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RIP: A Novel Protein Containing a Death Domain That Interacts with Fas/APO-1 (CD95) in Yeast and Causes Cell Death

Ben Z. Stanger,*† Philip Leder,*† Tae-Ho Lee,†‡ Emily Kim,†‡ and Brian Seed†‡
*Howard Hughes Medical Institute
†Department of Genetics
Harvard Medical School
Boston, Massachusetts 02115
‡Department of Molecular Biology
Massachusetts General Hospital
Boston, Massachusetts 02114

Summary

Ligation of the extracellular domain of the cell surface receptor Fas/APO-1 (CD95) elicits a characteristic programmed death response in susceptible cells. Using a genetic selection based on protein–protein interaction in yeast, we have identified two gene products that associate with the intracellular domain of Fas: Fas itself, and a novel 74 kDa protein we have named RIP, for receptor interacting protein. RIP also interacts weakly with the p55 tumor necrosis factor receptor (TNFR1) intracellular domain, but not with a mutant version of Fas corresponding to the murine *lpr^cg^* mutation. RIP contains an N-terminal region with homology to protein kinases and a C-terminal region containing a cytoplasmic motif (death domain) present in the Fas and TNFR1 intracellular domains. Transient overexpression of RIP causes transfected cells to undergo the morphological changes characteristic of apoptosis. Taken together, these properties indicate that RIP is a novel form of apoptosis-inducing protein.

Introduction

Although regulated cell death is known to be essential for the orderly development of metazoan organisms and is crucial to the proper functioning of the immune system in higher vertebrates, relatively little is known about the mechanisms by which cell death programs are executed. One important mediator of immunologically relevant cell death processes is the Fas antigen/APO-1 (CD95), originally identified as the target of monoclonal antibodies that could kill multiple cell types (Trauth et al., 1989; Yonehara et al., 1989). cDNA cloning and sequence analysis (Itoh et al., 1991; Watanabe-Fukunaga et al., 1992a; Oehm et al., 1992) showed Fas to be a member of a family of transmembrane receptors that includes the low affinity nerve growth factor (NGF) receptor, the tumor necrosis factor receptors (TNFR1 and TNFR2), and a variety of immune cell receptors including CD40, OX40, CD30, CD27, and 4-1BB (for review see Smith et al., 1994). Several members of this family besides Fas have been shown to regulate or induce cell death, in particular the p55 TNFR (TNFR1) (Tartaglia et al., 1991, 1993b) and the p75 TNFR (TNFR2) (Heller et al., 1992, 1993; Clement and Stamenkovic, 1994).

Disruption of Fas expression or function in lymphoproliferation (*lpr*) mutant mice leads to a progressive lymphadenopathy and autoimmune syndrome resembling human systemic lupus erythematosus (Watanabe-Fukunaga et al., 1992b). The residual cytotoxic activity of T cells derived from perforin-deficient mice is also dependent on the presence of at least one wild-type allele of the *lpr* locus (Kagi et al., 1994; Kojima et al., 1994; Lowin et al., 1994). Thus, the ability of Fas to induce cell death is important for the maintenance of at least two immunological processes in vivo: peripheral tolerance to self (Singer and Abbas, 1994; Crispe, 1994) and calcium-independent T cell cytotoxicity (reviewed by Henkart, 1994).

The mechanism by which Fas induces cell death is unknown, but it requires multivalent cross-linking of the receptor (Dhein et al., 1992) and is facilitated by concurrent inhibition of RNA or protein synthesis in some cell types. Other factors have been reported to modulate Fas activity (Klas et al., 1993), and under certain circumstances, Fas is capable of signaling activation rather than death (Alderson et al., 1993). Anti-Fas antibodies and TNF are both capable of signaling cell death in vitro with similar kinetics (Yonehara et al., 1989; Itoh and Nagata, 1993), and among members of the NGF/TNF receptor family, Fas and TNFR1 share the most significant cytoplasmic homology.

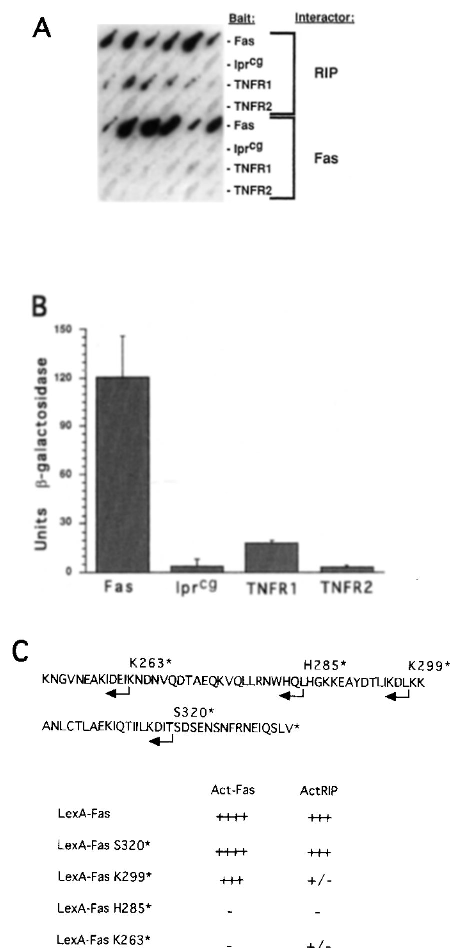
Deletion and mutational analyses have led to the identification of sequences within Fas that are required for the cell death response (Itoh and Nagata, 1993). It has been proposed that a death domain contained in a region of similarity between Fas and TNFR1 is essential for the initiation of apoptosis by both molecules, perhaps through an interaction with other intracellular proteins (Tartaglia et al., 1993a).

To study the events elicited by Fas ligation, we have exploited a yeast protein interaction system to identify a novel protein that interacts with the Fas cytoplasmic domain. This protein, which we have named RIP (for receptor interacting protein), contains a death domain homology region at its C-terminus and a kinase domain at its N-terminus. Overexpression of RIP leads to morphological changes characteristic of apoptosis. The ability of RIP to associate with the Fas intracellular domain in yeast and to promote apoptotic changes upon overexpression in mammalian cells suggests it may be an important element in the signal transduction machinery that mediates programmed cell death.

Results

Identification of cDNAs Encoding Protein Domains That Bind Fas

A cDNA library screen for proteins that interact with the intracellular domain of Fas was conducted with the help of a yeast genetic selection system (Gyuris et al., 1993; Zervos et al., 1993). A cDNA segment comprising virtually the entire cytoplasmic domain of human Fas (residues



	Act-Fas	ActRIP
LexA-Fas	++++	+++
LexA-Fas S320*	++++	+++
LexA-Fas K299*	+++	+/-
LexA-Fas H285*	-	-
LexA-Fas K263*	-	+/-

Figure 1. Specificity of RIP Interactions in Yeast

(A) RIP–Fas and Fas–Fas interactions. Cells of the S. cerevisiae strain EGY48/pRB1840 were sequentially transformed with the indicated LexA–fusion plasmid and either the Act–Fas or the Act–RIP library isolate. Independent colonies arising from the activator plasmid transformations were streaked on Ura^- His^- Trp^- plates containing X-Gal, galactose, and raffinose and photographed using a red filter.

