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# RIP mediates tumor necrosis factor receptor 1 activation of NF-κB but not Fas/APO-1-initiated apoptosis

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**The CD95 (Fas/APO-1) and tumor necrosis factor (TNF) receptor pathways share many similarities, including a common reliance on proteins containing 'death domains' for elements of the membrane-proximal signal relay. We have created mutant cell lines that are unable to activate NF-κB in response to TNF. One of the mutant lines lacks RIP, a 74 kDa Ser/Thr kinase originally identified by its ability to associate with Fas/APO-1 and induce cell death. Reconstitution of the line with RIP restores responsiveness to TNF. The RIP-deficient cell line is susceptible to apoptosis initiated by anti-CD95 antibodies. An analysis of cells reconstituted with mutant forms of RIP reveals similarities between the action of RIP and FADD/MORT-1, a Fas-associated death domain protein.**

*Keywords*: apoptosis/receptors/signal transduction/somatic cell mutant

## Introduction

Members of the tumor necrosis factor (TNF) receptor (TNFR) family (Smith *et al.*, 1994) play important roles in cell activation, differentiation and apoptosis. An analysis of the intracellular signal transduction pathways leading from four members of this family, TNFR1, TNFR2, CD40 and Fas/Apo-1/CD95, has revealed structural and functional similarities which suggest the existence of common mechanisms for the transmission of the ligand binding event to the cell interior. Receptor occupancy can lead to similar outcomes among this group: TNFR1 and CD95 are capable of inducing apoptosis (Tartaglia *et al.*, 1993; Clement and Stamenkovic, 1994; Zheng *et al.*, 1995), CD40 and TNF receptors mediate the activation of NF-κB (Berberich *et al.*, 1994; Cheng *et al.*, 1995; Rothe *et al.*, 1995b), and CD40 ligation facilitates Fas/APO-1-mediated apoptosis (Schattner *et al.*, 1995). Although the physiological context and structural similarities between the intracellular proteins identified to date suggest they primarily subserve parallel pathways with related but distinct endpoints, the pathways may share common members. In addition, proteins proposed to be involved in one pathway, based on the strength of interaction affinities inferred from yeast two-hybrid approaches or *in vitro* binding, may play a more fundamental role in another pathway.

The intracellular mediators identified thus far include the death domain-bearing molecules TRADD (Hsu *et al.*, 1995), MORT-1/FADD (Boldin *et al.*, 1995b; Chinnaiyan *et al.*, 1995) and RIP (Stanger *et al.*, 1995), the ring finger domain molecules TRAF1 (Rothe *et al.*, 1994), TRAF2 (Rothe *et al.*, 1994) and TRAF3/CD40bp/CRAF1/CAP1/LAP1 (Hu *et al.*, 1994; Cheng *et al.*, 1995; Mosialos *et al.*, 1995; Sato *et al.*, 1995), TANK (Cheng and Baltimore, 1996) and the IAP-related molecules CIAP1 and CIAP2 (Rothe *et al.*, 1995a). The death domain itself is a protein–protein interaction element that permits homotypic as well as heterotypic interactions (Boldin *et al.*, 1995a,b; Stanger *et al.*, 1995; Varfolomeev *et al.*, 1996). Coordination of Fas/APO-1 is known to lead to the formation of a complex at the cell membrane consisting of Fas/APO-1, FADD/MORT-1 and two other species (Kischkel *et al.*, 1995). Events at the TNFR interface are less well delineated. Overexpression studies suggest that TRADD recruits FADD, TRAF2 and RIP to TNFR1 (Chinnaiyan *et al.*, 1996; Hsu *et al.*, 1996a,b), and that members of this receptor complex can mimic TNF-induced biological activities. FAS/APO-1 and TNFR1 interact with death domain-containing proteins, and CD40 and TNFR2 interact with TRAF family proteins (Rothe *et al.*, 1994; Cheng *et al.*, 1995; Sato *et al.*, 1995). TRAF family members, in turn, associate with CIAP1 and CIAP2 in the TNFR2 complex (Rothe *et al.*, 1995a), which may act to antagonize the death pathway. Another molecule, TANK, has been shown to interact with TRAF family members, and elicits NF-κB activation when cotransfected with TRAF2 (Cheng and Baltimore, 1996).

