IκB-β Regulates the Persistent Response in a Biphasic Activation of NF-κB

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Summary

We have cloned the cDNA encoding IκB-β, one of the two major IκB isoforms in mammalian cells. The recombinant IκB-β protein interacts with equal affinity to p65 and c-Rel and does not exhibit a preference between these Rel proteins. Instead the primary difference between IκB-α and IκB-β is in their response to different inducers of NF-κB activity. One class of inducers causes rapid but transient activation of NF-κB by primarily affecting IκB-α complexes, whereas another class of inducers causes persistent activation of NF-κB by affecting both IκB-α and IκB-β complexes. Therefore, the overall activation of NF-κB consists of two overlapping phases, a transient phase mediated through IκB-α and a persistent phase mediated through IκB-β.

Introduction

The transcription factor NF-κB is important for the inducible expression of a wide variety of cellular genes, including cytokines, cytokine receptors, and stress proteins (reviewed by Baeuerle and Henkel, 1994; Kopp and Ghosh, 1994). In addition, it is a key element in the replication of viruses such as human immunodeficiency virus and cytomegalovirus. NF-κB is considered to be a heterodimer of proteins that belong to the Rel family of transcription factors. The members of the family in mammalian cells include the proto-oncogene c-rel, p50/p105 (NFKB1), p65 (RelA), p52/p100 (NFKB2), and RelB (reviewed by Baeuerle and Henkel, 1994; Kopp and Ghosh, 1994). All of these proteins share an ~300 amino acid region of homology that is known as the Rel homology domain. A common feature of the regulation of transcription factors belonging to the Rel family is their sequestration in the cytoplasm as inactive complexes with a class of inhibitory molecules known as IκBs (reviewed by Beg and Baldwin, 1993; Gilmore and Morin, 1993). Treatment of cells with different inducers, e.g., interleukin-1 (IL-1), tumor necrosis

factor α (TNFα), lipopolysaccharide (LPS), double-stranded RNA, or phorbol myristate acetate (PMA) results in the dissociation of the cytoplasmic complexes and translocation of free NF-κB to the nucleus. The dissociation of the cytoplasmic complexes is thought to be triggered by the phosphorylation and subsequent degradation of the IκB protein (Beg et al., 1993; Brown et al., 1993; Ghosh and Baltimore, 1990; Henkel et al., 1993; Mellitis et al., 1993; Scott et al., 1993; Sun et al., 1993).

There are two major biochemically characterized forms of IκB proteins in mammalian cells, IκB-α and IκB-β (Ghosh and Baltimore, 1990; Zabel and Baeuerle, 1990; Link et al., 1992). In addition, three other proteins have been cloned or implicated as IκBs: chicken pp40, the homolog of mammalian IκB-α (Davis et al., 1991); IκB-γ, a tissue-specific form that arises from an alternative splice, yielding the C-terminus of the p105 protein (Inoue et al., 1992); and the candidate oncogene Bcl-3 (Nolan et al., 1993; Ohno et al., 1990; Wulczyn et al., 1992). A common feature of all the cloned IκB proteins is the presence of multiple copies of a sequence motif known as ankyrin repeats (reviewed by Beg and Baldwin, 1993; Gilmore and Morin, 1993). However, while IκB-γ has been detected only in mouse pre-B cells (Ghosh et al., 1990; Inoue et al., 1992), Bcl-3 can only be detected in very low amounts in some tissues (S. G., unpublished data). In addition, both IκB-γ (Liou et al., 1992) and Bcl-3 (Franzoso et al., 1992; Naumann et al., 1993; Nolan et al., 1993; Wulczyn et al., 1992) are specific for NF-κB p50 dimers, and only IκB-α and IκB-β interact with p65 and c-Rel, thus indicating that the responsibility for regulating the prototypical NF-κB activity is primarily carried out by these IκB isoforms. IκB-α was cloned previously, and its regulation has been studied quite extensively (reviewed by Baeuerle and Henkel, 1994; Kopp and Ghosh, 1994). These studies indicated that IκB-α regulated NF-κB activity through a novel autoregulatory feedback loop. Signals that led to an induction of NF-κB activity resulted in the phosphorylation and rapid loss of IκB-α protein through proteolysis. However, the induced nuclear NF-κB caused the subsequent up-regulation of IκB-α mRNA levels due to the presence of NF-κB sites in the IκB-α promoter (de Martin et al., 1993; Le Bail et al., 1993). The newly synthesized IκB-α mRNA was translated, and the accumulated IκB-α protein helped to shut down the NF-κB response, thus ensuring that responsive genes were activated only transiently. While this model explained some aspects of the regulation of NF-κB activity in cells, it failed to explain how some inducers, particularly bacterial LPS, could cause persistent long-term activation of NF-κB for as long as 48 hr. Persistent activation of NF-κB might also occur during differentiation, either in early embryonic development or in the development of B cells or macrophages. Because a significant portion of the cytoplasmic Rel complexes are bound to IκB-β, it is possible that inducers like LPS or differentiation signals caused persistent NF-κB activation by affecting IκB-β complexes. However, the lack of a clone for IκB-β and

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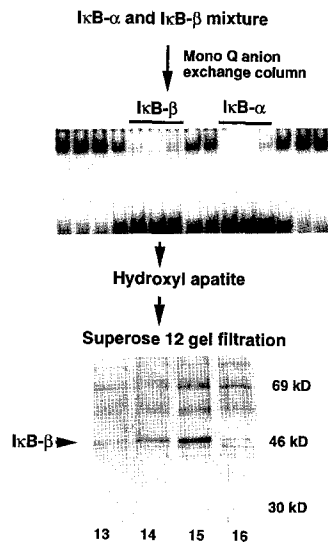
IκB-α and IκB-β mixture

Figure 1. Purification of IκB-β from Rabbit Lung Cytosol

The two IκB isoforms are resolved in the Mono Q column, where the peak that elutes earlier contains IκB-β. The purified fractions of IκB-β from the Superose-12 column were analyzed by silver staining.

reagents specific for the protein had prevented the determination of how and when complexes bound to IκB-β were activated. Therefore, to determine how the activity of the total NF-κB pool is regulated, we have cloned and characterized the cDNA encoding IκB-β.

We find that IκB-β resembles other members of the IκB family in containing six closely spaced ankyrin repeats. The recombinant protein binds to p65 and c-Rel and blocks DNA binding, but does not exhibit any apparent preference between either of these Rel proteins. However, the behavior of the two IκBs differs significantly when cells are stimulated with inducers of NF-κB activity. Some inducers, such as LPS, that cause a persistent activation of NF-κB activity result in the degradation of both IκB-α and IκB-β, while other inducers, such as PMA, that cause a transient induction of NF-κB activity affect only IκB-α. Therefore, the persistent activation of NF-κB in response to inducers occurs in a novel biphasic manner in which the early, transient phase is due to activation of IκB-α complexes and the later, persistent phase is due to activation of IκB-β complexes.

