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In Vivo Stimulation of IκB Phosphorylation Is Not Sufficient To Activate NF-κB

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NF-κB is a major inducible transcription factor in many immune and inflammatory reactions. Its activation involves the dissociation of the inhibitory subunit IκB from cytoplasmic NF-κB/Rel complexes, following which the Rel proteins are translocated to the nucleus, where they bind to DNA and activate transcription. Phosphorylation of IκB in cell-free experiments results in its inactivation and release from the Rel complex, but in vivo NF-κB activation is associated with IκB degradation. In vivo phosphorylation of IκBα was demonstrated in several recent studies, but its role is unknown. Our study shows that the T-cell activation results in rapid phosphorylation of IκBα and that this event is a physiological one, dependent on appropriate lymphocyte costimulation. Inducible IκBα phosphorylation was abolished by several distinct NF-κB blocking reagents, suggesting that it plays an essential role in the activation process. However, the in vivo induction of IκBα phosphorylation did not cause the inhibitory subunit to dissociate from the Rel complex. We identified several protease inhibitors which allow phosphorylation of IκBα but prevent its degradation upon cell stimulation, presumably through inhibition of the cytoplasmic proteasome. In the presence of these inhibitors, phosphorylated IκBα remained bound to the Rel complex in the cytoplasm for an extended period of time, whereas NF-κB activation was abolished. It appears that activation of NF-κB requires degradation of IκBα while it is a part of the Rel cytoplasmic complex, with inducible phosphorylation of the inhibitory subunit influencing the rate of degradation.

The NF-κB transcription factor plays a major role in the regulation of numerous cellular and viral genes induced in immune and inflammatory responses (4, 9, 19, 31, 37, 51). The factor, which is a heterodimer of Rel proteins, is retained in a latent form in the cytoplasm in most cells studied (1, 2, 19). Following cell stimulation with numerous immunomodulators and a variety of other agents (4), NF-κB is translocated to the nucleus where it binds to its cognate DNA-binding site and induces the transcription of its target genes (4, 19, 31). It is assumed that the major regulatory step involved in the activation of NF-κB is its release from a cytoplasmic inhibitory protein (1, 2, 6, 18, 60). This process is coupled to the translocation of the active Rel factor to the nucleus (4, 8, 31, 61).

Several proteins, collectively termed IκB and associated with the latent NF-κB/Rel factor in the cytoplasm, share the property of retaining NF-κB and preventing its translocation to the nucleus (6, 18, 27, 36, 44, 47). To date, the most extensively studied IκB is IκBα, the product of the human *MAD-3* gene or its homologs in different species (11, 13, 21, 27, 56). IκBα associates efficiently with all Rel proteins possessing transactivation properties: c-Rel, RelA, and RelB (3, 6, 8, 18, 56). Among the many proteins exhibiting IκB function, IκBα is the only inhibitor which in response to cell stimulation dissociates from the Rel complex, with kinetics matching NF-κB translocation to the nucleus (34, 36, 55). It was therefore suggested that the inducible activation of NF-κB is mainly regulated by NF-κB/IκBα dissociation (6, 18, 31).

Two processes have been implicated in NF-κB/IκBα dissociation: (i) phosphorylation affecting either IκBα or some of the Rel proteins and (ii) IκBα degradation. In vitro phosphorylation of IκB by several serine/threonine kinases prevents its binding to NF-κB (17, 52); transient in vivo phosphorylation of IκBα has been observed in several studies following cell stimulation (7, 10, 16, 34, 55). A more common finding is the rapid degradation of IκBα after cell stimulation, as monitored by immunoblotting (7, 10, 11, 16, 22, 34, 54, 55). In principle, the inducible degradation of IκBα could obviate the need for phosphorylation in NF-κB activation, because of the activation of an IκBα-specific protease. However, the two processes, phosphorylation and degradation of IκBα, may be linked. According to the most prevalent NF-κB activation model, IκBα phosphorylation results in its dissociation from the Rel proteins, thereby exposing it to degradation (6, 18, 31). This model is supported by studies showing that the half-life of both endogenous and transfected IκBα is prolonged by overexpression of RelA (43, 50, 54).

In our study we reevaluated the mechanism of NF-κB/IκB activation. We show that the phosphorylation of IκBα is induced by T-cell costimulation, in accordance with NF-κB activation requirements in T cells. The inducible phosphorylation of IκBα, like its degradation, can be prevented by preincubating the T cells with various nonrelated NF-κB-blocking reagents, suggesting its importance to NF-κB activation. However, inducible IκBα phosphorylation neither results in IκBα dissociation nor is it sufficient to activate NF-κB, and it must be complemented by selective proteolysis of IκBα. Accordingly, we have identified a family of protease inhibitors which do not interfere with the inducible phosphorylation of IκBα but, by inhibiting its degradation, prevent NF-κB activation.

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MATERIALS AND METHODS

Cell culture and reagents. Jurkat T cells were grown in RPMI 1640 supplemented with 10% fetal calf serum. Phorbol myristate acetate (PMA) and the

Ca^{2+} ionophore A23187 (Sigma) were used at concentrations of 10 ng/ml and 1 μM, respectively. Tosylamide phenylmethyl chloromethyl ketone (TPCK) and pyrrolidinedithiocarbamate (PDTC) (Sigma) were used at concentrations of 25 and 100 μM, respectively, for 30 or 60 min before costimulation of the cells. Cyclosporin A (CsA) (Sandoz) was used at a concentration of 400 ng/ml for 30 min prior to cell costimulation. *N*-acetyl-Leu-Leu-norleucinal (ALLN) and *N*-acetyl-Leu-Leu-methioninal (ALLM) were purchased from Calbiochem. (Benzyloxycarbonyl)-Leu-Leu-phenylalaninal (ZLLF) was provided by Signal Pharmaceuticals, Inc. (San Diego, Calif.). L-Transepoxysuccinic acid (E64) and *N*-acetyl-Leu-Leu-argininal (leupeptin) were purchased from Sigma.