The complexity of the present picture clearly illustrates the need for approaches that directly provide physiological information. One such approach is the analysis of mice with targeted deficiencies in the individual molecules; another is the study of somatic cell lines with lesions in the relevant signaling pathways. We report here that a mutant cell line selected for its inability to activate NF-κB does not express RIP, and that reconstitution with a RIP expression plasmid restores TNF signaling. Although RIP was initially identified based on its ability to physically associate with FAS/APO-1 in yeast, and overexpression of RIP induces apoptosis, the RIP-deficient Jurkat mutant remains susceptible to Fas/APO-1-mediated apoptosis.

## Results

Mutant cell lines deficient in the ability to respond to TNF-α (Figure 1) were created by mutagenesis and selection of a parental line, a Jurkat T cell derivative generated by transfection with a synthetic NF-κB multimer promoter directing the expression of the cell surface antigen CD14. CD14 expression in the parental line can be induced by treatment with TNF, anti-TNFR1 antibody or phorbol ester plus ionomycin (Figure 1). Mutant cell

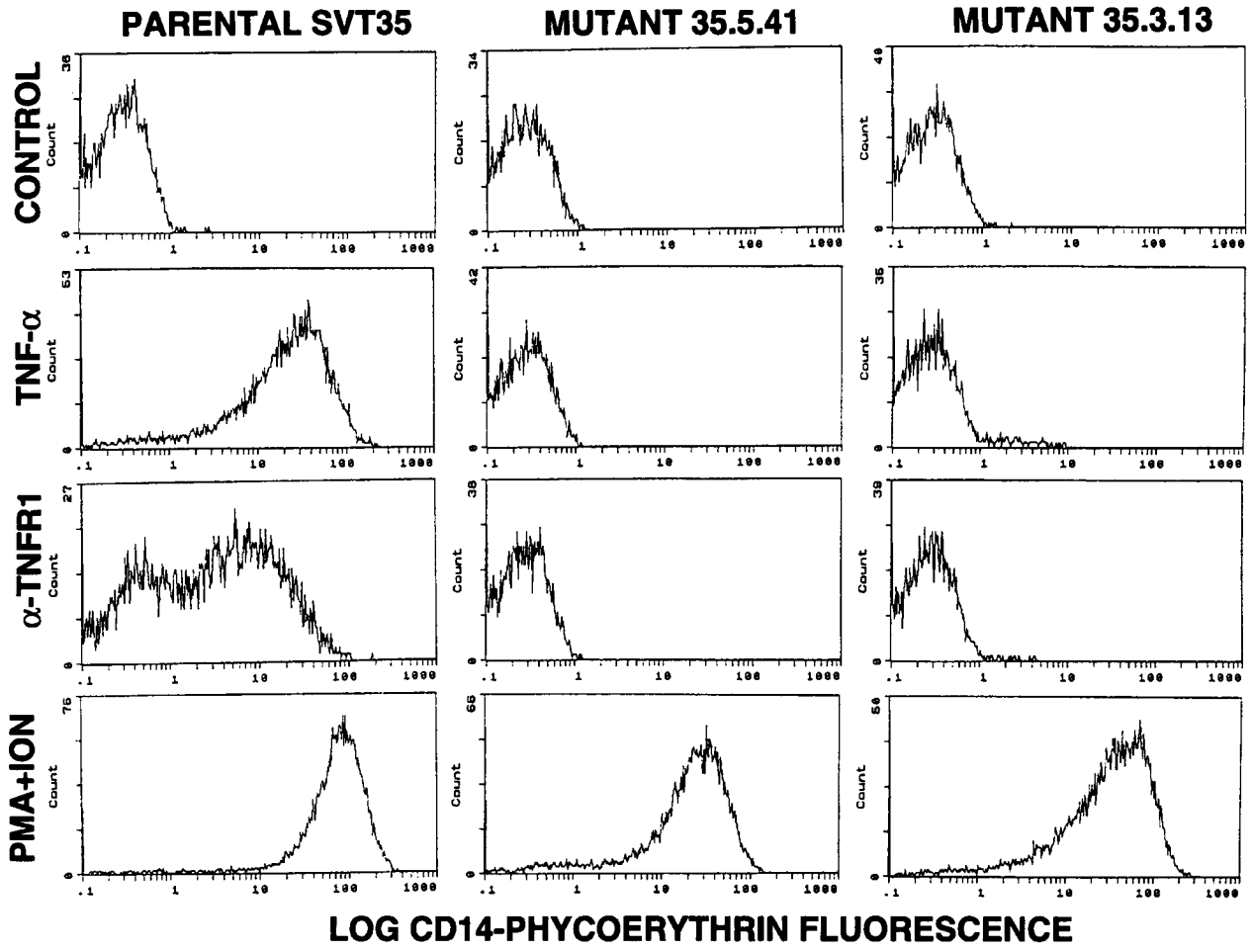


**Fig. 1.** The Jurkat T cell mutants 35.5.41 and 35.3.13 possess proximal TNF-α signaling defects. The parental Jurkat SVT35 clone was generated by transfecting the Jurkat J77 subline with an NF-κB–CD14 reporter construct. TNF-α signaling mutants were obtained after chemical mutagenesis of SVT35 cells with ICR191, followed by successive rounds of flow cytometric sorting for TNF-non-responsive cells as described in Materials and methods. Shown are cytometry profiles for anti-CD14 reactivity of parental Jurkat clone SVT35 (left column), mutant clone 35.5.41 (middle column) and mutant clone 35.3.13 (right column) which were either unstimulated (control) or stimulated for 8 h with 10 ng/ml TNF-α (TNF-α), with 10 μg/ml agonist anti-TNFR1 (α-TNFR1) or with a combination of 10 ng/ml PMA and 1 μM ionomycin (PMA+ION).