Results

Purification and Sequencing of IκB-β from Rabbit Lungs

The purification of IκB-β was carried out from rabbit lung cytosolic extracts that were previously demonstrated to contain significant quantities of NF-κB–IκB complexes (Ghosh and Baltimore, 1990; Ghosh et al., 1990). The purification takes advantage of the difference in chromatographic properties between the NF-κB–IκB complexes and free IκB proteins. The initial steps of the purification protocol led to a partially purified NF-κB–IκB (α and β) complex that was then dissociated with deoxycholate and separated into NF-κB and IκB pools using anion exchange

chromatography (Ghosh and Baltimore, 1990). The mixture of IκB-α and IκB-β isoforms was subjected to additional chromatographic steps that yielded a highly enriched fraction of IκB-β (Figure 1). The purified protein was fractionated on an SDS–polyacrylamide gel and transferred to nitrocellulose membrane. The protein on the membrane was stained with Ponceau S, and the deeply staining IκB-β band was excised. The protein was digested on the membrane with trypsin, and the eluted peptides were fractionated on a reverse-phase high pressure liquid chromatography column. Four peptides were sequenced, one of which was a mixture while the other three were pure peptides.

Molecular Cloning and Sequencing of IκB-β cDNA

The sequence of two of the peptides revealed that they were derived from ankyrin repeats. Degenerate polymerase chain reaction (PCR) primers based on these ankyrin-related peptide sequences were synthesized and used for PCR on cDNA from rabbit lung RNA as template. A 160 bp fragment was obtained upon cloning the products of the PCR that, when sequenced, was found to contain the complete sequence necessary to encode the peptides used to generate the primers. Northern blot analysis indicated that this 160 bp fragment was derived from a 1.3 kb mRNA that was present in rabbit lungs, as well as in the mouse B cell line LyD9 (data not shown). The 160 bp fragment was used as a probe to screen a 0.7–1.6 kb size-fractionated library of mouse LyD9 cDNA, and multiple clones were obtained. The sequence of these clones revealed an open reading frame of 359 amino acids that can encode a protein with a predicted molecular mass of 41 kDa that is smaller than the 45 kDa size of the purified protein (Figure 2A). The predicted pI of 4.6, however, is in close agreement with that of the purified protein (Link et al., 1992). The cDNA sequence includes the entire 160 bp sequence of the PCR fragment and also contains the third peptide sequence that had not been used for cloning, and thus the cloned cDNA most likely encodes the IκB-β protein that we had purified.

The primary sequence indicates that IκB-β contains six consecutive ankyrin repeats, an organization that is a hallmark of all cloned IκB proteins (Figure 2A) (Beg and Baldwin, 1993; Gilmore and Morin, 1993). Comparing the sequence of IκB-β with the other IκB proteins reveals that the similarity between ankyrin repeats at the same position in different IκBs is greater than between different repeats in the same IκB (Figure 2B) (Gilmore and Morin, 1993). The greater spacing between the third and fourth ankyrin repeats in IκB-β is similar to the arrangement in cactus, the IκB-like inhibitor of the Drosophila Rel homolog dorsal, and is not seen in the other IκB proteins (Geisler et al., 1992; Kidd, 1992). The C-terminal region is rich in the proline, glutamic acid, and serine residues that suggest that it may be a PEST domain, sequences that have been implicated to signal rapid protein turnover (Haskill et al., 1991). Although IκB-β contains the same number of serine/threonine residues as IκB-α, including a putative casein kinase II site, it lacks the protein kinase C (PKC) phosphorylation site present in the other isoform (Haskill et al.,

A

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M A G V A C L G K T A D A D E W C D S G L G S L G P D A A A P    31
G G P G L G A E L G P E L S W A P L V F G Y V T E D G D T A L    62
H L A V I H Q H E P F L D F L L G F S A G H E Y L D L Q N D L    93
G Q T A L H L A A I L G E A S T V E K L Y A A G A G V L V A E   124
                                    Peptide T15
R G G H T A L H L A C R V R A H T C A C V L L Q P R P S H P R   155
D A S D T Y L T Q S Q D C T P D T S H A P A A V D S Q P N P E   186
N E E E P R D E D W R L Q L E A E N Y D G H T P L H V A Y I H   217
                        Peptide T 27
K D A E M V R L L R D A G A D L M K P R P T C G R T P L H L A   248
V E A Q A A S V L E L L L K A G A D P T A R M Y G G R T P L G   279
      Peptide T41
S A L L R P N P I L A R L L R A H G A P E P E D G G D K L S P   310
C S S S G S D S D S D N R D E G D E Y D D I V A H S G R S L N   341
R Q P P S P A S K P L P D D P N P A                            359
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B

Consensus   -G-TPLH-AA--GH---V--LL--GA--N----

REPEAT 1

IκB-β	DGDTALHLAVIHQHEPFLDFLGFSAHEYLDL
IκB-α	DGDSFLHLAIIHEEKALTMELRQVKDLAFLN
IκB-γ	NGDVLHLAIIHLHAOLVRDLEVTSLISDDI
Bcl-3	DGDTFLHPVMQGNLPAVHRLNLFQQGGRELD
Cactus	DGDTFLHLATSGSVDVVAABRMAPHPCLLNI

REPEAT 2

IκB-β	LLQTPLHLARILGEASTVEKLYAAGAGVLVAER
IκB-α	LLQTPLHLAVITNQPEIAEALGAGCDPELRDF
IκB-γ	LYQTPLHLAVITKQEDVVTLLRNGADLSKLDR
Bcl-3	LRQTPLHLAVITTLPSVVRLLVTAGASPMLAR
Cactus	MAQTPLHLATAQPNIMRILLLAGAEPTVFBR

REPEAT 3

IκB-β	GGHTALHLACRVRAHTCACVLLQPRPHPRDAS
IκB-α	RGNTPLHLACEQGCLASVGVLILSCTPHLHSI
IκB-γ	WGNSALHLAAKEGHDRILSILLKSRKAAPLIDH
Bcl-3	HGITAHHLACEHRSPTCLFSLLLSAAPGTLDLE
Cactus	HGNIPLHLACIAGEKQCVHILBKFGATEIHEA

REPEAT 4

IκB-β	DGHTPLHVAVIHKDAEMVRLLRDAGADLNKFPIF
IκB-α	KGHTPLHLAAIHGYLGIVELLVSLGAINACFB
IκB-γ	GGLNAALHLAVMSNSLPCLLLVAAGABILACHQ
Bcl-3	DGIFLHVAVNTECQETVALILERGAIPBAVEI
Cactus	DGERCKLAREAGHIDIFPRLVSHGAINIAPBG