Antisera and proteins. Rabbit antisera to IκBα (MAD-3), RelA (p65), and NFKB1 (p50), a gift from J. DiDonato, were prepared against His-tagged human IκBα fusion protein, the mouse p65 C-terminal (last 125-amino-acid [aa]) fragment fused to glutathione *S*-transferase, and human p50 (36). The specificity of the reagents was confirmed by competition assays with recombinant proteins (14). Rabbit antiserum to Ltk was used as the control antiserum (5). The anti-CD3 (OKT3) monoclonal antibody was purified from culture supernatants on protein A-Sepharose. Anti-CD28 (clone 9.3) monoclonal antibody was a gift from Peter Linsley. Recombinant human IκBα (MAD-3) was provided by T. Henkel and P. Baeuerle.

Nuclear and cytoplasmic extracts. Following stimulation at 37°C in complete medium, cells were washed in cold phosphate-buffered saline, pelleted, and resuspended in hypotonic buffer {10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.6), 1 mM EDTA, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 10 mM KCl, 1 mM dithiothreitol, protease and phosphatase inhibitors (5)}. After 15 min on ice, Nonidet P-40 was added at a concentration of 0.6%, and the lysates were incubated for an additional 5 min on ice and spun down at 14,000 rpm in a microcentrifuge (Eppendorf) at 4°C. The supernatant was removed and used as cytoplasmic extract for immunoprecipitation and Western blot (immunoblot) studies. The nuclear pellet was resuspended in an equal volume of nuclear extract buffer (20 mM HEPES [pH 7.6], 0.2 mM EDTA, 0.1 mM EGTA, 25% glycerol, 0.42 M NaCl, 1 mM dithiothreitol, protease and phosphatase inhibitors) with frequent vortexing for 30 min at 4°C. After a microcentrifuge spin at 14,000 rpm for 15 min, the supernatant was collected and used as nuclear extract in the electrophoretic mobility shift assay (EMSA).

EMSA. The binding reaction mixture containing 10 mM HEPES (pH 7.9), 60 mM KCl, 0.4 mM dithiothreitol, 10% glycerol, 2 μg of bovine serum albumin, 1 μg of poly(dI-dC), 10,000 cpm of ^{32}P-labeled κB oligonucleotide (22), and nuclear extract (5 μg of protein) was incubated on ice for 30 min. In the antibody-supershift assay, the reaction mixture minus probe was incubated with 1 μl of antiserum for 15 min at room temperature. The ^{32}P-labeled oligonucleotide was then added, and incubation allowed to proceed for 30 min on ice. Products were analyzed on a 5% acrylamide gel made up in 1× TGE (50 mM Tris, 400 mM glycine, 2 mM EDTA).

Immunoblotting. Cytosolic extracts (50 μg of protein) were electrophoresed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were incubated with the primary antibody for 2 h (1:2,500 dilution for anti-IκBα and 1:2,000 for anti-p65 and anti-p50) and then with peroxidase-conjugated protein A (Amersham) at a 1:25,000 dilution for 30 min and analyzed according to the Amersham enhanced chemiluminescense system (ECL). IκB was dephosphorylated in dephosphorylation buffer containing calf intestinal phosphatase (both from Boehringer Mannheim) in the absence or presence of phosphatase inhibitors (*p*-nitrophenyl phosphate [PNPP], 20 mM; β-glycerophosphate, 20 mM; and Na_3VO_4, 1 mM) and then subjected to Western blot analysis.

Immunoprecipitation. Cytosolic extracts (500 μg of protein) were incubated for 18 h on ice with 2 μl of antiserum in 300 μl of hypotonic buffer containing protease and phosphatase inhibitors. Precipitates were collected on protein A-Sepharose (Pharmacia), washed in hypotonic buffer containing 0.1% Triton X-100, and analyzed by Western blotting, with anti-IκBα serum.

RESULTS

Physiological activation of T lymphocytes requires costimulation through the T-cell receptor/CD3 complex and CD28, which can be mimicked by suboptimal PMA concentrations in combination with the engagement of one receptor or Ca^{2+} ionophore (12, 30, 53, 57). Previous studies indicated that NF-κB activation is enhanced by costimulation (16, 28, 32, 51). We extended these studies to include the steps involved in NF-κB activation: from the posttranslational modification of IκBα through its release from the Rel complex and to the appearance of DNA-binding activity in the nucleus.

The costimulation requirements of NF-κB were evident in an EMSA. Whereas single stimuli were barely sufficient to induce NF-κB activity in EMSA, combinations of PMA with either Ca^{2+} ionophore, anti-CD3, or anti-CD28 and costimulation with the two antibodies enhanced NF-κB DNA-binding activity 10- to 20-fold over the level induced by a single stimulus (Fig. 1A). In defining the composition of NF-κB detected in EMSA, we tested the effect of antibodies and recombinant IκBα protein [which eliminates NF-κB but not $(p50)_2$ DNA binding (61)] on costimulation-induced κB-binding activity (Fig. 1B). While anti-RelA (p65) serum (lane 4) eliminated only the upper κB-binding complex, the recombinant IκBα protein (lane 2) eliminated the two upper complexes, and anti-p50 serum retarded all the κB-binding complexes (lane 5). We therefore concluded that the lower complex was composed of p50 homodimers, the middle one was a heterodimer of p50 and an additional unidentified protein, and the upper complex was a heterodimer of p50/RelA.

