* **344,** 678–682.
14. Sun, S.-C., Ganchi, P. A., Ballard, D. W. & Greene, W. C. (1993) *Science* **259,** 1912–1915.
15. Scott, M. L., Fujita, T., Liou, H.-C., Nolan, G. P. & Baltimore, D. (1993) *Genes Dev.* **7,** 1266–1276.
16. Miyamoto, S., Schmitt, M. J. & Verma, I. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5056–5060.
17. Henkel, T., Macheidt, T., Alkalay, I., Kronke, M., Ben-Neriah, Y. & Baeuerle, P. A. (1993) *Nature (London)* **365,** 182–185.
18. Chiao, P. J., Miyamoto, S. & Verma, I. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 28–32.
19. Adachi, Y., Murachi, T., Maki, M., Ishii, K. & Hatanaka, M. (1990) *Biomed. Res.* **11,** 313–317.
20. Rechsteiner, M. (1990) *Semin. Cell Biol.* **1,** 433–440.
21. Figueiredo-Pereira, M. E., Banik, N. & Wilk, S. (1994) *J. Neurochem.* **62,** 1989–1994.
22. Palombella, V. J., Rando, O. J., Goldberg, A. C. & Maniatis, T. (1994) *Cell* **78,** 773–785.
23. Croall, D. E. & Demartino, G. N. (1991) *Physiol. Rev.* **71,** 813–847.