MOLECULAR AND CELLULAR BIOLOGY, Mar. 1997, p. 1441–1449 Vol. 17, No. 3
0270-7306/97/$04.00+0

Characterization of a Mutant Cell Line That Does Not Activate NF-κB in Response to Multiple Stimuli

GILLES COURTOIS,[1] SIMON T. WHITESIDE,[1] CAROL H. SIBLEY,[2] AND ALAIN ISRAEL[1]*

Unité de Biologie Moléculaire de l'Expression Génique, UMR 321 CNRS, Institut Pasteur, 75724 Paris Cedex 15, France,[1] and Department of Genetics, University of Washington, Seattle, Washington 98195[2]

Received 5 September 1996/Returned for modification 28 October 1996/Accepted 10 December 1996

Numerous genes required during the immune or inflammation response as well as the adhesion process are regulated by nuclear factor κB (NF-κB). Associated with its inhibitor, IκB, NF-κB resides as an inactive form in the cytoplasm. Upon stimulation by various agents, IκB is proteolyzed and NF-κB translocates to the nucleus, where it activates its target genes. The transduction pathways that lead to IκB inactivation remain poorly understood. In this study, we have characterized a cellular mutant, the 70/Z3-derived 1.3E2 murine pre-B cell line, that does not activate NF-κB in response to several stimuli. We demonstrate that upon stimulation by lipopolysaccharide, Taxol, phorbol myristate acetate, interleukin-1, or double-stranded RNA, IκBα is not degraded, as a result of an absence of induced phosphorylation on serines 32 and 36. Neither a mutation in IκBα nor a mutation in p50 or relA, the two major subunits of NF-κB in this cell line, accounts for this phosphorylation defect. As well as culminating in the inducible phosphorylation of IκBα on serines 32 and 36, all the stimuli that are inactive on 1.3E2 cells exhibit a sensitivity to the antioxidant pyrrolidine dithiocarbamate (PDTC). In contrast, stimuli such as hyperosmotic shock or phosphatase inhibitors, which use PDTC-insensitive pathways, induce IκBα degradation in 1.3E2. Analysis of the redox status of 1.3E2 does not reveal any difference from wild-type 70Z/3. We also report that the human T-cell leukemia virus type 1 (HTLV-1)-derived Tax *trans*-activator induces NF-κB activity in 1.3E2, suggesting that this viral protein does not operate via the defective pathway. Finally, we show that two other IκB molecules, IκBβ and the recently identified IκBε, are not degraded in the 1.3E2 cell line following stimulation. Our results demonstrate that 1.3E2 is a cellular transduction mutant exhibiting a defect in a step that is required by several different stimuli to activate NF-κB. In addition, this analysis suggests a common step in the signaling pathways that trigger IκBα, IκBβ, and IκBε degradation.

The NF-κB/rel family of transcription factors plays a crucial role in the expression of numerous genes that participate in the immune or inflammatory response and in the adhesion processes (for a review, see references 3, 18, 51, and 59). In addition, these factors are required for the transcription of several viruses, among them human immunodeficiency virus type 1. NF-κB molecules are homo- or heterodimeric combinations of rel-related proteins. Members of this family are p50, p52, relA, relB, and c-rel (8, 9, 19, 24, 33, 37, 42–44, 46, 62). They all share a conserved domain, the rel homology domain, that participates in DNA binding and dimerization.

In most cell types, NF-κB resides in the cytoplasm in a latent form that is associated with the inhibitor IκB. IκB retains NF-κB in the cytoplasm by masking its nuclear localization signal. In response to multiple stimuli, including phorbol esters, cytokines, bacterial lipopolysaccharides (LPS), and viral products, the inhibitor is inactivated and NF-κB translocates to the nucleus, where it activates its target genes.

Three major IκB molecules, IκBα, IκBβ and IκBε, have been identified (21, 55, 61). Important progress has been made concerning the mode of inactivation of IκBα. It has been shown that the induced phosphorylation of IκBα on Ser 32 and 36 targets the molecule for ubiquitination and subsequent degradation by the proteasome complex (4, 7, 10, 11, 13, 16, 22, 32, 45, 54, 57, 60). In addition, by using proteasome inhibitors, it has been demonstrated that the phosphorylated form of IκBα remains associated with NF-κB, indicating that the proteolysis step is absolutely required to inactivate IκBα (1, 15, 17, 35, 56).

The recent cloning of IκBβ and IκBε has not yet allowed a detailed analysis of their mode of inactivation. Nevertheless, like IκBα, IκBβ and IκBε are degraded in response to stimulation (16, 31, 55, 61). In both cases, this degradation process involves the proteasome complex and requires two serine residues that are located in a sequence motif similar to that of Ser 32 and 36 of IκBα (16, 31, 61).

As indicated above, a large number of stimuli are able to activate NF-κB. However, if the final target of several of them is Ser 32 and 36 of IκBα, suggesting the involvement of a regulated kinase or phosphatase, the intermediate steps are poorly defined. Since various antioxidants are able to inhibit NF-κB activation (34, 49, 52), one of them could be redox regulated. Alternatively, oxidants themselves have been shown to activate NF-κB in certain cell types, suggesting that reactive oxygen intermediates (ROI), such as superoxides or H_2O_2, could be second messengers common to all NF-κB activators (47–49).

By analogy with procaryotic or lower eucaryotic systems, the isolation of mammalian cellular mutants, allowing a genetic analysis of the NF-κB activation process, would be very useful. Such a mutant, the murine pre-B 70Z/3-derived 1.3E2 cell line, was isolated several years ago by selecting cells unable to express surface immunoglobulin M after LPS treatment (29). When the activation of NF-κB by LPS or phorbol myristate acetate (PMA) was tested, no inducible DNA-binding activity could be detected in the nucleus. Importantly, such a defect was not a result of a problem of p50 or relA synthesis since, when the detergent deoxycholate was used, a latent form of

* Corresponding author. Mailing address: Unité de Biologie Moléculaire de l'Expression Génique, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France. Phone: 33 1 45 68 85 53. Fax: 33 1 40 61 30 40. E-mail: aisrael@pasteur.fr.

NF-κB was observed in the cytoplasmic compartment (39). The recessive nature of the defect was demonstrated by fusing 1.3E2 with wild-type (wt) 70Z/3. The resulting hybrid cell was able to activate NF-κB upon stimulation (reference 29 and unpublished results).

In this study, we have characterized the 1.3E2 cell line more thoroughly. We demonstrate that IκBα, IκBβ, and IκBε are not degraded in response to a large number of stimuli. Moreover, in the case of IκBα, we also show a lack of induced phosphorylation on Ser 32 and 36. These data suggest that 1.3E2 represents a cellular transduction mutant that is deficient in a crucial component of the NF-κB activation process. Such a component appears to be required for inducing IκBα, IκBβ, and IκBε degradation.