Parallel to testing the NF-κB DNA-binding activity, we investigated the fate of IκBα following cell treatment with various stimuli. Single stimuli, which failed to activate NF-κB, did not affect the migration or intensity of the IκBα signal in a Western blot. In contrast, T-cell costimulation resulted in the reduced intensity of the IκBα signal and the appearance of a slowly migrating form of IκBα (Fig. 1C). Alkaline phosphatase treatment of cytosolic extracts from stimulated cells reversed the slow migration of the novel IκBα band, indicating that it was a phosphorylated form of IκBα (Fig. 1D). Hence, on the basis of our results, phosphorylation of IκBα, in parallel to NF-κB activation, is induced according to the physiological requirements for T-cell activation.

Kinetic studies of the IκBα modification process revealed that shortly after the appearance of phosphorylated IκBα, starting at 4 min after cell stimulation with PMA and the Ca^{2+} ionophore, the nonmodified IκBα band was diminished (Fig. 2A). Upon densitometry analysis (Table 1), after a short lag, the intensity of the phosphorylated IκBα signal at each time point poststimulation nearly equaled the consecutive signal loss of the nonmodified band from the previous time point. Stimulation had no effect on the stability of p50 (Fig. 2A) or p65 (data not shown), emphasizing the selectivity of IκBα degradation. These features raise the possibility that the decay of the nonphosphorylated IκBα signal occurs subsequent to its modification, whereas the decay of the phosphorylated band is due to degradation.

To test the hypothesis that IκBα degradation requires prior phosphorylation, we used three different reagents which have been shown to block NF-κB activation: TPCK (11, 22, 34), PDTC (22, 48, 54), and CsA (32). TPCK, an alkylating agent protease inhibitor (23), and PDTC, a metal chelator antioxidant (20), completely blocked both the inducible modification and the degradation of IκBα (Fig. 2B). CsA is a powerful immunosuppressive drug and inhibitor of Ca^{2+}-dependent phosphatase calcineurin (49) and blocks T-cell costimulation (32, 53). Since IκBα phosphorylation and degradation require costimulation (Fig. 1), CsA was expected to interfere with the signaling process leading to IκBα modification. Indeed, CsA abolished more than 75% of the inducible IκBα phosphorylation and nearly all of the degradation (Fig. 2B). The fact that all three reagents, each with its individual mode of action, blocked both the inducible IκBα modification and its degradation suggests a linkage between the two events.

Since the stability of IκBα has been shown to be enhanced by complexing to RelA (p65), it was suggested that modification of IκBα could lead to its dissociation and destabilization (6, 18, 31, 43, 50, 54). Once dissociation is induced through IκBα phosphorylation, the released Rel proteins would be free to translocate to the nucleus and bind to κB sites. Subsequent degradation of the released IκBα would complement the dissociation, ensuring its irreversibility. To test the effect of in-

