I-Rel: a novel *rel*-related protein that inhibits NF- κ B transcriptional activity

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The NF- κ B transcription factor complex is comprised of two subunits, p50 and p65, that share significant homology to the *rel* oncogene. We have isolated a cDNA encoding a novel 66-kD *rel*-related protein, designated I-Rel. Unlike other *rel*-related proteins, I-Rel does not interact with DNA. I-Rel forms heterodimers with p50, however, and greatly attenuates its DNA-binding activity—an effect probably resulting from the presence of a domain inhibitory to DNA binding present within the 121 amino-terminal residues of I-Rel. In contrast, I-Rel does not associate with p65. Transfection experiments demonstrate that I-Rel suppresses NF- κ B-induced transcription, probably through its association with p50. Expression of I-Rel mRNA is induced by mitogenic stimulation and accumulates after the appearance of p50 transcripts. Our findings suggest that p50 and I-Rel are components of a feedback pathway where expression of I-Rel may modulate indirectly the expression of genes responsive to the NF- κ B transcription factor complex.

[Key Words: NF- κ B; transcription factor complex; *rel* oncogene]

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The NF- κ B transcription factor complex, characterized originally as an immunoglobulin κ light-chain enhancer-binding activity (Sen and Baltimore 1986a) is now known to be involved in the inducible expression of a large number of genes. Binding sites for NF- κ B have been identified in the regulatory elements of cytokine, cytokine receptor, major histocompatibility antigens, and several viral enhancer elements [for review, see Lenardo and Baltimore 1989; Gilmore 1990; Baeuerle and Baltimore 1991]. NF- κ B is composed of a 50-kD and a 65-kD protein subunit (Baeuerle and Baltimore 1989; Ghosh and Baltimore 1990). Identification and cloning of the individual genes encoding proteins that comprise the NF- κ B transcription factor complex permit insight into this important signal transduction pathway. For example, it is known that residues present within the amino terminus of both the p50 (Bours et al. 1990; Ghosh et al. 1990; Kieran et al. 1990) and the p65 (Nolan et al. 1991; Ruben et al. 1991) subunits of NF- κ B share considerable homology with the oncogene *c-rel*, as does the amino terminus of the *Drosophila* maternal morphogen *dorsal* (Steward 1987). The *v-rel* oncogene, identified originally in the avian reticuloendotheliosis retrovirus Rev T (Theilen et al. 1966), causes lymphoid cell tumors in birds (for review, see Rice and Gilden 1988). It is now clear that the *rel* family of proteins possesses transcriptional regulatory properties (Gelinas and Temin 1988; Hannink and Temin 1989; Rushlow et al. 1989; Bull et al. 1990; Kamens et al. 1990; Richardson and Gilmore 1991; Urban et al. 1991).

Residues within the *rel* homology region confer the ability to form homomeric and heteromeric protein complexes, a process prerequisite for DNA binding (Ballard et al. 1990; Ghosh et al. 1990; Kieran et al. 1990; Ruben et al. 1992). After association with DNA, certain combinations of these proteins elicit transcriptional activation, the best example being the association of p50 and p65 in NF- κ B (Kawakimi et al. 1988; Schmitz and Baeuerle 1991; Ruben et al. 1992). Other associations may serve different functions, as the association of *v-rel* with NF- κ B has been shown to suppress transcriptional activation (Ballard et al. 1990).

Earlier studies demonstrated that the entire NF- κ B signal transduction pathway is under stringent regulatory control. In resting cells, the p50–p65 complex exists in the cytoplasm in an inactive state bound with the repressor protein I κ B (Baeuerle and Baltimore 1988a,b) by interaction with the p65 subunit (Baeuerle and Baltimore 1989; Urban and Baeuerle 1990). The property of regulation by subcellular localization is shared by each of the *rel*-related family members. For example, the chicken *c-rel* protein is localized in the cytoplasm of avian fibroblasts, whereas the *v-rel* protein is nuclear in these cells (Capobianco et al. 1990). In transformed avian lymphoid cells, however, *v-rel* is localized primarily in the cytoplasm (Gilmore and Temin 1986). In addition, the pp40 phosphoprotein, which is identical to I- κ B β (Zabel and Baeuerle 1990), prevents DNA binding of both *c-rel* and NF- κ B by maintaining a cytoplasmic pool of these proteins (Davis et al. 1991; Kerr et al. 1991). Similarly, the *Drosophila* maternal morphogen *dorsal* is localized in

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the cytoplasm of cleavage stage embryos, whereas in the blastoderm it is relocated in a graded fashion to the ventral nuclei (Roth et al. 1989; Rushlow et al. 1989; Steward 1989). Translocation of NF- κ B to the nucleus can be induced by a variety of stimuli, including viral proteins (Ballard et al. 1988; Leung and Nabel 1988; Ruben et al. 1988), mitogens (Seiki et al. 1986; Bohnlein et al. 1988), and several cytokines (Lowenthal et al. 1989; Osborn et al. 1989). This process is thought to occur after dissociation of I κ B from p65, a process that may involve phosphorylation of I κ B (Ghosh and Baltimore 1990). p50 mRNA expression is also observed after NF- κ B activation (Bours et al. 1990). Induction of p50 mRNA synthesis may reflect the action of NF- κ B on the p50 promoter, as the p50 promoter contains two potential NF- κ B-binding motifs (Ten et al. 1992). Thus, the induction of additional p50 by translocation of NF- κ B to the nucleus may constitute an autoregulatory feedback pathway. During prolonged exposure of some cells to mitogens, the level of nuclear NF- κ B decreases (Sen and Baltimore 1986b). The signals that regulate this suppression remain to be established. I κ B provides one means of early control by maintaining an inactive cytoplasmic pool of NF- κ B. During prolonged periods of stimulation, however, an additional means of suppressing NF- κ B activity may be necessary as I κ B presumably remains inactive under these conditions.

This study presents the identification and partial characterization of a novel *rel*-related protein, designated I-Rel (for inhibitory Rel). By several criteria, I-Rel is unlike other *rel*-related proteins in that it is unable to associate with the κ B motif and possesses an additional 121 amino acids preceding the *rel* homology domain. I-Rel associates with p50, however, to form a heteromeric complex with a greatly attenuated ability to bind DNA. In contrast, I-Rel fails to associate with p65. Accumulation of I-Rel transcripts is observed at a time after induction of p50 mRNA, and transient cotransfection studies demonstrate that expression of I-Rel suppresses NF- κ B function. These findings raise the intriguing possibility that I-Rel functions as a feedback inhibitor to regulate NF- κ B function.

Results

Identification of I-Rel

Previously, we described the use of a polymerase chain reaction (PCR)-based approach, using degenerate oligonucleotides synthesized to two regions of extreme homology within the known *rel*-related proteins, to clone the p65 subunit of NF- κ B (Ruben et al. 1991). Individual clones containing the PCR-amplified products were sequenced. Of these, 49 were identical to NF- κ B p65, whereas one, although sharing considerable similarity to known *rel*-related gene products, was dissimilar to any of the family members described previously. This sequence was used as a probe to screen a cDNA library prepared from human Jurkat T-cell RNA. Seven individual phage clones were identified under high-stringency hybridiza-

tion conditions. DNA sequencing revealed that each contained the probe sequence. Two of the largest cDNAs were sequenced to completion.

