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I kappa B epsilon, a novel member of the IκB family, controls RelA and cRel NF-κB activity

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We have isolated a human cDNA which encodes a novel IκB family member using a yeast two-hybrid screen for proteins able to interact with the p52 subunit of the transcription factor NF-κB. The protein is found in many cell types and its expression is up-regulated following NF-κB activation and during myelopoiesis. Consistent with its proposed role as an IκB molecule, IκB-ε is able to inhibit NF-κB-directed transactivation via cytoplasmic retention of rel proteins. IκB-ε translation initiates from an internal ATG codon to give rise to a protein of 45 kDa, which exists as multiple phosphorylated isoforms in resting cells. Unlike the other inhibitors, it is found almost exclusively in complexes containing RelA and/or cRel. Upon activation, IκB-ε protein is degraded with slow kinetics by a proteasome-dependent mechanism. Similarly to IκB-α and IκB-β, IκB-ε contains multiple ankyrin repeats and two conserved serines which are necessary for signal-induced degradation of the molecule. A unique lysine residue located N-terminal of the serines appears to be not strictly required for degradation. Unlike IκB-α and IκB-β, IκB-ε does not contain a C-terminal PEST-like sequence. IκB-ε would, therefore, appear to regulate a late, transient activation of a subset of genes, regulated by RelA/cRel NF-κB complexes, distinct from those regulated by other IκB proteins.

Keywords: cRel/IκB-ε protein/NF-κB activity/RelA

Introduction

NF-κB is a multi-subunit transcription factor that is involved in the regulation of a large number of genes that control various aspects of the immune and inflammatory response [MHC class I genes, immunoglobulin κ light chain, interleukin 2 (IL-2) and its receptor, IL-6 and -8, granulocyte–macrophage colony-stimulating factor (GM-CSF), β-interferon, T-cell receptor β chain] as well as of several viruses, including HIV-1 (for a review, see Baeuerle and Henkel, 1994; Siebenlist *et al.*, 1994; Finco and Baldwin, 1995; Thanos and Maniatis, 1995; Verma *et al.*, 1995). In mammalian cells, NF-κB activity contains homo- and heterodimers of the rel/NF-κB family of proteins (p50 and p52, synthesized as p105 and p100 precursors respectively, p65/RelA, cRel and RelB) which specifically bind to κB motifs located in the promoters and enhancers of these target genes, resulting in transcriptional activation. All of these proteins share an ~300 amino acid region of homology known as the Rel homology domain (RHD), responsible for DNA binding, dimerization and interaction with inhibitory proteins, the IκB family. In most cell types, NF-κB is maintained in an inactive, cytoplasmic state in complexes with members of the IκB family (only B cells, some cells of the monocyte/macrophage lineage and certain neurons in the brain exhibit constitutive nuclear NF-κB activity).

The IκB proteins also comprise a distinct family (Haskill *et al.*, 1991; Beg and Baldwin, 1993; Gilmore and Morin, 1993; Thompson *et al.*, 1995): IκB-α and IκB-β, which are cytoplasmic in localization and inhibit both DNA binding and transactivation; and the product of the *bcl-3* proto-oncogene, which is nuclear and can either inhibit DNA binding or superactivate transcription via NF-κB, dependent upon the type of NF-κB complexes involved (Wulczyn *et al.*, 1992; Bours *et al.*, 1993; Fujita *et al.*, 1993; Nolan *et al.*, 1993). These inhibitors all contain multiple copies of a motif, the ankyrin repeat, which interacts with the RHD of the the rel/NF-κB proteins. In addition, the C-terminal portions of the p105 and p100 precursors of the p50 and p52 subunits of NF-κB also contain ankyrin repeats and these precursors have also been shown to behave as IκB proteins (Capobianco *et al.*, 1992; Rice *et al.*, 1992; Mercurio *et al.*, 1993; Naumann *et al.*, 1993a,b; Scheinman *et al.*, 1993; Sif and Gilmore, 1993; Sun *et al.*, 1994). Furthermore, it has been demonstrated that proteins corresponding to the C-terminal, ankyrin repeat-containing portions of p105 and p100 can be found in certain cell types and act as IκB molecules (IκB-γ and IκB-δ respectively; Inoue *et al.*, 1992; Dobrzanski *et al.*, 1995). Two other IκB-like proteins have been isolated recently, (IκB-R and IκB-L), but their association *in vivo* with NF-κB and thus their relevance to NF-κB signalling remains to be established (Albertella and Campbell, 1994; Ray *et al.*, 1995).

Treatment of cells with different inducers [including phorbol 12-myristate-13-acetate (PMA), tumour necrosis factor (TNF), IL-1, lipopolysaccharide (LPS), viral infection and many mitogens and cytokines] results in the liberation of active NF-κB and its subsequent translocation to the nucleus, there to activate transcription of its target genes. In the case of IκB-α, this inactivation of the inhibitor is a result of rapid phosphorylation and degradation of the IκB molecule (for a review, see Finco and Baldwin, 1995; Israël, 1995; Thanos and Maniatis, 1995; Verma *et al.*, 1995). Studies have demonstrated that treatment with various stimuli leads to phosphorylation on two critical serine residues (Brockman *et al.*, 1995; Brown *et al.*, 1995; Chen *et al.*, 1995; Traenckner *et al.*, 1995;

Whiteside *et al.*, 1995; Didonato *et al.*, 1996; Sun *et al.*, 1996). This process renders the molecule a substrate for ubiquitination upon, amongst others, two lysines in the N-terminus, thus targeting the molecule for degradation by the ubiquitin–26S proteasome pathway (Alkalay *et al.*, 1995; Chen *et al.*, 1995; Scherer *et al.*, 1995; Baldi *et al.*, 1996). This degradation is dependent upon C-terminal PEST sequences (Whiteside *et al.*, 1995). The IκB-β protein is also degraded following stimulation, with kinetics that vary between cell types but which are slower than found for IκB-α (Thompson *et al.*, 1995; R.Weil and A.Israël, unpublished results). The presence of corresponding serine and lysine residues in the N-terminus, and a C-terminal PEST-like region have led to the suggestion that degradation of IκB-β is controlled by a similar mechanism. Indeed, it has been demonstrated recently that mutation of the two serine residues to alanines inhibits the degradation of IκB-β in response to extracellular signalling and to intracellular Tax protein (Didonato *et al.*, 1996; McKinsey *et al.*, 1996; R.Weil and A.Israël, unpublished results).

By screening a human PBL-derived cDNA library using the yeast two-hybrid system for proteins able to interact with the p52 subunit of NF-κB, we have isolated a cDNA that encodes a novel IκB family member, which we have designated I kappa B epsilon (IκB-ε). Although the cDNA contains an open reading frame (ORF) capable of producing a 62 kDa protein, translation is initiated at a second methionine to form a 45 kDa protein which exists as multiple phosphorylated isoforms in resting cells. Sequence analysis of the cDNA reveals that IκB-ε protein strongly resembles other members of the IκB family: it contains six, closely spaced ankyrin repeats and two serine residues (at positions 18 and 22 after the second methionine) in a sequence context highly similar to those found around the serines of IκB-α and IκB-β necessary for ligand-induced degradation. It also contains a unique N-terminally located lysine (at position 6 after the second methionine), but has no PEST-like sequence in the C-terminal region. Unlike IκB-α, IκB-ε is found complexed predominantly with RelA and cRel in resting cells, and may thus control the expression of a distinct subset of NF-κB-regulated genes. Upon stimulation with PMA or LPS, IκB-ε is proteolytically degraded via a mechanism which is sensitive to antioxidants and inhibitors of proteasome activity. This degradation is preceded by a shift in electrophoretic mobility and is dependent upon the two N-terminal serine residues, but not upon Lys6.

Results

Isolation of IκB-ε cDNA

To search for novel proteins capable of interacting with NF-κB, we performed a yeast two-hybrid screen using the p52 subunit of NF-κB as bait. From 400 000 independent clones tested, 185 colonies grew on selective medium and turned blue when tested in filter lift assay. After re-screening, five of these were found to contain cDNAs which specifically interacted with the bait. Primary sequence analysis revealed these clones to encode three independent cDNAs. One of these was found to contain multiple repetitions of the ankyrin repeat sequence motif, highly related to those found in members of the IκB family. We have called this new protein IκB-ε. The two other cDNAs isolated during this screening will be described elsewhere.

Northern blot analysis indicated that IκB-ε is encoded by an mRNA of 2.2 kb (Figure 3) found in all cells thus far tested. The cDNA isolated from the two-hybrid screen was used to probe a cDNA library prepared from human fetal brain, and multiple clones were obtained. Two overlapping cDNAs were found to contain the complete ORF for IκB-ε.

Structure of IκB-ε

The primary sequence revealed an ORF of 500 amino acids encoding a protein with a predicted M_r of 55 kDa (Figure 1A) and which gives rise to a protein of 62 kDa when expressed *in vitro* or in cells (data not shown). The ORF encodes the entire fragment isolated from the two-hybrid screen.

Unlike the classical IκB-α and IκB-β, IκB-ε contains a long N-terminal extension that includes a region rich in G residues, reminiscent of the region of p105 responsible for processing (Lin and Ghosh, 1996). This region contains homology to a half-active site of the trypsin family of proteases (GDSG, amino acids 24–27), although the other half-site is not apparent. The relevance of this observation is unclear. However, closer inspection of the nucleotide sequence revealed that the first methionine in the ORF is not in a sequence context conducive for efficient translation, most noticeably the presence of a pyrimidine residue at position –3 (Figure 1B, IκB-ε MET1; Kozak, 1992). Further analysis of the ORF of IκB-ε revealed the presence of a second, in-frame ATG codon at position 140; this methionine is situated in a favourable sequence context for efficient initiation of translation (Figure 1B, IκB-ε MET140). Since we have found no evidence of a 62 kDa protein in cells, nor any to support a model in which the larger protein acts as a precursor of the 45 kDa protein which co-precipitates with rel proteins (see below and Discussion), we propose that the scanning ribosome skips over the first ATG codon and initiates translation at Met140. In support of this hypothesis, cDNAs which initiate translation at this methionine when translated *in vitro* or when expressed in transfected 70Z/3 cells give rise to proteins with a mol. wt of 45 kDa which co-migrate with the protein found bound to NF-κB complexes (see below and data not shown).