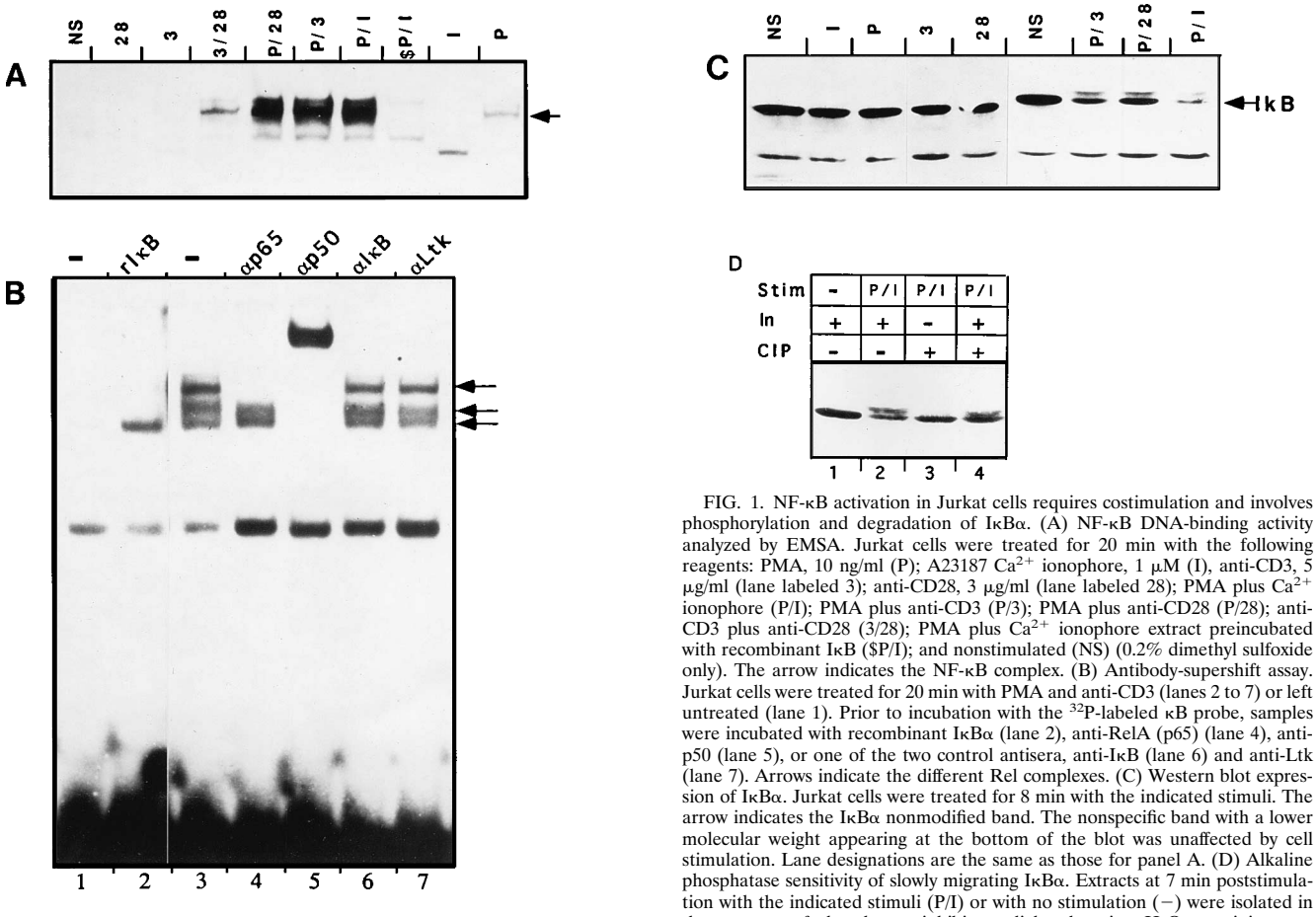

FIG. 1. NF-κB activation in Jurkat cells requires costimulation and involves phosphorylation and degradation of IκBα. (A) NF-κB DNA-binding activity analyzed by EMSA. Jurkat cells were treated for 20 min with the following reagents: PMA, 10 ng/ml (P); A23187 Ca^{2+} ionophore, 1 μM (I), anti-CD3, 5 μg/ml (lane labeled 3); anti-CD28, 3 μg/ml (lane labeled 28); PMA plus Ca^{2+} ionophore (P/I); PMA plus anti-CD3 (P/3); PMA plus anti-CD28 (P/28); anti-CD3 plus anti-CD28 (3/28); PMA plus Ca^{2+} ionophore extract preincubated with recombinant IκB ($P/I); and nonstimulated (NS) (0.2% dimethyl sulfoxide only). The arrow indicates the NF-κB complex. (B) Antibody-supershift assay. Jurkat cells were treated for 20 min with PMA and anti-CD3 (lanes 2 to 7) or left untreated (lane 1). Prior to incubation with the ^{32}P-labeled κB probe, samples were incubated with recombinant IκBα (lane 2), anti-RelA (p65) (lane 4), anti-p50 (lane 5), or one of the two control antisera, anti-IκB (lane 6) and anti-Ltk (lane 7). Arrows indicate the different Rel complexes. (C) Western blot expression of IκBα. Jurkat cells were treated for 8 min with the indicated stimuli. The arrow indicates the IκBα nonmodified band. The nonspecific band with a lower molecular weight appearing at the bottom of the blot was unaffected by cell stimulation. Lane designations are the same as those for panel A. (D) Alkaline phosphatase sensitivity of slowly migrating IκBα. Extracts at 7 min poststimulation with the indicated stimuli (P/I) or with no stimulation (−) were isolated in the presence of phosphatase inhibitors, dialyzed against H_2O containing protease inhibitors, and either were untreated (lanes 1 and 2) or were treated with calf intestinal phosphatase (CIP) alone (lane 3) or together with phosphatase inhibitors (In) (lane 4), and analyzed by Western blot.

ducible IκBα phosphorylation on its dissociation, we examined the composition of the Rel complex in T lymphocytes either before or after stimulation, by immunoprecipitation with anti-RelA serum and anti-p50 serum, followed by Western blot analysis with anti-IκBα (Fig. 3). An 18-h immunoprecipitation with anti-RelA serum (lane 4) or with anti-p50 serum (lane 7) of nonstimulated cell extracts resulted in the recovery of non-phosphorylated IκBα. However, following stimulation, considerable amounts of phosphorylated IκBα were immunoprecipitated along with the nonmodified IκBα by anti-RelA (lanes 5 and 6) or anti-p50 (lane 8). Similar results were obtained with another RelA antiserum (44), whereas control antisera did not precipitate any IκBα (data not shown and see Fig. 5B, control IP). It is therefore clear that following its induced phosphor-

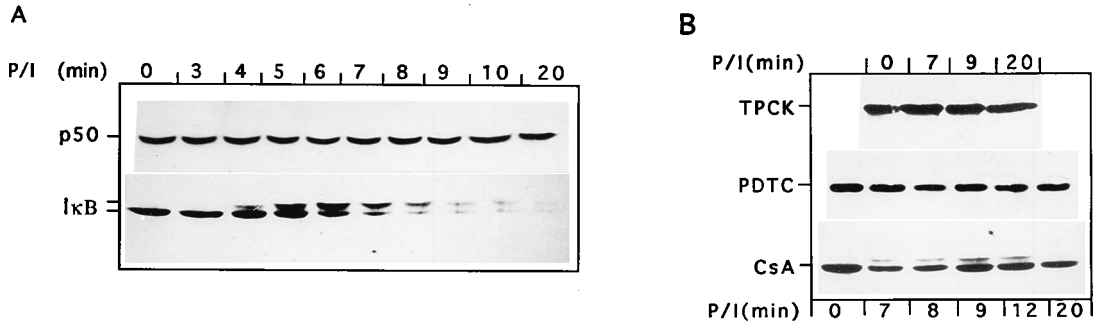

FIG. 2. Inhibition of IκBα phosphorylation blocks its inducible degradation. (A) Kinetics of IκBα modification by Western blot analysis. Jurkat cells were stimulated with PMA and the Ca^{2+} ionophore for the indicated times. (Lower section) Western blot with anti-IκBα serum. The positions of nonmodified and modified IκBα are indicated. (Upper section) Western blot with anti-p50 serum. The position of p50 is indicated. (B) IκBα modification in the presence of NF-κB-blocking reagents. Jurkat cells were treated prior to stimulation with TPCK, PDTC, or CsA and analyzed by Western blot. The duration of Jurkat stimulation with PMA and the Ca^{2+} ionophore is indicated above (for TPCK) and below (for PDTC and CsA) the panel.