REPEAT 5

IκB-β	CGRTPLHLAVEYAAASVLELLLKPGAIPTARMY
IκB-α	NGRTALHLAVELGNPDLVSLLLNCGADVNRVTV
IκB-γ	SGRTPLHLAVEYDNISLAGCLLLEFSDAHVDSTT
Bcl-3	SGRTPLISRFAVENNSLSMVQLLLQHFNVNAQMU
Cactus	SGRTPLHFAHFGCNEDLANFLILDECEKLNLETA

REPEAT 6

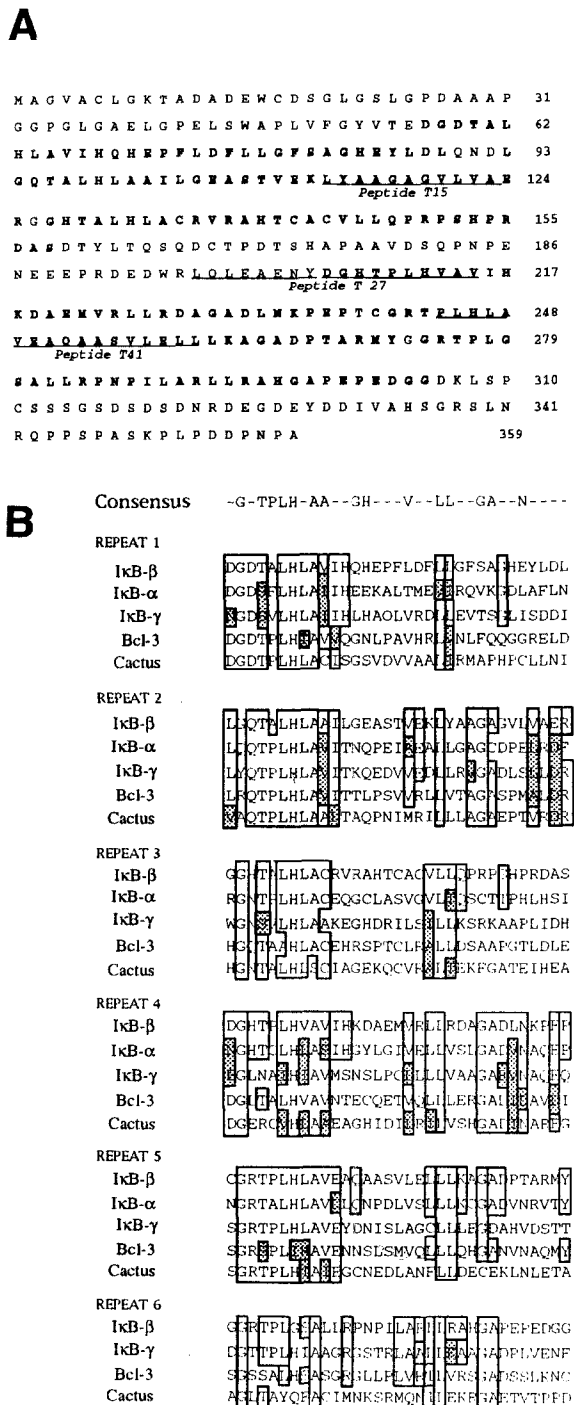|	
IκB-β	CGRTPLGTALLRPNPILARTIRAFGAPEPEIGG
IκB-γ	DGTTPLHIIAAGRISTRLARILRMADPIVENF
Bcl-3	SFSSADHLASGULLPLVFLLVRSIFSSLFNC
Cactus	AGLFAYQFACIMNKSRMQHILEKFGAETVTPPD

Figure 2. Predicted Sequence of the Mouse IκB-β Protein

(A) The predicted protein sequence of the clone 15f is presented. The six ankyrin repeat sequences are indicated in boldface. The peptide sequences obtained from the purified rabbit protein corresponding to the predicted sequence from the cDNA are indicated.

(B) Alignment of the ankyrin repeats of different IκBs: mouse IκB-β, human IκB-α (MAD3), mouse IκB-γ, human Bcl-3, and Drosophila cactus. The stippled boxes indicate similarity at identical positions.
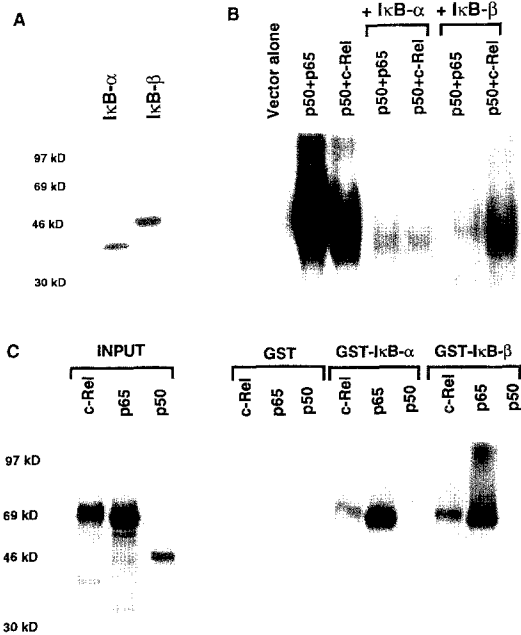

Figure 3. IκB-β Interacts with p65 and c-Rel but Not p50

(A) The cDNAs encoding the two IκBs were cloned in a pCDNA-3 vector under the control of a T7 promoter and were used to program rabbit reticulocyte-coupled transcription–translation systems. The translated proteins were run on SDS–polyacrylamide gels and visualized by fluorography.

(B) Inhibition of DNA binding by transfected p50–p65 and p50–c-Rel in COS cells upon cotransfection with IκB-α and IκB-β. COS cells were transfected with the pCDNA-3 vector alone, with p50 plus p65 (3 μg each), with p50 plus c-Rel (3 μg each), with p50 plus p65 plus IκB-α or IκB-β (3 μg plus 3 μg plus 5 μg), and with p50 plus c-Rel plus IκB-α or IκB-β (3 μg plus 3 μg plus 5 μg). In all cases, the total amount of DNA transfected was equalized to 11 μg by adding vector DNA.

(C) Specificity of GST–IκB proteins for p50, p65, and c-Rel. Each of the GST–fusion proteins (50 ng) was mixed with 6 μl of the ³⁵S-labeled and in vitro translated p50, p65, and c-Rel. The labeled Rel proteins bound to GST–IκBs were precipitated by binding to glutathione–agarose beads, washed extensively, and boiled in SDS sample buffer, and the eluted proteins were analyzed by SDS–PAGE. The input lanes contained 2.5 μl of lysate, whereas the GST lanes represent precipitated proteins from 5 μl of lysate.

1991). The structure of the IκB-β mRNA is unique in that it contains very short 5' and 3' untranslated regions (data not shown). In particular, the lack of any AUUUA sequences at the 3' end, unlike IκB-α mRNA, suggests that the IκB-β mRNA may be stable and not subject to rapid turnover (Haskill et al., 1991).