IκB-ε contains six consecutive ankyrin repeats, whose sequences closely resemble those found in other IκB proteins; the similarity between ankyrin repeats in the same position is greater than the similarity between different repeats in the same molecule (Figure 1C). It has been shown for IκB-α that its degradation is dependent upon phosphorylation on two serine residues situated N-terminally to the ankyrin repeats, and subsequent ubiquitination upon two lysine residues situated 10 amino acids N-terminally to the serines (Alkalay *et al.*, 1995; Brockman *et al.*, 1995; Brown *et al.*, 1995; Chen *et al.*, 1995; Scherer *et al.*, 1995; Traenckner *et al.*, 1995; Whiteside *et al.*, 1995; Baldi *et al.*, 1996; Didonato *et al.*, 1996; Sun *et al.*, 1996). Similar sequences have been found in IκB-β and, at least in the case of the serines, play a similar role in the regulation of its degradation in response to extracellular stimuli (Didonato *et al.*, 1996; McKinsey *et al.*, 1996).

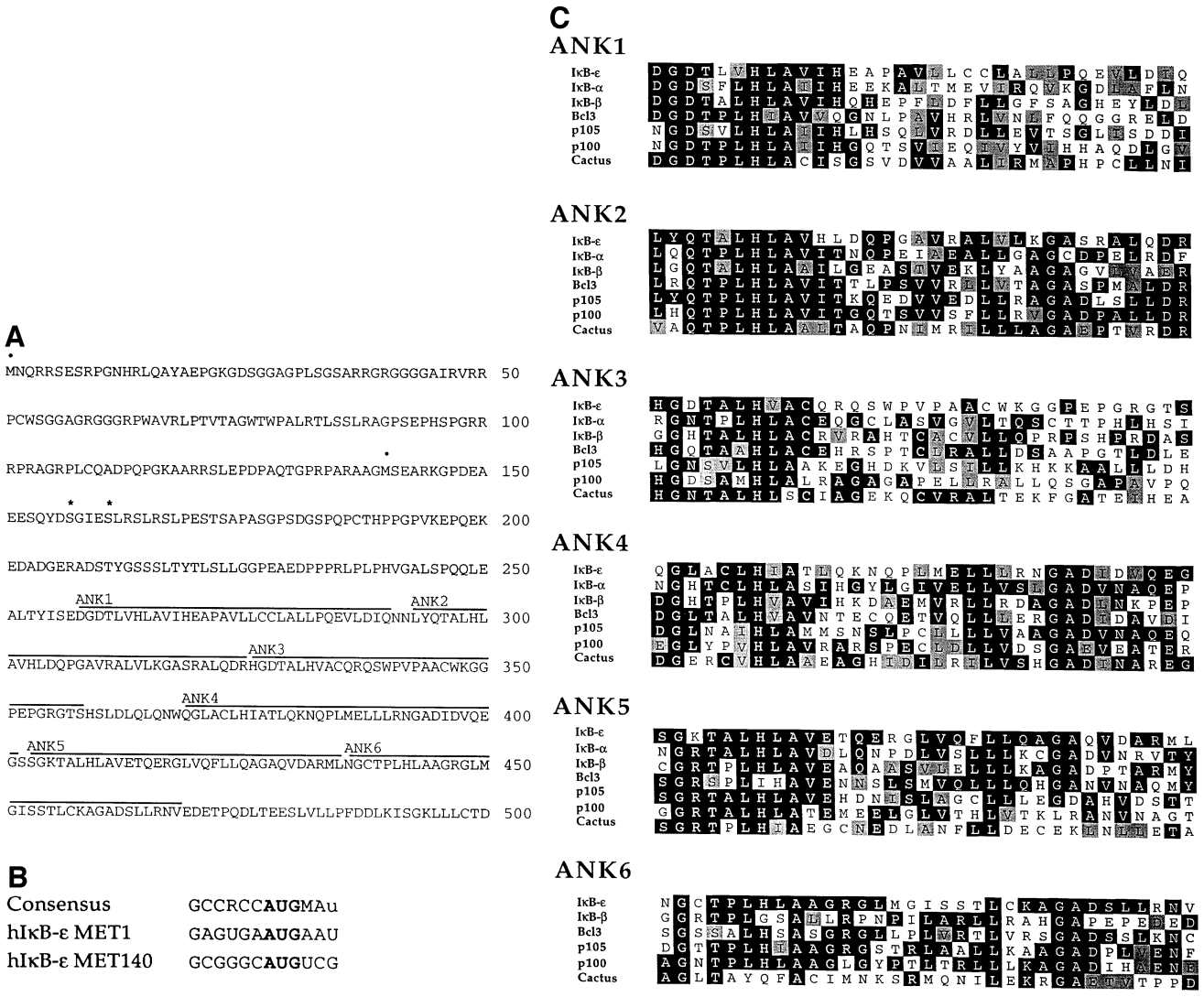

Fig. 1. Structure of IκB-ε. (**A**) Primary sequence of the IκB-ε open reading frame showing the position of the two initiator methionines (o), the two serine residues corresponding to those found in IκB-α, IκB-β and Cactus (*) and the six ankyrin repeats. (**B**) Alignment of sequences surrounding the putative initiation sites for translation of IκB-ε with the consensus for efficient initiation (Grunert and Jackson, 1994; Kozak, 1995). The AUG codons are shown in bold type. R stands for A or G, M for A or C. (**C**) Alignment of ankyrin repeat sequences of the IκB family. (**D**) Alignment of amino acids 142–169 of IκB-ε with corresponding sequences found in IκB-α (amino acids 17–44), IκB-β (amino acids 4–31) and Cactus (amino acids 59–86). Serines 32 and 36 and lysines 21 and 22 of IκB-α, as well as the corresponding residues in IκB-β, IκB-ε and Cactus, are shown by black boxes, conserved identities by heavy shading and conservative changes by light hatching.

IκB-ε also contains a pair of serines, in a similar sequence context to that found in IκB-α and IκB-β, preceded N-terminally by a lysine residue (Lys145, Figure 1D). In addition, IκB-α and IκB-β contain C-terminal regions rich in P, E, D, S and T residues, sequences implicated in the rapid turnover of proteins (Rodgers *et al.*, 1986). Indeed, this region is essential for IκB-α (Brown *et al.*, 1995; Rodriguez *et al.*, 1995; Whiteside *et al.*, 1995) and IκB-β (R.Weil and A.Israël, unpublished results) to be degraded in response to extracellular stimuli. Such a region is absent in IκB-ε, although putative PEST regions are to be found in the region N-terminal to the ankyrin repeats (amino acids 146–164, 167–190 and 208–236, PESTFIND software, Rodgers *et al.*, 1986).

IκB-ε inhibits transactivation by NF-κB

To investigate whether the IκB-ε could inhibit transactivation by NF-κB *in vivo*, cDNAs encoding IκB proteins were co-transfected with a κB-dependent luciferase reporter gene into Jurkat cells which subsequently were stimulated with either PMA and ionomycin (PMA/iono, Figure 2A) or TNF (data not shown). Levels of overexpressed IκB proteins are such that cells are unable to degrade all the exogenous IκB present, resulting in an

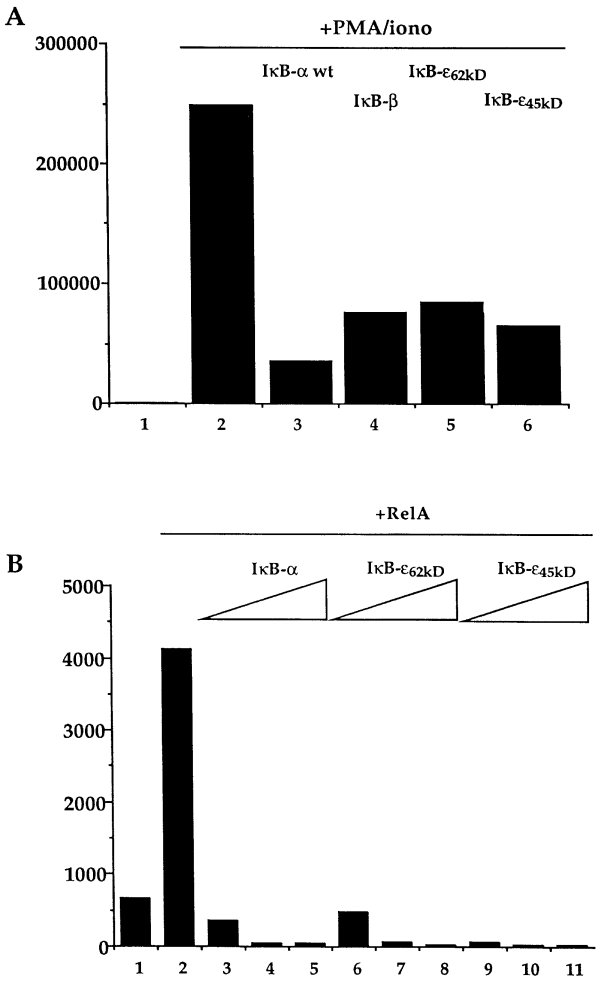

Fig. 2. Inhibition of κB-dependent transcription by IκB proteins. (**A**) Jurkat cells were transfected with 0.7 μg of the reporter plasmid Igκ-luc along with 1.2 μg of IκB-expressing plasmid as indicated. At 24 h after transfection, cells were stimulated with either 0.01% dimethylsulfoxide (lane 1) or 50 ng/ml PMA + 1 μg/ml ionomycin (lanes 2–6) for 4 h. (**B**) Jurkat cells were transfected with 0.7 μg of the reporter plasmid Igκ-luc (Munoz *et al.*, 1994) along with 0.7 μg of RcCMV (Invitrogen, lane 1), 0.7 μg of pCMV:RelA (lanes 2–14) and 0.3 (lanes 3, 6, 9 and 12), 0.6 (lanes 4, 7, 10 and 13) or 1.2 μg (lanes 5, 8, 11 and 14) of IκB-expressing plasmid as indicated. Luciferase activity was measured as described in Materials and methods and is indicated on the abscissa. The figures show the average of three independent experiments.