MATERIALS AND METHODS

Cells. wt 70Z/3 and 1.3E2 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 50 μM β-mercaptoethanol. Stable cell lines were prepared as described previously (60) with plasmids Rc/CMV-β-IκBα-wt (60) for stable cells expressing human IκBα (hIκBα), Rc/CMV-p105Rsa (24, 36) for cell lines expressing hp50, Rc/CMV-Flag-p65 (a kind gift of J. Didonato) for cell lines expressing Flag-p65, and cDNA3βIκB-ε45k (61) for cell lines expressing hIκBε.

Reagents. LPS, PMA, poly(I-C), Taxol, and D-sorbitol were from Sigma. Recombinant human interleukin-1β (IL-1β) was from Biogen (Geneva, Switzerland). Absence of endotoxin contamination in all these reagents, except LPS, was checked by a polymyxin B assay (50).

Antisera. The following rabbit antisera were used: anti-p50, 1141 and 1157 (38); anti-p65, 1226 (38); anti-IκBα, S5 (30); anti-IκBβ (55) and 30715 (59a); and anti-IκBε, 812 (61). Mouse monoclonal antibodies were anti-Flag (IBI/Kodak); anti-myc, 9E10; and antiphosphotyrosine, 4G10 (UBI).

Preparation of cell extracts. Cells were washed with phosphate-buffered saline (PBS) and then resuspended at 10^6 cells/10 μl in hypotonic solution (10 mM HEPES [pH 7.8], 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM NaF). After 10 min at 4°C, 3 μl of 10% Nonidet P-40 was added and the cells were centrifuged in a microcentrifuge for 20 s. The supernatant, containing the cytoplasmic fraction, was recovered. One volume of 2× Laemmli buffer containing 20% β-mercaptoethanol was added, and the sample was boiled for 5 min. It was kept at −20°C or analyzed immediately (see below). The nuclear pellet was briefly washed with hypotonic buffer and then resuspended in 40 μl of extraction buffer (50 mM HEPES [pH 7.8], 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol). After a 30-min incubation on ice with occasional agitation, the DNA was pelleted by centrifuging at 10,000 × *g* for 10 min. The supernatant, containing the nuclear fraction, was recovered and quickly frozen on dry ice. Samples were stored at −20°C.

Western blot analysis. Proteins from cytoplasmic extracts were fractionated on sodium dodecyl sulfate–10% polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell). After being blocked in 3% milk–PBS, the membranes were incubated in 0.3% milk/PBS containing a 1:1,000 dilution of the relevant antibody. When the 4G10 antibody was used, 5% bovine serum albumin instead of 3% milk was used. After 1 h at room temperature, the membranes were washed for 30 min with three changes in 0.05% Tween 20–PBS. Immobilized antigen-antibody conjugates were detected with horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse immunoglobulins (Biosys) in 0.3% milk–PBS. After three washes in 0.05% Tween 20–PBS, the blots were analyzed with an enhanced chemiluminescence detection system (ECL; Amersham).

Immunoprecipitations. Cytoplasmic extracts prepared from 10^7 cells were resuspended in 1 ml of TNT buffer (20 mM Tris [pH 7.5], 200 mM NaCl, 1% Triton X-100). Antibody (3 to 5 μl) was added, and the samples were gently rocked at 4°C for 1 h. A 50-μl volume of Pansorbin (Calbiochem), previously equilibrated in TNT, was added, and the incubation was continued for 30 min. After three washes in TNT (30 s in a microcentrifuge), the Pansorbin pellets were resuspended in 1× Laemmli buffer, containing 10% β-mercaptoethanol, for 15 min at room temperature to recover the antigen-antibody conjugate. After being boiled for 5 min, the samples were fractionated by SDS-polyacrylamide gel electrophoresis and analyzed as described above.

Electrophoretic mobility shift assays (EMSAs). A 5-μl volume of nuclear extracts was added to 15 μl of binding buffer [10 mM HEPES (pH 7.8), 100 mM NaCl, 1 mM EDTA, and 10% glycerol final] with 1 μg of poly(dI-dC) and 0.5 ng of ^{32}P-labeled MHC-1 probe (36) for 30 min at room temperature. For supershifting experiments, extracts were preincubated for 20 min in the same buffer with 1 μl of antibody before the probe was added. Samples were run on a 5% polyacrylamide gel in 0.5× TBE.

Transfection and luciferase assays. Cells were transiently transfected by a modified DEAE-dextran method. Briefly, cells washed in TBS were resuspended at 10^7 cells/ml in TBS containing 0.5 mg of DEAE-dextran (Pharmacia) per ml. Then 1 μg of Igκ-luc reporter plasmid (36), or total DNA when several plasmids were used, was added per 10^6 cells. After 45 min at room temperature, the cells were diluted with 10 volumes of TBS, centrifuged, and resuspended at 10^6 cells/ml in 10% FCS–RPMI. After incubation at 37°C for 24 h, the cells were stimulated for 4 to 6 h and finally lysed in a luciferase buffer (25 mM Tris-phosphate [pH 7.8], 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, 15% glycerol). Luciferase determination was carried out in a Berthold luminometer.

Analysis of ROI levels by fluorescence-activated cell sorting (FACS). Cells (10^6) in PBS were incubated with 5 μM 2′,7′-dichlorofluorescein (DCFH) diacetate (Fluka) in dimethylformamide at 37°C. Aliquots of 10^5 cells were then scanned on a FACSSORT (Beckton Dickinson) with excitation and emission settings of 495 and 525 nm, respectively (6).

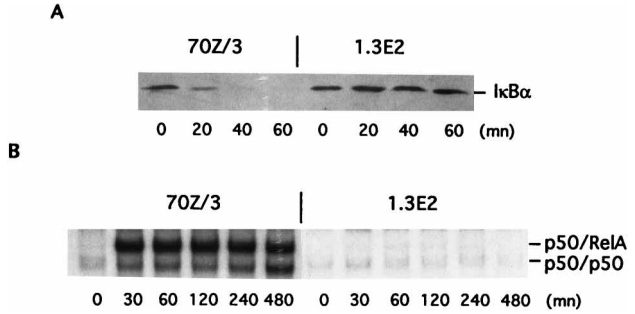

FIG. 1. LPS does not induce degradation of IκBα in 1.3E2 cells. (A) Western blot analysis of IκBα levels in wt 70Z/3 and 1.3E2 following exposure to 15 μg of LPS per ml for the indicated times. (B) Induction of NF-κB binding activity in wt 70Z/3 and 1.3E2 following exposure to 15 μg of LPS per ml. In each lane, nuclear extracts corresponding to approximately 0.5×10^6 cells were analyzed by EMSA with a ^{32}P-labeled MHC1-derived probe. The protein composition of the two inducible complexes was determined by preincubating the extracts with antibodies specifically directed against relA (no. 1226) or p50 (no. 1157) (data not shown).