IκB-β Interacts with p65 and c-Rel but Not p50

To begin characterizing the properties of the protein encoded by the cDNA, the clone was translated in vitro in rabbit reticulocyte lysate. Although the open reading frame predicts a protein of 41 kDa, the in vitro translated protein migrated with an apparent M, of 45 kDa, which is very similar to the size of the purified protein (Figure 3A). The small amount of the 45 kDa protein synthesized could efficiently inhibit the DNA binding of endogenous NF-κB in rabbit reticulocyte lysates (which is primarily p50–p65)

A

B

C
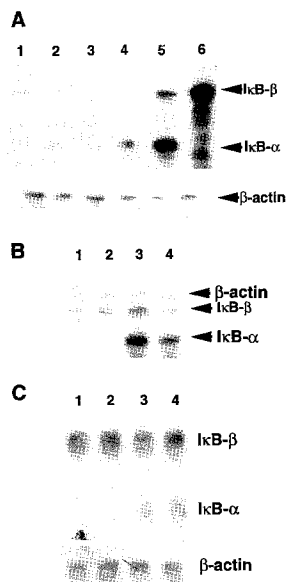
Figure 4. IκB-β mRNA Levels Are Not Regulated by NF-κB

(A) Ribonuclease protection analysis for distribution of IκB-α and IκB-β in different mouse tissues: brain (lane 1), heart (lane 2), liver (lane 3), lung (lane 4), spleen (lane 5), and testis (lane 6). Total RNA (10 μg) from each tissue (Clontech) was used for each lane. The probes for IκB-α and IκB-β in the ribonuclease protection assay were used for hybridization in the same RNA samples. Separate reactions with identical samples were used for β-actin.

(B) Expression of IκB-β and IκB-α in mouse B cell lines, HAFTL (pro-B) (lane 1), PD-31 (pre-B) (lane 2), WEHI 231 (early mature B) (lane 3), and S-194 (plasma) (lane 4). Total RNA (10 μg) made from the different cell lines was used for analysis using ribonuclease protection assay using antisense probes for IκB-α, IκB-β, and β-actin. The β-actin probe was labeled at lower specific activity to allow exposure on the same gel.

(C) PD-31 pre-B cells were stimulated with 2 μg/ml LPS for 4 hr and 12 hr and with 25 ng/ml PMA for 8 hr. The cells were then harvested, and total RNA was made using a guanidium-thiocyanate acid-phenol extraction procedure. RNA (20 μg) was analyzed in each lane, and the same blot was probed sequentially with IκB-β, β-actin, and IκB-α. Lane 1 is unstimulated, lane 2 is stimulated with PMA for 8 hr, and lanes 3 and 4 are stimulated with LPS for 4 and 12 hr, respectively.

(data not shown) (Davis et al., 1991). The translated IκB-β could also inhibit DNA binding of exogenous NF-κB (p50–p65 purified from rabbit lungs) when added to the lysates (data not shown). However, the low amount of in vitro translated IκB-β protein that was synthesized was not sufficient for carrying out a systematic quantitative analysis to determine whether IκB-β demonstrated any preference that was distinct from IκB-α for specific Rel proteins.

To determine whether the cloned cDNA was active in cells, the IκB-α and IκB-β cDNAs were cotransfected into COS cells with p50, p65, and c-rel cDNAs. Analysis of the extracts made from the transfected cells indicated that DNA binding by both p65 and c-Rel was inhibited by the IκB isoforms, although it appeared that IκB-β was less effective on c-Rel (Figure 3B). Inclusion of a reporter construct that contains a luciferase gene driven by two κB sites in these transfections indicated that both IκB isoforms could inhibit p65- and c-Rel-mediated transcription,
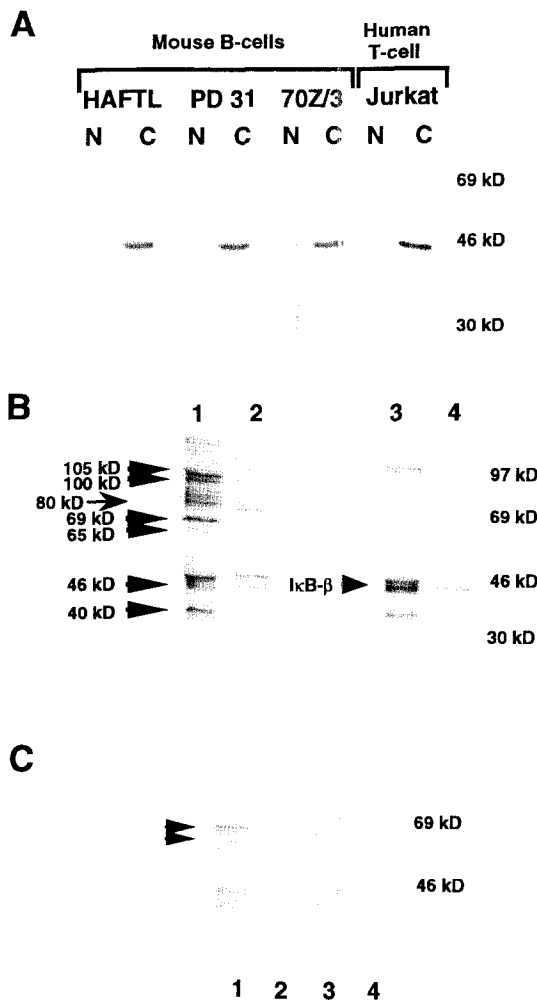
consistent with the results of the gel retardation assays (data not shown).

To obtain an independent confirmation of the relative affinities of IκB-α and IκB-β for p65 and c-Rel, we fused full-length IκB proteins to glutathione S-transferase (GST), produced them in bacteria, and tested them for their ability to bind Rel proteins. The GST–IκB proteins were mixed with in vitro translated, ^{35}S-labeled Rel proteins (Figure 3C). The IκB–Rel protein complexes were precipitated with glutathione–agarose and washed extensively, and the bound Rel proteins were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by fluorography. Both IκB-α and IκB-β efficiently and specifically interacted with and precipitated p65 and c-Rel, but not p50 (Figure 3C). In both instances, p65 was more efficiently precipitated than c-Rel.

IκB-β mRNA Is Widely Expressed in Different Tissues and Is Not Up-Regulated upon Induction of NF-κB

To determine whether IκB-β plays a unique function in certain tissues, the pattern of expression of mRNAs for the two IκB isoforms was analyzed using ribonuclease protection assays. Total RNA from mouse brain, heart, liver, lung, spleen, and testis was hybridized to probes for IκB-α and IκB-β. The results of the assay indicated that the mRNA encoding IκB-β was present in low but varying levels in all of the tissues examined, with a significantly higher level of expression in testis (Figure 4A). The IκB-α mRNA was also expressed in different tissues, with the exception that there was no expression in testis and increased expression in spleen. The increased expression of IκB-β in testis may indicate a distinct role for this IκB in testis differentiation or function. The pattern of expression observed was also further verified by carrying out Northern blot analysis, and the results were identical to those obtained from ribonuclease protection analysis (data not shown).