inhibition of induced transcription (data not shown). In the absence of co-transfected IκB protein, PMA/iono strongly transactivated the reporter gene (compare lanes 1 and 2). Co-transfection with IκB-α (lane 3), IκB-β (lane 4) and both forms of IκB-ε (lanes 5 and 6) resulted in strong inhibition of transactivation. In similar experiments using a co-transfected RelA-expressing plasmid as an activator (Figure 2B), it was clear that both forms of IκB-ε could also inhibit RelA-activated transcription (lanes 6–11) at least as efficiently as IκB-α (lanes 3–5). Similar results have been obtained for the inhibition of cRel-activated transcription (data not shown). A constitutive promoter (HSV tk) was unaffected by expression of any IκB proteins (data not shown). These experiments demonstrate that IκB-ε can efficiently inhibit the transactivation of the reporter gene by endogenous NF-κB induced with PMA/iono or by co-transfected RelA proteins. Studies in HeLa cells on the subcellular localization of transfected rel proteins in the absence or presence of co-transfected IκB-ε demonstrate that IκB-ε, like IκB-α and IκB-β, acts to retain rel proteins in the cytoplasm (data not shown).

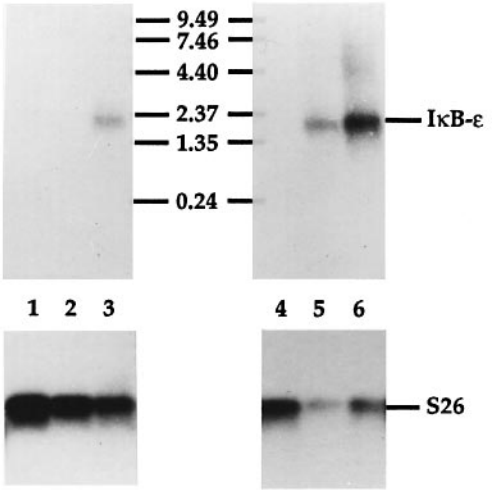

Fig. 3. Expression of IκB-ε. Poly(A)$^+$ RNA was prepared from Jurkat cells treated with 50 ng/ml PMA + 1 μg/ml ionomycin for 0 (lane 1), 30 (lane 2) and 120 min (lane 3) or from HL-60 cells (lane 4), HL-60 cells differentiated into granulocytes (4 days treatment with 1.6% dimethylsulfoxide, lane 5) or macrophages (2 days treatment with 10 ng/ml PMA, lane 6). Four μg of poly(A)$^+$ RNA was loaded per lane. The position of RNA standards is shown in the middle of the figure, and the positions of the IκB-ε (upper panel) and the S26 loading control (lower panel, Vincent *et al.*, 1993) messages are indicated.

IκB-ε mRNA is up-regulated following NF-κB induction and HL-60 cell differentiation

It has been shown that following stimulation of NF-κB, the expression of IκB-α mRNA is up-regulated, thus providing a negative feedback loop important for the transient nature of NF-κB activation. IκB-β expression is not regulated in the same manner. To address whether IκB-ε expression was regulated in a manner similar to that of IκB-α, we stimulated Jurkat T cells with PMA/iono, extracted poly(A)$^+$ mRNA at various times and analysed IκB-ε expression by Northern blot (Figure 3). The expression of IκB-ε mRNA is strongly up-regulated following 2 h of stimulation, suggesting a second, slower, negative feedback loop for tightly controlling NF-κB activity. We also studied the expression of the IκB-ε gene during the differentiation of HL-60 cells into macrophages or granulocytes. Following differentiation into granulocytes (lane 5) or macrophages (lane 6), we observed a strong induction in the expression of the IκB-ε mRNA.

IκB-ε protein exists as multiple, phosphorylated isoforms complexed predominantly to RelA and cRel proteins

For IκB-ε to function as a bona fide inhibitory molecule, it must be complexed to NF-κB molecules in resting cells. To demonstrate this, we precipitated different IκB-containing complexes from Jurkat, THP-1 and 70Z/3 cells and analysed associated proteins by Western blot with anti-cRel and anti-RelA antisera. In Jurkat, THP-1 and 70Z/3 cells (Figure 4A,

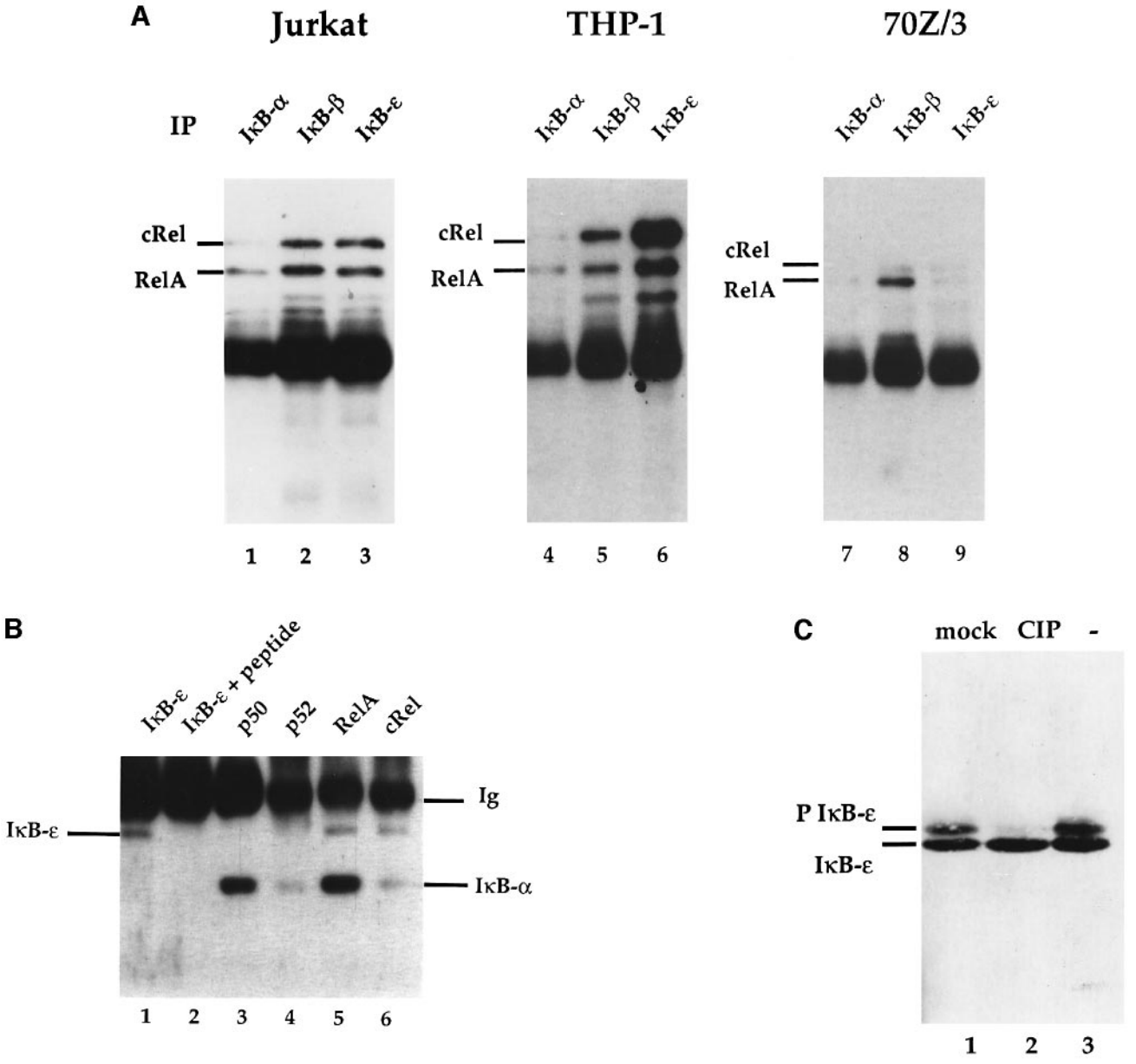

Fig. 4. (**A**) Association of RelA and cRel with IκB in the cytoplasm of resting cells. Resting Jurkat cells (lanes 1–3), THP-1 cells (lanes 4–6) or 70Z/3 cells (lanes 7–9) were lysed in hypotonic buffer containing 0.25% NP-40 and cytoplasmic extracts prepared. Extracts from 10^6 cells were precipitated with anti-IκB-α (IκBαN, lanes 1, 4 and 7), anti-IκBβ (lanes 2, 5 and 8) or anti-IκB-ε (IκBε891, lanes 3, 6 and 9), immune complexes collected and subjected to SDS–PAGE. RelA and cRel proteins were visualized by immunoblotting with RelAC and cRelC antisera. The positions of RelA and cRel proteins are indicated. In all cases, complete precipitation of the relevant IκB-containing complexes was verified by re-precipitation of the supernatant obtained from the first round of precipitation with the relevant anti-IκB antiserum and subsequent blotting with anti-RelA and anti-cRel antisera (data not shown). (**B**) IκB-ε is mostly associated with RelA and cRel in resting cells. Cytoplasmic extracts from resting THP-1 cells were precipitated with anti-IκBε (IκBε891, lanes 1), anti-IκBε blocked with its cognate peptide (IκBε891, lane 2), p50 (p105N, lane 3), p52 (p100N, lane 4), RelA (RelAC, lane 5) or cRel (cRelC, lane 6) antisera, and immune complexes blotted for IκB-ε (IκBε891) and IκB-α (IκBαS5) following SDS–PAGE. The positions of immunoglobulins (Ig) and IκB-ε and IκB-α proteins are indicated. (**C**) IκB-ε exists as multiple phosphorylated isoforms in resting cells. Cytoplasmic extracts from WEHI 231 cells were precipitated with cRelC antibodies and the immunocomplexes collected on protein A–Sepharose. Precipitates were then either mock-treated (lane 1), treated with alkaline phosphatase (lane 2) or left untreated (lane 3). Immune complexes were then eluted with cognate peptide and the eluted proteins separated on SDS–PAGE and IκB-ε proteins analysed by Western blotting with IκBε891 antiserum. The positions of IκB-ε and hyperphosphorylated IκB-ε (PIκB-ε) proteins are indicated.