RESULTS

LPS does not induce phosphorylation and degradation of IκBα in 1.3E2. It has recently been shown that degradation of IκBα is a prerequisite for NF-κB activation. We therefore analyzed the fate of IκBα after stimulation by LPS in the 1.3E2 mutant cell line. As shown in Fig. 1A, no obvious degradation of IκBα was detected after 1 h whereas the same treatment of wt 70Z/3 resulted in complete disappearance of IκBα within 40 min. Concomitantly, we did not observe any p50/relA, p50/p50 (Fig. 1B) or c-rel/relA (not shown) accumulation in the nucleus, as judged by EMSA. As a consequence, activation of a NF-κB-driven reporter plasmid appeared strongly reduced (see below, Fig. 6A).

Recent reports have demonstrated that stimulation of various cell lines with LPS, PMA, or tumor necrosis factor induces phosphorylation of IκBα on Ser 32 and 36 and that such a modification targets the inhibitor for rapid degradation by the proteasome. Although IκBα-induced phosphorylation is transient, it can be detected by using proteasome inhibitors which stabilize phosphorylated IκBα in the cytoplasm. In the case of the human protein, an upshifted band can be easily detected after migration on a denaturing SDS-polyacrylamide gel. Stable wt 70Z/3 and 1.3E2 clones expressing hIκBα (hereafter referred to as the Zhα and Ehα cell lines, respectively) were therefore prepared (Fig. 2A) and checked first for the response of the exogenous hIκBα to LPS. In each case, hIκBα responded identically to the endogenous mIκBα (Fig. 2B), indicating that IκBα was not mutated in 1.3E2. Preincubation of the cell lines with the proteasome inhibitor aLLnL, before LPS treatment, allowed the detection of a retarded phosphorylated hIκBα band in Zhα cells but not in Ehα cells (Fig. 2C, compare lanes 2 and 6).

Although this observation was strongly suggestive of a defect

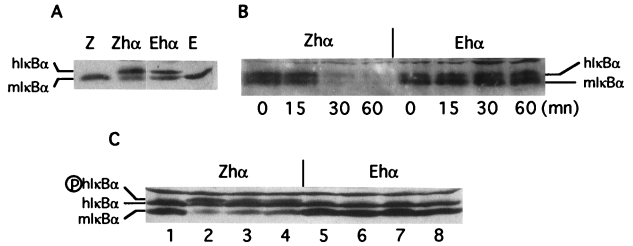

FIG. 2. LPS, PMA, and IL-1 do not induce phosphorylation of IκBα in 1.3E2 cells. (A) Western blot analysis of cell lines stably expressing hIκBα. Lanes: Z, wt 70Z/3 cells; Zhα, 70Z/3 cells expressing hIκBα; E, 1.3E2 cells; Ehα, 1.3E2 cells expressing hIκBα. The relative migrations of exogenous hIκBα and endogenous murine IκBα (mIκBα) are indicated. (B) Western blot analysis of IκBα levels in Zhα and Ehα following exposure to 15 μg of LPS per ml for the indicated times. (C) Analysis of hIκBα-induced phosphorylation in Zhα and Ehα cell lines. Cells were preincubated for 45 min with 100 μM aLLnL and then left untreated for 1 h (lanes 1 and 5) or stimulated with 15 μg of LPS per ml (lanes 2 and 6), 100 ng of PMA per ml (lanes 3 and 7), or 20 ng of IL-1 per ml (lanes 4 and 8). Cytoplasmic extracts from Zhα and Ehα were analyzed by Western blotting. The relative migrations of phosphorylated hIκBα (**P**-hIκBα), hIκBα and mIκBα are indicated on the left.

located at a step preceding IκBα phosphorylation, we could not formally dismiss the existence of a mutation in 1.3E2 at the level of p50 or relA that would affect the phosphorylation process. For instance, interaction of a kinase with p50 or relA could be required to phosphorylate IκBα. To solve this problem, we prepared stable wt 70Z/3 and 1.3E2 cell lines expressing human p50 (Fig. 3A) or tagged murine relA (Fig. 3C) and compared the fate of these exogenously introduced proteins to that of their endogenous counterparts.

An exogenous human p50 protein stably introduced either into wt 70Z/3 or into 1.3E2 cells behaved similarly to the endogenous p50; i.e., it translocated into the nucleus of 70Z/3 after LPS stimulation whereas it did not in 1.3E2 (Fig. 3B). This observation indicated that p50 was fully functional in 1.3E2. A similar conclusion was reached for relA by using a tagged protein and performing the same analysis (Fig. 3D): LPS treatment of wt 70Z/3 expressing Flag-relA resulted in a translocation of Flag-relA into the nucleus. In contrast, Flag-relA expressed in 1.3E2 did not respond to LPS.

Multiple stimuli are unable to activate NF-κB in 1.3E2. Since, in addition to LPS, a large number of stimuli are able to activate NF-κB in 70Z/3, we analyzed their effect on 1.3E2 cells. As with LPS, we monitored IκBα degradation (Fig. 4), induction of nuclear NF-κB binding activity (Fig. 5) and NF-κB transcriptional activation (Fig. 6A). Stimuli like PMA, IL-1, double-stranded RNA (dsRNA), and Taxol (26) were unable to induce IκBα degradation and induce NF-κB binding or transcriptional activities in 1.3E2. Moreover, an induced phosphorylated hIκBα band could not be observed in the Ehα

