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Stimulation-dependent IκBα phosphorylation marks the NF-κB inhibitor for degradation via the ubiquitin–proteasome pathway

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ABSTRACT **The nuclear translocation of NF-κB follows the degradation of its inhibitor, IκBα, an event coupled with stimulation-dependent inhibitor phosphorylation. Prevention of the stimulation-dependent phosphorylation of IκBα, either by treating cells with various reagents or by mutagenesis of certain putative IκBα phosphorylation sites, abolishes the inducible degradation of IκBα. Yet, the mechanism coupling the stimulation-induced phosphorylation with the degradation has not been resolved. Recent reports suggest a role for the proteasome in IκBα degradation, but the mode of substrate recognition and the involvement of ubiquitin conjugation as a targeting signal have not been addressed. We show that of the two forms of IκBα recovered from stimulated cells in a complex with RelA and p50, only the newly phosphorylated form, pIκBα, is a substrate for an *in vitro* reconstituted ubiquitin–proteasome system. Proteolysis requires ATP, ubiquitin, a specific ubiquitin-conjugating enzyme, and other ubiquitin–proteasome components. *In vivo*, inducible IκBα degradation requires a functional ubiquitin-activating enzyme and is associated with the appearance of high molecular weight adducts of IκBα. Ubiquitin-mediated protein degradation may, therefore, constitute an integral step of a signal transduction process.**

The transcription factor NF-κB is a heterodimeric Rel protein involved in many immune and inflammatory responses (1, 2). Originally identified as a B-cell-specific factor required for the expression of the immunoglobulin κ chain (3), NF-κB mostly appears in the nucleus after immunostimulation (1). Its cognate binding site has been identified in many immunoregulatory promoters/enhancers and viral regulatory sequences (1, 2). Stimuli, including viruses, cytokines, and antigens, were found to induce the translocation of NF-κB from the cytoplasm to the nucleus (1). Nuclear translocation is apparently the major step in the activation of NF-κB, which, once it enters the nucleus, is capable of binding its cognate site and initiating the transcription process (4–6).

A critical step in mobilizing NF-κB into the nucleus is its release from a cytoplasmic inhibitor. Several proteins collectively termed IκB share the ability to retain the factor in the cytoplasm (6). The most extensively studied is IκBα (7), a member of the ankyrin family of proteins (8). It interacts as a monomer with all tested dimeric NF-κB complexes through their Rel-homology domain, masking their nuclear localization sequence (2, 8).

Cell stimulation induces the dissociation of IκBα from the NF-κB heterodimers, thereby exposing the nuclear localization sequence and allowing nuclear translocation (1, 2, 6). Until recently, on the basis of *in vitro* phosphorylation studies (6, 8), IκBα dissociation was thought to be due to its phosphorylation. New *in vivo* data indicate that IκBα phosphorylation is indeed critical to the dissociation and nuclear translocation of NF-κB: treatment of cells with various reagents that block IκBα phosphorylation (9–13) or site-directed mutagenesis of certain putative phosphorylation sites (14, 15) abolishes NF-κB activation. However, IκBα phosphorylation is not sufficient to release NF-κB. NF-κB enters the nucleus only upon degradation of the inhibitor (9–13, 16–18). Pretreatment of stimulated cells with protease inhibitors, particularly certain peptide aldehydes, preserves the stimulation-induced, phosphorylated IκBα (pIκBα) in a complex with RelA (p65) and p50 in the cytoplasm and thus inhibits NF-κB activation (11–13, 18). There is a good correlation between the capacity of such peptide aldehydes to block stimulation-dependent IκBα proteolysis and their potency as proteasome blockers (12, 13, 19–21); yet there is no direct proof implicating the proteasome in pIκBα degradation. Furthermore, the mode of substrate recognition has not been addressed—i.e., does the protease distinguish between pIκBα and IκBα; is the substrate directly recognized by the protease or does it need to be further modified before being degraded. We show that the stimulation-dependent pIκBα is indeed targeted by the proteasome in a specific manner, but must undergo multiubiquitination prior to degradation.