Each of the characterized clones contained an insert of 2314 bp with a predicted open reading frame of 579 amino acids beginning with a methionine present at nucleotide 145 (Fig. 1). By Northern blot analysis, the mRNA for these clones appears to be ~2.3 kb, slightly smaller than p65 mRNA (2.6 kb), suggesting that these clones represent full-length messages. Analysis of the predicted protein, which we have designated I-Rel, revealed a domain from amino acids 122 to 425 that shares considerable homology with the other known *rel*-related gene products (Fig. 2). Comparison of the predicted amino acid sequence of I-Rel with other *rel*-related proteins revealed that it is most similar to p65 (51% identical amino acids) and *rel* (49% identity) within the *rel* homology domain. Homology between I-Rel and both *dorsal* and p50 within this domain is also significant (45% identity). Unlike previously identified *rel*-related proteins, however, I-Rel has an additional 121 amino acids preceding the *rel* homology domain. Within this region is a predicted α -helical structure between residues 40 and 68 that has the potential to form a leucine zipper-like motif (Landshultz et al. 1988). Another notable difference between I-Rel and other *rel*-related proteins is replacement of the putative serine phosphorylation site (RRXS) conserved within other *rel*-related proteins by the sequence QRLT. Also, the putative nuclear localization signal between amino acids 410 and 414 (KKAKR) differs from the nuclear localization signals of other *rel*-related proteins, in that it contains a hydrophobic residue within the basic region. There is a basic region between amino acids 433 and 438, however, that could serve as a potential localization signal. The carboxy-terminal region (i.e., residues following amino acid 425) is completely divergent with respect to any of the characterized *rel*-related proteins. Also, the carboxy-terminal region of I-Rel is considerably shorter than the carboxy-terminal regions of human *c-rel* and p65 by 135 and 95 amino acids, respectively. Similar to p65, however, this region contains a high percentage of proline residues (17%) and has an overall net negative charge. In vitro translation of the I-Rel RNA, derived by in vitro transcription of its cDNA (see below), results in a 66-kD protein. The predicted molecular mass based on the amino acid composition corresponding to the longest open reading frame is 62 kD.

I-Rel does not associate with DNA

Each of the *rel*-related proteins identified to date has demonstrated DNA-binding properties and, more specifically, has been shown to interact with the sequence originally identified as the NF- κ B-binding motif present upstream of the immunoglobulin light-chain enhancer (Sen and Baltimore 1986a). To examine whether I-Rel could associate with the κ B motif, the ability of in vitro translated I-Rel to interact with a 32 P-labeled κ B oligonucleotide was tested in a gel mobility-shift assay. No

Figure 1. Nucleotide and predicted amino acid sequence of the I-Rel cDNA. The nucleotide sequence corresponding to the beginning of the cDNA and the deduced amino acid sequence of the longest open reading frame is shown. The amino acids corresponding to the *rel* homology domain are underlined. Sequence data have been submitted to the EMBL/GenBank data libraries under accession no. M83221.

The precursor of p50, p105, shows no binding activity *in vitro* and must be truncated before demonstrating binding activity (Ghosh et al. 1990; Kieran et al. 1990). Therefore, I-Rel may also need to be processed in a similar manner before it can bind DNA. To examine this possibility, nucleotides encoding residues 1–425 of I-Rel

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CONSENSUS									
I-REL	MLRSGPASGP	SVPTGRAMPS	RRVARPPAAP	ELGALGSPDL	SSLSLAVSRS	TDELEIIDEY	IKENGFGLDG	GQPGP	
p65									
hcrel									
Mouserel									
p50									
Dorsal									
	1								75
CONSENSUS		gaa.l..p..	p....	p...l.	q...s....P	yveIIEQP.q	rgmrFRYkCE	GrSaG	
I-REL	GEGPLRLVSR	GAASLSTVTL	GPVAPPATPP	PWGCPLGLRV	SPAPGPGPQP	HLVITEQPKQ	RGMPFRYEC	GRSAG	
p65				MDLFLPLIF	PAEPAQASGP	YVEIIEQPKQ	RGMRFRYKCE	GRSAG	
hcrel					..MASGLYNP	YIEIIEQPRQ	RGMRFRYKCE	GRSAG	
Mouserel					..MASSGYNP	YVEIIEQPRQ	RGMRFRYKCE	GRSAG	
p50		MAE	DDPYLGRPEQ	MFHLDPSLTH	TIFNPEVFQP	QMALPTADGP	YLQILEQPKQ	RGFRFRYVCE	GPSHG
Dorsal	...MFPNQNN	GAAPGQGP	PAV	DGQQSLNNG	LPAQQQQQLA	QSTKNVRKKP	YVKITEQFAG	KALRFRYEC	GRSAG
	76								150
CONSENSUS	sipge.Stdn	nktyPsi.im	nyyG.gkvr.	t..lVtk.dp	y.phpHdLVG	Kd.Crdgyye	aefgperrp.	lsFqN	
I-REL	SILGESSTEA	SKTLPAIELR	DOGGLREVEV	TACLWVKDWP	HRVPHSLVVG	KD.CTDGICR	VRLRPHVSPR	HSFNN	
p65	SIPGERSTDT	TKTHPTIKIN	GYTGGTVRI	S...LVTKDPP	HRPHPHSLVG	KD.CRDGYFE	AELCPDRCI	HSFQN	
hcrel	SIPQEHSTDN	NRTYPSINIM	NYGGRGVRI	T...LVTKNDP	YKPHPHDLVG	KD.CRDGYFE	AEFGNERRP	LFFQN	
Mouserel	SIPGERSTDN	NRTYPSVQIM	NYGGRGVRI	T...LVTKNDP	YKPHPHDLVG	KD.CRDGYFE	AEFGNERRP	LFFQN	
p50	GLPGASSEKN	KKSYPQVKIC	NYVGPAKIV	Q...LVTKNGN	IHLHAHSLVG	KH.CEDGICT	VTAGPKDMV	VGFAV	
Dorsal	SIPGVNSTPE	NKTYPTIEIV	GYKGRAVVVV	S...CVTKDTP	YRPHPHNLVG	KEGCKGVCT	LEINSETMR	AVFSN	
	151								224
CONSENSUS	LGIGcVvKkK.	v.eai...ri.	ag.inpfN..		...vpeeql	.die.....	D	lnvVR	
I-REL	LGIGCVRKKE	IEAAIERKIQ	LG.IDFYN..		...AGSL	KNHQ.....	EVD	MNVVR	
p65	LGIGCVKRRD	LEQAISQRIQ	TN.KNPFQ..		...VPIEE.		...QRGDYD	LNAV	
hcrel	LGIRCVKKE	VKEAIIIRIK	AG.INPFN..		...VPEKQL	NDIE.....	DCD	LNVVR	
Mouserel	LGIRCVKKE	VKGAILRIS	AG.INPFN..		...VGEQQL	LDIE.....	DCD	LNVVR	
p50	LGILHVTKKK	VFETLEARMT	EACIRGYNPG	LLVHPDLAYL	QAEGGGDRQL	GDREKELIRQ	AALQQTKEMD	LSVVR	
Dorsal	LGIGCVKRRD	IEAALKAREE	IR.VDPFKTG	FSHRF.....			...QPSSID	LNSVR	
	225								267
CONSENSUS	lcFq.fi.pd	ehGnftralp	PvvsnpIyDn	rapntaeLrI	cRvnkn.gsv	.GgdeifLLC	dKVQkDdIev	rFvI.	
I-REL	LCFQASY.RD	QQGQMRR.MD	PVLSEPVYDK	KSTNTSELRI	CRINKESGPC	TGGEELYLLC	DKVQKEDISV	VFSR.	
p65	LCFQVTV.RD	PSGRPLR.LP	PVLPHPIFDN	RAPNTAELKI	CRVNRNSGSC	LGGDEIFLLC	DKVQKEDIEV	YFTG.	
hcrel	LCFQVFL.PD	EHGNLTALP	PVVSNPIDN	RAPNTAELRI	CRVNKNCGSV	RGDEIFLLC	DKVQKDDIEV	RFVL.	
Mouserel	CVFMFFL.PD	EDGNFTTAVP	PVVSNPIDN	RAPNTAELRI	CRVNKNCGSV	RGDEIFLLC	DKVQKDDIEV	RFVL.	
p50	LMFTAFL.PD	STGSEFTRLE	PVVSDAIYDS	KAPNASNLKI	VRMDRTAGCV	TGGEIYLLC	DKVQKDDIQI	RFYEE	
Dorsal	LCFQVMESE	QKGRFTSPLP	PVVSPEIFDK	KA...MSDLVI	CRLCSCSATV	FGNTQIILLC	EKVAKEDISV	RFEE	
	268								339
CONSENSUS	...n.WEar	gdFsgaDVHR	QVAIVFKTPP	ycd...itePv	tVkmqLrRps	DgevSepm.F	rYlPdekDpy	g.k.K	
I-REL	...ASWEGR	ADFSQADVHR	QVAIVFKTPP	YEDLEIVEPV	TVNVFLQRLT	DGVCSELPF	TYLPRDHSY	GVDKK	
p65	...PGWEAR	GSFSQADVHR	QVAIVFKTPP	YADPSLQAPV	RVSMQLRRPS	DRELSEPMF	QYLPDTPDRH	RIEEK	
hcrel	...NDWEAK	GIFSQADVHR	QVAIVFKTPP	YCK.AITEPV	TVKMQLRRPS	DQEVSGSMD	KYLPDEKDTY	GMKAK	
Mouserel	...NDWEAR	GVFSQADVHR	QVAIVFKTPP	YCK.AITEPV	TVKMQLRRPS	DQEVSESMDF	RYLPDEKDAY	ANKSK	
p50	EENGGVWEGF	GDFSPTDVHR	QVAIVFKTPK	YKDINITKPA	SVFVQLRRKS	DLETSEPKPF	LYYPEIKDKE	EVQRK	
Dorsal	KNGQSVWEAF	GDFQHTDVHK	QTAITFKTPR	YHTLDITEPA	KVFIQLRRPS	DGVTSEALPF	EYVPMDSIPA	HLRRK	
	340								410
CONSENSUS	rqkttldfQk	llqd.g.ag							
I-REL	AKRGMPDVLG	ELNSSDPHG							
p65	RKRTYETFKS	IMKKSPPNG							
hcrel	KQKTTLLFQK	LCQDHVETP							
Mouserel	KQKTTLLFQK	LLQDCGHFT							
p50	RQKLMPNFSD	SFGGGSGAG							
Dorsal	RQKTGGDPMH	LLLQQQKQ							
	411	429							