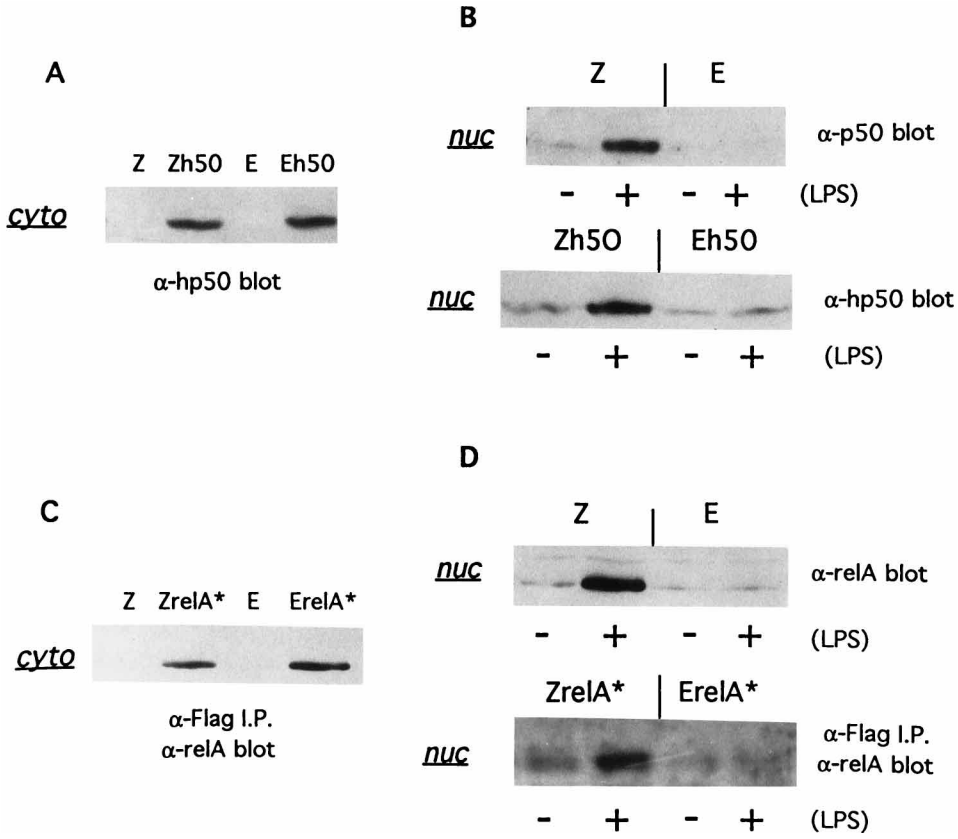

FIG. 3. p50 or relA subunits of NF-κB are functional in 1.3E2. (A) Western blot analysis of cell lines stably expressing human p50 (hp50). Lanes: Z, wt 70Z/3 cells; Zh50, 70Z/3 cells expressing hp50; E, 1.3E2 cells; Eh50, 1.3E2 cells expressing hp50. The antibody used (no. 1141) specifically recognizes hp50 but does not recognize murine p50 (mp50). (B) Nuclear accumulation of hp50 in 70Z/3 but not in 1.3E2 following LPS stimulation. A Western blot analysis of endogenous mp50 (with antiserum 1157) (upper panel) or exogenous hp50 (with antiserum 1141) (lower panel) was carried out on nuclear extracts. (C) Western blot analysis of cell lines stably expressing Flag-relA (relA*). Lanes: Z, wt 70Z/3 cells; ZrelA*, 70Z/3 cells expressing relA*; E, 1.3E2 cells; ErelA*, 1.3E2 cells expressing relA*. Cytoplasmic extracts were first immunoprecipitated with an anti-Flag antibody and then analyzed by Western blotting with an anti-relA antibody (no. 1226). (D) Nuclear accumulation of relA* in 70Z/3 but not in 1.3E2 following LPS stimulation. A Western blot analysis of endogenous murine relA (upper panel) or exogenous relA* (with antiserum 1226) after immunoprecipitation with anti-Flag (lower panel) was carried out on nuclear extracts.

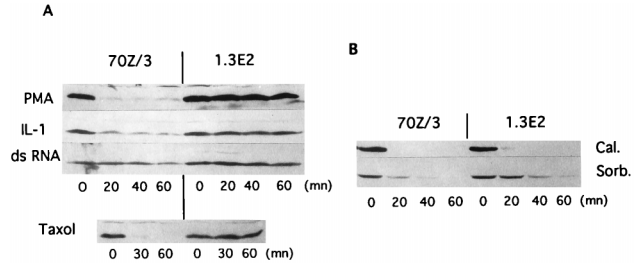

FIG. 4. Several different stimuli do not induce IκBα degradation in 1.3E2. (A) Western blot analysis of IκBα levels in wt 70Z/3 and 1.3E2 following exposure to 100 ng of PMA per ml, 20 ng of IL-1 per ml, 0.1 mg of poly(I-C) per ml, or 50 μM Taxol for the indicated times. (B) Same analysis, with 300 nM calyculin A (Cal.) or 0.4 M sorbitol (Sorb.).

clone following treatment with PMA or IL-1 (Fig. 2C, lanes 7 and 8), whereas it was clearly detected in Zhα (Fig. 2C, lanes 3 and 4). In contrast, two phosphatase inhibitors, calyculin A and okadaic acid, as well as hyperosmotic shock with 0.4 M sorbitol (14), were able to activate NF-κB in 1.3E2, although the rate of IκBα degradation by sorbitol was somewhat lower than in 70Z/3 (Fig. 4 and data not shown). Interestingly, the use of calyculin A allowed us to also show that in the mutant cell line, the degradation step per se was fully functional. Indeed, the proteasome inhibitor aLLnL blocked IκBα degradation induced by this reagent in both wt 70Z/3 and 1.3E2 (data not shown).

As indicated above, IκBα degradation triggered by LPS, PMA, or IL-1 requires inducible phosphorylation on Ser 32/36, a step that is sensitive to the antioxidant pyrrolidine dithiocarbamate (PDTC). We therefore tested the Ser 32/36 requirement and the sensitivity to PDTC of the stimuli that were still able to activate NF-κB in 1.3E2. Using a 70Z/3 cell line stably expressing a human IκBα doubly mutated on Ser 32 and 36, we observed that in addition to LPS, PMA, IL-1, and Taxol, calyculin A was unable to induce degradation of the mutant IκBα

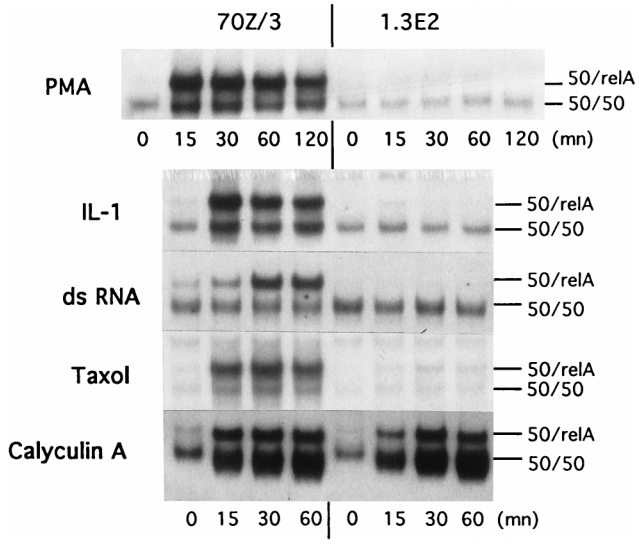

FIG. 5. Defective activation of NF-κB in 1.3E2. Induction of NF-κB-binding activity in wt 70Z/3 and 1.3E2, following exposure to 100 ng of PMA per ml, 20 ng of IL-1 per ml, 0.1 mg of poly(I-C) per ml, 50 μM Taxol, or 300 nM calyculin A was analyzed by EMSA, as described in the legend to Fig. 1B. Note the unusual migration of both p50-relA and p50-p50 heterodimers when calyculin A was used (see the text for an explanation).

molecule (Fig. 7A). In the case of dsRNA, no unambiguous involvement of Ser 32/36 was demonstrated, due to the weakness of the response to this stimulus (Fig. 4). Concerning hyperosmotic shock, we have recently shown that sorbitol does not induce IκBα degradation through Ser 32/36 in 70Z/3 (14).

Treatment of 70Z/3 cells with the antioxidant PDTC could inhibit IκBα degradation induced by all the stimuli tested except for calyculin A (Fig. 7B) and hyperosmotic shock (14). Therefore, while calyculin A requires Ser 32/36 for degradation, both calyculin A and osmotic shock are insensitive to the presence of PDTC. This indicates that the stimuli that are able to activate NF-κB in 1.3E2 do not use the redox-sensitive IκBα degradation pathway.