(B) Quantitative β-gal assay. Three colonies from each Act–RIP/LexA–fusion protein pair were used to inoculate a galactose-containing liquid culture. The β-gal activity of lysates prepared from each culture was measured and normalized to the total protein concentration of the lysate. 5 units (nmoles/min/mg protein) represents the limit of detection of β-gal activity in this system.

(C) Pairwise association between LexA–Fas deletion variants and Act–Fas or Act–RIP. The indicated yeast strains were constructed by transformation, and the production of β-gal was tested by a chromogenic colony assay using X-Gal. The extent of color development of individual colony streaks was scored visually, with ++++ indicating dark blue and − indicating the growth of white colonies only; +/− indicates the presence of faint blue flecks in some of the colonies bearing the indicated Act/LexA pair.

192–329 of the Fas precursor) was fused to the 3′ end of the coding region for the bacterial repressor LexA, and a yeast expression plasmid containing this gene fusion was introduced into a reporter strain harboring the Saccharomyces cerevisiae LEU2 and Escherichia coli β-galactosidase (β-gal) coding sequences under the control of a synthetic promoter bearing LexA-binding sites. The resulting yeast strain was transformed with a transcriptional activator fusion protein library prepared from mRNA isolated from the Jurkat (human T cell leukemia) cell line, which is known to undergo apoptosis when subjected to treatment with anti-Fas antibody. Transformants were plated on selective (Leu-deficient) plates containing galactose, which induces the *GAL1* promoter that directs transcription of the library insert. Leu prototrophs were transferred to plates containing X-Gal and galactose, and colonies giving a dark blue color were recovered and analyzed further.

To test the specificity of the interaction between the candidate interaction partners and Fas, the library plasmids were reintroduced into a second strain harboring a LexA–Fas fusion gene (LexA–lpr^{cg}) in which the Fas portion had been mutated by substitution of Asn for Val at position 254 of the Fas precursor sequence. This mutation was expected to exhibit a molecular phenotype similar to that of the murine lpr^{cg} allele, which is formed by substitution of Asn for Ile at the homologous position. None of the candidate plasmids showing evidence of strong interaction with LexA–Fas were capable of interacting with LexA–lpr^{cg} (Figure 1A; data not shown).

Restriction site and sequence analysis of the cDNA inserts of the candidate clones showed that they fell into only two classes, each of which consisted of an incomplete cDNA. One of the inserts encoded the C-terminal residues 222–335 of the intracellular domain of Fas itself, and the other encoded a protein, subsequently named RIP, without overt relationship to previously described polypeptides. For simplicity in the following discussion, we will refer to the transcriptional activator fusion proteins expressed by the two classes of library isolates as Act–Fas and Act–RIP.

The specificity of the interaction partners was further tested by using the library plasmids to transform yeast harboring expression plasmids encoding LexA fusions with intracellular domains of various cell surface receptors (Figure 1A). Although no interaction was detected in most cases (data not shown), weak promoter activity was discerned following introduction of the yeast plasmid encoding Act–RIP into strains harboring LexA–TNFR1 intracellular domain. By contrast, no activity was seen when Act–RIP was introduced into strains harboring LexA–TNFR2 intracellular domain. Yeast transformed with Act–Fas displayed promoter activity in strains harboring LexA–Fas intracellular domain, but showed no activity in strains harboring any other LexA–intracellular domain (Figure 1A).

To measure this effect more precisely, β-gal assays were performed on lysates of yeast harboring various pairs of LexA–intracellular domain and Act–RIP. Lysates from yeast bearing LexA–Fas and Act–RIP contained about 30- to 40-fold more β-gal activity than strains bearing Act–RIP and either LexA–lpr^{cg} or LexA–TNFR2 (Figure 1B). Lysates prepared from yeast harboring LexA–TNFR1 and Act–RIP expressed β-gal activity at about 10% of the level seen in lysates prepared from yeast bearing LexA–Fas and Act–RIP (Figure 1B).

One explanation for the failure to detect an interaction between Act–RIP and LexA–lpr^{cg} or LexA–TNFR2 could have been that the LexA fusion proteins were poorly ex-

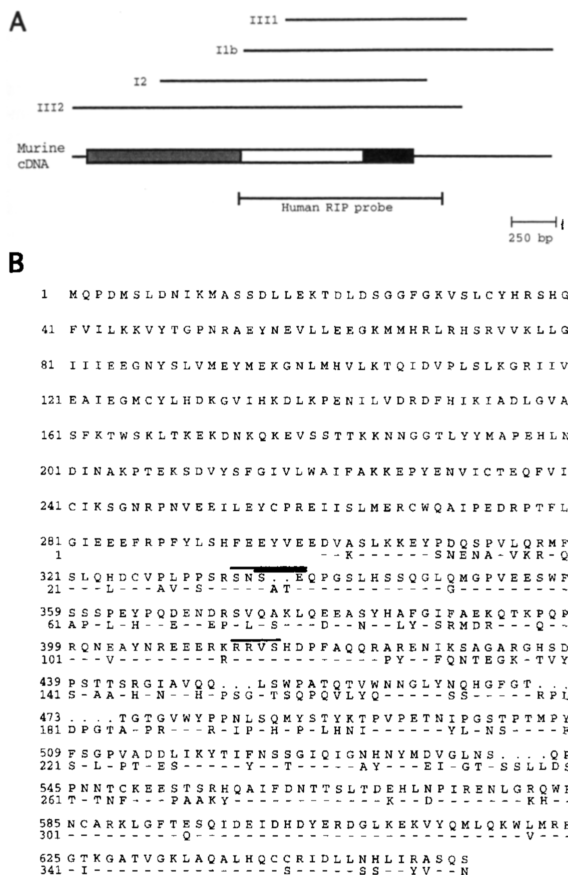


Figure 2. Structure of RIP cDNAs and Deduced Amino Acid Sequence

(A) Schematic diagram of RIP cDNA. The inserts of clones isolated from a mouse thymus cDNA library are symbolized as lines above a bar diagram of the composite sequence, which depicts the regions encoding the kinase domain (stippled box), the death domain (closed box), and a region of unknown function predicted to have high α-helical content (open box). Also shown is the region of human RIP that was used as a probe to isolate murine cDNAs. The original human RIP isolate encompassed sequences encoding the death domain and about 100 residues upstream.

(B) Inferred polypeptide sequence. The mouse sequence consists of a contiguous open reading frame proceeding from a translational initiation consensus. The human sequence predicted from a cDNA fragment is shown below the mouse; identical residues are indicated by a dash, and gaps indicated by a period. The conserved consensus sequences for casein kinase II (S–X–X–E) and cAMP- or cGMP-dependent protein kinase (R–X–X–S) are overlined (reviewed by Kennelly and Krebs, 1991).

pressed. To address this possibility, a portion of each lysate used to measure enzyme activity was subjected to gel electrophoresis and blot transfer, followed by detection with anti-LexA antiserum. LexA fusion proteins of the appropriate size were detected in each of the lysates, and both the LexA–lpr^{cg} and LexA–TNFR2 fusion proteins were found to be more abundantly expressed than LexA–Fas or LexA–TNFR1 (data not shown), making it unlikely that failure to detect interaction in vivo could be attributed to degradation or inadequate synthesis of the LexA chimeras.

To localize the sequences in Fas/APO-1 that are responsible either for self-interaction or for interaction with RIP, we prepared a set of LexA–Fas C-terminal deletion chimeras and tested their ability to support interaction with either Act–Fas or Act–RIP, as measured by β-gal colony assay (Figure 1C). These studies showed that the 16 C-terminal residues of Fas are not required for interaction with either RIP or Fas itself, but that removal of additional residues severely compromises the association with RIP.