Figure 2. Homology of the amino terminus of I-Rel with other *rel*-related proteins. The amino-terminal region of I-Rel is aligned with other *rel*-related proteins, including human p65 (Ruben et al. 1991), human *c-rel* (Brownell et al. 1989), mouse *rel* (Grumont and Gerondakis 1989), mouse p50 (Ghosh et al. 1990), and *dorsal* (Steward 1987). The numbers that appear below the sequence correspond to the amino acid positions in I-Rel. Uppercase letters correspond to identity among each of the proteins.

were incorporated into a bacterial expression vector (pDS I-Rel Δ 3'). Purification of the truncated I-Rel protein was achieved by the addition of 6 histidines at the amino terminus, which facilitates purification using a nickel chelate affinity matrix (Gentz et al. 1989). For control purposes, a similar strategy was used in parallel for purification of p50 (amino acids 1–377) and p65 (amino acids 1–309), as described previously (Ruben et al. 1992). High-level expression of each of the proteins was obtained (Fig. 3). Strong association with the κ B probe was observed using either purified bacterially expressed p50 or p65 (Fig. 4A). However, no association of the κ B motif with I-Rel(Δ 3') was observed. To provide for the possibility that the denaturation and renaturation involved in purification may be deleterious to I-Rel function, crude bacterial extracts were also used in the binding studies and provided similar results (Fig. 4B).

One possibility for the lack of binding is that I-Rel recognizes a DNA motif other than the canonical κ B consensus sequence. To examine this possibility, an oligonucleotide degenerate at 18 contiguous nucleotides was prepared. Incubation of the degenerate oligonucleotide probe using either purified p50 or p65 or a protein obtained directly from sonicated bacterial extracts demonstrated strong association in the gel-shift assay (Fig. 4). No association of κ B DNA or the degenerate probe with I-Rel(Δ 3'), however, was evident (Fig. 4). Taken together, these findings strongly suggest that I-Rel does not associate with DNA and, therefore, clearly differs from other known *rel*-related proteins.

Formation of nonfunctional p50/I-Rel heterodimeric complexes

Previous studies have established that the association of

rel-related proteins with DNA requires multimer formation mediated through residues within the *rel* homology domain (Ghosh et al. 1990; Kieran et al. 1990; Ruben et al. 1992). To test whether the inability of I-Rel to associate with DNA might reflect lack of a multimerization function, the ability of I-Rel to associate with p50 and p65 was examined. Because pure p50 and p65 form stable homodimers, it was necessary to first denature and renature them with an increasing amount of I-Rel. The resulting complexes were assayed for DNA binding using a gel mobility-shift assay. Similar experiments performed in parallel included renaturation of p50 with p65 and renaturation of p50 with p65 Δ [p65 Δ is encoded by an alternatively spliced form of p65 mRNA and is deficient in multimerization (Ruben et al. 1992)]. As reported earlier (Ruben et al. 1992), p50 readily formed a heteromeric complex with p65 but not p65 Δ (Fig. 5A, cf. lanes 9–12 with 13–15). Interestingly, renaturation of p50 with increasing amounts of I-Rel resulted in loss of DNA binding (lanes 1–4), indicative of the formation of an inactive p50/I-Rel heteromeric complex. This effect was specific for p50 as renaturation of p65 in the presence of an increasing amount of I-Rel had no effect on the ability of p65 to bind the κ B probe (lanes 5–8). These results indicate that I-Rel may associate with p50 and not p65. With long exposure, a weak but detectable slower migrating complex was seen upon renaturation of p50 with the highest level of I-Rel (lane 4). This may reflect residual low-affinity binding of the p50/I-Rel complex to DNA.

For I-Rel to function as an effective suppressor of NF- κ B activity, it must be capable of competing with p65 for binding to p50. To examine this possibility, bacterially produced p65 and p50 were combined at a concentration that promotes heterodimer formation and I-Rel was added at increasing concentrations. The protein mixture was denatured followed by gradual renaturation and used in the gel mobility-shift assay (Fig. 5B). As increasing levels of I-Rel were added, a decrease in p50–p65 complex formation was observed (lanes 3–6), indicating that I-Rel can compete with p65 for binding to p50.