The levels of IκB-α and IκB-β mRNA were then examined in cell lines representing different stages of mouse B cell development, particularly because mature B cells are among the few cell types in which NF-κB is constitutively active (Sen and Baltimore, 1986). Expression of IκB-α mRNA is greatly increased in mature B cells because the nuclear NF-κB in these cells up-regulates the expression of the gene in an autoregulatory fashion (Figure 4B). By contrast, the level of IκB-β mRNA was not significantly altered in mature B cells, suggesting that its expression was not subject to up-regulation by nuclear NF-κB (Figure 4B). To prove that the expression of IκB-β mRNA was indeed independent of nuclear NF-κB, we treated pre-B cells with PMA and LPS and determined the levels of IκB-α and IκB-β mRNAs by Northern blot analysis. After 4 and 12 hr of induction by LPS, there was a significant increase in the level of IκB-α mRNA while the level of IκB-β mRNA remained relatively unaltered (Figure 4C). Since the 3′ untranslated region of the IκB-β mRNA does not contain any AUUUA sequences, which signal rapid RNA turnover and are found in transcripts encoding IκB-α, it is likely that IκB-β mRNA has a long half-life (Haskill et al., 1991). Therefore, these results suggest that,

A

Mouse B-cells | Human T-cell

HAFTL PD 31 70Z/3 Jurkat
N C N C N C N C

69 kD
46 kD
30 kD

B

1 2 3 4

105 kD
100 kD
80 kD
69 kD
65 kD
97 kD
69 kD

46 kD
40 kD
IκB-β
46 kD
30 kD

C

69 kD
46 kD

1 2 3 4

Figure 5. IκB-β Is Present in Complexes Containing p65 and c-Rel

(A) Immunoblot analysis of nuclear and cytosolic fractions from different cell lines using the rabbit IκB-β antiserum.

(B) Immunoprecipitations were carried out on 2 × 10⁷ metabolically labeled Jurkat cells (with [³⁵S]methionine and [³⁵S]cysteine; ICN Tran³⁵S-label) using the antiserum to IκB-β (lanes 1 and 3) and the corresponding preimmune serum (lanes 2 and 4). The bands that appear only with the immune serum are indicated (lanes 1 and 3). Immunoprecipitations from boiled samples neutralized with Nonidet P-40 (lanes 3 and 4) were carried out similarly to the other samples (lanes 1 and 2)

(C) Immunoprecipitations were carried out as in (B) on metabolically labeled cells with immune (lanes 1 and 3) and preimmune (lanes 2 and 4) sera. The immunoprecipitates were then boiled with 0.5% SDS buffer and then neutralized with 1% Triton X-100–containing buffer. Secondary immunoprecipitations were then carried out with antibodies to c-Rel (lanes 1 and 2) and p65 (lanes 3 and 4). The secondary immunoprecipitates were fractionated on an SDS–polyacrylamide gel and detected by fluorography.

unlike IκB-α, IκB-β may not be utilized for regulating rapid responses but for responding to persistent signals that yield a more permanent change.

IκB-β Exists In Vivo as a Complex with p65 and c-Rel

To examine the regulation of IκB-β in greater detail, we raised rabbit polyclonal antiserum against the GST–IκB-β

fusion protein. This antiserum recognized one predominant band of ~45 kDa on immunoblots of both cytosolic extracts from different cells (Figure 5A) and purified fractions from rabbit lung (data not shown). The size of the protein detected was identical to the size of the purified IκB-β protein. In most cells, the majority of IκB-β protein was in the cytoplasm (Figure 5A). The small amount in the nucleus might be an artifact of the extraction procedure, although the IκB-α protein in these same cells was exclusively cytosolic (data not shown). An unexpected finding from these experiments was that the level of IκB-β protein in cells appeared to be at least equal to, if not greater than, IκB-α (using antibodies of equivalent affinity; determined by titrating the antibodies against known amounts of purified recombinant bacterial proteins), and the lower estimates of its abundance reported previously may have been due to greater losses during purification.

The IκB-β protein was purified as a complex with p50–p65 from human placenta and rabbit lungs, and therefore it was likely that it would exist in cells as a complex with p50–p65 (Davis et al., 1991; Ghosh and Baltimore, 1990; Link et al., 1992; Zabel and Baeuerle, 1990). The experiments described above indicated that it was able to bind equally well with both p65 and c-Rel. Therefore, in lymphoid cells, which contain both p65 and c-Rel, IκB-β should be in complexes with both Rel proteins. To test this hypothesis, we performed immunoprecipitations on Jurkat cell extracts using the IκB-β antiserum and its corresponding preimmune serum. The immune serum coprecipitated polypeptides of ~40, ~46, ~65, ~70, ~105, and ~110 kDa that resembled the sizes of IκB-α, IκB-β, p65, c-Rel, NF-κB p100, and NF-κB p105, respectively (Figure 5B) (an additional polypeptide of ~80 kDa does not correspond to any known Rel protein). Because IκB-β does not label efficiently and overlaps with p50 and immunoglobulin heavy chain, it was not clearly resolved in these gels. A similar pattern was observed upon immunoprecipitating IκB-α from Jurkat cells (R. J. P. and S. G., unpublished data) and WEHI 231 cells (Rice and Ernst, 1993). Immunoprecipitations carried out on extracts that were boiled previously with SDS and neutralized with Nonidet P-40 contained only p40 and IκB-β, but not the other proteins, indicating that they were noncovalently associated with IκB-β in cell extracts (Figure 5B) (p40 probably cross-reacted with the antiserum). To prove that IκB-β is present in complexes that contain p65 and c-Rel, we carried out immunoprecipitations as before, which were then boiled in SDS buffer to release the associated polypeptides and reimmunoprecipitated with antibodies to p65 and c-Rel. Such experiments revealed that only the IκB-β antiserum, and not the preimmune serum, coimmunoprecipitated the p65 and c-Rel proteins (Figure 5C).