Cloning and Structure of Murine RIP

Because the RIP cDNA insert identified by genetic selection in yeast did not encode an open reading frame bearing a consensus translational initiation sequence, additional human cDNA libraries were screened by hybridization for a full-length clone. None were identified, however, and several of the resulting isolates appeared to terminate at approximately the same 5′ terminus, suggesting that secondary structure in the mRNA might have prevented extension of the cDNA by reverse transcriptase. Although the largest clone spanned approximately 1 kb of sequence, preliminary RNA blot hybridizations revealed a transcript of approximately 4.2 kb expressed in cell lines of diverse provenance, including tumors of lymphoid, hepatic, renal, neuronal, cervical, intestinal, muscular, and skeletal origin.

To isolate a full-length clone, the human RIP coding sequence was used to probe a mouse thymus cDNA library. Four distinct overlapping clones were identified, ranging in size from 1 kb to 2.4 kb, as shown in Figure 2A. Restriction analysis and sequencing revealed that one of these clones, III2, extended further 5′ than the others. The 2.3 kb insert of III2 contains a 1968 nt open reading frame beginning with a translational initiation consensus sequence (Kozak, 1987) and predicting a polypeptide of 656 amino acids with a M_r of 74,000 (Figure 2B).

The N-terminal region of RIP bears an extended homology to protein kinases that begins a few residues after the presumptive initiating Met and extends to the vicinity of residue 300. Quantitative sequence comparisons based on a word match algorithm (Altschul et al., 1990) predict that this domain is most similar overall to the Tyr subclass of protein kinases, with the highest relatedness seen to the mouse *lck* gene product (Marth et al., 1985). However, in the key subdomains that discriminate most closely between Tyr and Ser/Thr substrate specificity, the DLKPEN sequence (corresponding to kinase subdomain VI) and the GTLYYMAPE sequence (kinase subdomain VIII), RIP appears to match the Ser/Thr family consensus (Figure 3A; Hanks and Lindberg, 1991; Taylor and Radzio-Andzelm, 1994). Residues that are conserved in kinases of both subclasses (for example, kinase subdomain IX; Figure 3A) are also well conserved in RIP.

The sequence predicted by the longest of the human cDNA fragments consists of 375 amino acids corresponding to the region just C-terminal to the kinase domain of murine RIP and shares 67% sequence identity with the murine sequence over this length. Within this domain, the first 270 amino acids following the kinase domain have no striking homology to other proteins, although a small subdomain is highly conserved between mouse and human proteins (residues 391–427 of the murine sequence) and has a relatively high representation of Arg (R), Gln

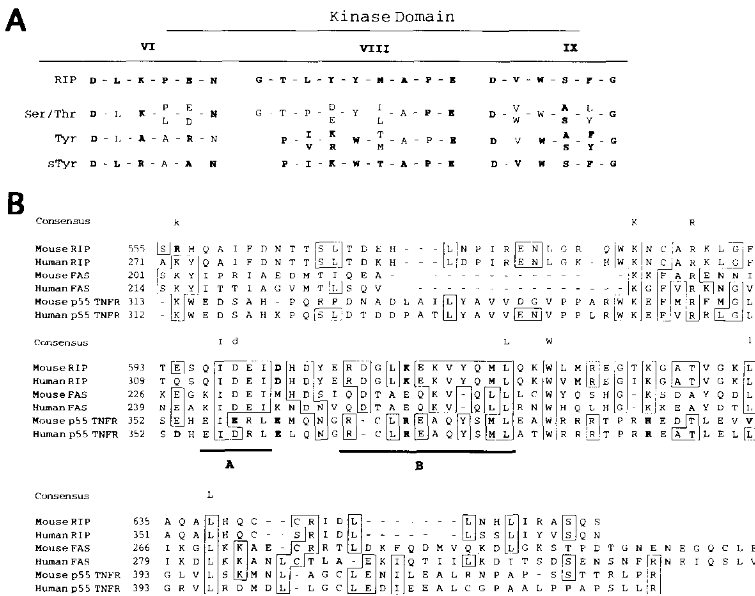


Figure 3. RIP is Similar to Proteins with Kinase and Death Domains

(A) Comparison of RIP to the catalytic domains of Ser/Thr and Tyr kinases and the Src subfamily of Tyr kinases (sTyr). Consensus sequences were described by Hanks and Lindberg (1991) and represent analysis of 70 Ser/Thr kinases, 26 Tyr kinases, and 8 sTyr kinases. Consensus residues found in over 95% of sequences analyzed are bolded.

(B) Sequence alignment of the RIP C-terminus with the death domains and C-termini of Fas and TNFR1. Gaps are indicated by dashes. Consensus residues conserved in all six sequences are capitalized, whereas positions at which a charge-conserved residue is found in one of the sequences are shown in lower case. Noteworthy charge conservations are bolded. The regions denoted (A) and (B) represent two portions of the Fas cytoplasmic domain completely conserved between mouse and human.

(Q), and Glu (E) (18/37 residues in both sequences). The sequence of this region is similar to portions of the trichohyalin family of hair structural proteins, which contain RQE-rich repeats that form highly stabilized α helices (Lee et al., 1993).

The RIP C-Terminus Has Death Domain Homology

The 98 C-terminal amino acids of RIP share 87% sequence identity between mouse and human, suggesting they subserve some regulatory function (Figure 2B). Comparable domains of approximately 90 residues close to the C-termini of Fas and TNFR1 have been shown to play a role in the transduction of apoptotic signals to receptive cells and have been termed death domains for this reason (Tartaglia et al., 1993a). Pairwise comparisons of the death domain sequences aligned in Figure 3B showed the highest relatedness between human RIP and human TNFR1 (59% similarity and 30% identity), which are significantly more similar than human Fas and human TNFR1 (42% similarity and 23% identity). The interspecies conservation of the RIP death domain (84% identity between mouse and human) exceeds that of the TNFR1 (68% identity) and Fas (49% identity) death domains.

Constitutive and Inducible Expression of RIP mRNA

Preliminary RNA blot hybridization experiments demonstrated the existence of an RNA species of approximately 3.8 kb in a variety of cell lines. To more precisely assess mRNA abundance in tissues, a quantitative ribonuclease protection assay was employed. Use of a labeled antisense RNA probe corresponding to the 3′ terminus of the cDNA gave rise to a ribonuclease-resistant species of the expected size in all adult tissues tested (Figure 4A). An in vitro labeled RNA antisense to the mRNA for the ribosomal large subunit protein L32 was used as an internal standard to allow normalization to the amount of RNA loaded in each lane. Analysis of the protected RNA showed that RIP mRNA levels varied by less than 2- to 3-fold between most tissues (data not shown); lung showed the highest expression, whereas tongue showed the least.

The possibility that RIP mRNA might be regulated as a consequence of activation in T cells was also explored. Dissociated murine splenocytes were stimulated in vitro with the lectin–concanavalin A (ConA), and total RNA prepared at various times following addition of lectin was analyzed for the presence of RIP sequences by RNA blot analysis. Little or no RIP RNA could be detected in unstimulated splenocytes, but a single 3.8 kb species appeared in unfractionated splenocytes that had been exposed to lectin for 2 hr or longer (Figure 4B). Since RIP mRNA is detectable by ribonuclease protection in the spleen as a whole (Figure 4A), the inability to detect RIP mRNA in splenocytes treated with ConA for less than 2 hr is probably due to the lower sensitivity of RNA blot analysis, although this discrepancy could also have resulted from RIP expression exclusively in the fibrous tissue of the spleen.