lines deficient in the TNF-mediated induction of CD14 were selected following multiple rounds of ICR191 mutagenesis and selection by cell sorting. Among the resulting clones were several that responded to phorbol ester/ionomycin but not to TNF (Figure 1 and data not shown). A screen of TNF-non-responsive cell lines with a monoclonal antibody raised against the C-terminal half of RIP revealed that one of the mutants expressed undetectable levels of RIP protein (Figure 2). The RIP-deficient line was fully responsive to phorbol ester and ionomycin (Figure 1), but did not respond to TNF or anti-TNFR1 antibodies. No response to anti-TNFR2 antibodies was detected in parental or mutant cells, and immunoblot analyses showed that TNFR1 but not TNFR2 was present in the parental and mutant cell lines (Figure 2). The parental Jurkat line does not undergo apoptosis in response to TNF (data not shown).

To explore the role of RIP in cell death pathways, we examined the rate and dose dependence of cell death induced by anti-CD95 antibodies in the parental and RIP-deficient cell lines. A slight diminution in the susceptibility to anti-CD95 was observed (Figure 3a) when apoptosis was scored by the accumulation of annexin-reactive, propidium-impermeant cells (Martin *et al.*, 1995; Vermes *et al.*, 1995), but a nucleosomal ladder was found to form with equal rapidity in both parental and mutant cell lines (Figure 3b). Thus no dramatic change in susceptibility to anti-CD95 can be detected in the RIP-deficient mutant.

To establish whether the elimination of RIP was responsible for the cellular phenotype, we cointroduced a murine RIP expression plasmid and an NF-κB–luciferase reporter construct into the mutant cells and measured luciferase activity after treatment with TNF, anti-TNFR1 antibody or phorbol ester plus ionomycin. Because the cells retain their ability to respond to phorbol ester plus ionomycin, the response to TNF pathway induction can be internally normalized as the fraction of luciferase activity observed relative to the activity induced by phorbol ester plus ionomycin. The reintroduction of RIP into the mutant cell line reconstituted the response to TNF and anti-TNFR1, but had little effect on the response to phorbol ester/ionomycin (Figure 4 and data not shown).

To determine which elements of RIP are required to reconstitute the TNF signaling defect, we made the mutated