MATERIALS AND METHODS

Cell Culture and Reagents. Jurkat T cells were grown in RPMI 1640 medium, C_3F_6 B cells and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM), and the Chinese hamster cell line E36 and its mutant clone E36ts20 (ts20) (22) were grown at 37°C and 33°C, respectively, in minimal essential medium (MEM). Recombinant human tumor necrosis factor α (TNF-α; Genzyme) was used at a concentration of 200 units/ml, recombinant mouse interleukin 1β (IL-1β; Boehringer Mannheim) was used at 100 units/ml, *N*-acetyl-Leu-Leu-norleucinal (ALLN; Sigma) was used at 100 μM, and benzyloxycarbonyl-Leu-Leu-phenylalaninal (ZLLF; Signal Pharmaceuticals, San Diego) was used at 6 μM.

Protein Extracts and Purification. Rabbit reticulocyte lysates were prepared and fractionated on DEAE cellulose as described (23). Proteolytic extracts were prepared from unstimulated C_3F_6 B cells and Jurkat T cells essentially as described (12), except omitting Nonidet P-40 and protease inhibitors.

***In Vitro* Proteolysis Assay.** The substrate IκBα was immunopurified in a complex with p50 and RelA by using RelA antiserum (13). Rabbit antiserum to Ltk (12) was used as a

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Abbreviations: pIκBα, phosphorylated IκBα; TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; MeUb, methylated ubiquitin; ATPγS, adenosine 5-γ-thiotriphosphate; ALLN, *N*-acetyl-Leu-Leu-norleucinal; ZLLF, benzyloxycarbonyl-Leu-Leu-phenylalaninal.

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control for nonrelevant immunocomplexes. Washed immunoprecipitates were added to a 50-μl proteolytic reaction mixture (23) supplemented with 20 mM *p*-nitrophenyl phosphate, 20 mM glycerol 2-phosphate, and 20 nM okadaic acid. Reticulocyte extract (30 μl); reticulocyte lysate fractions Fr II (160 μg), Fr I (1600 μg), or E2-F1 [purified from Fr I (24); 0.1–0.5 μg]; or C_3F_6 or Jurkat cell cytoplasmic extract (50 μg) was then added, and the reaction mixtures were incubated for 2 h at 37°C. Following incubation, washed precipitates were analyzed by immunoblotting with anti-IκBα.

***In Vitro* Ubiquitin-Conjugation Assay.** Cytoplasmic extract (25 μg) prepared from either E36ts20 or the parent E36 cells was added to a 20-μl reaction mixture containing 50 mM Tris·HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM dithiothreitol, 3×10^6 cpm of ^{125}I-labeled ubiquitin, and with or without 4 mM adenosine 5′-γ-thiotriphosphate (ATPγS). Following incubation for 1 h at 37°C, the reaction mixtures were separated by SDS/PAGE.

Immunoblotting. Ubiquitin conjugates, IκBα forms, and p65 were detected by immunoblotting (12) with specific rabbit antisera (13, 25).

RESULTS

Following a short stimulation with phorbol ester and calcium ionophore, a ternary complex composed of p50, RelA (p65), and newly-phosphorylated IκBα (pIκBα) was isolated from T-lymphocyte cytoplasmic extracts by using either anti-p65 or anti-p50 antibodies (12). In the presence of the peptide aldehyde inhibitor ALLN, the recovered complex contained both pIκBα and IκBα (Fig. 1, lane 1). This is consistent with the recent observation that IκBα phosphorylation is not sufficient to induce the inhibitor's dissociation from the latent NF-κB. On the basis of this finding, we and others hypothesized that stimulation-dependent phosphorylation of IκBα is required to target IκBα for degradation (9–13, 17, 18). To test this hypothesis, we subjected the immunopurified ternary complex to *in vitro* proteolysis. The degradation system contained cytosolic extracts derived from either B (C_3F_6) or T (Jurkat) lymphocytes or from the well-characterized reticulocyte lysate system (23). Incubation of the IκBα complexes with ATP-depleted proteolytic extracts did not lead to significant IκBα degradation (Fig. 1, lanes 1, 2, 5, 6, 9, and 11). In contrast, incubation of the complexes in the presence of ATP resulted in the specific degradation of pIκBα (Fig. 1, lanes 3, 7, 10, and 12), independent of the pIκBα to IκBα ratio. Although IκBα from unstimulated cells is phosphorylated to some extent under basal conditions (ref. 13 and our unpublished results),