A Polypeptide of the Expected Molecular Mass Is Expressed In Vivo

To examine the distribution of RIP protein in vivo, a rabbit antiserum was prepared against a fusion protein consisting of the 250 C-terminal residues of murine RIP fused to E. coli maltose-binding protein. The antiserum specificity was validated by immunoprecipitation of RIP synthesized in vitro. Following in vitro transcription and translation of the RIP open reading frame, a single labeled product of approximately 74 kDa was observed that could be specifically immunoprecipitated with the rabbit antiserum, but not with serum from unimmunized animals (Figures 5A and 5B). The specificity of the antiserum for RIP was also documented by its inability to immunoprecipitate an irrelevant protein (Photinus pyralis luciferase) similarly translated in vitro. Immunoprecipitation of a lysate of metabolically labeled NIH 3T3 cells with the rabbit antiserum

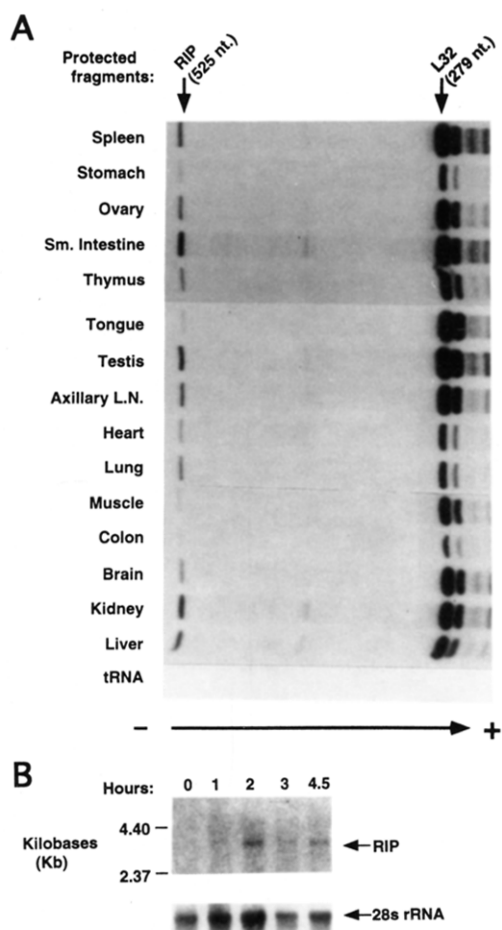


Figure 4. RIP Expression

(A) RNase protection analysis of total RNA harvested from adult tissues. Each RNA (10 μg) was incubated with labeled antisense RNAs complementary to the 3′ end of the RIP cDNA sequence and to sequences within the mRNA for the ribosomal large subunit protein L32 (normalization control). Following digestion of the unhybridized RNA, the protected fragments were separated on a 6% denaturing polyacrylamide gel. tRNA (50 μg) was substituted for the sample RNA to assess the adequacy of RNase digestion.

(B) T cell activation induces RIP mRNA. An RNA blot prepared from 8 μg samples of total RNA from splenocytes treated with ConA for the indicated period of time was hybridized with labeled RIP cDNA or a 28S rDNA probe and washed as described in the Experimental Procedures.

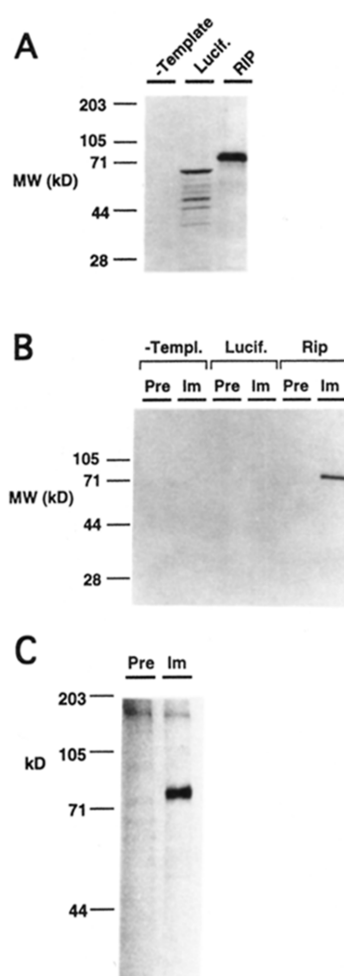


Figure 5. Immunoprecipitation of RIP

(A) An insert fragment from RIP cDNA clone III2 was transcribed and translated in vitro, and the reaction products were analyzed on a 10% SDS–polyacrylamide gel. Included as controls were samples in which either no RNA, or an RNA encoding P. pyralis luciferase, was translated.

(B) Immunoprecipitation of the in vitro translation products of (A) with an antiserum raised against the C-terminus of RIP, or with preimmune serum.

(C) Immunoprecipitation of an [^{35}S]Met-labeled lysate of NIH 3T3 cells with the antiserum described in (B).

revealed the presence of a single protein species with the same molecular mass as that revealed by in vitro translation of RIP (Figure 5C).

Detection of RIP in Transiently Transfected BHK Cells

To determine whether RIP protein could have a direct effect on cell viability, BHK cells grown on coverslips were transiently transfected with an epitope-tagged version of RIP (RIP-Myc) and reacted with anti-RIP antiserum or an anti-Myc monoclonal antibody. Weak expression was detected with both antibodies. The pattern of immunoreactivity was heterogeneous, with both diffuse cytoplasmic as well as punctate perinuclear patterns observed (Figures 6A and 6B). DNA staining with Hoechst 33258 showed that many of the RIP-expressing cells had apoptotic nuclei, a feature not seen when vector or Fas control expression plasmids were used (data not shown). However, a number of RIP-expressing cells could be found that had normal-appearing nuclei; conversely, apoptotic cells having no detectable RIP staining were also seen.

The concordance of cell death and RIP expression suggested the ability to detect RIP-Myc protein might be compromised by the death of the cells in which it was being expressed. To test this and the possible role of individual RIP domains in apoptosis, two additional epitope-tagged constructs were prepared: one lacking C-terminal sequences, including the death domain (RIP-MycΔdeath),