The viral transactivator Tax can activate NF-κB in 1.3E2. The human T-cell leukemia virus type 1 (HTLV-1)-derived Tax protein has been shown to activate NF-κB in various cells (5, 27, 41), but the way it operates remains unclear. Although some authors have reported that Tax acts by inducing IκBα degradation through Ser 32 and 36 phosphorylation (23, 28, 53), we have proposed that it could also work independently of IκBα, most probably by releasing NF-κB subunits retained in the cytoplasm by the p105 precursor of p50 (36). In support of this idea, it has recently been reported that Tax was able to increase the processing of p105 by interacting both with this precursor and with two different subunits of the proteasome, HsN3 and HC9 (40). In 70Z/3 cells, cotransfection of a NF-κB reporter plasmid with a Tax expression vector resulted in a five- to eightfold increase in luciferase production (Fig. 6B). A similar level of induction was also observed in 1.3E2, suggesting that Tax does not use the pathway that is deficient in this cell line.

IκBβ and IκBε are also resistant to degradation in 1.3E2. The recent cloning of two new IκB molecules, IκBβ and IκBε (55, 61), prompted us to analyze their fate in wt 70Z/3 and 1.3E2. In contrast to the results obtained by Thompson et al. (55), we were able to show that IκBβ could be degraded not only in response to LPS but also in response to PMA, IL-1, Taxol, and calyculin A in 70Z/3 (Fig. 8A and data not shown). We have confirmed that this corresponds to a bona fide difference between two 70Z/3 clones. When we analyzed 1.3E2, we found that all these stimuli were unable to induce IκBβ degradation. Thus, the only difference that we noticed between the fates of IκBβ and IκBα in 1.3E2 was in response to calyculin A. Nevertheless, when we tested the effect of PDTC on IκBβ degradation induced by this stimulus, we observed an inhibitory effect that had not been noticed with IκBα (Fig. 7B and data not shown). PDTC was also inhibitory for IκBβ degradation induced by LPS, PMA, and Taxol in wt 70Z/3 (data not shown).

Concerning IκBε, a more limited analysis was carried out but revealed important information. Since endogenous IκBε was difficult to detect in 70Z/3, we prepared stable wt 70Z/3 and 1.3E2 cell lines expressing a myc-tagged IκBε. As shown in Fig. 8B, whereas LPS was able to induce degradation of tagged IκBε, no such degradation was seen in 1.3E2. A similar result was obtained with PMA (data not shown). Therefore, in addition to that of IκBα, the degradation of IκBβ and IκBε is deficient in 1.3E2.

1.3E2 does not appear to be a mutant with a redox defect. Since all the stimuli that do not activate NF-κB in 1.3E2 use a redox-regulated pathway, we wondered whether the oxidative state of 1.3E2, before or after stimulation, could be perturbed. A FACS protocol, which measures the level of cytoplasmic ROIs with the DCFH dye, was used. Surprisingly, under standard culture conditions (10% FCS–RPMI), 70Z/3 cells exhibited a high ROI level (Fig. 9A) and this level was only weakly

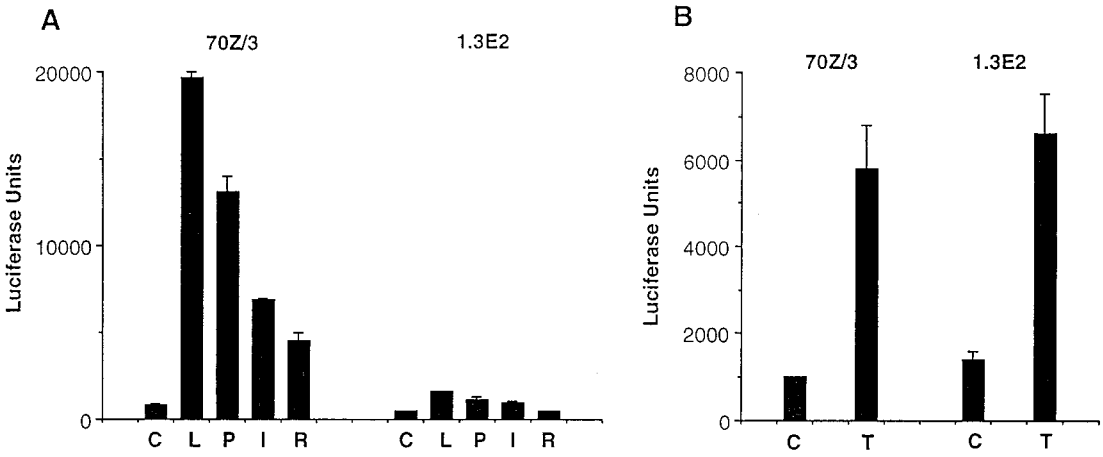

FIG. 6. (A) Defective NF-κB transcriptional activity in 1.3E2. wt 70Z/3 and 1.3E2 were transiently transfected with an Igκ-Luc reporter plasmid and stimulated 24 h later with 15 μg of LPS per ml (L), 100 ng of PMA per ml (P), 20 ng of IL-1 per ml (I), or 0.1 mg of poly(I-C) per ml (R) for 6 h. Cellular extracts were prepared, and the luciferase activity was compared to that of nonstimulated cells (C). A representative experiment is shown. For each stimulus at least four experiments, in duplicate, were carried out. (B) The HTLV-1-derived Tax *trans*-activator activates NF-κB in 1.3E2. wt 70Z/3 and 1.3E2 were transiently transfected with an Igκ-Luc reporter plasmid, together with either an empty expression vector (C) or a vector expressing Tax (T) (36). The luciferase activity of cellular extracts was determined 24 h later. A representative experiment is shown. Three other experiments were carried out in duplicate and gave similar results.

modified by H_2O_2 whereas it remained unaffected by PMA (Fig. 9B). A similar observation was made with 1.3E2 cells, excluding the possibility that the basal redox state of the mutant cell was perturbed. To analyze a putative production of ROIs during the stimulation process, we tried to grow 70Z/3 and 1.3E2 cells in 2% FCS–RPMI. This treatment resulted in a large drop in the internal ROI level in both cell lines (Fig. 9B, compare bars 1 and 6 to bars 3 and 8). Importantly, this ROI level could not be substantially modified by PMA or IL-1 (lanes 4 and 9 and data not shown) whereas it could be strongly increased by 500 μM H_2O_2 (lanes 5 and 10). In this case, a level similar to that observed with cells grown at high serum concentrations was obtained. Such an observation allowed us to investigate whether an oxidative stress was able by itself to activate NF-κB in 70Z/3 or to correct the defect of 1.3E2, by preincubating the cells with H_2O_2 before adding LPS. In neither case were we able to detect any induced NF-κB binding activity, as judged by EMSA, or induced transcriptional activity, as judged by a luciferase assay (data not shown). These studies, therefore, did not reveal any redox perturbation in 1.3E2. They also indicated that a redox stress was unable by itself to induce NF-κB.