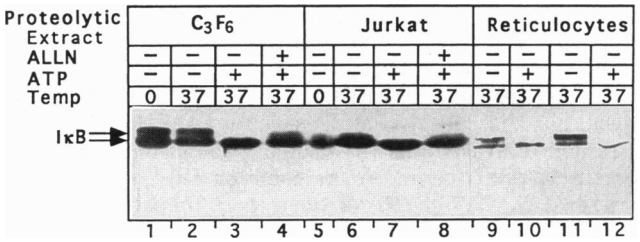

FIG. 1. Stimulation-dependent pIκBα is specifically degraded by an *in vitro* proteolytic system and requires ATP. ATP-dependent selective pIκBα degradation by various proteolytic extracts. The substrate IκBα was immunopurified in a complex with p50 and RelA by using RelA antiserum from 0.5–1 mg of cytoplasmic extract prepared from ALLN-treated, stimulated (15 min) Jurkat cells (12). The immunocomplexes were incubated for 2 h at 37°C with the indicated proteolytic extract in the absence (−) or presence (+) of ATP and an ATP-regenerating system (23) with or without the addition of 50 μM ALLN, as indicated. Controls were incubated on ice (lanes 1 and 5). Western blot analysis with anti-IκBα serum: upper arrow indicates pIκBα; lower arrow, IκBα.

this form was neither degraded nor did it interfere with pIκBα degradation. Therefore, it is the inducible hyperphosphorylation of IκBα which marks the protein for degradation in an ATP-dependent manner. *In vivo* experiments demonstrated that pIκBα is stabilized by the presence of certain peptide aldehyde proteasome inhibitors but not by calpain inhibitors, thus advocating the involvement of the proteasome in pIκBα proteolysis (11–13, 18, 20). This finding was substantiated in our *in vitro* experiments where one such inhibitor, ALLN (19, 21), afforded considerable (>50%) protection against pIκBα proteolysis *in vitro* (Fig. 1, lanes 4 and 8), whereas pIκBα proteolysis was not affected by calpain inhibitors (data not shown). Proteasome activity requires ATP and is blocked by ATPγS (26). Indeed, substitution of ATP with ATPγS inhibited pIκBα proteolysis (data not shown). Collectively, our results indicate that the 26S proteasome is responsible for pIκBα degradation. Since the proteasome can operate in tandem with or independent of the ubiquitin system (27), it was important to address the role of ubiquitin-conjugation in pIκBα proteolysis.

To this end, we fractionated reticulocyte extracts on DEAE cellulose (23) and examined the effect of the unadsorbed material (Fr I) and the high-salt eluate (Fr II) on pIκBα degradation. Fr I, which contains ubiquitin and a few components of the ubiquitin system but lacks proteasome (23), had no effect on pIκBα proteolysis (Fig. 2*A*, lane 1). Fr II contains the 26S proteasome and many of the ubiquitin-conjugating enzymes, including the ubiquitin-activating enzyme E1, several ubiquitin-conjugating enzymes (E2s), and the three known E3 ubiquitin ligases (27). Yet, it did not affect pIκBα degradation when supplemented with ubiquitin (Fig. 2*A*, lane 4). On the other hand, the combination of Fr II and Fr I induced the specific and efficient degradation of pIκBα in an ATP-dependent manner, sparing both the unmodified IκBα and p65 (Fig. 2*A*, lanes 2 and 3). The finding that Fr I was necessary