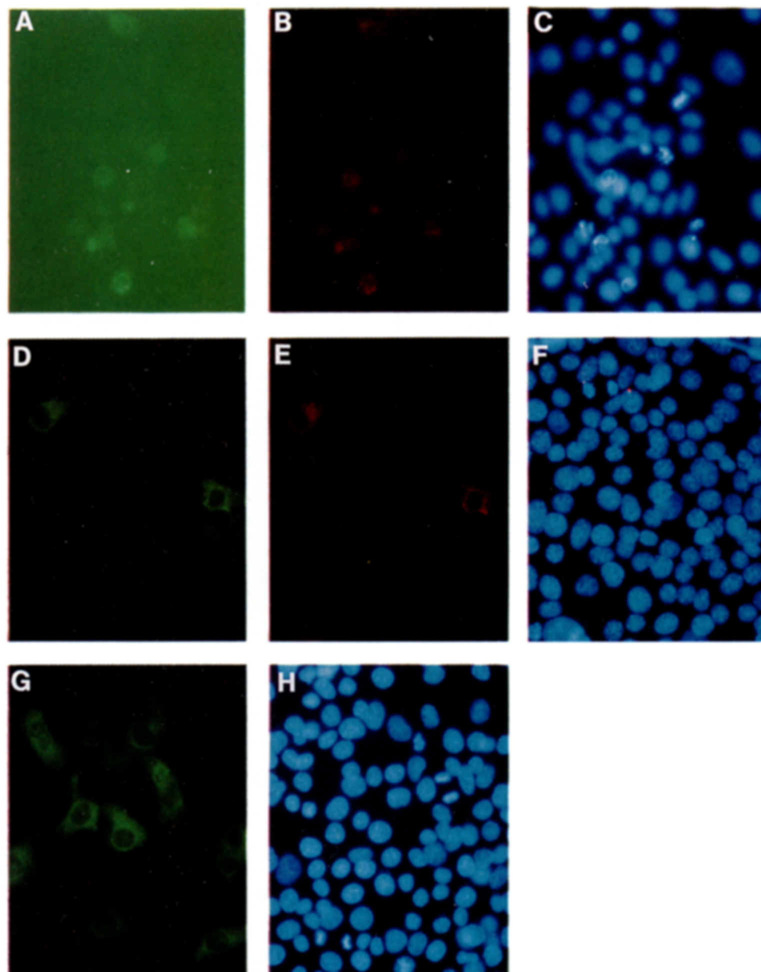


Figure 6. RIP Immunofluorescence

BHK cells were transiently transfected with plasmids expressing RIP-Myc (A–C), RIP-MycΔkinase (D–F), or RIP-MycΔdeath (G–H) and reacted with anti-Myc monoclonal antibodies (A, D, and G), anti-RIP polyclonal antibodies (B and E), or Hoechst 33258 (C, F, and H). Cells transfected with RIP-MycΔdeath plasmid did not react with anti-RIP antisera (data not shown).

and one lacking ~200 amino acids in the kinase domain (RIP-MycΔkinase). Both deletion mutants showed greater immunoreactivity with anti-Myc antibodies (Figures 6D and 6G) than the full-length construct. Only RIP-MycΔkinase was detected by the anti-RIP antiserum (Figure 6E), as expected from the deletion of its epitope from RIP-MycΔdeath.

RIP Overexpression Leads to Cell Death

To determine whether RIP was inducing cell death, we marked the transfected cells by cotransfection with β-gal. Cells were transfected with pairs of expression plasmids encoding RIP-Myc and β-gal (Price et al., 1987) at a 1:3 ratio of β-gal plasmid to RIP plasmid. After histochemical detection of β-gal activity, cells transfected with β-gal and RIP-Myc expression plasmids were found to contain a large proportion of intensely staining, shrunken blue cells that exhibited membrane blebbing and loss of adherence (Figures 7A and 7E). By contrast, transfection with β-gal, either alone (Figure 7D) or in combination with RIP-MycΔdeath (Figure 7C), had no adverse effect upon nuclear morphology and resulted in a predominantly cytoplasmic β-gal staining pattern. Cotransfection of β-gal with RIP-MycΔkinase likewise gave cytoplasmic staining of healthy-appearing cells, although a number of shrunken, blebbed cells were also seen (Figure 7B).

To quantitate these results, cells from three independent transfections were examined, and the morphologically apoptotic blue cells were enumerated as a fraction of total blue cells (Table 1). Over 57% of blue cells arising from cotransfection of RIP-Myc and the β-gal showed morphological changes consistent with apoptosis, whereas only 1%–2% of blue cells that had been transfected with β-gal plasmid or in combination with RIP-MycΔdeath exhibited such a phenotype. However, a consistent frequency of apoptotic changes was seen in cotransfections involving RIP-MycΔkinase, with almost 11% of the blue cells appearing to have undergone cell death. The fraction of cells showing morphological changes was positively correlated with the ratio of RIP-Myc or RIP-MycΔkinase plasmid to β-gal plasmid, so that increasing RIP:β-gal plasmid ratios gave higher percentages of dead blue cells (data not shown).

Additional transfection experiments were performed using cells derived from the human embryonic kidney 293 cell line (Pear et al., 1993). As with the previous assay, cell death was observed in these cells when either the RIP-Myc or RIP-MycΔkinase plasmid was used. DNA frag-

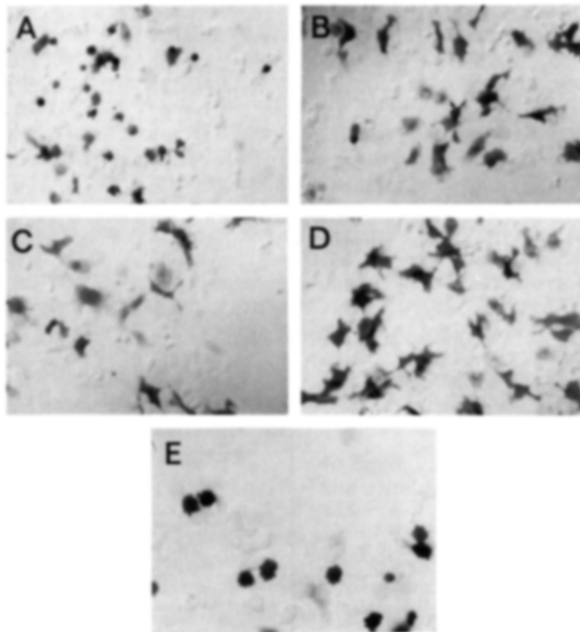


Figure 7. Apoptotic Changes in Cells Transfected with RIP and β-Gal Expression Plasmids

BHK cells were transfected on coverslips, fixed after 12 hr, and incubated in a buffer containing X-Gal to visualize β-gal activity. Transfections were carried out with plasmids encoding: (A) RIP-Myc and β-gal, (B) RIP-MycΔkinase and β-gal, (C) RIP-MycΔdeath and β-gal, or (D) β-gal alone. (E) Higher magnification of cells shown in (A).

mentation, a hallmark of apoptotic cell death, was detected in situ in cells transfected with these plasmids using the method of TdT-mediated dUTP-biotin nick end labeling (TUNEL; Gavrieli et al., 1992); no significant cell death or DNA fragmentation was observed with the RIP-MycΔdeath or vector plasmids (data not shown).

Discussion

In this study, a genetic selection in yeast for proteins capable of interacting with the intracellular domain of Fas/APO-1 identified cDNAs encoding two proteins from a human T cell cDNA library: Fas itself and a novel kinase-related protein, RIP, containing a death domain. Neither protein interacts with a humanized variant of the murine lpr^{cg} allele, a point mutant that confers much the same phenotype on affected mice as does the original *lpr* allele (Matsuzawa et al., 1990). The finding that the intracellular domain of the lpr^{cg} variant is incapable of interacting with the wild-type Fas cytoplasmic domain in yeast suggests that oligomerization of Fas mediated by cytoplasmic sequences may be a prerequisite for activity. However, it has not been established whether oligomerization of Fas is necessary for interaction with RIP, or whether the same subdomain exploited for oligomerization is also required for heteromeric association with RIP. The discovery of the Fas–Fas interaction in a library screen is consistent with a recent demonstration that TNFR1 and Fas are capable of both self- and cross-association in yeast (Boldin et al.,

Table 1. Quantitation of RIP-Induced Cell Death

Construct	Percent Dead ± SEM (number of cells scored)
RIP-Myc plus β-gal	57.3 ± 1.7 (618)
RIP-MycΔkinase plus β-gal	10.9 ± 1.1 (660)
RIP-MycΔdeath plus β-gal	2.0 ± 0.7 (661)
β-gal alone	1.6 ± 0.2 (639)

The cotransfections described in Figure 7 were repeated in triplicate, and the cells were fixed and analyzed for *lacZ* expression 16 hr after transfection as detailed in the Experimental Procedures. The data are expressed as the mean percentage of blue cells exhibiting signs of apoptosis as a fraction of the total number of blue cells counted (shown in parentheses).