TABLE 1. Results of densitometric analysis of IκBα[a]

Stimulation (min)[b]	No pretreatment				Pretreatment with ALLN			
	OD[c]			% PIκBα	OD			% PIκBα
	PIκBα	IκBα	Total		PIκBα	IκBα	Total	
0	0	1.098	1.098	0	0	0.599	0.599	0
3	0	1.110	1.110	0				
4	0.090	1.104	1.194	7.5	0	0.783	0.783	0
5	0.520	1.011	1.531	34				
6	0.625	0.472	1.097	57	0.311	0.407	0.718	43
7	0.297	0.138	0.435	68				
8	0.103	0.020	0.123	84	0.498	0.066	0.564	88
9	0.019	0	0.019	100				
10	0.018	0.004	0.022	82	0.636	0.066	0.702	90.5
12					0.491	0.035	0.526	93
20	0.016	0.004	0.020	80	0.543	0.081	0.624	87

[a] Analysis of the autoradiograms presented in Fig. 2A (no inhibitor) and Fig. 5A (ALLN) was done with the Bio-Rad densitometer (model GS-670). PIκBα, phosphorylated IκBα; IκBα, unmodified IκBα. Total, sum of the results for the two forms of IκBα; % PIκBα, percentage of phosphorylated IκBα of the total.
[b] Cells were stimulated with PMA and the Ca^{2+} ionophore for the times (in minutes) indicated.
[c] OD, optical density units.

ylation, the affinity of IκBα to RelA is not significantly reduced, with the phosphorylated IκBα remaining associated with the NF-κB complex in cytoplasmic extracts for at least 18 h.

Since the detectable modification of IκBα is hardly sufficient to induce its dissociation from NF-κB, another event is required for the observed IκBα loss following stimulation. On the basis of previous studies from our laboratory and from other groups, proteolysis is the most likely event (7, 10, 11, 16, 22, 34, 54, 55). It specifically targets IκBα, as we detected no significant loss of the IκBα-associated proteins, RelA and p50 (Fig. 2A and data not shown). It is therefore conceivable that IκBα proteolysis occurs in the cytoplasm and not in any specified cellular compartment, such as lysosomes, where selective degradation is unlikely to occur. Two major classes of cytoplasmic proteases which could eliminate IκBα are calpains (Ca^{2+}-dependent cysteine proteinases [35]) and the proteasome, a multicatalytic proteinase complex (38, 42). Several peptide aldehydes which are both calpain and proteasome inhibitors (15, 58), ALLN, ALLM, and ZLLF, are reversible nonalkylating protease inhibitors and, as such, not likely to disturb posttranslational modification events (58). We therefore tested their effect on IκBα processing. Pretreatment of Jurkat cells with the inhibitors variably blocked IκBα degradation (Fig. 4, lower section) but did not interfere with inducible IκBα phosphorylation at 50% inhibitory concentrations (IC_{50}). The most effective was ZLLF, with an IC_{50} of 10 μM; ALLN protected IκBα from degradation at an IC_{50} of 25 μM; and the least effective was ALLM, which even at 300 μM only partially influenced IκBα degradation. None of the inhibitors had any effect on RelA (p65), which remained constant regardless of cell stimulation (Fig. 4, upper section). The capacity to protect IκBα from degradation is not a general property of peptide aldehydes, since leupeptin, a peptide aldehyde and a potent calpain inhibitor (33), had no effect on IκBα-inducible degradation (Fig. 4, right panel). To further distinguish between the effects of the protease inhibitors of calpains and the proteasome on IκBα, we examined the influence of another calpain inhibitor, E64, on the stability of IκBα. Following stimulation (Fig. 5A, right panel), E64 did not interfere with the inducible phosphorylation and degradation of IκBα, even at the high concentration of 300 μM. Hence, the two potent calpain inhibitors, leupeptin and E64, have no effect on IκBα degradation.

The kinetics of PMA- and Ca^{2+} ionophore-inducible IκBα phosphorylation and degradation were studied in the presence of ALLN (Fig. 5A, lower section). At 100 μM ALLN, there was no significant degradation of IκBα at 20 min poststimulation, whereas in the absence of the inhibitor more than 98% of the protein was eliminated (Table 1). Furthermore, ALLN treatment resulted in the accumulation of phosphorylated IκBα. Between 8 and 20 min poststimulation, more than 85% of the stabilized IκBα appeared phosphorylated, in contrast to 30 to 70% phosphorylation in the absence of the inhibitor at 5 to 7 min poststimulation, where considerable IκBα was still preserved (Fig. 2A and 5A; Table 1). ALLN had no detectable effect on either RelA or p50 (Fig. 5A), nor did it affect the association of IκBα with RelA (Fig. 5B). Following preincubation with ALLN, RelA immune complexes from two late poststimulation time points (10 and 20 min; lanes 2 and 3, respectively), at which there is hardly any detectable IκBα in the absence of the inhibitor, contained considerable amounts of both nonphosphorylated and phosphorylated IκBα.