Several other signaling cascades are active in 1.3E2. Under standard growth conditions, the growth rate of 1.3E2 is slightly higher than that of wt 70Z/3 (data not shown). This indicates that the basal metabolism of this cell line is not perturbed. To confirm this view, we analyzed various transduction cascades involving kinases of the mitogen-activated protein kinase family (for reviews, see references 12, 25, and 58). Two distinct sets of experiments were carried out. In the first set, the signal-induced tyrosine phosphorylation of ERK2 and p38 was analyzed. In 70Z/3, ERK2 and p38 can be phosphorylated on tyrosine in response to PMA and sorbitol, respectively (20). Cytoplasmic extracts were therefore prepared from stimulated wt 70Z/3 and 1.3E2 cells, run on a denaturing gel and analyzed by Western blotting with the antiphosphotyrosine antibody 4G10. As shown in Fig. 10, ERK2 and p38 were phosphorylated on tyrosine to the same extent in wt 70Z/3 and 1.3E2.

The second set of experiments used transfection of wt 70Z/3 and 1.3E2 cells with reporter plasmids carrying multicopies of the tetradecanoyl phorbol acetate (TPA)-responsive element (TRE) or serum responsive element (SRE). AP-1, the transcription factor that recognizes TRE, has been shown to respond to JNK activation (12, 25, 58) whereas TCF and SRF, the SRE-binding proteins, can be targets of ERK, p38, or JNK, depending on the stimulus tested. Despite repeated attempts, we were unable to observe any substantial activation of the TRE-Luc plasmid by PMA in wt 70Z/3, precluding a comparison with 1.3E2. In contrast, an SRE-Luc plasmid was responsive to PMA or sorbitol to similar extents in wt 70Z/3 and 1.3E2 cells (Fig. 10). These results indicated that at least two different MAPK pathways were fully functional in 1.3E2.

DISCUSSION

A hallmark of NF-κB is its remarkable capacity to respond to a wide range of stimuli. Only recently has it been shown that all these stimuli act by inactivating IκB molecules. In the case

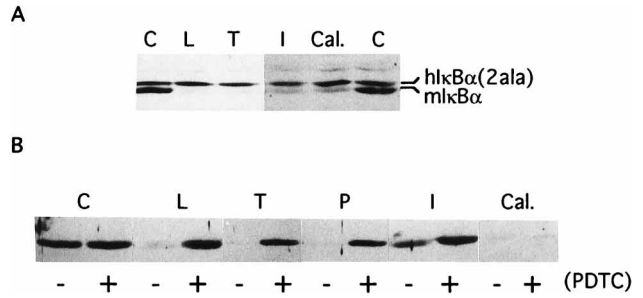

FIG. 7. The stimuli that do not activate NF-κB in 1.3E2 act on Ser 32/36 of IκBα and use a PDTC-sensitive pathway. (A) Role of Ser 32/36 in the degradation process. 70Z/3 cells expressing a doubly mutated (Ser 32/36 → Ala 32/36) hIκBα molecule were mock stimulated (lanes C) or treated with 15 μg of LPS per ml (lane L), 50 μM Taxol (lane T), 20 ng of IL-1 per ml (lane I), or 300 nM calyculin A (lane Cal.) for 1 h. Degradation of hIκBα was monitored by Western blotting. The relative migrations of exogenous hIκBα and endogenous mIκBα are indicated on the right. (B) Effect of the antioxidant PDTC on IκBα degradation. wt 70Z/3 cells were preincubated with 100 μM PDTC for 45 min and then left untreated (lane C) or stimulated with 15 μg of LPS per ml for 40 min (lane L), 50 μM Taxol for 30 min (lane T), 100 ng of PMA per ml for 20 min (lane P), 20 ng of IL-1 per ml for 30 min (lane I), or 300 nM calyculin A for 20 min (lane Cal). IκBα levels were monitored by Western blotting.

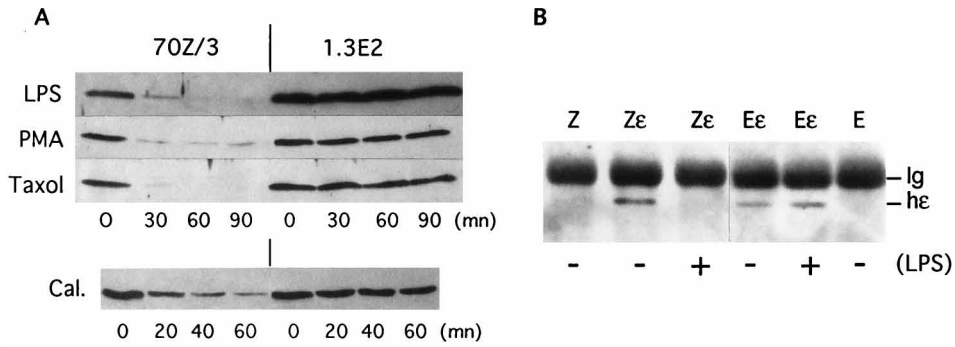

FIG. 8. Degradation of IκBβ and IκBε is defective in 1.3E2. (A) Western blot analysis of IκBβ levels in wt 70Z/3 and 1.3E2 following exposure to 15 μg of LPS per ml, 100 ng of PMA per ml, 50 μM Taxol, or 300 nM calyculin A for the indicated times. (B) Western blot analysis of myc-hIκBε levels in wt 70Z/3 and 1.3E2. wt 70Z/3 (lane Z), 1.3E2 (lane E), and cells stably expressing myc-hIκBε (lanes Zε and Eε) were pre-incubated for 45 min with 10 μg of cycloheximide per ml and then stimulated for 90 min with 15 μg of LPS per ml. Cytoplasmic extracts were immunoprecipitated with anti-ε antibody (no. 812) and analyzed with an anti-myc antibody. Ig, heavy chain of immunoglobulin G.

of IκBα, for most stimuli, inactivation proceeds according to the following sequence. First, a phosphorylation event takes place on Ser 32/36, and then the molecule is ubiquitinated on Lys 21/22 and subsequently degraded by the 26S proteasome. It is still not known whether Ser 32/36 phosphorylation results from the activation of a kinase or from the inactivation of a phosphatase. It also remains to be determined whether one or several distinct kinases (or phosphatases) participate in the IκBα phosphorylation process.

In this study, we have shown that the mutant cell line 1.3E2 does not degrade IκBα in response to LPS, Taxol, PMA, IL-1, or dsRNA. More specifically, we have demonstrated an absence of phosphorylation on Ser 32/36 after stimulation with these agents. Since neither IκBα, p50, nor relA appears to be mutated, the 1.3E2 cell line represents a transduction mutant that is defective in a step shared by at least five different stimuli. If a single kinase is responsible for Ser 32/36-induced phosphorylation, such a kinase could be mutated in 1.3E2. Alternatively, in the case of the inactivation of a phosphatase, the inactivation step could be defective. Finally, a more upstream component could be mutated but it would have to be common to all the signal transduction pathways which are inactive in inducing degradation of IκBα in 1.3E2.