1995). In accord with the notion that the death domain may itself mediate such interactions, we find here that RIP can also associate with the intracellular domain of TNFR1.

Despite conservation of sequence, structure, and function between Fas and TNFR1, downstream effects of the two receptors have been dissociated in some cell types (Wong and Goeddel, 1994; Schulze-Osthoff et al., 1994), whereas responses in other cells suggest that elements of their signaling pathways may be shared (Clement and Stamenkovic, 1994; Schulze-Osthoff et al., 1994). The finding that RIP interacts with both Fas and TNFR1 in yeast is attractive for its intimation of a simple explanation for the shared manifestations of receptor ligation.

Deletion analysis of Fas has previously demonstrated that the 15 C-terminal amino acids are not required for signaling, but that larger deletions abolish the ability of Fas to induce apoptosis (Itoh and Nagata, 1993). Consistent with these findings, this study has shown that in yeast RIP binds well to a Fas molecule lacking the 16 C-terminal amino acids, but that larger deletions of the Fas cytoplasmic domain greatly reduce or abolish this interaction.

The human RIP fragment identified by yeast interaction was used to clone a murine cDNA that appears to span the entire coding region. The authentic 5′ and 3′ ends of the RIP mRNA have yet to be defined, however. Sequencing of the 3′ ends of cDNA clones III1, III2, and I1b yielded about 1.4 kb of putative untranslated sequence including an AAUAAA transcription termination signal, but no poly(A) tail (data not shown). Genomic sequence obtained from the 5′ end suggests the presence of at least one more noncoding 5′ exon (data not shown). Of the discrepancy between the 3.4 kb of identified sequence and the approximately 3.8 kb of RNA identified in blot hybridization experiments, the fraction that can be attributed to poly(A) is uncertain.

RIP mRNA is expressed at low levels in all tissues. A pattern of widespread expression has also been observed for Fas (Leithauser et al., 1993; B. Z. S. and P. L., unpublished data), although in contrast with RIP, *fas* mRNA is found at higher levels in thymus, liver, lung, and heart. Like *fas* mRNA, RIP mRNA is induced in splenocytes after activation with ConA. Although a requirement for RIP in Fas-mediated killing has not been established, coordinate induction of Fas and RIP may contribute to increased sus-

ceptibility of T cells to Fas-mediated cell death following activation (Owen-Schaub et al., 1992).

The N-terminus of RIP has strong homology to kinases of both Ser/Thr and Tyr kinase subfamilies. In the two interacting loops that appear to control hydroxyamino acid recognition (Hubbard et al., 1994; Taylor and Radzio-Andzelm, 1994), RIP closely resembles a Ser/Thr kinase. In particular, it lacks the Ala–X–Arg or Arg–X–Ala motif in subdomain VI and the Pro–X–X–Trp motif in subdomain VIII that closely correlate with Tyr substrate specificity (Hanks et al., 1988). However, among sequences available at the time of submission, RIP shows the greatest global similarity to murine *lck*, a Tyr kinase of the Src family. The relatedness to Tyr kinases is especially apparent among the framework residues outside the putative active site. For example, RIP has a tryptophan at position 269 that is present in all Tyr kinases analyzed by Hanks and Quinn (1991), but absent from all Ser/Thr kinases examined except Mos. The presence of structural motifs from both Tyr and Ser/Thr kinases has also been noted for the soybean kinase GmPK6 (Feng et al., 1993), with which RIP shares high global similarity. RIP contains a small number of amino acids that differ from the conserved residues of either Ser/Thr or Tyr kinases, for example a Gly at position 24 that is present in the majority of kinases from both classes. However, at residues that are conserved in all protein kinases, RIP follows the consensus.

We have found that overexpression of RIP results in the induction of a cell death program morphologically similar to apoptosis. Deletion of the C-terminal region of RIP spanning the segment of death domain homology eliminated the apoptotic response, but deletion of the kinase domain did not entirely quench activity. It may be that the overexpression of the death domain itself is lethal to transfected cells, consistent with the observation by Boldin et al. (1995) that expression of the free intracellular domain of TNFR1 has a cytocidal effect on HeLa cells. The induction of apoptosis by transient transfection suggests that the level of expression or ability of death domain–bearing proteins to self-associate may be subject to some form of regulation to prevent the spontaneous initiation of apoptosis in the absence of ligand or other eliciting stimulus.

The notion that a stoichiometrically limiting intracellular effector is involved in the pathway of Fas-mediated apoptosis is supported by the recent demonstration that maximal signaling by Fas and the TNF receptors requires an intermediate level of receptor cell surface expression (Clement and Stamenkovic, 1994). This result is consistent with a model in which the initiation of signal transduction is critically dependent on the aggregation-mediated focal accumulation of a limiting cytoplasmic ligand, which can be diluted to the point of inefficacy by overexpression of the cell surface receptor. The data presented here that RIP is not encoded by a highly expressed RNA, but that the transcript accumulates rapidly in response to T cell activation, in turn admits the possibility that RIP may be limiting for receptor-mediated apoptosis. However, other factors may also interact with the Fas intracellular domain, as suggested by the findings that Fas can transmit activation as well as death signals (Alderson et al., 1993).

Still unclear is the mechanism by which formation of a multimeric Fas complex would result in signal transduction. Although enzymatic activity has not been demonstrated for RIP, the possibility that it encodes a protein kinase is attractive given the proposed participation of at least one Tyr kinase in Fas signaling (Eischen et al., 1994). However, results with the mutant plasmid RIP-MycΔkinase suggest that any kinase potential RIP has may not be required for its induction of cell death.

In preliminary experiments, a monoclonal antibody recognizing murine Fas has been found to specifically coimmunoprecipitate a polypeptide of about 59 kDa that reacts with anti-RIP antiserum (data not shown). This 59 kDa protein may represent a proteolytic fragment of RIP or a related protein that is recognized by anti-RIP antibodies. Although the evidence that RIP binds to Fas in yeast favors a model in which RIP acts directly downstream of Fas in a death pathway, it is also possible that RIP has other actions, or that the normal physiological role of the molecule lies in a pathway initiated by other cues. The creation of mice or cell lines homozygously deficient in RIP should help to address this point. In addition, the elucidation of any target(s) of action of RIP may also facilitate our understanding of the role(s) RIP plays in the cell death response.

Experimental Procedures

Plasmid Construction

The yeast interaction system was modified from that described by Gyuris et al. (1993) by engineering the LexA expression plasmid to remove an internal MluI site and to insert MluI and NotI sites downstream from the DNA portion encoding the C-terminus of the gene. The resulting distal polylinker has the site sequence MluI–PmeI–NotI–EcoRI in the frame in which the MluI site encodes Thr and Arg (frame 1). Complete sequences are available from the authors (B. S.) upon request.