The accumulation of phosphorylated IκBα following stimulation in the presence of ALLN raised the question of whether the stabilized IκBα is still active and retains NF-κB in the cytoplasm. Nuclear extracts corresponding to two late post-

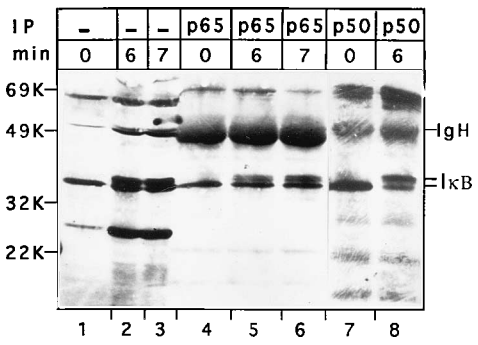

FIG. 3. IκBα phosphorylation does not lead to its dissociation from the NF-κB complex. Jurkat cells were stimulated with PMA and Ca^{2+} ionophore for the indicated times (in minutes). Immunoprecipitates (IP) with anti-p65 serum (lanes 4 to 6) or with anti-p50 serum (lanes 7 and 8); lanes 1 to 3, pre-IP reference samples. The positions of the immunoglobulin heavy chain (IgH) and IκBα are indicated on the right; the positions of molecular mass markers (in daltons) are indicated on the left.

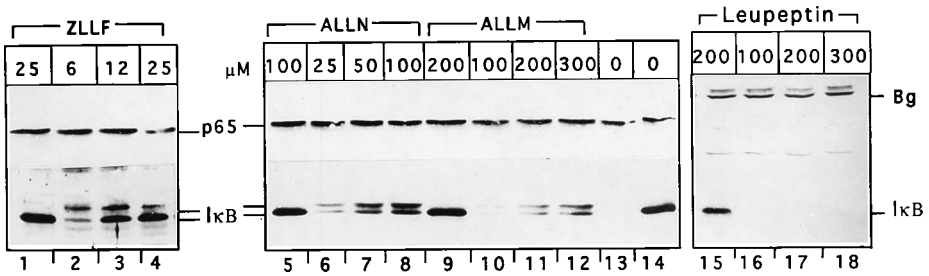

FIG. 4. Specific peptide aldehyde inhibitors do not interfere with inducible phosphorylation of IκBα but block its subsequent degradation. Cells were pretreated for 1 h with ZLLF, ALLN, ALLM, and leupeptin at the indicated concentrations (lanes 1 to 12 and 15 to 18) or with dimethyl sulfoxide (0.1%) alone (lanes 13 and 14). Lanes: 1, 5, 9, 14, and 15, unstimulated cells; 2 to 4, 6 to 8, 10 to 13, and 16 to 18, cells stimulated with PMA and Ca^{2+} ionophore for 20 min. Upper section, Western blot with anti-p65 serum; lower section, Western blot with anti-IκBα serum. Bg indicates the position of a nonspecific band which was unaffected by cell stimulation.

stimulation time points were tested for the effect of ALLN on in vivo-inducible NF-κB activity (Fig. 5C). The presence of the protease inhibitor in the culture medium resulted in almost total abrogation of NF-κB activation, whereas the addition of ALLN to the binding reaction mixture did not interfere with DNA binding of NF-κB. Hence, it is conceivable that ALLN blocks NF-κB activation by inhibiting the release of IκBα, presumably by preventing IκBα proteolysis.

DISCUSSION

The NF-κB activation system is a model for rapid cellular induction of latent transcription factors. The basic phenomenon behind the activation process, documented by Baeuerle and Baltimore (1, 2), is the coupling of inducible NF-κB activity to the dissociation of an inhibitory component in the cytoplasm (1–3). Several posttranslational modification events appear to participate in the activation of the transcription factor, converting it from a latent cytoplasmic factor to an active nuclear one (6, 18). Early studies emphasized the putative role of protein phosphorylation in the dissociation process, particularly the in vitro inactivation of IκB through phosphorylation (17, 52), with later reports of transient in vivo IκBα phosphorylation (7, 10, 16, 34, 55). Recent work has centered on another posttranslational modification process, the rapid inducible degradation of IκBα (7, 10, 11, 16, 22, 34, 54, 55).

In our study, we monitored the fate of IκBα during T-lymphocyte activation. NF-κB is an essential component in the regulation of interleukin-2 expression, one of the hallmarks of T-cell activation (26, 28, 57). T cells require two stimuli for the induction of interleukin-2 expression (12, 30, 53, 57). Indeed, earlier studies suggested that T-cell costimulation with phorbol ester and the Ca^{2+} ionophore is required for optimal induction of NF-κB (16, 32). We extended these studies to the effect of other costimuli on NF-κB, in parallel to their influence on IκBα. T-cell stimulation with suboptimal concentrations of phorbol ester in combination with either anti-CD3 antibodies, antibodies to CD28, or the Ca^{2+} ionophore induced phosphorylation and degradation of IκBα along with NF-κB activation. By contrast, single stimuli failed to induce either NF-κB DNA-binding activity or any modification of IκBα. The costimulation dependence of the phosphorylation and degradation of IκBα