As I-Rel is unable to bind DNA, it was not possible to determine the specific activity of the bacterially produced protein prepared by the denaturation–renaturation process. An alternate approach toward examining the function of I-Rel used I-Rel protein produced in an *in vitro* reticulocyte translation system (Fig. 6). Cotranslation of the RNA encoding the full-length species of I-Rel with RNA encoding p50 resulted in a similar level of expression for both proteins (Fig. 6A). Incubation of the translation reaction containing p50 alone with the κ B probe gave the expected size complex in the mobility-shift assay, whereas no complex was observed with addition of the translation containing only I-Rel (Fig. 6B). In the binding reaction containing the cotranslation of p50 and I-Rel there was a significant reduction in the amount of p50 binding to the κ B probe, in agreement with results obtained with the bacterially derived protein. Furthermore, inhibition occurred at an apparent excess of p50 to I-Rel (Fig. 6A). In the binding reaction containing p50 and I-Rel there was also an increase in

Figure 3. Purification of I-Rel from bacteria. *E. coli* expressing the I-Rel(Δ 3'), I-Rel(Δ 5'), p50(1–377), and p65(1–309) proteins were induced with IPTG during mid-logarithmic growth. The individual proteins were purified using a nickel chelate affinity matrix (see Materials and methods), analyzed on a 10% PAGE gel, and visualized by staining with Coomassie blue.

Figure 4. I-Rel does not bind DNA. (A) Purified p50[1-377], p65[1-309], and I-Rel[Δ3'] were incubated with a ³²P-labeled κB probe (κB) or a degenerate probe containing 18 randomly substituted nucleotides (D) in a gel mobility-shift assay. (B) Cleared bacterial extracts expressing the indicated protein were used in the mobility-shift assay with either the κB probe (5 × 10⁴ cpm) (κB) or degenerate probe (D) (1 × 10⁶ cpm).

the endogenous κB-binding activity. This may reflect an increase in free probe owing to the sequestration of p50 by I-Rel, which can now be bound by endogenous κB-binding activity. It is unlikely that I-Rel is present with the endogenous complex as no effect on the mobility of this complex was seen with the addition of an I-Rel-specific antibody (Fig. 6B).

The potential protein–protein associations involving I-Rel were examined more directly and under more stringent conditions in coimmunoprecipitation experiments (Fig. 7). As each of the proteins being examined shares considerable homology within the *rel* homology domain, expression vectors for several of the proteins were made fusing the amino-terminal sequences in-frame with a sequence encoding a 10-amino-acid epitope of the influenza hemagglutinin antigen (HA) (Kolodziej and Young 1991). DNA encoding the epitope-tagged HAp50, HAp65, or HA I-Rel proteins and untagged I-Rel, I-Rel(Δ3'), or p50 proteins was transcribed in vitro using T7 RNA polymerase. The RNAs encoding the tagged proteins were cotranslated with RNA coding for the indicated untagged protein in rabbit reticulocyte translation lysates. The epitope-tagged proteins were then immunoprecipitated using the monoclonal anti-HA antibody (Kolodziej and Young 1991). Each of the proteins was expressed efficiently in this system (see Fig. 7). Cotranslation of either full-length or truncated I-Rel with HAp50 resulted in coimmunoprecipitation of I-Rel with p50 (see Fig. 7A). In addition, HA I-Rel was able to coimmunoprecipitate p50 consistent with results obtained with HAp50 and I-Rel. In contrast, no association with either full-length or truncated I-Rel was evident after cotranslation and immunoprecipitation with either HAp65 or HAp65(1-309) (Fig. 7B). HAp65, however, efficiently coimmunoprecipitated p50, demonstrating that HAp65 is functional. These data support the findings obtained by gel mobility-shift analysis and suggest that

I-Rel forms inactive heteromeric complexes with p50 and is unable to associate with p65.

The ability of I-Rel to form homodimers was also examined. RNA encoding HA I-Rel was cotranslated with untagged I-Rel(Δ3') and immunoprecipitated using anti-HA antibody. Although HA I-Rel was able to coimmunoprecipitate p50, it was unable to coimmunoprecipitate I-Rel(Δ3'), suggesting that I-Rel lacks the ability to form homodimers. This could explain, in part, the apparent lack of DNA binding observed for I-Rel (see Fig. 4).

The amino-terminal domain of I-Rel is inhibitory to DNA binding

As I-Rel differs from other *rel*-related proteins by 121 amino acids preceding the *rel* homology domain, we examined whether this region might interfere with DNA binding. A further truncation of I-Rel(Δ3') [I-Rel(Δ5')] that lacks amino acids 1–121 and thus contains residues 122–425, corresponding to the *rel* homology domain, was made [I-Rel(Δ5'); see Fig. 3]. Consistent with results obtained using I-Rel(Δ3'), I-Rel(Δ5') also did not bind on its own (see Fig. 5C, lane 4). In contrast to results obtained upon renaturation of I-Rel with p50, no inhibition of p50 binding was obtained upon renaturation with I-Rel(Δ5') (see Fig. 5C). Rather, I-Rel(Δ5') formed a heteromeric complex with p50 that was capable of interacting with κB DNA (faster migrating complex below p50). The faster migrating complex must represent the p50–I-Rel(Δ5') heterodimer, as I-Rel(Δ5') was unable to interact with κB DNA in the absence of p50 (see Fig. 5C, last lane). These results suggest that the amino-terminal 121 residues of I-Rel contain a domain that is inhibitory to DNA binding when I-Rel associates with p50. The inability of I-Rel(Δ5') to interact with DNA on its own suggests the contribution of a second domain for DNA

Figure 5. Association of I-Rel and I-Rel ($\Delta 5'$) with p50 in a mobility-shift assay. (A) The purified proteins indicated were denatured and renatured together as described in Materials and methods. Renatured proteins were incubated with a 32 P-labeled κ B probe and analyzed on 4% nondenaturing polyacrylamide gel. The bars indicate increasing ratios of the respective proteins mixed with a constant amount (10 ng) of the indicated protein. (B) Purified p50 and p65 (truncated derivative, amino acids 1–309) were mixed at a ratio favorable for heterodimer formation and were denatured together with an increasing amount of I-Rel followed by gradual renaturation. Renatured proteins were incubated with a 32 P-labeled κ B probe and analyzed on 4% nondenaturing polyacrylamide gel. (C) Purified I-Rel($\Delta 5'$) was denatured either alone or in the presence of p50 followed by gradual renaturation. Renatured proteins were incubated with a 32 P-labeled κ B probe and analyzed on a 4% nondenaturing polyacrylamide gel.

binding and may reflect its inability to form a homodimer (Fig. 7A).

I-Rel lacks a carboxy-terminal transcriptional activation domain

Earlier studies have established that the carboxy-terminal regions of *rel*, *dorsal*, and p65 possess transcriptional activation domains (Gelinas and Temin 1988; Hannink and Temin 1989; Rushlow et al. 1989; Bull et al. 1990; Kamens et al. 1990; Richardson and Gilmore 1991; Urban et al. 1991; Ruben et al. 1992). The carboxy-terminal sequence of I-Rel contains a large percentage of proline residues (17%) similar to p65 (Ruben et al. 1991, 1992) and could possibly contribute to an activation function, as proline-rich regions have been identified in several other mammalian transcription factors including CTF/NF- κ B (Mermoud et al. 1989), AP-2 (Williams et al. 1988), and Jun/AP-1 (Struhl 1988). The presence of a transcriptional activation domain in I-Rel corresponding to the

region containing the transcriptional activation domain in other *rel*-related proteins was examined by construction of a chimeric protein containing amino acids 1–147 (DNA-binding domain) of the yeast transcriptional activator GAL4 (Sadowski and Ptashne 1989) and amino acids 404–579 of I-Rel. Use of the GAL4 DNA-binding domain and the GAL4 upstream activating sequence (UAS) was required, as a DNA target element responsive to I-Rel has not been identified. The transcriptional activity of the chimeric GAL4/I-Rel protein was assessed by cotransfection of a GAL4/I-Rel expression vector with a chloramphenicol acetyl transferase (CAT) reporter plasmid containing the GAL4 UAS sequence upstream of the mouse mammary tumor virus (MMTV) promoter (GMCS Δ GRE/UAS) (Kakidani and Ptashne 1988). Whereas significant stimulation was evident with cotransfection of a GAL4 chimeric protein containing the carboxy-terminal activation domain of p65, no significant stimulation was obtained with the GAL4/I-Rel(405–579) chimeric protein (Fig. 8). Similarly, fusion

of the carboxy-terminal portion of I-Rel with the DNA-binding domain of p50 was inactive with a CAT reporter plasmid driven by a κ B motif (not shown), whereas a chimeric protein containing the p50 DNA-binding domain and p65 activation domain demonstrated significant transcriptional activity (Ruben et al. 1992). These results do not rule out the possibility of a transcriptional activation domain present within another region of I-Rel.