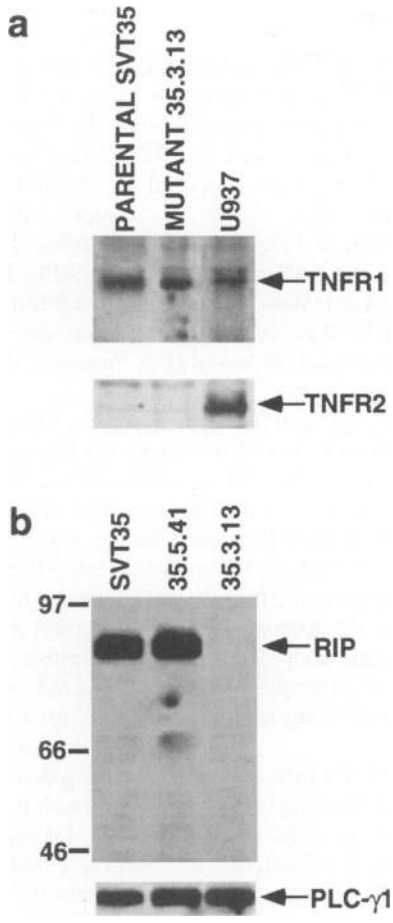


**Fig. 2.** (**a**) Immunoblots of parental SVT35 and mutant 35.3.13 Jurkat T cell lines indicate that these cell lines express the 55 kDa TNFR1 but not the 75 kDa TNFR2. The U937 cell line has been shown previously to express both TNF-α receptors (Kohno *et al.*, 1990; Loetscher *et al.*, 1990) and was included as a positive control. (**b**) Immunoblotting with a human RIP monoclonal antibody demonstrates that the Jurkat TNF-α signaling mutant, 35.3.13, does not express detectable levels of RIP. The membrane was reprobed with a PLC-γ1 antiserum to ensure equivalent loading of the lanes.

and deleted versions of murine RIP shown schematically in Figure 5a. Transient transfection of COS cells showed that deletion of the intermediate domain considerably improves RIP expression, as detected in anti-*myc* tag blots of total cell lysates (Figure 5b). To verify that the RIP kinase activity was compromised by the two mutations (K45R and D138N), we performed an *in vitro* kinase assay with tagged RIP kinase domain expressed in COS cells (Figure 5c). A dimerized version of the kinase domain produced an apparent doubling of the molecular weight of the phosphorylated band, demonstrating that the RIP substrate is indeed itself, and not some associated band of similar molecular weight. A phosphoamino acid analysis of the autophosphorylated kinase domain of RIP showed approximately equimolar levels of serine and threonine phosphorylation (Figure 5d). Thus the ATP binding domain of RIP supports phosphate transfer, even though it does not retain all of the residues commonly associated with protein kinases.

Transfection of the deficient clone showed that recon-

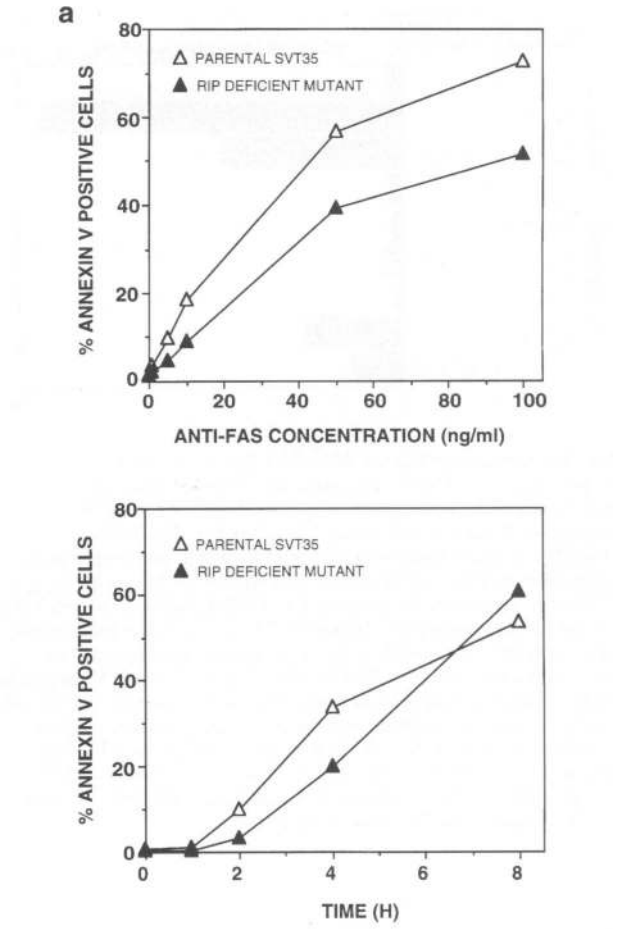


**Fig. 3.** The Fas/APO-1 apoptotic pathway is unaffected in the RIP-deficient clone. (**a**) Dose–response and time course of phosphatidylserine redistribution induced by agonistic anti-CD95 antibody in parental and RIP-deficient cells. Parental and RIP-deficient mutant cells were incubated with the indicated concentrations of agonistic anti-CD95 antibody for 4 h or with 50 ng/ml of the same antibody for the indicated times. Cells were stained with annexin V–FITC and propidium iodide, and analyzed by flow cytometry. The *y*-axis shows the percentage of living cells that stained negative with propidium iodide but were positive for annexin V, as a measure of cells undergoing apoptosis. (**b**) Time course of DNA fragmentation induced by anti-CD95 antibody in parental and RIP-deficient clone cells. $2\times10^6$ cells were incubated with 100 ng/ml of an agonistic anti-CD95 antibody for the times shown. Cells were then harvested and low molecular weight DNA extracted as described in Materials and methods.