lanes 3, 6 and 9 respectively), we found that both cRel and RelA could be co-precipitated with IκB-ε under native conditions. This precipitation was specific to the epitope used as no cRel nor RelA were precipitated when the antibody was blocked with an excess of its cognate peptide (data not shown). We have found IκB-ε associated with NF-κB in all cell types thus far tested (human and mouse B cells, murine pre B cells, human monocytes, granulocytes and fibroblasts; see below and data not shown). In parallel experiments, we precipitated complexes containing IκB-α or IκB-β: interestingly, in THP-1 cells (Figure 4A, lanes 4–6), the amount of cRel and RelA associated with IκB-ε is greater than that associated with either IκB-α or IκB-β (compare lane 6 with lanes 4 and 5), whereas in Jurkat cells

(lanes 1–3), the amounts of RelA and cRel precipitated by IκB-β and IκB-ε are comparable, suggesting that IκB-ε is an important regulator of NF-κB activity in these cells. In both cases, very little RelA or cRel was precipitated by IκB-α. For all three IκB proteins, secondary precipitation of the supernatant obtained after collection of the immune complexes was performed to verify that all IκB-containing complexes had been precipitated (data not shown). In contrast, a similar analysis in 70Z/3 cells (lanes 7–9) revealed that the majority of RelA (>90%) was associated with IκB-β. Both IκB-β and IκB-ε precipitated comparable quantities of cRel. Once again, IκB-α precipitated very little RelA or cRel. To analyse which other rel family proteins are associated with IκB-ε, we precipitated rel family proteins with specific anti-peptide antisera and looked for the presence of IκB-ε and IκB-α by Western blot assay. As shown in Figure 4B, antibodies raised against IκB-ε specifically precipitated a protein of 45 kDa from extracts of resting THP-1 cells (lanes 1 and 2). This protein was found in NF-κB complexes containing either RelA (lane 5) or cRel (lane 6), but not p50 or p52 (lanes 3 and 4). In contrast, IκB-α was found predominantly associated with complexes containing p50 and RelA. IκB-ε proteins are detected more weakly than IκB-α because of the weaker reaction of the IκB-ε antisera than those raised against IκB-α (at least 50-fold, our unpublished observations). We have obtained similar results using Jurkat or differentiated HL-60 cells (data not shown). In control experiments, *in vitro* co-translation of rel and IκB-ε proteins demonstrated that all antibodies used were able to interact efficiently with all IκB-ε/rel protein complexes (data not shown).

During these studies, we noticed that IκB-ε is detectable as multiple isoforms in murine cells (Figures 4C and 6). One possibility is that the upper forms represent hyperphosphorylated forms of the lower protein. To test this hypothesis, we precipitated cRel-containing complexes from WEHI 231 cells, and then treated the precipitated proteins with alkaline phosphatase. Proteins were then eluted with cognate peptide and analysed by SDS–PAGE followed by Western blotting with an anti-IκB-ε antibody. As shown in Figure 4D, the upper forms of IκB-ε associated with cRel disappear upon treatment with alkaline phosphatase (lane 2). It must be noted that the phosphorylated forms of the IκB-ε are much more readily detectable in WEHI 231 cells than in any of the human cell lines used (see Figures 3–6). The reason for this apparent discrepancy is, as yet, unclear.

IκB-ε is proteolytically degraded in response to inducers of NF-κB activity

Since IκB-ε has all the properties consistent with its function as a regulator of NF-κB activity, we went on to investigate whether IκB-ε activity was regulated during the induction of NF-κB. To this end, we prepared cytoplasmic extracts from Jurkat and THP-1 cells treated with PMA and ionomycin (PMA/iono) and bacterial LPS respectively, in the presence or absence of cycloheximide (CHX). As shown in Figure 5A (upper panel), IκB-ε is degraded in Jurkat cells by PMA/iono with relatively slow kinetics (IκB-α is degraded after 15 min, data not shown) and starts to be resynthesized after 2 h of treatment (lanes 1–5), in accordance with the results from Northern blotting studies (Figure 3). Induction in the presence of CHX abolishes this resynthesis (Figure 5A, upper panel, lanes 6–10), while CHX alone has no effect on IκB-ε protein levels, even after 2 h (lanes 11–14). In THP-1 cells (Figure 5A, lower panel and Figure 5B), treatment with LPS also led to the disappearance of IκB-ε, with kinetics similar to those found for IκB-α and IκB-β (Figure 5B). Similar results were obtained in WEHI 231 cells stimulated with LPS (data not shown). Interestingly, after 30 min of treatment with LPS, a novel, more slowly migrating form of the protein was discernible (Figure 5A, lower panel, lanes 2 and 7 and B, upper panel, lane 2), suggestive of an inducible phosphorylation taking place before degradation. After 4 h of treatment, IκB-ε levels re-increased in a CHX-sensitive manner (Figure 5A, lower panel, compare lanes 1–5 and 6–10).

Since the degradation of IκB-α and IκB-β in response to inducers of NF-κB activation has been shown to pass via the ubiquitin–26S proteasome pathway, we also tested the effects of an inhibitor of proteasome activity, *N*-acetyl-Leu-Leu-norleucinal (ALLN), upon IκB-ε degradation. Treatment with LPS/CHX for 2 h resulted in a complete disappearance of the protein in WEHI 231 (Figure 6, lane 2) and THP-1 (human pre-monocyte cells, lane 8). In all cases, this degradation was inhibited by pre-treatment of the cells with ALLN (Figure 6, lanes 3 and 6). Furthermore, the stabilized forms of IκB-ε present in extracts from cells stimulated in the presence of ALLN migrated more slowly than those present in the extracts from untreated cells, further supporting the hypothesis that IκB-ε, like IκB-α, is inducibly phosphorylated in response to extracellular signals before being degraded (see below).

IκB-ε requires serines 157 and 161 to be degraded

To study the domains of IκB-ε responsible for its ligand-induced degradation, we constructed stable 70Z/3 cell lines expressing the 45 kDa form of IκB-ε. By precipitating NF-κB complexes, we were able to show that, as is the case for THP-1 and Jurkat cells, the transfected IκB-ε was associated predominantly with RelA- and cRel-containing complexes (Figure 7, compare lanes 4 and 5 with lanes 3 and 6). In addition, we assayed whether the exogenous protein was degraded in response to inducers of NF-κB activity. As shown in Figure 8A, IκB-ε was degraded in response to treatment of cells with either PMA or LPS after 60 min of treatment. CHX was included in the induction protocol to avoid resynthesis of IκB-ε proteins as a result of induction of the cytomegalovirus (CMV) promoter used to drive expression of the transfected protein (data not shown). This inducible degradation could be blocked by pre-treatment of cells with either ALLN (Figure 8B, lane 7) or the anti-oxidant pyrollidine dithiocarbamate (PDTC) (lane 6). Identical results were obtained for cell lines expressing the 62 kDa form of the protein (data not shown).

We constructed stable cell lines expressing mutant IκB-ε proteins and analysed the ability of the transfected proteins to be degraded in response to extracellular signalling events. We have shown that IκB-ε contains at its N-terminus two serine residues (Ser157 and Ser161, or Ser18 and Ser22 after the second methionine) in a sequence context highly similar to that found in IκB-α and IκB-β (Figure 1D). We therefore mutated these two residues to alanines and analysed whether the mutated protein