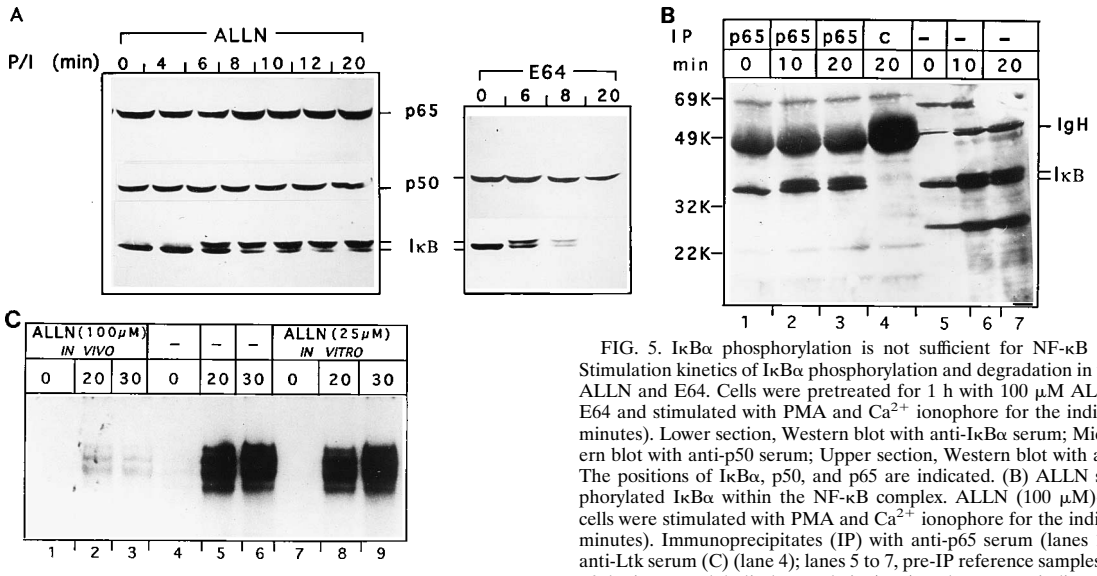

FIG. 5. IκBα phosphorylation is not sufficient for NF-κB activation. (A) Stimulation kinetics of IκBα phosphorylation and degradation in the presence of ALLN and E64. Cells were pretreated for 1 h with 100 μM ALLN or 300 μM E64 and stimulated with PMA and Ca^{2+} ionophore for the indicated times (in minutes). Lower section, Western blot with anti-IκBα serum; Midsection, Western blot with anti-p50 serum; Upper section, Western blot with anti-p65 serum. The positions of IκBα, p50, and p65 are indicated. (B) ALLN stabilizes phosphorylated IκBα within the NF-κB complex. ALLN (100 μM)-treated Jurkat cells were stimulated with PMA and Ca^{2+} ionophore for the indicated times (in minutes). Immunoprecipitates (IP) with anti-p65 serum (lanes 1 to 3) or with anti-Ltk serum (C) (lane 4); lanes 5 to 7, pre-IP reference samples. The positions of the immunoglobulin heavy chain (IgH) and IκBα are indicated on the right; the positions of the molecular mass markers (in daltons) are indicated on the left. (C) ALLN inhibits in vivo NF-κB activation in EMSA. The durations of stimulation (in minutes) with PMA and Ca^{2+} ionophore are indicated. Lanes: 1 to 3, ALLN-treated cells; 4 to 9, untreated cells. The DNA-binding reaction was performed in the absence (lanes 4 to 6) or in the presence (lanes 7 to 9) of ALLN.

and NF-κB activation indicates that the two processes, phosphorylation and degradation, are involved in physiological NF-κB activation.

Effective costimulation of Jurkat cells resulted in the phosphorylation of a fraction of IκBα within 4 to 5 min. After an additional 1 or 2 min, the fraction of newly phosphorylated IκBα equaled or exceeded the nonphosphorylated fraction, while both IκBα forms co-decayed within a period of 3 to 4 min. When the kinetics of IκBα modification was monitored at intervals of 1 min or less, the decay of nonmodified IκBα seemed attributable mainly to its phosphorylation. It is thus conceivable that, following a short lag, the rate of IκBα phosphorylation is equal to the rate of degradation of the newly phosphorylated form, resulting in the observed codecay of both forms. According to this scenario, selective inhibition of the proteolysis event should lead to accumulation of phosphorylated IκBα, an outcome which was indeed observed in our experiments. The nonmodified form of IκBα recovered in the presence of some of the protease inhibitors could be attributed to dephosphorylation occurring either in vivo or artificially during preparation of the cell extracts. This could also account for some of the variation in the ratio of the two forms in different experiments.

Several protease inhibitors have the capacity to block in vivo IκBα degradation and NF-κB activation (22). However, whereas some of the previously reported inhibitors (such as TPCK) interfere with IκBα phosphorylation, the effect of the peptide aldehyde inhibitors described here is mainly limited to inhibition of IκBα proteolysis. IκBα phosphorylation is initiated and proceeds normally at peptide aldehyde concentrations which block NF-κB activation. Therefore, the two modification processes affecting IκBα following cell stimulation are not reciprocally linked, with phosphorylation able to proceed in the absence of degradation but blockage of phosphorylation apparently abolishing degradation. The two NF-κB-blocking reagents, PDTC and CsA, which are neither protease nor kinase inhibitors, interfered with both the phosphorylation of IκBα and its degradation, suggesting that the two processes are coupled. Nevertheless, on the basis of our studies, we cannot conclude that phosphorylation is a prerequisite to degradation. It should be possible to resolve this issue upon identification of phosphorylation site mutants of IκBα which escape inducible degradation or the demonstration of selective proteolysis of the phosphorylated IκBα.