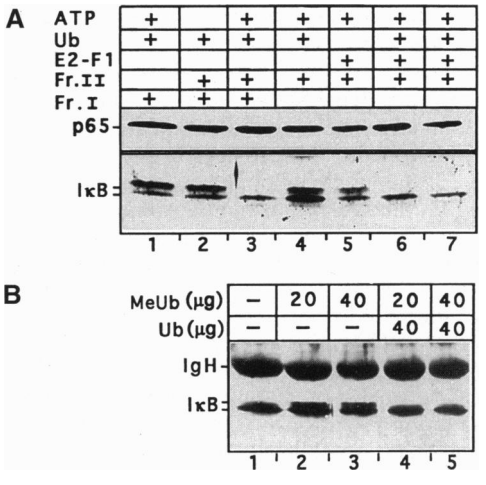

FIG. 2. *In vitro* proteolysis of pIκBα via the ubiquitin pathway. (*A*) pIκBα degradation requires specific ubiquitin-proteasome components derived from rabbit reticulocyte lysate. IκBα immunocomplexes from stimulated cells were mixed with Fr I (lane 1), Fr II (lane 4), both Fr I and Fr II (lanes 2 and 3), or Fr II supplemented with either 0.5 μg (lanes 5 and 6) or 0.1 μg (lane 7) of E2-F1. The mixtures were incubated in the absence or presence (+) of ATP and an ATP-regenerating system, with (+) or without exogenous ubiquitin (Ub). The samples were Western blotted and analyzed with either anti-RelA (p65) serum or anti-IκBα serum. (*B*) pIκBα degradation requires polyubiquitination. IκBα immunocomplexes from stimulated cells were mixed with a combination of Fr I and Fr II, without (lane 1) or with (lanes 2–5) the indicated amounts of methylubiquitin (MeUb) and with the indicated amounts of native ubiquitin (Ub) (lanes 4 and 5) and analyzed by Western blotting with anti-IκBα serum. IgH, immunoglobulin heavy chain.

to complement Fr II while ubiquitin alone was insufficient indicates that the poor proteolytic capacity of Fr II is due not only to the lack of ubiquitin but also to the absence of an additional component(s). One such component could be the recently purified ubiquitin-conjugating enzyme E2-F1 (24). This 18-kDa Fr I protein facilitates ubiquitin-dependent degradation of p53 and of some other non-"N-end rule" protein substrates (24, 27). Indeed, when supplemented with E2-F1 purified to homogeneity and ubiquitin, Fr II induced efficient degradation of pIκBα (compare Fig. 2*A*, lane 5 without ubiquitin with lanes 6 and 7 with ubiquitin). The specific activity of E2-F1 was at least 16,000-fold greater than that of Fr I in pIκBα degradation, with 0.1 μg of E2-F1 as effective as the amount of Fr I necessary for complete pIκBα degradation (1600 μg) (Fig. 2*A*, lanes 3 and 7). Reconstitution of the specific proteolytic activity by Fr II and purified E2-F1 does not rule out the possibility that additional E2 enzymes are involved in the process. Once highly specific antibodies against E2-F1 are available, it should be possible to deplete E2-F1 from Fr I and assess the residual proteolytic activity. Nevertheless, it is clear that pIκBα degradation requires ubiquitin and specific components of the ubiquitin system.

We addressed the need for pIκBα polyubiquitination by using MeUb in the proteolytic reaction (28). MeUb, in which all the lysine residues serving as potential polyubiquitination sites are modified, competes with the endogenous ubiquitin for the substrate and does not promote the formation of high molecular weight substrate–ubiquitin conjugates. In fact, MeUb works as a chain terminator in the polyubiquitin reaction. Low molecular weight ubiquitin conjugates produced in the presence of MeUb are poor substrates for the 26S proteasome (28). Indeed, in the presence of MeUb, we found nearly complete inhibition of pIκBα degradation (Fig. 2*B*, lanes 2 and 3). Inhibition was alleviated upon the addition of excess native ubiquitin (Fig. 2*B*, lanes 4 and 5), a finding in accordance with previous reports that show a reversal of the MeUb inhibitory effect on proteolysis (28) and with our own results that demonstrate the reversal of its chain-terminator effect in the polyubiquitination reaction (data not shown).