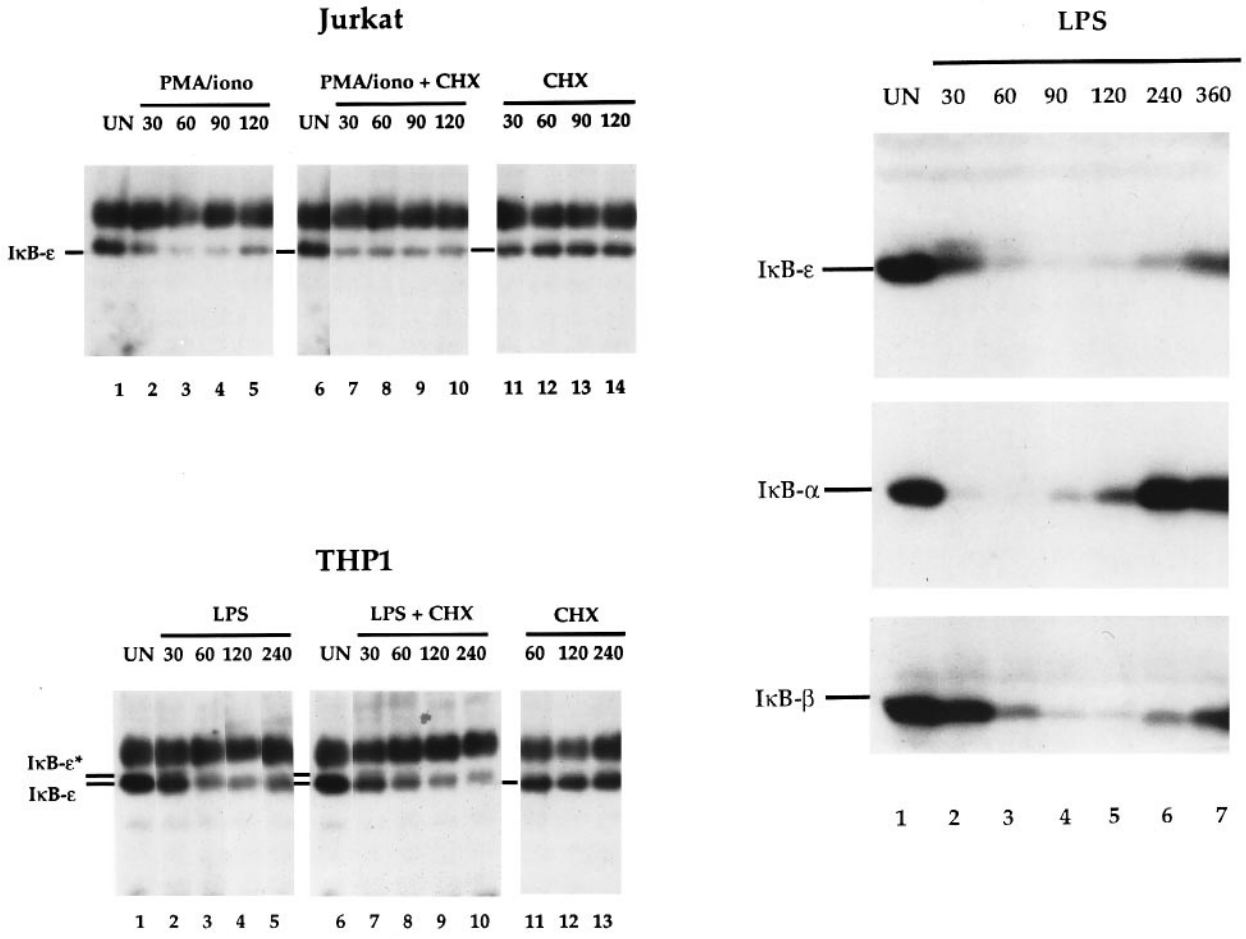

Fig. 5. Degradation of IκB-ε. (**A**) Upper panel: cytoplasmic extracts were prepared from 10^7 Jurkat cells either untreated (lanes 1 and 6) or treated with 50 ng/ml PMA plus 1 μg/ml ionomycin (lanes 2–5 and 7–10) in the absence (lanes 2–5) or presence (lanes 7–10) of 10 μg/ml CHX for the times indicated. Cells were also treated with 10 μg/ml CHX alone (lanes 11–14) for the times indicated. Extracts were boiled in 1% SDS, 0.5% β-mercaptoethanol, diluted 10-fold in TNT buffer and precipitated with anti-IκBε891. Immune complexes were precipitated, washed and then separated by SDS–PAGE. Proteins were blotted onto Immobilon and IκB-ε proteins revealed by Western blotting with IκBε891 antiserum. The positions of IκB-ε proteins are indicated. Lower panel: cytoplasmic extracts were prepared from 10^7 THP-1 cells either untreated (lanes 1 and 6) or treated with 1 μg/ml LPS (lanes 2–5 and 7–10) in the absence (lanes 2–5) or presence (lanes 7–10) of 10 μg/ml CHX for the times indicated. Cells were also treated with 10 μg/ml CHX alone (lanes 11–13) for the times indicated. IκB-ε proteins were revealed as described above. The positions of native (IκB-ε) and the more slowly migrating form of IκB-ε (IκB-ε*) are indicated. (**B**) Cytoplasmic extracts were prepared from 3×10^6 THP-1 cells either untreated (lane 1) or treated with 1 μg/ml LPS (lanes 2–7) for the times indicated above the figure. Proteins were separated on SDS–PAGE and blotted for IκB-ε (IκB-ε891, upper panel), IκB-α (IκBαS5, middle panel) or IκB-β (lower panel). Blots were revealed with [^{125}I]protein A. The positions of the IκB proteins are indicated.

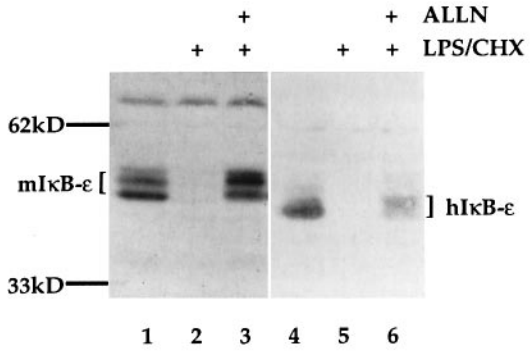

Fig. 6. Degradation of IκB-ε occurs via the 26S proteasome. Cytoplasmic extracts were prepared from WEHI 231 cells (lanes 1–3), or THP-1 cells (lanes 4–6) either untreated (lanes 1 and 4), treated with 1 μg/ml LPS + 10 μg/ml CHX for 120 min (lanes 2 and 5) or treated with 1 μg/ml LPS + 10 μg/ml CHX for 120 min following pre-treatment with 100 μM ALLN for 45 min (lanes 3 and 6). The positions of IκB-ε proteins are indicated.

associated with NF-κB could be degraded in response to LPS. As shown in Figure 8C, whereas the wild-type protein was degraded following 90 min of treatment with LPS/CHX (lane 4), the mutated protein remained stable (lane 9). We also tested whether the lysine at position 145 (position 6 after the second methionine) was important, since the corresponding lysine residues of IκB-α (Lys21 and Lys22) are required for degradation, but mutation of this residue had no effect (lane 14). The double mutant containing mutation of the two serines and the lysine was also stable (data not shown).

IκB-ε protein levels are up-regulated in IκB-α-deficient fibroblasts

Finally, since IκB-ε expression is controlled by NF-κB, we analysed the expression of IκB-ε in fibroblasts derived from mice homozygously deficient for IκB-α (Klement *et al.*, 1996). To our surprise, we found that, unlike the situation found for IκB-β, IκB-ε protein levels are strongly

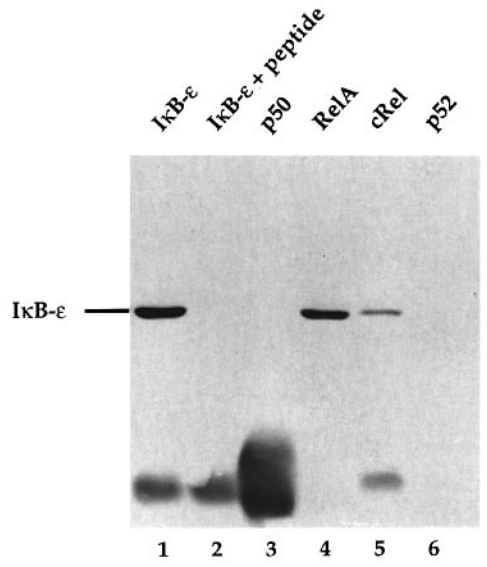

Fig. 7. Transfected IκB-ε is associated solely with RelA and cRel in 70Z/3 cells. Cytoplasmic lysates were prepared from 70Z/3 cells stably expressing the 45 kDa form of IκB-ε containing a myc 9E10 epitope at its N-terminus and were precipitated with the following antisera: lane 1, IκBε891; lane 2 IκBε891 blocked with its cognate peptide; lane 3, anti-p50, lane 4, anti-RelAC, lane 5, anti-mcRelC; lane 6, anti-p100N. Immune complexes were collected and subjected to SDS–PAGE followed by Western blotting with 9E10 monoclonal antibody. The position of the tagged IκB-ε proteins is indicated. The signals at the bottom of the figure come from the Ig light chain proteins.

increased in these cells (Figure 9). These cells do not contain any constitutively activated NF-κB complexes in their nuclei, and other NF-κB-regulated gene products such as cRel or p105 are similarly not affected (Klement *et al.*, 1996). This suggests some sort of compensatory mechanism and cross-talk between IκB family members (see below).

Discussion

In an attempt to identify novel proteins capable of interacting with the p52 subunit of NF-κB, we have isolated a cDNA which encodes a protein containing six ankyrin repeats highly homologous to those found in the IκB family of proteins. Further analysis of the protein encoded by this cDNA reveals it to be a novel IκB family member; when overexpressed in cells, the protein can inhibit transactivation from a NF-κB-driven promoter both by co-transfected rel proteins and by inducers of NF-κB activity. In addition, the overexpressed protein is capable of retaining NF-κB proteins in the cytoplasm of transfected HeLa cells (data not shown). Since there are, to date, four cytoplasmic inhibitors of NF-κB activity (IκB-α/MAD3, IκB-β, IκB-γ/the C-terminal moiety of p105 and IκB-δ/the C-terminal moiety of p100), we have called this new IκB protein IκB-ε. We have shown that, in resting cells, IκB-ε exists as multiple, differentially phosphorylated isoforms which form complexes predominantly with RelA and cRel (Figure 4). This might seem contradictory with the bait used for screening (p52); however, the situation in yeast, where only p52 and IκB-ε are present, is clearly different from the situation in cells where all members of the families are present and the actual associations are dependent on the respective affinities of the proteins for each other. However, using *in vitro* co-translation followed by immunoprecipitation, we could demonstrate that IκB-ε and p52 can associate (data not shown).