LPS and IL-1 Lead to the Loss of Both IκB-α and IκB-β Protein While PMA and TNFα Affect Only IκB-α

To examine the fate of IκB-β upon activation of NF-κB, we treated 70Z/3 pre-B cells with LPS or IL-1 and analyzed subcellular fractions by immunoblotting and gel retardation assays. Immunoblotting analysis revealed that both
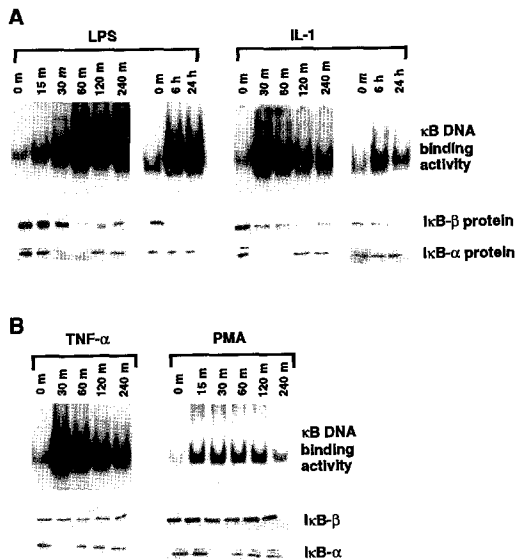
A

κB DNA binding activity

IκB-β protein

IκB-α protein

B

κB DNA binding activity

IκB-β

IκB-α

Figure 6. LPS and IL-1, but Not PMA and TNFα, Cause the Degradation of IκB-β

70Z/3 cells (for LPS, IL-1, and PMA) and Jurkat cells (for TNFα) were treated with the different inducers for different lengths of time. Nuclear extract (8 μg) was used for gel retardation assays, while 25 μg of the cytoplasmic extract was used for immunoblotting.

(A) 70Z/3 cells were stimulated with either 10 μg/ml LPS or 0.05 U/ml IL-1 for the indicated periods of time. The band seen on immunoblots with IκB-β antiserum after 120 and 240 min of LPS stimulation is different from the band seen at earlier timepoints as it migrates slightly faster; however, this band also disappears upon further stimulation (6 and 24 hr). Two closely spaced proteins can be distinguished in the IκB-β immunoblots of the IL-1-treated samples.

(B) Jurkat cells were stimulated with TNFα (1 ng/ml) while 70Z/3 cells were treated with 25 ng/ml of PMA.

inducers led to a loss of IκB-α protein, which was rapidly degraded upon stimulation (~30 min) but reappeared within 2 hr (Figure 6A). By contrast, with both inducers the 45 kDa IκB-β band was less affected at early timepoints (30 min), but then its levels decreased gradually and almost disappeared by 2 hr (Figure 6A). Increasing the period of stimulation to 6 hr and 24 hr did not alter the overall pattern; the level of IκB-α protein was similar to unstimulated controls, while levels of IκB-β was greatly reduced. This experiment highlighted subtle differences in the pattern of IκB-β degradation upon stimulation with LPS and IL-1. Persistent LPS stimulation led to complete loss of IκB-β protein while persistent IL-1 stimulation caused a dramatic decrease in IκB-β levels, although some residual IκB-β could be detected even after 24 hr of stimulation. The significance of these differences, however, is not yet clear. On immunoblots, the IκB-β antibody occasionally detected a closely spaced doublet of bands in different cells, and upon stimulation the upper band was lost preferentially (seen in Figure 6A). The upper band may represent a phosphorylated form that is targeted for degradation. Gel retardation assays indicated that nuclear NF-κB DNA-binding activity appeared as soon as IκB-α was lost and continued to be detected for the length of the assay (~24 hr), even though newly synthesized IκB-α accumulated

and reappeared within 1 hr (Figure 6A). Therefore the nuclear NF-κB detected at later timepoints may be released from IκB-β complexes.

Treatment of 70Z/3 cells with PMA or of Jurkat cells with TNFα leads to the rapid but transient induction of NF-κB activity. Typically, the activity peaks within 30 min and gradually decays thereafter, reaching baseline levels within 4–6 hr. Therefore, it differs from LPS induction of NF-κB, which increases with slower kinetics but then, in the continuing presence of the inducer, persists for over 36 hr. Because the persistent induction of NF-κB by LPS was accompanied by the sequential degradation of both IκB-α and IκB-β, we wanted to determine whether the transient induction by PMA or TNFα affected only the IκB-α complexes. To test this possibility, we treated 70Z/3 cells with PMA and treated Jurkat cells with TNFα. Subcellular extracts from these cells were then analyzed by immunoblotting and gel retardation assays. The kinetics of NF-κB activation and decay were similar to previous reports: a peak around 30 min followed by significant reduction of the signal by 4 hr (Figure 6B). The immunoblot of the cytoplasmic fractions indicated that both inducers caused a loss of IκB-α protein within 30 min followed by its synthesis and reappearance by 1 hr. However, with both inducers there was no effect on IκB-β, thus implicating degradation of IκB-β in the persistent activation of NF-κB. These results also strongly suggested that the activation of the two IκBs involved distinct signaling pathways.

TPCK and PDTC Block the Degradation of IκB-β

To determine whether different signaling pathways targeted the two IκBs, we tested a number of inhibitors and examined their effect on LPS-induced degradation of IκB-α and IκB-β. The degradation of IκB-α upon stimulation of cells is not affected by the protein synthesis inhibitor cycloheximide, suggesting that no new protein synthesis is required (Henkel et al., 1993; Sun et al., 1993). Since activation of IκB-β may occur through a distinct pathway, we wanted to determine whether cycloheximide affected its degradation. We pretreated 70Z/3 cells with 25 μg/ml cycloheximide for 30 min, a concentration that blocks all protein synthesis in these cells, and then stimulated them with LPS. Gel retardation assays showed that cycloheximide itself induced some NF-κB activity but did not alter the pattern of NF-κB activation in these cells (Figure 7). As expected, the IκB-α that disappeared from cycloheximide-treated cells did not reappear since no new protein synthesis had taken place. IκB-β also disappeared with a kinetics similar to noncycloheximide-treated cells, suggesting that activation of both IκBs occurs through distinct signaling pathways that do not require any new protein synthesis.

We then tested the chymotrypsin inhibitor N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), which has been demonstrated to block the activation of NF-κB and the accompanying disappearance of IκB-α with all known inducers, leading to the suggestion that it inhibits the protease responsible for IκB-α degradation (Henkel et al., 1993; Mellitis et al., 1993). However, recent studies suggest that TPCK inhibits NF-κB activation by interfering

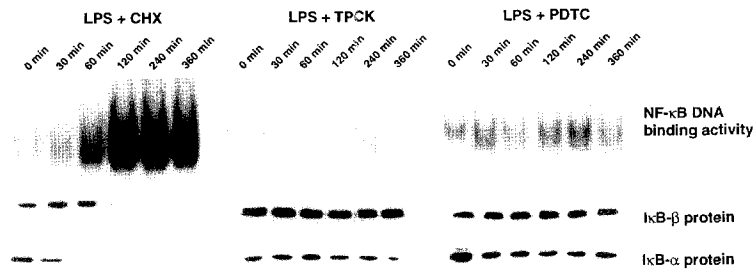Figure 7. Effect of Cycloheximide, TPCK, and PDTC on Turnover of IκB-α and IκB-β