On the basis of the ubiquitin dependence observed in the *in vitro* experiments, we examined the role of the ubiquitin system in pIκBα degradation *in vivo*. In the presence of proteasome inhibitors, pIκBα accumulates in stimulated cells at the expense of IκBα (11–13, 18, 20). Polyubiquitin conjugates are mostly transient adducts with an extremely short half-life due to the combined action of the proteasome and isopeptidases (23, 29). Using ubiquitin-specific antibodies (25), we found accumulation of high molecular weight protein–ubiquitin conjugates in proteasome-inhibited HeLa cells (Fig. 3*C*). If pIκBα undergoes polyubiquitination *in vivo*, and provided that the rate of polyubiquitination exceeds that of ubiquitin trimming by the isopeptidases, then pIκBα polyubiquitin conjugates would be expected to pile up in proteasome-inhibited cells. Indeed, in the presence of ZLLF, one of the most efficient proteasome inhibitors (19), there was stimulation-dependent accumulation of high molecular weight IκBα cross-reactive proteins (Fig. 3*A*). Although it was not possible to immunoprecipitate these high molecular weight proteins, probably due to the complexity of the multiubiquitin coat, several features attest to the fact that these proteins most likely represent pIκBα–polyubiquitin conjugates. (*i*) Their accumulation was strictly dependent on the IκBα-stabilizing effect of the inhibitor (Fig. 3*A*). (*ii*) These proteins were not detected prior to stimulation and their level of accumulation peaked 12–15 min poststimulation, corresponding to the maximal conversion of IκBα to pIκBα (Fig. 3*B*). The transient nature of polyubiquitin adducts enables their detection only upon very long exposure of the autoradiogram (compare the intensity of the nonconjugated IκBα signal in Fig. 3 *A* and *B*). Under such long-term exposure, other cross-reactive bands appeared but were mostly

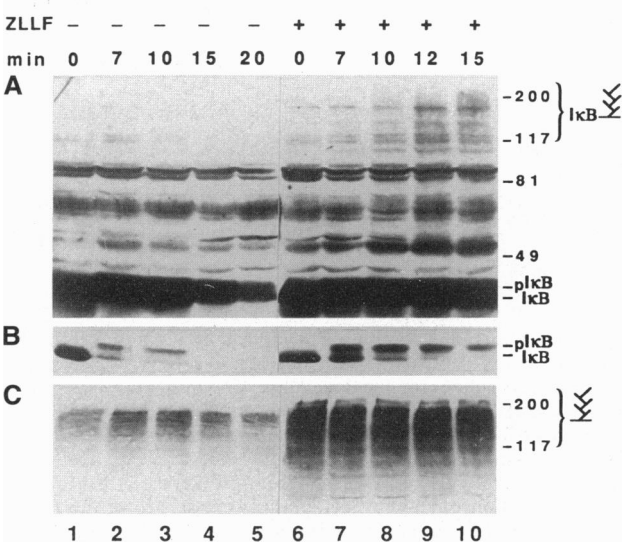

FIG. 3. Stimulation-dependent accumulation of high molecular weight IκBα forms in the presence of the proteasome inhibitor ZLLF. Following preincubation with (lanes 6–10) or without (lanes 1–5) ZLLF, HeLa cells were stimulated with TNF-α for the indicated intervals (min). (*A*) Anti-IκBα immunoblot (prolonged exposure). IκBα (IκB), pIκBα (pIκB), and the pIκBα-ubiquitin conjugates (marked by a symbol of a tree) are indicated. The identities of the other bands are not known. They are probably nonspecific and appear only after prolonged exposure. (*B*) Anti-IκBα immunoblot (short exposure). (*C*) Anti-ubiquitin immunoblot. Polyubiquitinated proteins are indicated by a symbol of a tree.

unaffected by ZLLF and were not influenced by stimulation. (*iii*) In the presence of ZLLF, the molecular weight spectrum of the stimulation-induced IκBα cross-reactive proteins corresponded to the spectrum of random polyubiquitinated proteins, as detected with the aid of anti-ubiquitin serum (Fig. 3*C*). However, while there were hardly any high molecular weight bands reactive with anti-IκBα before stimulation, the anti-ubiquitin reactive bands accumulated independent of cell stimulation.