Whether essential to degradation, and at variance with some prevailing models, the detectable IκBα phosphorylation per se is not sufficient to inactivate IκBα. This conclusion is inferred from two observations. First, shortly after stimulation, while both nonmodified and newly phosphorylated IκBα are present in the cytoplasm, both forms are captured in a complex with RelA and p50. In the presence of protease and phosphatase inhibitors, both complexes proved stable in vitro for at least 18 h, indicating that the affinity of the newly phosphorylated IκBα to RelA is not significantly different from that of the nonmodified form. Second, upon cell stimulation in the presence of the peptide aldehyde inhibitors, complexes of phosphorylated IκBα and RelA persist in the cytoplasm at least 20 min poststimulation, while no active NF-κB is found in the nucleus. We may therefore conclude that phosphorylated IκBα is bound to RelA in the cytoplasm as long as it is not subject to the cell's proteolytic machinery. This concept is not peculiar to NF-κB activation following costimulation in T cells. Similar findings with HeLa cells following stimulation with tumor necrosis factor alpha have been reported by DiDonato et al. (14).

Assuming that phosphorylation is indeed an essential step in NF-κB activation, what then is the role of the inducible phosphorylation of IκBα? Although not directly addressed in our present study, it is possible that phosphorylation singles out IκBα for degradation. Phosphorylation at a cdc-2 site has recently been shown to contribute to the destabilization of the RAG-2 protein in the S phase of the cell cycle (29). Similar to the cell cycle-regulated kinase which sensitizes the RAG-2 protein to degradation, cell stimulation may induce a kinase responsible for tagging IκBα for degradation. The newly marked protein would then be recognized by a cytoplasmic protease, facilitating subunit-specific degradation in a fashion similar to the degradation of the yeast α2 repressor (24) and sparing the other subunits of the complex. Although we favor this model, which is based solely on the detectable protein modifications found in our study, we cannot rule out more complex models for the dissociation of NF-κB/IκBα. For example, following the inducible phosphorylation (reflected in the slow migration of IκBα in SDS-polyacrylamide gels) a second nondetectable event may ensue, leading to the release of IκBα and its subsequent degradation. Such an event may modify IκBα itself or one of the other subunits of the complex. Should the event also be sensitive to some unknown effects exerted by the peptide aldehydes, it would even provide an alternative explanation to the stabilization of IκBα by the reagents.

The selectivity of the proteolytic event toward IκBα indicates that it occurs in the cytoplasm where the complexes of NF-κB and phosphorylated IκBα are found prior to their dissociation and not in a nonselective proteolytic compartment, such as lysosome. Two major classes of proteases are known to operate outside of the lysosome: the calpains (Ca^{2+}-dependent cysteine proteinases) and the proteasome, particularly the ATP-dependent, 26S multisubunit proteinase, which can operate in a ubiquitin-dependent or -independent fashion (42). We previously reported a series of cell-penetrating protease inhibitors which, concomitant with NF-κB activation, block the loss of IκBα (22). We then concluded that IκBα degradation is a necessary step in NF-κB activation and have substantiated this claim in the present study. However, some of the previously used protease inhibitors are capable of alkylating cell proteins (23). Since it was impossible to detect the phosphorylated IκBα with the previously used antibodies, we could not have observed the effect of TPCK on IκBα phosphorylation. With the aid of new IκBα-specific antiserum, we have now demonstrated that TPCK, an alkylating agent which is incapable of blocking the proteasome (23, 25), can totally abolish the inducible phosphorylation of IκBα. It thus probably blocks proteolysis indirectly, by interfering with the signaling of IκBα modification.

Although some of the previously reported inhibitors, such as 3,4-dichloroisocoumarin, may stabilize IκBα by blocking the proteasome (39, 40, 58), the peptide aldehyde protease inhibitors used in the present work are much more selective (39, 58) and particularly effective against calpains and the proteasome (15, 45, 46, 58). However, the IC_{50} values that we obtained for the inhibitors studied correlate between IκBα stabilization and the proteasome inhibition far better than with calpain inhibition (15, 45, 58). After the submission of our manuscript, Palombella et al. (41) reported the inhibition of IκBα degradation in MG-63 cells by the proteasome inhibitor MG115, a peptide aldehyde related to ALLN. Furthermore, E64 and leupeptin, two efficient calpain inhibitors (33) which do not inhibit the proteasome (45, 58), had no effect on IκBα stabilization in our experiments, even at a concentration of 300 μM. Also, unlike calpains (35, 59), the proteasome degrades its substrates to completion, similarly to the processing of IκBα, without generating discrete peptide cleavage products (38).

Finally, the proteasome apparently has the capacity to sense conformational changes in protein substrates (40). It could, therefore, distinguish the inducible phosphorylation of IκBα which affects the protein's migration in a gel mobility. Further studies are necessary to show whether the proteasome is indeed implicated in NF-κB activation following cell stimulation, particularly whether it is a function of the ATP-dependent 26S proteasome and requires IκBα ubiquitination.

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ADDENDUM IN PROOF

After submission of our manuscript, two papers (E. B.-M. Traenckner, S. Wilk, and P. A. Baeuerle, EMBO J. **13:**5433–5441, 1994, and S.-C. Sun, J. Elwood, C. Béraud, and W. C. Greene, Mol. Cell. Biol. **14:**7377–7384, 1994) provided evidence to support our suggested model of NF-κB activation. Similar conclusions are drawn by DiDonato et al. (14).

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