The little we know about the signaling pathways that are activated in response to LPS, Taxol, PMA, IL-1, or dsRNA does not provide any obvious common intermediate. Nevertheless, activation of NF-κB by the five stimuli listed above is inhibited by preincubating the cells with the antioxidant PDTC, indicating the requirement of a redox-regulated step. Two models have been proposed to explain the effect of antioxidants on NF-κB activation. In the first, the stimuli that activate NF-κB are able to generate ROIs, and these ROIs, acting as second messengers, modulate the phosphorylation of IκBα (47–49). Antioxidants are believed to act by neutralizing such intermediates. If this model is correct, a possibility would be that LPS, PMA, IL-1, etc., act on the same molecule to generate an ROI and that this molecule is inactive in 1.3E2. In this case, it should be possible to recover NF-κB activation in 1.3E2 by artificially raising the ROI level. Unfortunately, despite our repeated attempts, such recovery was not observed by treating cells with H_2O_2.

In the second model proposed to explain the effect of anti-

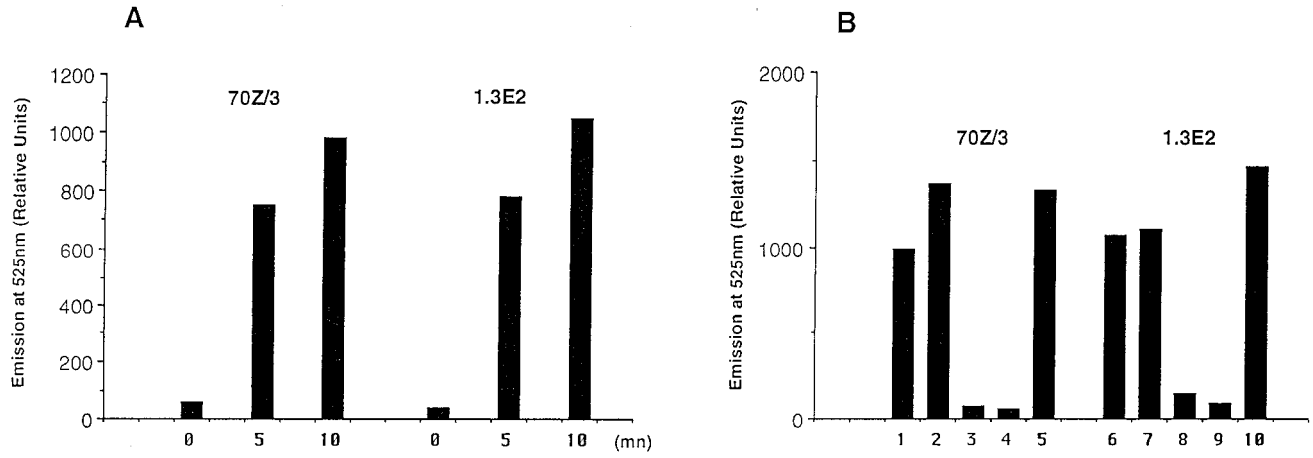

FIG. 9. Analysis of ROI levels in 70Z/3 and 1.3E2. (A) Kinetics of DCFH fluorescence. 70Z/3 and 1.3E2 cells were incubated for the indicated times with 5 μM DCFH at 37°C and then analyzed by FACS. Three experiments were performed, and the results of a representative experiment are shown. (B) Effect of growth conditions and stimulation on the ROI levels. Cells were grown in 10% FCS–RPMI (bars 1, 2, 6, and 7) or 2% FCS–RPMI (bars 3 to 5, and 8 to 10) and stimulated with 100 ng of PMA per ml (bars 4 and 9) or 500 μM H_2O_2 (bars 5 and 10) for 30 min. After a 5-min incubation at 37°C with 5 μM DCFH, the cells were analyzed by FACS.

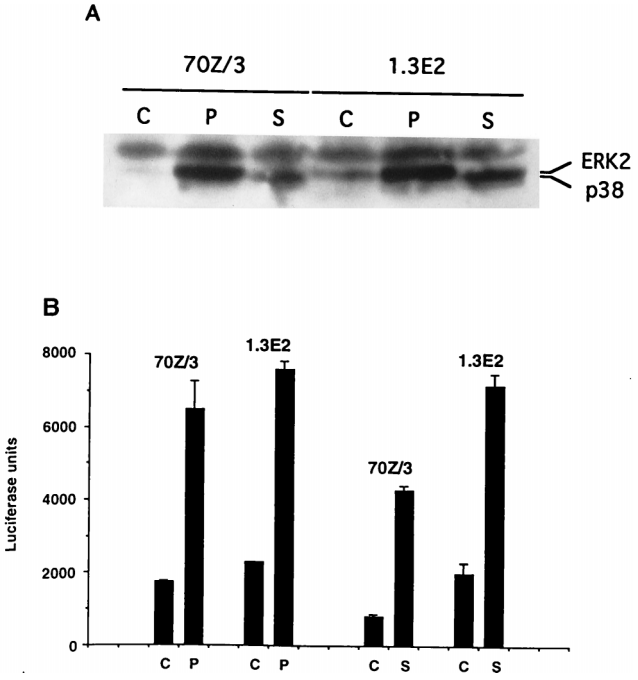

FIG. 10. MAPK activation pathways are functional in 1.3E2. (A) Tyrosine phosphorylation of ERK 2 and p38 in response to a 15-min exposure to 100 ng of PMA per ml (lanes P) or 0.4 M sorbitol (lanes S). Lanes C contain mock-stimulated cells. Cytoplasmic extracts were run on a 10% acrylamide gel and analyzed after Western blotting with the 4G10 antibody. (B) Transcriptional activation of the reporter construct SRE-Luc. SRE-Luc was transiently transfected into wt 70Z/3 or 1.3E2 and then mock stimulated (bars C) or stimulated with 100 ng of PMA per ml (bars P) or 0.4 M sorbitol (bars S). The experiment was repeated twice, with similar results.

oxidants on NF-κB activation, some undefined step in the transduction pathway that leads to IκBα degradation is thought to depend on the redox state of the cytoplasm (2). In this case, antioxidants would act by globally modifying the redox state of the cell. As presented above, we have not been able to detect any significant difference between the redox states of wt 70Z/3 and 1.3E2. We have observed that under standard growth conditions, both wt 70Z/3 and 1.3E2 exhibit the same high ROI level. All these observations, therefore, do not favor the view that 1.3E2 is a redox-deficient mutant.

Another important feature of 1.3E2 is the absence of IκBβ and IκBε degradation in response to stimuli like LPS, PMA, IL-1, or Taxol. This suggests some common requirement for the degradation of the three major NF-κB inhibitors. Although numerous questions remain as to how the IκBβ and IκBε inhibitors are inactivated, four facts are worth noting. First, degradation of the three inhibitors, in response to the stimuli listed above, is PDTC sensitive. Second, in each case, proteolysis is carried out by the proteasome complex. Third, IκBβ and IκBε exhibit a two-serine motif (Ser 19 and 23 for β; Ser 157 and 161 for ε) very similar to the one containing Ser 32 and 36 in IκBα. Mutagenesis of both serines in this motif inhibits IκBβ and IκBε degradation in response to LPS (16, 59a, 61). Fourth, in our cell system, IκBα, IκBβ, and IκBε appear to respond to the same set of stimuli (Results and data not shown), although some quantitative differences can be noted. All these observations point to a similar mode of degradation for the three inhibitors. Since we already know, from the IκBα analysis, that proteolysis by the proteasome is not defective in 1.3E2 but induced phosphorylation is abolished, a similar problem may affect IκBβ and IκBε. In this case, IκBβ and IκBε could be the targets of the same kinase(s) [or phosphatase(s)] as IκBα. Alternatively, different kinases (or phosphatases) could be recruited but their activation would be under the same control. In any case, 1.3E2 analysis reveals an unexpected functional coupling between IκBα, IκBβ, and IκBε inactivation.