70Z/3 cells were preincubated with 25 μg/ml cycloheximide, 25 μM TPCK, or 25 μM PDTC for 30 min before stimulating them with 10 μg/ml LPS for the indicated periods. Nuclear and cytoplasmic extracts were made and analyzed by immunoblotting and gel retardation assays.

with a common element in the signaling pathways that affect IκB (R. J. P. and S. G., unpublished data). Preincubation of 70Z/3 cells with TPCK for 30 min, followed by LPS treatment for different lengths of time, indicated a complete block in LPS induction of NF-κB activity (Figure 7). Immunoblot analysis on the cytoplasmic extracts of the treated cells revealed that neither IκB-α nor IκB-β was affected in the presence of TPCK, a result in keeping with the lack of any induced NF-κB activity. Similar results were seen using a pyrrolidone derivative of dithiocarbamate (PDTC), an antioxidant that blocks the activation of NF-κB in response to various inducers through an as-yet-uncharacterized mechanism involving oxidative free radicals (Schreck et al., 1991; Sun et al., 1993). In 70Z/3 cells treated with LPS, 25 μM PDTC almost completely blocked both the induction of NF-κB when examined by gel retardation assays and the degradation of IκB-α and IκB-β when examined by immunoblot analysis (Figure 7). Thus, although the pathways that lead to activation of IκB-α and IκB-β complexes are different, they involve some common steps that are sensitive to TPCK and PDTC.

Discussion

In this paper we report the cloning and characterization of the second major isoform of IκB in mammalian cells. In contrast with previous reports, we find that IκB-α and IκB-β display similar inhibitory activities and are present in equal amounts in cells as complexes containing similar proteins (Kerr et al., 1991, 1992). Instead, the major difference between the two IκB isoforms lies in their responses to different inducers of NF-κB activity. Some inducers elicit a transient activation by affecting only IκB-α complexes while other inducers yield a more permanent change by affecting both IκB-α and IκB-β complexes. We also find that nuclear NF-κB is unable to induce transcription of IκB-β mRNA, unlike IκB-α, suggesting that NF-κB released from IκB-β is not down-regulated by an autoregulatory feedback mechanism. Therefore, the activation of NF-κB occurs in a novel biphasic fashion in which, after stimulation with persistent inducers such as LPS, NF-κB is first released from IκB-α complexes and then from IκB-β complexes.

Activation of NF-κB is likely to be persistent in a tissue that lacks IκB-α, e.g., testis, because there will be no feedback inhibition through increased synthesis of IκB-α. However in cells that contain both IκB isoforms, activation of NF-κB can occur in a biphasic manner only if the NF-κB

released from IκB-β complexes is resistant to newly synthesized IκB-α. This might be achieved if, upon release from IκB-β, the NF-κB enters the nucleus via a pathway that is insensitive to any free cytosolic IκB-α (or IκB-β) or if the NF-κB released from IκB-β complexes does not interact efficiently with IκB-α. An alternate explanation may be that both IκB-α and IκB-β complexes are affected at the same time, but the NF-κB released from IκB-β complexes persists for longer periods in the nucleus. The dissociation of NF-κB–IκB-β complexes might require a concomitant modification of the NF-κB protein, e.g., phosphorylation, and the phosphorylated NF-κB can be retained for longer periods in the nucleus. A similar scenario is probably observed in the activation of the morphogen dorsal during Drosophila embryogenesis, when the dorsal protein that is dissociated from its inhibitor cactus is modified by phosphorylation (Wasserman, 1993). Genetic evidence suggests that modification of dorsal occurs concomitantly or subsequently to the modification of cactus, by the upstream kinase pelle (Wasserman, 1993). This phosphorylated dorsal protein persists in the nucleus for sufficiently long periods of time to allow events in differentiation to occur. The overall similarity of IκB-β with cactus, both in the number of ankyrin repeats as well as in the spacing of the repeats, hints that a common mechanism may be used for dissociating their cytoplasmic complexes.

The availability of clones for the two major isoforms of IκB should allow us to determine how Rel transcription factors are differentially regulated. Previous studies had indicated that IκB proteins were the targets of signaling pathways that led to the activation of NF-κB (Ghosh and Baltimore, 1990; Kerr et al., 1991; Link et al., 1992). Therefore, the presence of two IκB molecules should allow greater flexibility in regulating the activity of Rel transcription factors because the two inhibitors can respond differentially to different signals. Our results have identified fundamental differences between signaling through LPS and IL-1 versus TNFα and PMA that were not appreciated previously. As most of the biological effects of PMA are believed to be mediated through activation of PKC, it appears that direct phosphorylation of IκB-β by PKC is not sufficient to cause its degradation and that LPS and IL-1 are able to activate a secondary signaling pathway. Therefore, a cell containing Rel protein complexes with both IκB-α and IκB-β could respond very differently to different signal transduction pathways. If the pathway targets only IκB-α, e.g., with PMA, the result would be the phosphorylation and degradation of IκB-α and a rapid and transient induc-

tion of NF-κB. On the other hand, persistent activation of NF-κB with LPS, IL-1, or differentiation signals may result in phosphorylation and degradation of both IκB-α and IκB-β, and if the NF-κB released from IκB-β complexes is retained longer in the nucleus, it would not be subject to feedback inhibition through increased synthesis of IκB-α.

The sequence of IκB-β does not reveal many surprises; it contains six consecutive ankyrin repeats, has an abundance of acidic amino acids, and contains a putative PEST domain similar to the other major IκB isoform, IκB-α (Davis et al., 1991; Haskill et al., 1991). However, the number of ankyrin repeats in IκB-β may be indicative of its distinct role. Whereas IκB-α, with five repeats, binds specifically to p65, both Bcl-3 and IκB-γ, with seven repeats each, bind specifically to p50 (cactus, which is specific for dorsal, has six repeats). Therefore, the six repeats in IκB-β might signify a target other than p65. However, both IκB-α and IκB-β were purified as complexes with p50–p65 NF-κB from rabbit lung cytosol and human placenta (Ghosh and Baltimore, 1990; Zabel and Baeuerle, 1990) and, as demonstrated in this report, appear to have similar affinities for Rel proteins. It would thus appear that the critical difference in the number of repeats and specificity lies between either five or six repeats (IκB-α and IκB-β) or seven repeats (IκB-γ and Bcl-3). On the other hand, the six repeats in IκB-β might allow a specific interaction with p65 or c-Rel that could only be broken by modifying both NF-κB and IκB-β through phosphorylation or dephosphorylation and would explain how NF-κB released from IκB-β complexes could persist for longer periods in the nucleus. Unequivocal determination of this model would require characterizing the phosphorylation status of nuclear versus cytoplasmic NF-κB.