Induction of I-Rel mRNA

Earlier studies have established that several of the *rel*-related gene members are subject to differential expression after exposure of cells to mitogens or cytokines (Bours et al. 1990; Molitor et al. 1990). The kinetics of I-Rel expression after mitogenic stimulation were examined by Northern blot analysis (Fig. 9). Jurkat T lymphocytes were exposed to PMA, PHA, and cycloheximide, and RNA was isolated at various time intervals after exposure. Northern blots were hybridized with specific probes for I-Rel, p50, or p65 under high-stringency conditions. Consistent with earlier reports, induction of p50 mRNA synthesis accompanied mitogen stimulation and was evident as early as 2 hr after stimulation (Fig. 9). In contrast, synthesis of p65 mRNA was found to be constitutive and only slightly induced after mitogen stimulation. I-Rel mRNA synthesis was also induced but delayed relative to the induction of p50 mRNA synthesis. Alternatively, if I-Rel is induced at the same time as p50 induction it must be expressed at a much lower level. These data are consistent with p50 and I-Rel belonging to a class of mitogen-inducible genes.

Expression of I-Rel inhibits NF- κ B function

The ability of I-Rel to inhibit the interaction of p50 with

DNA in vitro predicts that in vivo expression of I-Rel should diminish NF- κ B activity through formation of inactive p50/I-Rel heteromeric complexes. To examine this possibility, an I-Rel expression vector was constructed by placing the I-Rel cDNA under control of the cytomegalovirus (CMV) early promoter (CMV I-Rel). The effect of I-Rel on NF- κ B function was assessed in transient cotransfection assays using CMV I-Rel and CMVp50/p65, together with a CAT reporter plasmid under transcriptional control of either the natural -427/-225 regulatory sequence of the interleukin-2/receptor α (IL-2R α) subunit containing one κ B motif (Fig. 10A) (Ruben et al. 1988) or a synthetic sequence containing four tandem κ B sequences (H κ B-4CAT; Leung and Nabel 1988) (Fig. 10B). Plasmid CMVp50/p65 expresses a fusion protein that contains the p50 DNA-binding domain and p65 activation domain (Ruben et al. 1992). Previous studies have established that the chimeric p50-p65 protein recognizes the same motif as p50 and provides stronger stimulation than that observed with cotransfection using the individual p50 and p65 expression vectors (Ruben et al. 1992). In cotransfections receiving only the p50-p65 expression vector with the CAT reporter, induction of CAT activity was observed (Fig. 10A,B). In contrast, inclusion of the I-Rel expression vector in the cotransfection with CMVp50/p65 led to a marked decrease of CAT activity. Similar results were obtained using either the authentic IL-2R α promoter (Fig. 10A) or the H κ B-4CAT reporter containing four copies of the κ B motif (Fig. 10B).

As a further means of addressing I-Rel function in vivo, the ability of I-Rel to suppress endogenous NF- κ B activity after mitogenic stimulation was examined. For this experiment, Jurkat T lymphocytes were transfected with H κ B-4CAT (Leung and Nabel 1988), in the absence or presence of CMV I-Rel. At 30 hr post-transfection, the cells were stimulated with PMA and assayed for CAT

Figure 7. Coimmunoprecipitation of I-Rel with p50 and p65. The RNAs corresponding to the protein products shown were used to program rabbit reticulocyte lysates. The lanes depict translation reactions before immunoprecipitation (–) or after immunoprecipitation with the anti-HA sera (+).

activity the following day. CAT activity in transfected cells that received the H κ B-4CAT reporter alone was markedly induced after mitogenic stimulation, relative to the nonstimulated cells (Fig. 10C). In those cells receiving the CMV I-Rel vector, induction of CAT activity after mitogenic stimulation decreased as the amount of CMV I-Rel transfected was increased, with only slight induction evident when 3 μ g of CMV I-Rel was used. This is consistent with the cotransfection data demonstrating the ability of I-Rel to inhibit NF- κ B function. As an indication of the specificity for inhibition, the ability of I-Rel expression to affect activation of the human immunodeficiency virus long terminal repeat (HIV LTR) by the *trans*-activator Tat (Rosen et al. 1985) was examined. No inhibition was observed upon cotransfection of CMV I-Rel with the HIV LTR CAT reporter in the presence of a Tat expression vector (Fig. 10D). Similarly, cotransfection of I-Rel with a Rous sarcoma virus (RSV) LTR-driven CAT reporter had only a minimal effect on CAT gene expression (not shown). These findings strongly

suggest that expression of I-Rel selectively inhibits NF- κ B activity in this system.

Discussion

We have identified a novel *rel*-related gene product designated I-Rel (for inhibitory *rel*) with properties quite dissimilar to *rel*-related proteins identified previously. I-Rel, as with other members of the *rel* family, contains a region of ~300 amino acids that shares extensive identity with other *rel*-related proteins. The I-Rel cDNA, however, encodes an additional 120 amino acids at its amino terminus, and the divergent carboxy-terminal region is considerably shorter than other known *rel*-related proteins. The most notable feature is the inability of I-Rel to bind the κ B motif, in contrast to previously identified *rel* family members, each of which has been shown to associate with κ B DNA (Ballard et al. 1990; Ghosh et al. 1990; Kieran et al. 1990; Ip et al. 1991; Nolan et al.

Figure 8. I-Rel lacks a carboxy-terminal transcriptional activation domain. The sequence coding for amino acids 295–551 of p65 or 404–579 for I-Rel was amplified by PCR and cloned in-frame with the binding domain of GAL4 (amino acids 1–147). In transient cotransfection assays, the plasmid DNAs indicated were transfected into COS7 cells together with a CAT reporter plasmid containing the GAL4-responsive UAS sequence upstream of the MMTV promoter lacking the GRE (Kakidani and Ptashne 1988). Cells were harvested 48 hr post-transfection, and CAT assays were performed. The CAT assays shown represent a 30-min reaction.

1991; Ruben et al. 1991; Urban et al. 1991). In addition, I-Rel lacks the ability to form a homodimer as observed with the other *rel*-related proteins. Moreover, each of these proteins, with the possible exception of *v-rel*, can elicit transcriptional activation under certain conditions (Gelinas and Temin 1988; Hannink and Temin 1989; Rushlow et al. 1989; Bull et al. 1990; Kamens et al. 1990; Urban et al. 1991; Richardson and Gilmore 1991), as best exemplified by the strong transcriptional activity elicited by the NF- κ B transcription factor complex.