To confirm the ubiquitin dependence of pIκBα proteolysis *in vivo*, we tested stimulation-dependent IκBα degradation in the E36ts20 cell line, which carries a temperature-sensitive mutation in the E1 ubiquitin-activating enzyme (22). Although the mutant cells must be maintained at 33°C, at 37°C the mutant E1 enzyme is active for at least several hours (unpublished results). At 37°C, stimulation-dependent IκBα degradation in the mutant cell line (induced by IL-1) was similar in magnitude and kinetics to that observed in the wild-type parent line, E36 (Fig. 4*A*). Following a 45-min incubation at the nonpermissive temperature (43°C), the signaling of IκBα degradation in both cell lines declined but recovered within 2 h when cells were returned to 37°C. Both cell lines responded to IL-1 by degrading IκBα; upon blocking the degradation with the proteasome inhibitor ALLN, both cell lines accumulated stimulation-induced pIκBα (data not shown). However, the response of the mutant cell line at the nonpermissive temperature was clearly different from that of the wild type. Whereas the wild-type cells responded to IL-1 by degrading 80% of the IκBα by 11 min (Fig. 4*A*, lane 2) (as determined by densitometric analysis using p65 as an internal control), no degradation was evident in the E1 mutant cells at the corresponding time (lane 5). Upon long fluorographic exposure (data not shown), some accumulation of pIκBα was observed at 11 min in the mutant cells, while there was no trace of pIκBα in the wild-type cells. After 25 min of stimulation, IκBα degradation was evident in both cell lines, although it was somewhat more efficient in the wild-type cells (Fig. 4*A*, lanes 3 and 6). The most significant difference in the IκBα response between the

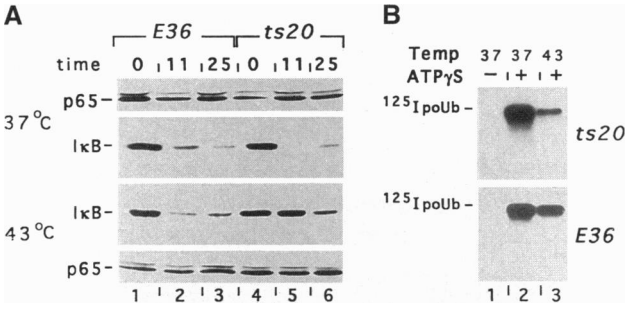

FIG. 4. Functional ubiquitin-activating enzyme E1 is essential to *in vivo* stimulation-dependent IκBα degradation. (*A*) E36ts20 and the parental E36 cells were incubated for 45 min at 37°C (permissive temperature) or at 43°C (nonpermissive temperature). Following a 2-h recovery at 37°C, cells were stimulated with IL-1 for the indicated intervals (min), and their cytoplasmic extracts were analyzed by immunoblotting with anti-IκBα and anti-p65 sera. (*B*) *In vitro* ubiquitin-conjugation capacity of cytoplasmic extracts from the two cell lines after culture at either the permissive (37°C) or the nonpermissive temperature (43°C). Conjugation reactions were carried out in the presence of ^{125}I-labeled ubiquitin with (+) or without (−) ATPγS. The positions of ^{125}I-labeled polyubiquitinated proteins (^{125}IpoUb) are indicated.