Three kinds of stimuli, the osmotic shock, the phosphatase inhibitors, and the viral transactivator Tax, are still able to activate NF-κB in 1.3E2. We have shown that degradation of IκBα in response to osmotic shock does not require Ser 32 and 36 (14), suggesting an alternative mode of NF-κB activation that is still functional in 1.3E2. In the case of calyculin A and okadaic acid, the two phosphatase inhibitors we have used, our results were more surprising. We have indeed shown that IκBα degradation by both inhibitors requires Ser 32 and 36 and is proteasome dependent but is not sensitive to PDTC. How can we reconcile this observation with the data reported above? It could indicate that the phosphatase inhibitors are able to indirectly activate the kinase(s) or to directly inhibit the phosphatase(s) involved in IκBα degradation without using the PDTC-sensitive step. Consequently, the defect in 1.3E2 would be located before the Ser 32/36 phosphorylation step. Alternatively, and this is the hypothesis we favor, the action of the phosphatase inhibitors does not reflect the physiological mode of NF-κB activation. Indeed, incubation of 70Z/3 with calyculin A or okadaic acid results in a broad hyperphosphorylation of numerous cellular proteins (see the aberrant migration of p50/relA and p50/p50 dimers in Fig. 5), among them IκBα (data not shown), and such a hyperphosphorylation could inadvertently occur on Ser 32 and 36, triggering the degradation of IκBα. In this case, the translocation of p50/relA in 1.3E2 would only confirm the existence of potentially functional IκB/NF-κB complexes in the cytoplasm of the mutant cell line but would not bring any valuable information regarding the normal NF-κB activation process. Interestingly, however, calyculin A induces IκBβ degradation in wt 70Z/3 but not in 1.3E2, and PDTC blocks this degradation. This demonstrates that the PDTC-insensitive pathway that is observed with IκBα is not active on IκBβ and suggests that calyculin A can act at several levels in 70Z/3.

We have no obvious explanation for the observation that the viral transactivator Tax is fully active in 1.3E2. If, like LPS or PMA, it acts via IκBα phosphorylation, this would mean that the phosphorylation step is still functional in 1.3E2 and, consequently, would locate the defect at a step upstream. Alternatively, the NF-κB activation that we observed with Tax could result from an alternative pathway, such as a change in p105 processing (36, 40). Unfortunately, 70Z/3 cells are difficult to transfect and the transdominant effect of an IκBα doubly mutated on Ser 32/36 on NF-κB activation by Tax has not yet been possible to evaluate.

A summary of our observations is depicted in Fig. 11. We propose that in the 1.3E2 cell line, a component shared by several distinct stimuli to induce the degradation of IκBα, IκBβ, and IκBε is mutated. This component itself or the pathway in which it participates is sensitive to antioxidants. In contrast, other pathways that do not use this step are fully functional.

In conclusion, we believe that complementation of the 1.3E2 cell line will allow the characterization of an intermediate that plays a crucial role in NF-κB activation. More importantly, since 1.3E2 is fully viable, despite a perturbed NF-κB activation process, such an intermediate may represent a putative target for immunosuppressive or anti-inflammatory drugs. Finally, 1.3E2 should help to identify new targets of NF-κB by

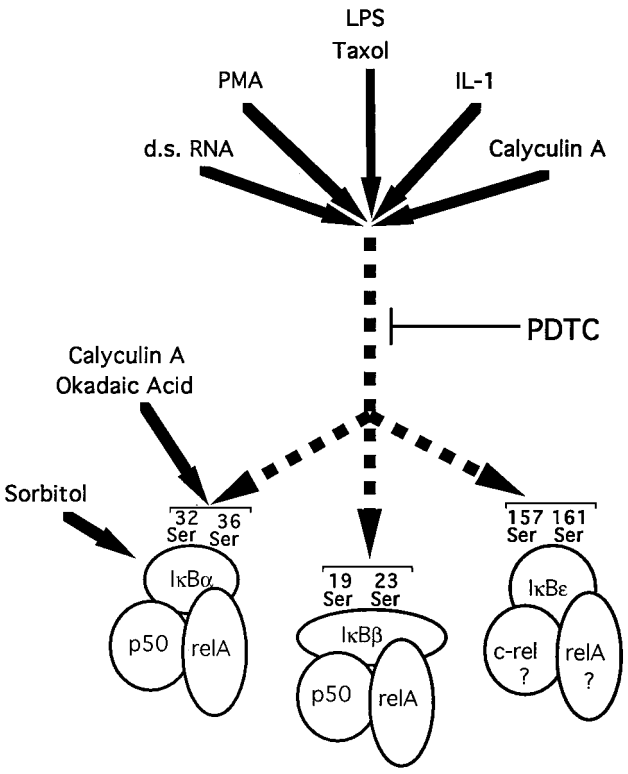

FIG. 11. Proposed location of the defect in 1.3E2. The various data reported in this study, together with some not shown, have been assembled to give a simplified overview. The dotted arrow indicates the pathway that is defective in 1.3E2. The dual effect of calyculin A is explained in the text. Moreover, to simplify the figure, dsRNA is shown to act on IκBβ and IκBε, but this point remains obscure, since this stimulus is a weak activator of NF-κB. Finally, since IκBε does not appear to interact with p50-containing NF-κB molecules (61), a tentative association with molecules like c-rel/relA has been represented.

comparing gene induction between this mutant and wt 70Z/3. Experiments are in progress to address these issues.

ACKNOWLEDGMENTS

We are grateful to R. Hay (St. Andrews, United Kingdom), for the kind gift of anti-IκBα antibody, J. DiDonato and M. Karin (UCSD, San Diego, Calif.) for the Flag-p65 expression vector and anti-IκBα antibody, R. Weil (BMEG, Institut Pasteur) and S. Ghosh (Yale University, New Haven, Conn.) for anti-IκBβ antibodies, and N. Rice (NCI Frederick, Frederick, Md.) for anti-p50 and anti-p65 antibodies. We thank R. Weil for useful discussions and help with immunoprecipitations. We also thank J. C. Epinat and F. Logeat for critical reading of the manuscript. G.C. thanks E. Muñoz (University of Cordoba, Cordoba, Spain) for encouragements and valuable comments during the initial part of this work.

S.T.W. is the recipient of a Wellcome Travelling Fellowship. This research was supported in part by grants from the ANRS, ARC, and Ligue Nationale contre le Cancer to A.I.

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