The distinct behavior of the two IκBs following stimulation fulfills the promise of differential regulation suggested by the existence of multiple IκB isoforms. Although the two major IκB isoforms interact with the same Rel proteins, they are activated by distinct signaling pathways, probably eliciting very different physiological responses. While the IκB-α response is used for responding immediately to transient situations of stress, the persistent response through IκB-β may be utilized for situations of chronic inflammation, infection, stress, or differentiation. Future studies will allow us to address whether similar modification events are responsible for the activation of the two IκB isoforms and whether the degradation of IκB-β occurs through the same pathway as IκB-α.

Experimental Procedures

Purification of IκB-β from Rabbit Lungs

The purification of IκB-β was carried out from a total of 4 kg of rabbit lungs, with four individual purifications of 1 kg each. The initial steps in the purification were as reported in Ghosh and Baltimore (1990) for the purification of IκB-α. The present purification differed from that carried out previously as the phenyl–Sepharose step was eliminated and an FPLC hydroxyl apatite column (Pentax) was used instead of the conventional resin (Figure 1). The peak fractions containing IκB-β activity (50 μl) were analyzed by SDS–PAGE, and the proteins were visualized by silver staining. The yield of the pure protein after the final Superose-12 gel filtration column was ~2 μg, of which ~1.8 μg was used for SDS–PAGE and electroblotting to nitrocellulose mem-

brane. Following transfer, we estimated that ~1 μg of protein (20 pmol) was available for digestion with trypsin and sequencing.

Sequencing the Purified IκB-β Protein

The 45 kDa protein band on nitrocellulose was digested with trypsin, releasing peptides that were separated by narrow-bore HPLC as described previously (Ghosh et al., 1990). Six peaks were observed over the trypsin autolytic background. Using microanalytical techniques, sequence information was obtained from three peptides. The sequences of the three peptides were T15, LYAAxA(G)VCVAE with 1.4 pmol yield; T27, LQLEAENYDGxTPLxVA(v) at 1.6 pmol; and T41, PLHLAVEAQAAD(V)LELL at 1.5 pmol. Sequences are in the single letter annotation; x indicates that no residue could be identified at this position, parentheses indicate identifications with a lower degree of confidence, and amino acids shown in lowercase and parentheses were present at very low levels.

Cloning IκB-β cDNA from Mouse LyD9 Pro-B Cell cDNA Library

Degenerate PCR primers, containing EcoRI and BamHI restriction sites, were synthesized using sequences from the peptides T27 and T41 in both orientations. Rabbit lung total RNA was used to synthesize the cDNA template for PCR, using random hexamers for priming. Of the reverse-transcribed reaction mixtures, ~1/10 was used for the PCRs with different combinations of the primers; 5′ T27 plus 3′ T41 and 5′ T41 plus 3′ T27. The entire PCR mix was purified and digested with the two unique restriction enzymes and then ligated into a Bluescript vector digested with the same enzymes. A few clones were obtained from both ligations, and miniprep DNAs isolated from them were sequenced. One of the clones from the 5′ T27 plus 3′ T41 contained a 160 bp insert that had sequences that could encode the peptides used to design the primers. On Northern blots, this insert, when used as a probe, hybridized to a single band of 1.3 kb from both rabbit lung and mouse LyD9 RNA (data not shown). This result strongly suggested that there was only a single species of mRNA in mouse that was homologous to the 160 bp PCR product from rabbit. Therefore, we used this insert as a probe to screen different libraries, either obtained from commercial sources or made from mouse LyD9 or 22D6 cell lines. No clones were obtained from multiple screenings of multiple libraries that were all size selected for larger cDNAs (>1 kb), and therefore we made a new cDNA library in λgt10 with cDNA from the mouse pro-B cell line LyD9. The cDNA was size selected from 0.7 kb to 1.6 kb to increase the proportion of mRNAs encoding IκB-β, which has a size of 1.3 kb. Upon screening 10⁶ clones from this library, we obtained 15 positives, which all contained the same cDNA. We selected one of them, 15f, for subsequent analysis.

Expression of GST–IκB Fusions

Fusions of IκB-α and IκB-β with GST were generated by PCR-assisted cloning into the GEX-2t vector. The constructs in DH5α cells were grown overnight in small cultures. The overnight cultures were diluted 1:50 in media and grown to a density of ~0.3 OD$_{600}$ when they were induced with 0.4 mM IPTG for 4 hr. The cells were then harvested and lysed by freeze–thawing followed by sonication. The soluble extract was used to purify the GST–fusion proteins. For using IκB-β as antigen, the extract was first purified by glutathione–agarose affinity chromatography followed by FPLC Mono Q anion exchange and Sephacryl gel filtration chromatography.

Immunoprecipitation Analysis

Cells (2 × 10⁷) were labeled with Tran³⁵S-label (ICN Pharmaceuticals) for 45 min in RPMI containing 5% dialyzed fetal calf serum. Following labeling, cells were lysed in 1.2 ml of TNT buffer (20 mM Tris–HCl, 200 mM NaCl, and 1% Triton X-100) and centrifuged, and the supernatant was collected. The supernatant (300 μl) was used for each immunoprecipitation, and the volume of it was made up to 1 ml with TNT buffer. Then, 5 μl of the preimmune or immune serum and 20 μl of a 1:1 slurry of protein A–Sepharose was added and incubated overnight at 4°C. The samples were then centrifuged and the protein A–Sepharose washed five times with TNT buffer, and finally the pellet was boiled for 5 min in 2× SDS sample buffer and loaded onto SDS–polyacrylamide gels. Following electrophoresis, the gels were fixed

and incubated in Amplify (Amersham). The dried gels were then exposed for fluorography.

Western Blot Analysis

Western blot analysis was generally carried out using 25 µg of cellular extracts. Proteins were electroblotted from SDS–polyacrylamide gels onto PVDF membrane. The membrane was blocked with 5% Blotto, and the primary antibody was added in Blotto. Subsequent washes and incubation with the secondary antibody were done in TTBS. Proteins detected by the primary antibody were visualized by the use of an enhanced chemiluminescence assay (ECL) using reagents from Amersham and by exposure to film. The IκB-α antibodies used were either affinity-purified rabbit polyclonal antibody raised against a peptide (Santa Cruz Biotechnology) or affinity-purified antibody against the recombinant full-length protein. The p50 and IκB-β antibodies were rabbit polyclonal antisera raised against purified recombinant proteins.

Ribonuclease Protection Assays

Ribonuclease protection assays were carried out according to protocols and reagents from Ambion (RPA II kit). Antisense probes of 250, 200, and 150 bases for β-actin, IκB-β, and IκB-α were made by in vitro transcription using T7 RNA polymerase. The labeled probes were excised from a polyacrylamide gel and eluted. The probes were then hybridized overnight to 10 µg of total RNA for each sample at 42°C. The samples were then digested with RNase and analyzed by gel electrophoresis.

Acknowledgments

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GenBank Accession Number

The accession number for the sequence reported in this paper is U19799.