The recent identification and cloning of the p50 and p65 subunits of NF- κ B revealed the similarity between these proteins and other known *rel*-related proteins. This led to the observation that the other *rel*-related proteins also bind to the κ B motif (Ghosh et al. 1990; Kieran et al. 1990) and that binding is dependent on dimer formation. Although it is known that the *rel* homology domain is important for dimerization and DNA-binding functions (Ghosh et al. 1990; Kieran et al. 1990; Nolan et al. 1991; Ruben et al. 1992), it contains no striking similarity to characterized DNA binding and protein association motifs such as the leucine zipper, helix-loop-helix (HLH), zinc finger, or homeo box. It is therefore difficult to predict what features of the peptide structure of I-Rel may prevent DNA binding. In light of recent results demonstrating that each subunit of a p50–p65 heterodimer contacts one half-site of the κ B motif (Urban et al. 1991), the fact that p50/I-Rel(Δ 5') can form a heterodimer that binds DNA suggests that I-Rel contains a functional DNA-binding domain. It is likely that the lack of DNA binding observed with I-Rel reflects the inability of I-Rel to form homodimers.

The ability of I-Rel to associate with p50 and abolish

its DNA-binding activity as a heteromeric complex can be interpreted in several ways. The simplest explanation is that I-Rel contains a domain inhibitory to DNA binding. Consistent with this prediction, a truncated I-Rel protein lacking the amino-terminal 121 residues preceding the *rel* homology domain forms a heterodimer with p50 that is capable of binding κ B DNA. Analysis of the amino-terminal inhibitory domain of I-Rel reveals that residues 40–68 resemble a leucine zipper-like motif (Landschultz et al. 1988; Ryseck et al. 1992). It is possible that an association may occur between the putative leucine zipper motif of I-Rel and the DNA-binding domain of p50, thus resulting in loss of DNA binding. It has been shown recently that there is a direct interaction between the leucine zipper of c-Jun and the HLH motif of MyoD (Bengal et al. 1992). Alternatively, the association of I-Rel with p50 may alter the conformation of p50, thus preventing DNA interaction. A recent report by Ryseck et al. (1992) described the cloning of RelB, the apparent murine homolog of I-Rel. These investigators, however, concluded that the RelB–p50 complex associates with κ B DNA. The discrepancy between the findings obtained with RelB and I-Rel is not easily explained. One possibility is that RelB and I-Rel are indeed functionally equivalent and what these investigators may have observed are the remnants of a RelB–p50 complex that has a weak affinity for DNA as we have seen (see Fig. 5A). Alternatively, as the RelB clone expressed by Ryseck et al. (1992) lacks the 19 terminal amino acids present in I-Rel, it remains possible that these residues harbor the inhibitory properties of I-Rel.

The association of I-Rel with p50 and not p65 or itself raises several possibilities concerning the ability of *rel*-related proteins to interact with one another. It is assumed generally that different *rel*-related proteins can

Figure 9. Induction of NF- κ B and I-Rel with mitogens. Jurkat T lymphocytes were treated with PMA and PHA, and RNA was isolated from the cells at the indicated times and used for Northern blots. The RNA was electrophoresed on 1% denaturing agarose gels, and the RNA was transferred to durulose filters. The blots shown depict hybridization with 32 P-labeled probes specific for p50, p65, or I-Rel. Overnight exposures are shown. Similar effects were observed following exposure of cells to TNF α .

Figure 10. I-Rel is an inhibitor NF- κ B in T lymphocytes. Jurkat T cells were cotransfected with a CAT reporter plasmid containing (the IL-2R α regulatory sequence [nucleotides 421/–225] (A), or a plasmid with a synthetic sequence containing four copies of the κ B motif upstream of the SV40 promoter (H κ B-4CAT; Leung and Nabel 1988) and increasing amounts of CMV I-Rel (B). Cells were harvested 48 hr post-transfection, and CAT assays were performed. (C) Cells were transfected with either the H κ B4 reported plasmid alone or with CMV I-Rel and stimulated with PMA 30 hr after transfection. (D) Cells were transfected with plasmid pU3R-1 (contains the HIV LTR driving expression of the CAT gene), plasmid pHTat (encodes the HIV *trans*-activator Tat), and CMV I-Rel. Cells were harvested at 40 hr post-transfection for CAT assays. CAT assays shown represent a 30-min reaction. In each transfection, the amount of CMV promoter was kept constant to avoid spurious results reflecting competition for transcription factors.

interact with each other, as exemplified by the association of p50 and p65 in NF- κ B and *v-rel* with *c-rel* or p50 (Simek and Rice 1988; Bacuerle and Baltimore 1989; Ghosh and Baltimore 1990; Lim et al. 1990; Kerr et al. 1991). On the basis of the findings reported here, we suggest that other *rel*-related proteins may form selective associations (i.e., each *rel*-related protein may only associate with a subset of *rel*-related proteins). Precedent for this possibility is provided by the selective interactions among the family of transcription factors that associate through a leucine zipper motif. For example, Fos forms heterodimers with Jun-related proteins but does not form homodimers (Franza et al. 1988; Rauscher et al. 1988) and ATF-3 forms heterodimers with ATF-2 but not with ATF-1 (Hai et al. 1989).

Whether the inability of I-Rel to associate with p65 reflects subtle alterations between residues within a single multimerization domain or the presence of multiple dimerization domains with distinct specificities for individual *rel*-related proteins remains to be established. The ability of I-Rel to associate with p50 and not p65

could be explained by either of the above possibilities. Similarly, we have recently described the identification of a naturally occurring variant form of p65 arising from an alternatively spliced p65 mRNA, designated p65 Δ . p65 Δ lacks the ability to form homodimers or associate with p50 but retains the ability to associate with p65 (Narayanan et al. 1992; Ruben et al. 1992). The ability of both I-Rel and p65 Δ to establish selective associations with the individual *rel*-related family members would be consistent with the idea that subtle differences within the multimerization domains provide the opportunity for higher order regulation.

I-Rel provides another example of a regulatory protein that inhibits transcriptional activity through heterodimer formation with a related transcriptionally active family member. For example, the protein Id lacks the basic residues adjacent to the HLH domain essential for specific DNA binding in the protein MyoD (Benezra et al. 1990). Id can associate with several transcriptionally active HLH proteins, however, and attenuate their ability to bind DNA as a heterodimeric complex (Benezra et

al. 1990). In neuron-specific gene activation, the protein I-POU lacks two residues essential for DNA binding but forms a stable complex with another POU domain protein, CF1-A, and prevents CF1-A from binding to the DNA recognition element important for *trans*-activating the dopa-decarboxylase gene (Treacy et al. 1991). Furthermore, by analogy with the selective ability of I-Rel to associate with p50 and not p65, I-POU does not associate with Pit-1, which shares considerable homology with the POU-specific binding domain present in CF1-A (Treacy et al. 1991).