Receptor cytoplasmic tails were amplified by PCR from cDNA libraries and cloned as MluI–NotI or BssHII–NotI fragments using the following oligos: Fas, 5′-CGC GGG ACG CGT AAG GAA GTA CAG AAA ACA TGC-3′ and 5′-CGC GGG GCG GCC GCT CTA GAC CAA GCT TTG GAT TTC-3′; TNFR1, 5′ CGC GGG GCG CGC TAC CAA CGG TGG AAG TCC AAG-3′ and 5′ CGC GGG GCG GCC GCT GCC CGC AGG GGC GCA GCC TCA-3′; TNFR2, 5′-CGC GGG ACG CGT AAG AAG CCC TTG TGC CTG CAG-3′ and 5′-CGC GGG GCG GCC GCT TTA ACT GGG CTT CAT CCC AGC-3′. The Fas cytoplasmic domain used in the library screen diverges at the Glu 5 residues prior to the C-terminus and continues an additional 25 residues through vector sequences to the C-terminus. In all subsequent analysis, these residues were found not to contribute detectably to either Fas–Fas or Fas–RIP interaction. A mutant Fas bait protein analogous to the *lpr*^{cg} point mutation was made by mutating the Val at position 254 of human Fas to Asn using the following oligos in a recombinant PCR reaction: 5′-CGA AAG AAT GGT AAC AAT GAA GCC-3′ and 5′-GGC TTC ATT GTT ACC ATT CTT TCG-3′. In the resulting construct, residues 330 and 331 were also converted from Glu and Ile to Gly and Asn, respectively. Fas deletion mutants were made by PCR with the Fas 5′ oligo and 3′ oligos substituting a stop codon for the amino acid at the indicated position; products were subcloned into the LexA expression plasmid as described above. Oligos used were the following: (S320*) 5′-CGC GGG GCG GCC GCT TTA AGT AAT GTC CTT GAG GAT GAT-3′; (K299*) 5′-CGC GGG GCG GCC GCT TTA GAG ATC TTT AAT CAA TGT GTC-3′; (H285*) 5′-CGC GGG GCG GCC GCT TTA AAG TTG ATG CCA ATT ACG AAG-3′; (K263*) 5′-CGC GGG GCG GCC GCT TTA GAT CTC ATC TAT TTT GGC TTC-3′.

A Myc-tagged version of RIP (RIP-Myc) was made by digesting RIP clone III2 with TfiI and ligating a HindIII–TfiI adaptor to the 5′ end and a TfiI–NotI adaptor containing the Myc epitope and a stop codon to the 3′ end. The sense (145) and antisense (146) oligonucleotides com-

prising the 3′ adaptor were as follows: 145, 5′-ATT CGT GCC AGC CAG AGC GGC ATG GAG CAG AAG CTC ATC TCA GAA GAA GAC CTC GCG TAA GC-3′ and 146, 5′-GGC CGC TTA CGC GAG GTC TTC TTC TGA GAT GAG CTT CTG CTC CAT GCC GCT CTG GCT GGC ACG-3′. The resulting insert was cloned into the HindIII and NotI sites of pcDNA I (Invitrogen). To make RIP-MycΔkinase, a PCR reaction was performed using a 5′ RIP primer (160, 5′-CCC AAG CTT GTT GGA GAT TCT GAG CAA TC-3′) and an internal kinase domain primer (161, 5′-CCC GAT CTG CAG GTC ATG TAA GTA GCA CAT GCC-3′). The resulting product was cloned into the HindIII and PstI sites of RIP-Myc resulting in the deletion of RIP residues 132–323. RIP-MycΔdeath was made by PCR using a T7 primer and a Myc tag-containing primer (151, 5′-CCC CTC GAG TTA GAG GTC TTC TTC TGA GAT GAG CTT TTG CTC TTT CTT TAA ACT TGC CAC-3′). The amplified RIP-MycΔdeath sequence, lacking amino acids 309–656, was subcloned as a HindIII–XhoI fragment into pcDNA I. Myc tags were located at the C-terminus of all proteins, and thus, detection of the Myc epitope requires translation of the entire cloned sequence. The BAG retrovirus vector encoding β-gal has been described (Price et al., 1987).

Yeast Strains and Library Screen

Yeast transformation with library DNA was performed by the method of Schiestl and Gietz (1989) as follows. Recipient cells, EGY48/pRB1840 (Gyuris et al., 1993) bearing LexA–fusion protein plasmids, were grown overnight in YPAD medium to a density of approximately 10^7 cells/ml, then diluted in 100 ml of warmed YPAD to a density of 2 × 10^6 cells/ml and regrown to 10^7 cells/ml. The cells were harvested and washed in water, resuspended in 1 ml of water, transferred to a sterile microcentrifuge tube, and pelleted. The pellet was resuspended in 0.5 ml of 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.1 M Li acetate (pH adjusted to 7.5 with acetic acid and passed through 0.2 μ filter). 50 μl of the resulting suspension was mixed with 1 μg of transforming DNA and 50 μg of single-stranded salmon sperm DNA, after which 0.3 ml of a solution of 40% polyethylene glycol-4000 in Tris, EDTA, LiOAc was added and mixed thoroughly, followed by incubation at 30°C with agitation for 30 min. After a heat pulse at 42°C for 15 min, the cells were pelleted in a microcentrifuge, and the pellets were resuspended in 1 ml of Tris, EDTA, diluted, and plated on selective medium. Library screening and recovery of plasmids were performed as described by Gyuris et al. (1993).

For assessing the interaction of RIP and Fas with other bait proteins, cells of the yeast strain EGY48/pSH18-34 were transformed with the indicated bait construct and selected on Ura^- His^- glucose plates. These bait strains were subsequently used for transformation of the RIP or Fas library plasmids and plated on Ura^- His^- Trp^- glucose plates. Several colonies from each bait/interactor combination were picked and plated in duplicate on Ura^- His^- Trp^- X-Gal plates containing either 2% glucose or 2% galactose, 1% raffinose. The LexA–TNFR2 bait strain gave a weak blue color reaction when grown on galactose, indicating spontaneous transcriptional activation by the fusion protein.

β-Gal Assays

Assays of crude extracts were carried out as described (Rose et al., 1990). Cells bearing the appropriate bait and interaction plasmid were grown to saturation overnight at 30°C in minimal Ura^- His^- Trp^- medium with 2% glucose. The next day, cells were diluted 1:50 into medium containing 2% galactose and 2% raffinose and allowed to grow overnight. Cells were spun and resuspended in breaking buffer (100 mM Tris [pH 8], 20% glycerol [v/v], 1 mM DTT). Cells were lysed in the presence of 10 mM PMSF by vortexing with acid-washed beads. Lysates were cleared by centrifugation, and an aliquot was incubated in Z buffer (Miller, 1972) with 0.67 mg/ml o-nitrophenyl-β-D-galactoside substrate. Reactions were stopped with Na_2CO_3 when an appropriate level of color had developed. Protein concentrations were determined by Bradford assay. β-Gal units were calculated by the equation: specific activity (nmoles/min/mg) = (OD_{420}·378)/(time [min]·vol [ml]·concentration [mg/ml]).

cDNA Cloning

Additional clones overlapping the primary RIP isolate were sought in two libraries: an expression library prepared in the CDM8 plasmid vector using mRNA isolated from the human cytolytic T cell line WH3 and a commercially available human leukocyte library in λ phage purchased from Clontech (HL1169a). Both libraries were screened by filter replica hybridization, using radiolabeled probes derived from the insert isolated by interaction screening, as well as from subsequent inserts identified by hybridization.