Structure of IκB-ε

Examination of the structure of the IκB-ε protein reveals it to contain many of the features of other IκB molecules. The IκB-ε protein contains six copies of the structural motif known as the ankyrin repeat found in all other members of the IκB family. It is known that IκB-β also contains six such repetitions, as does the *Drosophila* IκB protein Cactus. IκB-α would seem to contain only five, whereas the cytoplasmic precursors p100 and p105, as well as the nuclear IκB molecule Bcl3, contain seven. The relevance of this different number of ankyrin repeats is not clear. It may be that the number of ankyrin repeats, as well as their primary structure, is important in determining the specificity and affinity of interaction between the different IκB proteins and the numerous different NF-κB complexes found in cells. What is clear from our studies is that IκB-ε is found almost exclusively complexed to RelA and cRel proteins, whereas IκB-α is complexed with more classical p50/RelA NF-κB complexes (Figure 4B). It remains to be established which sequences are responsible for these different interactions.

IκB-ε also contains structural homology to IκB-α and IκB-β in the region N-terminal to the ankyrin repeats (Figure 1D). More specifically, IκB-ε contains two serine residues spaced by three amino acids that are found in a sequence context highly similar to those found in IκB-α and IκB-β. These two serine residues have been shown to play a critical role in the regulation of the activity of these two molecules (Brockman *et al.*, 1995; Brown *et al.*, 1995; Chen *et al.*, 1995; Traenckner *et al.*, 1995; Whiteside *et al.*, 1995; Didonato *et al.*, 1996; McKinsey *et al.*, 1996). The presence of an acidic residue N-terminal to the second serine resembles the sequence found in IκB-α more than that of IκB-β, where a glycine residue is present. In addition, IκB-ε also contains a lysine residue situated ~10 residues N-terminally to the two serine residues. In the case of IκB-α, there are two lysine residues similarly situated which have been shown to be the major sites for ubiquitination following signal-induced phosphorylation, since mutation of these sites inhibits inducible degradation (Scherer *et al.*, 1995; Baldi *et al.*, 1996). IκB-β also contains a lysine residue similarly situated, although whether this represents the primary site for inducible ubiquitination is less clear (R.Weil and A.Israël, unpublished data). The presence of similarly positioned serine and lysine residues in IκB-ε suggests that the signalling events reponsible for the degradation of this protein are controlled by a mechanism similar to that for IκB-α and IκB-β (see below).

A further region of structural importance found in IκB-α and IκB-β is the C-terminal PEST domain, whose presence has been shown to be necessary for inducible degradation (Brown *et al.*, 1995; Rodriguez *et al.*, 1995; Whiteside *et al.*, 1995), although conflicting data can be found in the recent literature (Aoki *et al.*, 1996; Sun *et al.*, 1996). In IκB-α, this region is heavily phosphorylated in resting cells, probably by casein kinase II, and these phosphorylations seem to be mainly responsible for the

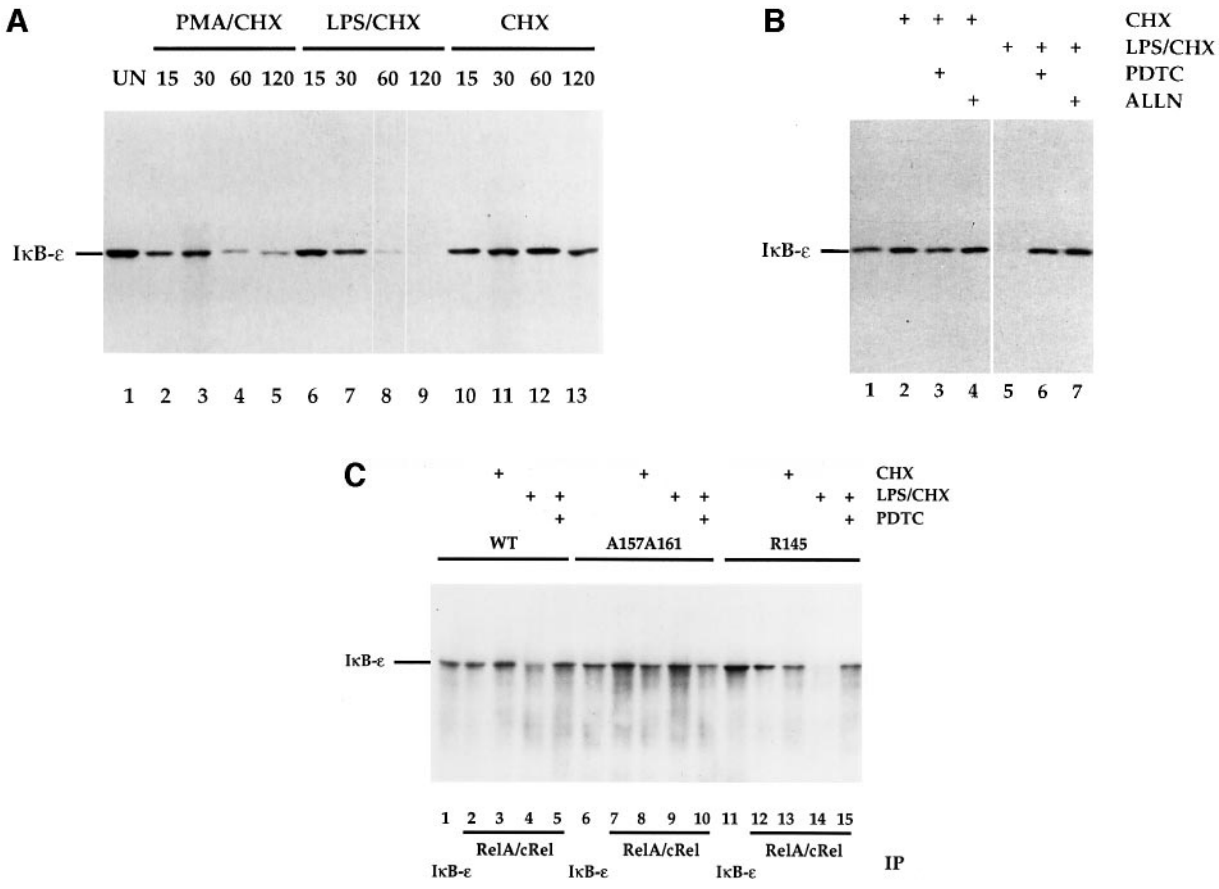

Fig. 8. Degradation of transfected IκB-ε proteins. (**A**) 70Z/3 cells stably expressing the 45 kDa form of IκB-ε (amino acids 140–500) containing a 9E10 myc epitope at its N-terminus were treated as indicated and cytoplasmic extracts prepared. Lysates were precipitated with anti-RelAC and anti-mcRelC, and immune complexes were collected and separated by SDS–PAGE. Transfected IκB-ε proteins were detected by Western blotting with anti-9E10 monoclonal antibody. Cells were either untreated (lane 1), or treated with 10 ng/ml PMA + 1 μg/ml CHX (lanes 2–5), 1 μg/ml LPS + 1 μg/ml CHX (lanes 6–9) or 1 μg/ml CHX (lanes 10–13). (**B**) Cytoplasmic lysates were prepared from the same cells as in (A) treated as follows: lane 1, untreated; lanes 2–4, 1 μg/ml CHX for 90 min; lanes 5–7, 1 μg/ml LPS + 1 μg/ml CHX for 90 min. Where indicated, cells were pre-treated with either 500 μM PDTC (lanes 3 and 6) or 100 μM ALLN (lanes 4 and 7) for 45 min before induction. Transfected IκB-ε proteins were detected as in (A). (**C**) Cytoplasmic lysates were prepared from 70Z/3 cells stably expressing either the wild-type or mutated forms of the 45 kDa form of IκB-ε containing a myc epitope at its N-terminus, and treated as follows: lanes 1, 2, 6, 7, 11 and 12, untreated; lanes 3, 8 and 13, 1 μg/ml CHX for 90 min; lanes 4, 5, 9, 10, 14 and 15, 1 μg/ml LPS + 1 μg/ml CHX for 90 min. Where indicated, cells were pre-treated with 500 μM PDTC (lanes 5, 10 and 15) for 45 min before induction. Extracts were precipitated with either anti-IκBε812 (lanes 1, 6 and 11) or anti-RelAC and anti-mcRelC (remaining lanes), immune complexes collected and subjected to SDS–PAGE and transfected IκB-ε proteins were visualized by immunoblotting with anti-9E10 monoclonal antibody. Transfected IκB-ε proteins were as follows: lanes 1–5 (WT), wild-type IκB-ε amino acids 140–500; lanes 6–10 (A157A161), wild-type IκB-ε amino acids 140–500 containing mutation of serines 157 and 161 to alanines; lanes 11–15 (R145), wild-type IκB-ε amino acids 140–500 containing mutation of Lys145 to arginine. The positions of the epitope-tagged IκB-ε proteins are indicated.

turnover of the molecule in unstimulated cells (Lin *et al.*, 1996; McElhinny *et al.*, 1996; Schwartz *et al.*, 1996). Analysis of the sequence of IκB-ε shows that the region C-terminal to the last ankyrin repeat is much shorter than those found in the other two inhibitors, and not to be particularly rich in P, E, D, S or T residues. IκB-ε degradation would thus appear to be independent of a C-terminal PEST sequence (see below). Closer examination reveals this C-terminal domain to contain one casein kinase II site (IκB-α and IκB-β contain six and four respectively). Indeed, we have shown that IκB-ε is phosphorylated constitutively in uninduced cells, although the site(s) and nature of this phosphorylation remain to be elucidated.