The ability of I-Rel to inhibit p50 function selectively adds a further level of complexity to the NF- κ B signal transduction pathway. The regulatory mechanisms of the inhibitory heterodimers mentioned above, and including I-Rel-p50, may be dependent on the interaction between shared regions of extensive homology. This contrasts with the negative regulation of *rel* family members based on cytoplasmic sequestration exemplified by association of NF- κ B p65 with I- κ B (Baeuerle and Baltimore 1988a,b), *dorsal* with cactus, and *rel* with pp40 (Davis et al. 1991; Kerr et al. 1991). In the case of NF- κ B, stimulation with mitogens, or various cytokines, leads to dissociation of I κ B and translocation of NF- κ B to the nucleus. The molecules that maintain the cytoplasmic localization of the *rel*-related proteins all share a common feature in that they contain multiple repeat elements that share extensive similarity to the ankyrin repeat structures. Proteins bearing ankyrin repeats appear to play a prominent role in cell growth and differentiation (Lux et al. 1990), as well as mediating heterodimer formation between the GABP α and β subunits (Thompson et al. 1991). It has been suggested that these repeat elements interact with the cytoskeleton in maintaining cytoplasmic partitioning of proteins associated with them (Lux et al. 1990), including the inactive *rel* protein-inhibitor complexes.

NF- κ B is a pleiotropic transcriptional activator. Although no solid evidence is currently available, it can be easily envisioned that continual activation of gene expression by NF- κ B could be detrimental to the cell. For example, the genes known to be regulated by NF- κ B include cytokines (Liebermann and Baltimore 1990; Shimizu et al. 1990) and the IL-2R α subunit (Bohnlein et al. 1988; Leung and Nabel 1988; Ruben et al. 1988), which are involved in T-cell activation. Therefore, prolonged expression of these genes could establish an autocrine loop leading to continual stimulation of the cell. It has been suggested that one potential pathway leading to adult T-cell leukemia/lymphoma after HTLV-I infection is the activation of NF- κ B by the Tax *trans*-activator protein (Leung and Nabel 1988; Ruben et al. 1988). Similarly, expression of *v-rel* is known to elicit phenotypic changes in certain cell lineages (Rice and Gilden 1988). Therefore, during prolonged exposure of cells to those stimuli that elicit NF- κ B activation (i.e., exposure to mitogens, cytokines, and various viral regulatory proteins) a means for suppressing the signaling pathway is probably required.

As mentioned above, the ability of I κ B to associate

with the p65 subunit to maintain an inactive cytoplasmic pool of NF- κ B provides one means for regulating NF- κ B activity. This might provide the primary means for regulation of NF- κ B function in a resting cell. Upon stimulation, I κ B is rendered inactive by phosphorylation, allowing NF- κ B to translocate to the nucleus initiating transcriptional activation. In this respect, modified I κ B is no longer functional as a repressor. Thus, a second level of control may be required to prevent the continual activation of NF- κ B-responsive genes in activated cells. Consistent with this possibility is the observation (Sen and Baltimore 1986b) that during prolonged exposure of cells to mitogens NF- κ B is suppressed even though these stimuli should inactivate I κ B.

The kinetics of I-Rel expression, as well as the results obtained in the transfection studies, demonstrating that I-Rel expression inhibits NF- κ B function, suggest that I-Rel could provide a second level of control through the formation of nonfunctional heterodimers with p50. This, in turn, would provide an additional and distinct mechanism for blocking NF- κ B activity. Furthermore, the structural dissimilarity between I-Rel and other *rel*-specific inhibitory molecules (i.e., I κ B, pp40) suggests that I-Rel function may not be affected by the same stimuli that regulate the other inhibitory molecules. The observation that I-Rel mRNA accumulates at a time after induction of p50 mRNA would provide time for activation of NF- κ B-responsive genes before suppression of the pathway begins.

Although our studies have focused on the ability of I-Rel to associate with p50, these experiments do not rule out the possibility that I-Rel can associate with other *rel*-related proteins to elicit positive or negative effects on transcriptional activation. For example, *v-rel* binds to the κ B enhancer and inhibits NF- κ B function (Ballard et al. 1990). If I-Rel can associate with *v-rel* to prevent its interaction with DNA as a heterodimer, under these conditions I-Rel would have a positive effect on transcriptional activation.

The identification of I-Rel provides an additional level of control for the NF- κ B signal transduction pathway. It is anticipated that as new *rel*-related family members are identified, different and selective combinations of protein associations will be seen. It is likely that the individual associations will have varied effects depending on the combination of proteins involved and their respective target sequences.

Materials and methods

Isolation and analysis of cDNA clones

Degenerate oligonucleotide primers were designed based on a highly conserved upstream region, 5'-TT(TC)(CA)G(AC)TA(C-T)(GA)(AT)(GA)TG(TC)GA(GA)GG-3', and downstream region, 5'-TG(TC)GA-(GC)AA(GA)GT(GT)(GC)(CA)(GAC)AA(GA)GA-3', within the *rel* homology domain. These primers were used in a PCR reaction with Jurkat cDNA as template and the following cycle: initial denaturation for 6 min at 94°C, denaturation for 1 min at 94°C, annealing for 1.5 min at 55°C, and extension for 2 min at 72°C for 35 cycles. During the initial annealing step, a

temperature of 45°C was used. The PCR amplicons were cloned into the *HindIII*–*XbaI* site of plasmid BL SK (Stratagene), and the inserts of 50 clones were sequenced by the chain termination method (Sanger et al. 1977) with T3 and T7 primers, using Sequenase II (U.S. Biochemical).

The *HindIII*–*XbaI* fragment obtained from the unique amplicon was used as a probe to screen a Jurkat T-cell cDNA library in λ ZAPII (Stratagene). From a total of 600,000 plaques screened under stringent hybridization (42°C, 50% formamide) and wash conditions (65°C, 0.1 $\times$ SSC), seven positive plaques were obtained through three rounds of purification. The BL SK phagemid containing the cDNA (BL I-Rel res) was rescued as described (Short et al. 1988) and sequenced by the chain termination method (Sanger et al. 1977). Primers were synthesized to permit overlapping sequencing of the cDNA in both directions. Computer analysis and assembly of sequence information were carried out using the GCG programs (University of Wisconsin, Madison, WI).

Plasmid construction

A pDS expression plasmid was used for expression of I-Rel in *Escherichia coli* (Gentz et al. 1989). Expression is driven from a bacteriophage T5 promoter under control of a *lac* operator. Primers were synthesized corresponding either to the region surrounding the initiator methionine (145–163 bp) or to amino acid residue 123 at the beginning of the *rel* homology domain (508–525 bp) to fuse I-Rel in-frame with the 6 histidine moiety present in the pDS plasmid, and the I-Rel cDNA was amplified using each of these primers for the 5' primers and a primer corresponding to amino acid 425 (1399–1413 bp) as the 3' primer. The amplified fragment was cloned into the *BamHI*–*XbaI* sites of the vector pDS I-Rel(Δ 3').

For in vitro transcription of I-Rel(Δ 3'), the *EcoRI*–*XbaI* fragment of pDS I-Rel(Δ 3') was cloned into Bluescript SK (Stratagene) to make BL I-Rel(Δ 3'). For in vitro transcription of full-length I-Rel, the *BamHI*–*XbaI* fragment from BL I-Rel res was exchanged with the *BamHI*–*XbaI* fragment of BL I-Rel(Δ 3') to make BL I-Rel. The expression vector CMV I-Rel was constructed by subcloning the *HindIII*–*XbaI* fragment of BL I-Rel between a CMV promoter– β -globin intron and an SV40 poly(A) signal. The pCMVp50/p65 chimeric protein was generated by fusing the sequence encoding amino acids 1–370 of p50 to the sequence corresponding to amino acids 309–550 of p65 as described (Ruben et al. 1992). The GAL4/I-Rel chimeric proteins were constructed by first amplifying the fragment of I-Rel corresponding to amino acids 404–579, restricting the amplified fragment with *BamHI* and *XbaI*, and cloning the fragment in-frame with the GAL4 sequence corresponding to amino acids 1–147 of the GAL4 DNA-binding domain in plasmid pSG424 (Sadowski and Ptashne 1989). Plasmid H κ B-4CAT contains four tandem copies of the κ B sequence from the HIV-1 enhancer (Leung and Nabel 1988). Plasmid IL-2R–421/–225 corresponding to the IL-2/ α -promoter has been described previously (Ruben et al. 1988). Epitope-tagged I-Rel, p50, and p65 were constructed by PCR using a 5' primer encoding the amino acid sequence MYPYDVPDYA corresponding to the influenza HA protein (Kolodziej and Young 1991), followed by cloning the amplified product into Bluescript SK.