To isolate murine RIP cDNA clones, 1.2 kb of human RIP sequence was subcloned into two halves and radiolabeled using the random hexamer method (Feinberg and Vogelstein, 1983). An oligo(dT)-primed mouse thymus cDNA library from a (C57Bl/6 × CBA)F1 mouse (Stratagene) was plated out and 10^6 plaques were screened using each of the human fragments on duplicate GeneScreen filters (DuPont). Hybridization conditions were 5× SSPE, 10× Denhardts, 2% SDS, 0.1 mg/ml herring sperm DNA at 55°C overnight. Filters were washed in 2× SSC, 0.1% SDS at 55°C with several changes over 1 hr. Plaque purified phage were isolated with three rounds of screening (Sambrook et al., 1989), and in vivo excision was carried out using Exassist phage and SOLR recipient cells (Stratagene). Seven independent clones were isolated that fell into the four classes in Figure 2A. The coding sequence of RIP was obtained as a composite from the cDNA clones sequenced on both strands using Sequenase T7 polymerase (United States Biochemical). Several nucleotide polymorphisms were detected between the multiple clones, only one of which resulted in an amino acid difference: a Thr to Ile at position 473. Sequence comparisons were done with Genetics Computer Group, Inc. or MegAlign (DNAStar, Inc.) software using default parameters.

RNase Protections and RNA Blot Hybridization

Tissue RNA samples were prepared from wild-type FVB mice (Taconic) by guanidinium thiocyanate lysis and centrifugation through a CsCl cushion (Chirgwin et al., 1979). An antisense probe for RIP made from cDNA clone III1 linearized with SpeI was synthesized using T7 polymerase with an in vitro transcription kit (Stratagene), with the addition of 20 μM cold rUTP and 100 μCi [^{32}P]UTP (New England Nuclear). The ribosomal L32 probe was synthesized from an XbaI-linearized template at 10% the specific activity of the RIP probe. The use of L32 as an internal control for RNA loading has been described elsewhere (Shen and Leder, 1992). The in vitro transcription products were slightly larger than the protected fragments of 525 nt and 279 nt, respectively, as expected. RNase protection was carried out as described by Melton et al. (1984).

For measuring RIP in activated T cells, spleens were removed from adult animals and dissociated using a stainless steel mesh. Cells (~10^7 per timepoint) were exposed to ConA (10 μg/ml) for 0–4.5 hr, and RNA was prepared as described above. Approximately 8 μg of total RNA from each sample was loaded on a 0.9% agarose, 4% formaldehyde gel, electrophoresed, and transferred to nylon filters (GeneScreen, DuPont) by capillary transfer (Sambrook et al., 1989). The blot was hybridized with either an RIP probe corresponding to the C-terminal half of the protein (cDNA III1) or with a probe detecting ribosomal 28S RNA and incubated overnight in 40% formamide, 4× SSC, 10% dextran sulfate, 7 mM Tris (pH 7.6), and 20 μg/ml salmon sperm DNA at 42°C. Blots were washed at 50°C in 1× SSC, 0.1% SDS (RIP probe) or 60°C in 0.1× SSC, 0.1% SDS (28S probe).

RIP Antisera and Immunoprecipitations

Rabbit polyclonal antisera recognizing RIP were prepared by Pocono Rabbit Farm and Laboratory, Inc. using a fusion protein containing the C-terminal 250 amino acids of mouse RIP fused to maltose-binding protein (MBP; New England Biolabs). MBP–RIP was purified by amylose chromatography and acrylamide gel elution. For immunoprecipitations, RIP was first transcribed in vitro using 15 μg of full-length RIP template in a reaction containing 3.3 mM each ATP, GTP, CTP, and UTP, 280 U of RNase inhibitor, 400 U of Sp6 RNA polymerase, and 1× Sp6 buffer (Boehringer Mannheim Biochemicals). Translations were carried out using a reticulocyte lysate kit containing a luciferase positive control (Promega) using approximately 4 μCi [^{35}S]Met per reaction. Reaction products were diluted to 1 ml in a modified RIPA buffer (0.5% NP-40, 0.5% sodium deoxycholate, 0.025% SDS, 50 mM Tris, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM sodium orthovanadate [pH 7.6]) containing protease inhibitors and precleared with normal rabbit serum and protein A–Sepharose. Reactions were split in half, 5 μl of preimmune or immune serum was added, and samples

were allowed to rotate for 1 hr at 4°C. Complexes were precipitated with 40 μl of protein A–Sepharose (1:1), washed with modified RIPA buffer, and resolved by SDS–PAGE.

For immunoprecipitation from metabolically labeled cells, a 150 mm plate of subconfluent NIH 3T3 cells was incubated overnight in Met-deficient DMEM (GIBCO/BRL) supplemented with [^{35}S]Met (~100 μCi/ml), 5% dialyzed fetal calf serum, 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cells were lysed for 15 min on ice in lysis buffer (250 mM NaCl, 50 mM HEPES-KOH, [pH 7.5], 5 mM EDTA, 0.1 mM sodium orthovanadate, 50 mM NaF, 0.1% Triton-X, 100 μg/ml PMSF, 2 μg/ml pepstatin, and 10 μg/ml each aprotonin and leupeptin), scraped, and spun at 15,000 × g for 10 min. Cleared lysate was precleared as above, followed by addition of antibodies (3 μl of preimmune or immune rabbit serum). Immune complexes were harvested with protein A–Sepharose, and beads were washed with lysis buffer before resuspension in denaturing sample buffer. Following SDS–PAGE, the gel was enhanced with enlightening solution (DuPont) and dried for autoradiography.

Transfections and Immunofluorescence

BHK cells were plated the night before transfection at a density of 10^4 cells per 18 mm round coverslip (VWR Scientific). $CaPO_4$ precipitates were made, and cells were reacted with antibodies for immunofluorescence as described by Heald et al. (1993). In most cases, cells were analyzed 18–20 hr after transfection. The 9E10 anti-Myc antibody (Evan et al., 1985) was obtained as an ascites from the Harvard Cell Culture facility and used at 20 μg/ml. RIP antiserum was used at a dilution of 1:200. FITC anti-mouse and Texas red anti-rabbit secondary antibodies were obtained from Jackson Immunoresearch. Cells were examined with a Zeiss Axiophot fluorescent microscope.

β-Gal activity in cells was visualized by fixing cells with 0.5% glutaraldehyde for 15 min followed by extensive washing in PBS with 5 mM $MgCl_2$. Cells were stained in PBS containing 20 mM each $K_3Fe(CN)_6$ and $K_4Fe(CN)_6 \cdot 3H_2O$, 1 mM $MgCl_2$, and 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) until a suitable color developed, usually for 2–3 hr. To enumerate the fraction of blue cells that had undergone apoptotic changes, cells were transfected with one of the four plasmid combinations described and fixed 16 hr after transfection. Blue cells were included for analysis only if their morphological status could be scored unambiguously.

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