mutant and wild-type cells is therefore manifested by the kinetics of IκBα degradation, which is remarkably delayed in the E36ts20 cells at the nonpermissive temperature. The ubiquitin-conjugation capacity of the E1 mutant cell line at 37°C is at least as good as that of the parental line (Fig. 4*B*, lane 2). After incubation at 43°C and 2 h of recovery at 37°C, the conjugation capacity of the mutant cells dropped to 15% of their capacity at 37°C, whereas the wild-type cells maintained 65% conjugation capacity (Fig. 4*B*, lanes 2 and 3). Therefore, although stimulation-induced IκBα proteolysis does occur in the E1 mutant cells, it is far less efficient. Collectively, the results of the two *in vivo* experiments confirm the involvement of the ubiquitin–proteasome pathway in stimulation-dependent pIκBα proteolysis, as observed in our *in vitro* proteolysis experiments.

DISCUSSION

NF-κB is a transcription regulator retained outside the nucleus in most resting cells but rapidly translocated into the nucleus upon cell stimulation (1, 2). Mobilization of NF-κB is mostly associated with two posttranslational events that result in the targeting of the inhibitory IκBα subunit: specific phosphorylation of IκBα, supported by the slower migration of pIκBα in SDS/polyacrylamide gels, and subsequent IκBα degradation, allowing NF-κB translocation into the nucleus (2). Although recent experiments indicate that such translocation can be abolished by blocking either the inducible phosphorylation step or the subsequent proteolysis (9–18, 20), the relationship between the two events has not been clarified. Likewise, the nature of the proteolytic system which targets IκBα following its stimulation-dependent phosphorylation has not been resolved, since the protease inhibitors that block IκBα degradation are not strictly specific (30). Moreover, the observed facilitated proteolysis of IκBα could also be attributed to concomitant activation of the protease system.

To address these questions, an *in vitro* proteolysis system was constructed which could distinguish, with high fidelity, between the two closely related ternary complexes isolated from stimulated cells: one containing newly phosphorylated IκBα (pIκBα) and NF-κB and the other composed of NF-κB and seemingly unmodified IκBα. Incubation of the proteolytic system, composed of cytosolic extracts of unstimulated B or T lymphocytes or of a reticulocyte extract with mixed immunopurified IκBα complexes (IκBα/RelA/p50 and pIκBα/RelA/p50), resulted in specific ATP-dependent proteolysis of pIκBα. IκBα, on the other hand, was neither degraded nor did it affect the rate of pIκBα degradation, indicating that it is not recognized by the proteolytic system. Furthermore, there is apparently no requirement for activation of the proteolytic system derived from unstimulated cells or reticulocytes. We found no advantage in using proteolytic extracts from stimulated Jurkat cells (data not shown). Although we cannot exclude modification and activation of one or more components of the proteolytic system *in vivo*, our findings in the *in vitro* system clearly indicate that the main purpose of stimulation is to modify IκBα, thereby rendering it susceptible to conjugation and subsequent degradation.

Construction of the *in vitro* proteolytic system from reticulocyte extracts allowed the addition or subtraction of different components of the system, particularly of the ubiquitin pathway. Several observations demonstrate the role of the ubiquitin pathway in pIκBα proteolysis. First, although reticulocyte Fr II contains most of the conjugating enzymes and a full assemblage of the 26S proteasome (23), it was incapable of degrading pIκBα in our experiments. Degradation required the presence of Fr I components, particularly ubiquitin and the specific ubiquitin-conjugating enzyme E2-F1. Second, pIκBα proteolysis required high-order polyubiquitination: proteolysis was abolished upon inclusion of the ubiquitin analogue MeUb, which supports only monoubiquitin conjugation (28); inhibition was alleviated by the addition of excess free ubiquitin. In an effort to detect high molecular weight pIκBα conjugates, we substituted ATPγS for ATP and added ubiquitin-aldehyde (Ubal), a specific inhibitor of certain isopeptidases to the reaction mixture (29). Under these conditions, adducts should accumulate as conjugation proceeds since degradation of conjugates by the 26S proteasome and by isopeptidases is greatly reduced (29). Nevertheless, IκBα conjugates were barely detectable (data not shown), possibly due to continuous activity of isopeptidases insensitive to Ubal (24, 29).