Cell culture and transfection

Jurkat T cells were maintained in RPMI-1640 medium containing 10% FCS and 50 μ g/ml of Gentamicin (GIBCO). For transient transfection assays, 5 $\times$ 10⁶ cells were transfected by the DEAE–dextran procedure (Queen and Baltimore 1983). Cells

were transfected with 2 μ g of reporter plasmid and 1–4 μ g of the CMV I-Rel expression vector. Cells were harvested 48 hr after transfection, and CAT assays were performed as described previously (Gorman et al. 1982). For PMA induction of Jurkat cells, the cells were transfected with H κ B-4CAT (1.5 μ g); 30 hr after the transfection, PMA was added at 50 ng/ml. Cells were harvested the following day. COS7 cells were maintained in IMDM + 10% FCS supplemented with 4500 μ g/ml of glucose. For transient transfection assays 2 $\times$ 10⁵ cells were plated on 35-mm plates, and a modified DEAE–dextran protocol (Dillon et al. 1990) was used to transfect the cells the following day. Cells were transfected with 1 μ g of the GAL4 UAS reporter construct and 2 μ g of the GAL4/I-Rel chimeric expression vectors.

In vitro transcription and translation and coimmunoprecipitation analysis

I-Rel, p50, or p65 cDNAs present in the Bluescript expression vector was linearized with *XbaI*, and 1 μ g was used as template for in vitro transcription with T7 RNA polymerase. The in vitro-transcribed RNA was used to program a rabbit reticulocyte translation lysate (Promega) that contained [³⁵S]methionine. Protein products were analyzed on a 10% SDS–polyacrylamide gel and fluorographed. For the coimmunoprecipitation analysis, 1 μ l of RNA corresponding to protein containing the HA tag was cotranslated with 1 μ l of RNA corresponding to I-Rel, p50, or p65 lacking the tag sequence. For immunoprecipitation, 4 μ l of lysate was mixed with 150 μ l of immunoprecipitation buffer [20 mM HEPES (pH 7.5), 250 mM NaCl, 4 mM EDTA, and 0.1% NP-40]. Two microliters of anti-HA immune sera (Babco) was added to the reaction followed by the addition of protein G agarose beads (Pharmacia). The samples were incubated at 4°C for 3 hr and washed four times with immunoprecipitation buffer. Agarose beads were heated to 80°C for 10 min before gel loading. Protein products were analyzed on a 10% SDS–polyacrylamide gel and fluorographed.

Expression and purification of bacterial-expressed proteins

A pDS expression plasmid was used for expression of I-Rel, p50, and p65 in *Escherichia coli* (Gentz et al. 1989). Briefly, expression was driven from a bacteriophage T5 promoter under control of a *lac* operator. Residues corresponding to the DNA-binding domain of each protein (see above) were fused in-frame with the 6 histidine moiety present in the pDS plasmid. Expression of these proteins was induced in mid-logarithmic cultures of *E. coli* by the addition of 1 mM IPTG. After 4 hr of incubation, cells were pelleted and lysed in 6 M guanidine hydrochloride (pH 8.0). The cleared lysate was adsorbed to a nickel chelate affinity resin, and proteins were eluted with a pH step gradient of 6 M guanidine-HCl. Purified protein was renatured slowly by dialyses against H buffer [20 mM HEPES (pH 7.9), 0.2 mM EDTA, 1 mM DTT, 0.1% NP-40, and 0.5 mM PMSF] plus 300 mM KCl containing 3, 1.5, 1, 0.5 M, and no guanidine-HCl, respectively. For corenaturation experiments, proteins were diluted 1 : 10 in 6 M guanidine-HCl and mixed at a ratio of 1 : 1, 1 : 5, and 1 : 20 before dialysis.

For preparation of crude *E. coli* lysate containing I-Rel, p50, and p65 proteins, mid-logarithmic cultures were induced with 1 mM IPTG for 2.5 hr, pelleted, and resuspended in H buffer without NP-40. Cells were lysed by sonication with two 30-sec cycles, and lysates were cleared by centrifugation and frozen at –70°C.

Electrophoretic mobility-shift assays

Binding reactions were carried out as described previously (Dayton et al. 1989), with the addition of DTT (1 mM) and NP-40 (0.1%) to the binding buffer. Bacterial protein (~20 ng) and ³²P-labeled probe (0.5 ng; 50,000 cpm) were used in the binding reactions. Nondenaturing gels (4%) were electrophoresed at 4°C in Tris-borate buffer (0.5× TBE) for 2 hr. The κB probe used in the binding reactions contains the sequence 5'-GGATCCT-CAACAGAGGGGACTTCCAGGCCA-3', which corresponds to the κB motif present in the immunoglobulin light-chain enhancer. The sequence of the degenerate primer was 5'-TGCCTAGAGGATCCGTGAC(X)₁₈CGGAATTCCTTAAGA-AGCTTCCGAGCG-3', where X equals A, G, C, or T. For labeling the degenerate oligonucleotide, a primer complementary to the 3' sequence (5'-CGCTCGGAAGCTTGAATTC-3') was annealed to the random oligonucleotide. The primer was then extended with DNA polymerase Klenow fragment using 40 μCi of each [³²P]dNTP followed by addition of 250 μM unlabeled dNTPs to ensure complete synthesis. In binding reactions containing the degenerate oligonucleotide probe, 20 ng was used.

Northern blot analysis

Jurkat T cells were cultured in RPMI/10% FCS with gentamicin (50 μg/ml) to a density of 1×10^6 to 1.5×10^6 cells/ml. Cells were then stimulated with PHA (1 μg/ml), PMA (50 ng/ml), and cycloheximide (10 μg/ml). At 0, 2, 4, 8, and 12 hr poststimulation, a 30- to 50-ml aliquot was removed and total RNA was isolated using RNeasy Lysis Buffer (Qiagen). RNA (10 μg) was size fractionated on a 1.2% agarose/2% formaldehyde gel using a MOPS electrophoresis buffer, transferred to Duralose UV membrane (Stratagene) using a positive pressure system (Stratagene), and UV-cross-linked (Stratalinker; Stratagene). Hybridization to ³²P-labeled probe DNA (1×10^6 cpm/ml) was performed under stringent conditions [50% formamide, 5× SSC, 1× Denhardt's solution, 14 mM Tris-HCl (pH 7.5), 10% dextran sulfate at 42°C], and the blots were subsequently washed using stringent conditions (0.1× SSC/0.1% SDS at 65°C). Probe DNA was isolated from p65, p50, I-Rel, or GAPDH cDNA and ³²P-labeled to a specific activity of 1×10^9 to 2×10^9 cpm/μg using a random oligonucleotide-primed DNA synthesis kit (Boehringer Mannheim). Autoradiography was performed using Kodak X-Omat film and an intensifying screen at -70°C.

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