The most startling difference between IκB-ε and the other IκB molecules is the presence of the long N-terminal extension giving rise to an ORF of 500 amino acids and a predicted mol. wt of 55 kDa. However, the IκB-ε protein exists in cells as a 45 kDa species (and the corresponding hyperphosphorylated forms). Closer analysis of the DNA sequence revealed the first ATG codon to lie in a sequence context highly unfavourable for translation, there being a pyrimidine residue at the critical –3 position (Kozak, 1992), whereas the second ATG codon is situated in a much more favourable context (Figure 1B). In addition, when cDNAs that initiate translation at this second ATG codon are expressed either *in vitro* (data not shown) or *in vivo* (Figure 8), they give rise to a protein with a molecular weight indistinguishable from that of endogenous IκB-ε, suggesting that translation does indeed initiate at this second ATG codon *in vivo*. An alternative possibility would be that the 45 kDa protein is the result of a proteolytic processing of the 62 kDa form. Indeed, the N-terminal region is rich in glycine residues and as such resembles the region in p105 responsible for the maturation of p105 into p50 (Lin and Ghosh, 1996). However, when the long, 62 kDa form of IκB-ε is expressed either *in vitro* or *in vivo* from constructs in which the ATG codon has

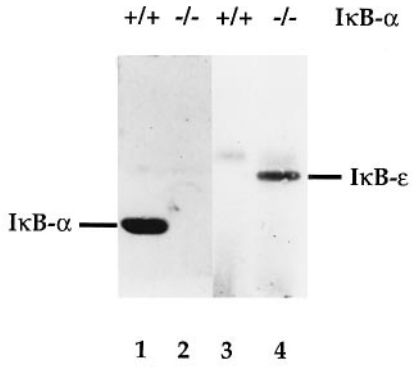

Fig. 9. IκB-ε is up-regulated in IκB-α –/– fibroblasts. Cytoplasmic extracts were prepared from murine embryo fibroblasts isolated from either IκB-α wild-type (+/+, lanes 1 and 3) or homozygous null (–/–, lanes 2 and 4) 13- to 15-day embryos. For IκB-α (lanes 1 and 2), lysates were separated on SDS–PAGE and blotted with anti-mIκBα. For IκB-ε, lysates were precipitated with IκB-ε891, immune complexes eluted with cognate peptide, separated on SDS–PAGE and blotted with IκB-ε891. The positions of the IκB-α and IκB-ε proteins are indicated to the left and right of the figure respectively.

been situated in a sequence context ensuring highly efficient initiation of translation, no protein at 45 kDa is apparent (data not shown). Also, we have been unable to demonstrate the existence of a 62 kDa protein in various cell types, even if cells are treated with protease inhibitors that have been shown to block the maturation of p105 and p100 (data not shown). Furthermore, since we have been unable to distinguish between the long, 62 kDa form and the short, 45 kDa form in any assay thus far performed (interaction with NF-κB proteins, inhibition of DNA binding, cytoplasmic retention, inhibition of transactivation or kinetics of degradation), it seems illogical to us that a precursor protein would be synthesized that has the exact same properties as its product. We thus prefer the hypothesis that IκB-ε is synthesized as a 45 kDa protein with translation initiating at the second ATG codon at position 140 in the ORF. We cannot, however, exclude the possibility that the long 62 kDa form is expressed in some cell types or under some physiological conditions that we have not yet tested.

IκB-ε-associated rel proteins

When we analysed the amounts of the four ubiquitously expressed rel proteins associated with IκB-ε, we were surprised to find that IκB-ε was associated almost exclusively with RelA and cRel, whereas IκB-α was found in complexes containing p50 and RelA (Figures 4B and 7). In most cell lines tested, IκB-ε was associated with as much, if not more, RelA and cRel than either IκB-α or IκB-β. In fact, IκB-α is associated with a small percentage of the cytoplasmic RelA and cRel present, whereas IκB-β and IκB-ε were associated with comparable and larger amounts. We thus believe that IκB-ε represents a major regulator of NF-κB activity in certain cell types and in response to certain stimuli. One exception that we have found has been the murine pre-B cell line 70Z/3, in which almost all the RelA protein is associated with IκB-β. It must be noted that these cells contain little endogenous IκB-ε (data not shown). It would thus seem that in the lymphocyte cell lines tested, IκB-β and IκB-ε represent the two major isoforms of IκB responsible for tethering cRel and RelA proteins in the cytoplasm of unstimulated cells. It remains to be established how the various NF-κB molecules are shared out between the different IκB molecules present in the cytoplasm. An interesting possibility would be that the availability of IκB molecules might dictate which NF-κB complexes can be found in the cytoplasm.

Regulation of IκB-ε activity

As is the case for both IκB-α and IκB-β, IκB-ε activity is regulated by proteolytic degradation. Following treatment with inducers of NF-κB activity, IκB-ε is degraded with relatively slow kinetics when compared with IκB-α and thus seems to resemble IκB-β in this respect. Pre-treatment with inhibitors of proteasome activity results in the appearance of hyperphosphorylated forms of IκB-ε, suggesting that degradation may depend on inducible phosphorylation events, perhaps on the two serines (see below). However, unlike IκB-β, and like IκB-α, the IκB-ε gene is inducible in Jurkat cells by treatment with PMA and ionomycin. This observation is confirmed by the reappearance of IκB-ε in these cells after 120 min of treatment. It would seem, therefore, that IκB-ε is sharply regulated, with the period of time between degradation and resynthesis being relatively short. For this and similar stimuli, IκB-ε could control the transient induction of an NF-κB activity, but with slower kinetics than those seen for IκB-α.

The presence of hyperphosphorylated isoforms of IκB-ε in cells may also be important for the regulation of its activity, although how this might be so remains unclear, since we have shown that all isoforms are associated with rel proteins and are degraded in response to the stimuli thus far tested.

The mechanism of IκB-ε degradation would seem to be very similar to that of IκB-α: following stimulation with LPS in THP-1 cells, a slower migrating form of the protein becomes apparent (Figure 5A, lower panel, lanes 2 and 7), which is stabilized following treatment with the proteasome inhibitor ALLN and subsequent inhibition of degradation (Figure 6 and data not shown). Both these results are suggestive of a mechanism similar to that responsible for the inducible degradation of IκB-α as discussed above. Since Ser157 and Ser161 of IκB-ε lie in a sequence context which closely resembles that found around Ser32 and Ser36 of IκB-α, it is tempting to speculate that inducible phosphorylation on these two serines signals IκB-ε to be degraded. Indeed, mutation of these two serines to non-phosphorylatable alanines abolishes IκB-ε degradation in response to LPS (Figure 8C). The presence of a lysine residue (Lys145) at a position similar to those in IκB-α shown to be the main sites for phosphorylation-induced ubiquitination (Lys21 and Lys22), suggests a similar mechanism for targeting the protein for degradation. However, Lys145 does not seem to be a primary target for ubiquitination, since its mutation has no effect upon LPS-induced degradation (Figure 8C). Differently to IκB-α and IκB-β, IκB-ε contains no PEST-like region C-terminal to the ankyrin repeats. Deletion of this short C-terminal region renders IκB-ε unstable and the truncated protein is no longer able to interact with rel proteins (data not shown). The absence of a C-terminal PEST region may explain the slow kinetics of degradation of IκB-ε.

Why do multiple IκB proteins exist?

From studies upon the kinetics of degradation of IκB-α and IκB-β proteins in response to extracellular stimuli of NF-κB activity, it has been proposed that the two inhibitors play a complementary role in the regulation of NF-κB activity: IκB-α is responsible for a fast, transient activation of NF-κB, being degraded rapidly in response to extracellular signalling events and resynthesized shortly afterwards in a NF-κB-dependent manner. On the other hand, IκB-β, whose gene expression does not seem to be regulated by NF-κB, is responsible for the biphasic response of NF-κB to certain stimuli (Thompson *et al.*, 1995), as well as for the regulation of slower NF-κB-controlled events (Molitor *et al.*, 1990). IκB-ε would appear to share some properties of both these inhibitors: it is degraded with slower kinetics (like IκB-β) but is also resynthesized in response to some stimuli and thus probably acts to autoregulate NF-κB activity, as is the case for IκB-α. It seems likely, therefore, that in human cells, IκB-ε is responsible for controlling a third type of NF-κB response: those that are slow but still transient in nature. The question remains of how NF-κB that is liberated from IκB-ε (as well as IκB-β) with slower kinetics is not retained by newly synthesized IκB-α. As discussed below, since IκB-ε interacts with a subset of NF-κB complexes, it may be that these proteins have a low affinity for IκB-α. Alternatively, it is possible that following IκB-β or IκB-ε degradation, NF-κB is modified in such a way (e.g. phosphorylation) that it becomes inaccessible to inhibition by other IκB molecules.

IκB-ε also differs from previously identified inhibitors in that it seems to control the regulation of a different type of NF-κB complex, those containing just RelA and/or cRel (see, for example, Ganchi *et al.*, 1993). Although classical NF-κB is composed of heterodimers between p50 and RelA/cRel (those recognized by IκB-α and IκB-β), and is responsible for the regulation of many of the genes containing κB sites, there are precedents for the existence of RelA/cRel-containing complexes controlling gene expression: in the case of the IL-8 and tissue factor (TF) promoters, it has been shown that the NF-κB sites responsible for induction of these genes are unable to bind, or to be transactivated by, p50/RelA NF-κB complexes (Kunsch and Rosen, 1993; Oeth *et al.*, 1994). However, these sites can bind to and are transactivated by homodimers or heterodimers of cRel and RelA. It has been shown that there exists a subset of genes inducible in activated macrophages and endothelial cells whose promoters are controlled by NF-κB sites which only bind RelA/cRel heterodimers *in vitro* (ICAM1, GM-CSF) (Parry and Mackman, 1994). Furthermore, studies on IκB-α-deficient mice have shown that the expression of some genes expressed in haematopoetic tissues, including GM-CSF, is not elevated (Beg *et al.*, 1995). The authors explain this observation by suggesting that factors other than NF-κB control the expression of these genes, but it is equally likely that other IκB molecules are responsible. It is interesting to note that the expression of these genes is activated in cell lines which contain large amounts of IκB-ε (e.g. macrophages, T cells, B cells, but not pre-B cells nor fibroblasts; S.T.Whiteside and A.Israël, in preparation). Thus in addition to controlling slow, transient responses, we propose a model in which IκB-ε also regulates a specific subset of NF-κB-responsive genes—those whose promoters are regulated by NF-κB complexes that contain only RelA and/or cRel proteins. Future studies of the genes which are up-regulated in mice deficient for IκB-β or IκB-ε will help to clarify these points. It is important to note that IκB-ε expression is up-regulated during differentiation of HL-60 cells into macrophages or granulocytes (Figure 3), suggesting an important role for this protein in these cells.