The role of the ubiquitin system in targeting IκBα for degradation was confirmed in two *in vivo* experiments: (*i*) In the presence of a potent proteasome inhibitor, parallel to stimulation-dependent accumulation of pIκBα, there was accumulation of high molecular weight IκBα cross-reactive proteins, most likely pIκBα–polyubiquitin conjugates. (*ii*) At the nonpermissive temperature, the ubiquitin-activating temperature-sensitive mutant enzyme (E1) was incapable of supporting the normal, high rate of stimulation-induced proteolysis of IκBα exhibited by the parental nonmutant cells. The reduced degradation rate of IκBα in the mutant cells at the nonpermissive temperature resulted in some accumulation of pIκBα, but far below that of pIκBα in the presence of proteasome inhibitors (data not shown). This is consistent with the leaky phenotype of the mutant cells in which conjugation cannot be completely inhibited. Nevertheless, this experimental system is the only available tool for studying a direct linkage between ubiquitination and degradation *in vivo*.

Collectively, our data implicate the ubiquitin–proteasome system in the specific degradation of stimulation-dependent pIκBα. The *in vivo* association of polyubiquitin conjugates with pIκBα suggests that modification of IκBα in the stimulated cell is required for polyubiquitination rather than for the subsequent degradation by the proteasome. According to this scenario, at least one component of the ubiquitin pathway recognizes the modified IκBα and stimulates its conjugation to ubiquitin. Candidate components may be a specific ubiquitin–protein ligase (E3), and/or a putative E3 assistant protein, similar to the human papillomavirus E6 protein (31). Upon acquiring sufficient ubiquitin multimers, the pIκBα–polyubiquitin conjugates become a substrate for the 26S proteasome and are degraded to completion. It is remarkable how these events target a single subunit of a ternary complex without disturbing the other subunits, which are subsequently

mobilized into the nucleus. Our studies did not address the relationship between ubiquitination and pIκBα–NF-κB dissociation. Ubiquitination could induce dissociation with subsequent degradation of ubiquitinated pIκBα. Alternatively, it is possible that the ubiquitinated pIκBα remains bound to NF-κB until specifically proteolyzed. Either way, proteolysis is essential to effective dissociation, since inhibition of the latter step *in vivo* results in stabilization of the pIκBα–NF-κB complex (11–13, 18).

Ubiquitin conjugation has been observed before in response to extracellular stimuli. The ζ subunit of the T-cell receptor underwent ubiquitination in response to receptor cross-linkage (32) and antigen-induced engagement of the immunoglobolin E receptor (FcεRI) resulted in multiubiquitination of its β and γ chains (33). Platelet-derived growth factor (PDGF) binding induced multiple ubiquitination and degradation of the PDGF receptor (34) and Kit underwent rapid polyubiquitination and degradation upon binding to its ligand Steel (35). Thus, by targeting receptors for proteolysis, ubiquitination may serve as a means of downregulation.

The ubiquitin proteasome pathway has also been implicated in several intracellular regulatory processes (27). Among these is the processing of the NF-κB1 precursor, which requires ubiquitin conjugation (20, 36, 37). However, the mode of substrate recognition has been investigated in only a few cases. A small number of proteins are targeted for conjugation and subsequent degradation via their free N-terminal amino acids (N-end rule pathway; ref. 38). Several distinct destabilizing primary sequences have also been identified, such as the "destruction box" in cyclins, where substrate phosphorylation is also involved (39–41); the δ region in JUN (42); and certain regions of the MATα2 repressor (43). However, inducible, ubiquitin-dependent proteolysis was not found to be an integral part of a signal-transduction process linked to inducible modification of the target protein in any of the above examples. The *in vitro* proteolytic system, with its remarkable specificity, provides an opportunity to identify specific components of the system responsible for recognition of the substrate. Their identification and characterization could enable specific modulation of NF-κB activity and its application to the control of inflammatory and immune responses.

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