Another interesting observation concerning the IκB-α-deficient mice is the fact that while the level of IκB-ε is undetectable in fibroblasts from wild-type mice, it is strongly increased in fibroblasts from IκB-α –/– mice (Figure 9). Since this increase cannot be explained by activation induced by constitutively nuclear NF-κB complexes, this suggests some sort of compensatory mechanism, and the sustained NF-κB response observed when these fibroblasts are stimulated with TNF could be due to the degradation of IκB-ε, since TNF treatment does not seem to induce IκB-β degradation in these cells (Klement *et al.*, 1996), although release of NF-κB from other inhibitors (e.g. p100 or p105) is also possible.

It is intriguing to speculate that the different IκB molecules may control the regulation of different genes by binding to and inhibiting different populations of NF-κB molecules in the cytoplasm. (It may be the case that further IκB molecules which regulate other subsets of NF-κB activity remain to be identified.) Such specificity of regulation would make different IκB proteins good targets for pharmacological agents which specifically inhibit subsets of NF-κB responses. What is clear, however, is that different cell types exhibit different 'fingerprints' of RelA and cRel proteins associated with the various IκB molecules (Figure 4A). This difference is probably important in determining the type of NF-κB response to occur following activation by a given stimulus and, subsequently, which genes are transactivated.

Materials and methods

Yeast transformation and two-hybrid screening

Cells were transformed using the lithium acetate method (Gietz *et al.*, 1992). For screening of the library, cells already transformed by the pAS2/p52 plasmid (see below) were retransformed with 100 μg of library DNA (Harper *et al.*, 1993) and double transformants were selected on ura$^-$leu$^-$trp$^-$his$^-$ glucose plates supplemented with 25 mM 3-aminotriazole (3-AT). Five days after transformation, the surviving cells were tested for β-galactosidase activity using a filter lift assay. Colonies were transferred onto nylon filters (Amersham Hybond-N), permeabilized by freezing in liquid nitrogen and thawed at room temperature. Filters were then overlaid on Whatmann 3MM paper saturated with an X-gal (5-bromo, 4-chloro, 3-indolyl, β-D-galactopyranoside, Sigma) solution (0.04% in Z-buffer, Breeden and Nasmyth, 1987) and incubated at room temperature. The time required for colour development to occur ranged from 5 to 20 min.

Cells

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum. 70Z/3, Jurkat, THP-1 and HL-60 cells were maintained in RPMI supplemented with 10% fetal calf serum (FCS) and 50 μM β-mercaptoethanol. HL-60 cells were differentiated into monocytes or into granulocytes as described in the legend to Figure 3. 70Z/3 cells stably expressing forms of IκB-ε were obtained as described in Whiteside *et al.* (1995), using plasmids cDNA3βIκB-ε62kNT or cDNA3βIκB-ε45kNT linearized with *Pvu*I.

Antisera

Of the antisera used, the following have already been described (Rice *et al.*, 1992; Whiteside *et al.*, 1995; Klement *et al.*, 1996): anti-p50,

105N (1141) and anti-p50 (#3); anti-p52, 100N (1267); anti-RelA, RelAN (1207) and RelAC (1226); anti-cRel, cRelC (265); mcRelC (1051); anti-IκB-α, IκBαN (1309) and anti-mIκBα (751).

Anti-IκB-ε: IκBε812 and IκBε891, were raised against amino acids 225–240 and 191–211 respectively of the human IκB-ε protein. The anti-IκB-α antiserum S5 (IκBαS5), a gift of R.T.Hay (St Andrews, UK), was raised against recombinant human IκB-α. The anti-IκB-β antisera, raised against recombinant murine IκB-β, were a kind gift of R.Weil (Biologie Moléculaire de l'Expression Génique, Institut Pasteur).

Plasmids

Plasmid pAS2/p52 was constructed by cloning p52 between the *Bam*HI and *Xho*I sites of pAS2 (Durfee *et al.*, 1993). The human lymphocyte cDNA library (Durfee *et al.*, 1993) and plasmid pAS2 were kind gifts from Dr S.Elledge (Baylor College, TX).

cDNA clones isolated from λZAP library screenings were excised *in vivo* using R408 helper phage. A full-length IκB-ε clone was constructed by fusing two overlapping cDNAs at the unique *Bsp*HI site situated at residue 260 and then cloned into pBluescriptSK (Stratagene). IκB-ε-coding sequences from amino acids 1–500 or 140–500 were amplified by PCR and cloned into pT7βplink or pT7βplinkTAG (Pollock and Treisman, 1990). Cassettes containing the β-globin 5′-untranslated region and IκB-ε-coding sequences subsequently were transferred into pcDNA3 (InVitrogen) using *Hin*dIII and *Xba*I to construct cDNA3βIκB-ε62k and cDNA3βIκB-ε45k (for use in transfection studies, Figure 2) and their 9E10-tagged counterparts cDNA3βIκB-ε62kNT and cDNA3βIκB-ε45kNT (used in establishing stable lines, Figures 7 and 8). Mutants of serines 157 and 161 and Lys145 were obtained by PCR, and then cloned into pT7βplinkTAG and subsequently into pcDNA3 (as described for the wild-type sequences) to obtain plasmids for use in stable lines (Figure 8C). Plasmids encoding IκB-α and RelA proteins have already been described (Munoz *et al.*, 1994; Whiteside *et al.*, 1995). A plasmid capable of expressing murine IκB-β under the control of the CMV promoter was a kind gift of Dr R.Weil (Biologie Moléculaire de l'Expression Génique, Institut Pasteur). Plasmid maps and construction details are available upon request.

Northern blotting

Poly(A)$^+$ mRNA was prepared using standard techniques and migrated through formaldehyde–agarose gels. RNA was blotted onto Hybond-N+ (Amersham) and pre-hybridized overnight. A fragment containing the whole IκB-ε cDNA was radiolabelled by random priming using a Boehringer Mannheim kit and hybridized to filters in Church buffer (Church and Gilbert, 1984) at 65°C. Filters were washed three times in 0.1× SSC; 0.1% SDS at 60°C for 15 min, rinsed once in phosphate-buffered saline (PBS) then exposed to autoradiographic film at –80°C with an intensifying screen.

Immunoprecipitations

For co-precipitation of proteins, extracts from 10^7 cells were diluted with 1 ml of TNT buffer (20 mM Tris, pH 8.0, 200 mM NaCl and 1% Triton X-100), 4 μl of antibody was added and immune complexes precipitated using protein A–Sepharose (Sigma). For elution by cognate peptide, the beads were resuspended in 100 μl of TNT buffer containing 10 μg of peptide and rotated overnight at 4°C; tubes were then centrifuged and the supernatant used for subsequent precipitation. Where boiling is indicated, beads were resuspended in TNT buffer containing 1% SDS, 0.5% β-mercaptoethanol and heated at 100°C for 10 min. Samples were then diluted with 1 ml of TNT buffer, centrifuged and the supernatant used for subsequent analysis. For analysis of immune complexes by SDS–PAGE, washed beads were boiled in 30 μl of denaturing Laemmli buffer, centrifuged for 30 s in a microfuge and the supernatant loaded onto the gel.

Cellular extracts

To prepare crude cytoplasmic extracts, 3×10^6 cells were washed twice with PBS and then lysed in 50 μl of Dignam buffer A supplemented with 5 mM NaF, 2.5 mM Na_3VO_4, 2.5 mM orthophosphate, 0.25% NP-40 and protease inhibitors as described in Whiteside *et al.* (1992). After centrifugation for 2 min at top speed in a microfuge, the supernatant was transferred to a clean tube for further analysis. The nuclear pellet was resuspended in 100 μl of Dignam buffer C supplemented with 5 mM NaF, 2.5 mM Na_3VO_4, 2.5 mM orthophosphate and protease inhibitors as described in Whiteside *et al.* (1992) and incubated on ice for 30 min with occasional vortexing. Extracts were clarified by centrifugation in a microfuge for 10 min at top speed at 4°C. Protein concentrations were determined by a Bradford assay (Bio-Rad).

Transfections

293T cells were transfected by calcium phosphate precipitation and extracts prepared 2 days following transfection. HeLa cells were transfected with Lipofectamine (Gibco BRL) according to the manufacturer's instructions and prepared for indirect immunofluorescence 2 days after transfection. Jurkat cells were transfected by DEAE–dextran. Briefly, 2 μg of DNA were placed into the well of a 96-well tissue culture dish; to this were added 2×10^6 cells in 100 μl of Tris-buffered saline and 100 μl of 1 mg/ml DEAE–dextran (Pharmacia). After 45 min at room temperature, cells were collected by centrifugation and transferred into 2 ml of RPMI/10% FCS in 24-well plates. Cells were harvested the following day and lysed in 100 μl of luciferase buffer (25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM dithiothreitol, 1% Triton X-100, 15% glycerol). Luciferase activity was quantified using a Berthold luminometer.

Immunoblots

Proteins were transferred onto Immobilon (Millipore) and filters were blocked in PBS supplemented with 5% skimmed milk (Regilait). Immunoblots were incubated with sera diluted 1:1000 in PBS/5% milk/0.1% Tween-20 and revealed either with the Amersham ECL system, or by incubation in [^{125}I]proteinA (Amersham) diluted in PBS/5% milk/0.1% Tween-20 and subsequent autoradiography.

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