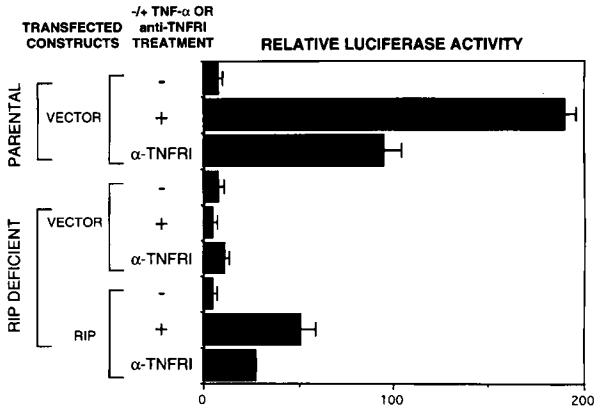


**Fig. 4.** Expression of murine RIP in the deficient clone restores responsiveness to TNF-α. The indicated cell types were transfected with a mixture of DNAs containing a 3:1 ratio of vector/RIP expression plasmid to NF-κB luciferase reporter, SV40κB–luc. Immediately after transfection, the cells were split into four aliquots. One was untreated, and the others were treated for 18 h with 20 ng/ml TNF-α or 5 μg/ml of an agonistic anti-TNFR1 antibody, or with PMA (10 ng/ml) and ionomycin (1 μM) for 7 h. At 24 h after transfection, cells were harvested and their luciferase activities measured. The values shown are ratios of TNF-α-induced activity to PMA/ionomycin activity, obtained in at least two independent experiments. The plasmid used also expressed synGFP S65T under cytomegalovirus promoter control. A portion of the cells was removed and analyzed by flow cytometry to establish transfection efficiency. Transfection of RIP significantly diminished the apparent transfection efficiency, probably as a consequence of RIP-induced apoptosis.

stitution of the NF-κB activation pathway was unaffected by the mutation or deletion of the kinase domain (Figure 6), and that neither the death domain nor the domain intermediate between the death and kinase domains showed an autonomous capacity to reconstitute signaling (Figure 6). The forms of RIP that reconstitute the parental phenotype contain an intact intermediate domain, which decreases the level of RIP in transfected cells (Figure 5b); thus it seems likely that high levels of RIP are not required for the normal conduction of signals from the receptor.

Within the RIP intermediate domain lies a charge-rich element consisting of multiple negatively charged residues followed by multiple positively charged residues. Deletion of this element caused the complete loss of TNF signaling activity in the reconstituted mutant cell line (Figure 7), but did not result in a significant diminution of spontaneously induced NF-κB activity in 293 cells (Figure 8). Interestingly, the death domain of RIP, by itself unable to recover the parental phenotype in the RIP-deficient clone (Figure 6), induced a significant NF-κB activation when overexpressed in 293 cells (Figure 8). Thus the pathway(s) induced by overexpression of RIP in a non-lymphoid cell line differs from those reconstituted by RIP in a RIP-deficient mutant cell line. Surprisingly, deletion of the death domain results in the constitutive expression of the NF-κB reporter in reconstituted mutant cells (Figure 6), and constitutive expression is diminished in this setting by the addition of TNF (Figure 6). These data suggest that the RIP death domain may function as a negative regulatory element in its natural setting.

## Discussion

Through analysis of a mutant cell line defective in TNF-dependent NF-κB activation we have found that RIP, a protein serine/threonine kinase originally identified on the basis of its association with Fas/APO-1 in yeast, is required for the TNF response triggered by TNFR1. The RIP-deficient cell line shows no major impairment in responsiveness to Fas/APO-1 crosslinking. This is consistent with our inability to detect Fas/APO-1-RIP interactions in mammalian systems or to identify truncated forms of RIP that negatively regulate the Fas/APO-1 pathway when overexpressed (F.X.Pimentel-Muiños, data not shown).

Using the RIP-deficient cell line, we have found that the kinase domain is not necessary for the reconstitution of responsiveness to TNF-α. However the findings that the kinase domain is catalytically active (Figure 5d; Hsu *et al.*, 1996b) and that kinase deletions and point mutants have blunted activity in the apoptosis assay (Stanger *et al.*, 1995; F.X.Pimentel-Muiños, unpublished observations) suggest that the kinase domain may exert an agonistic effect on some cell death pathways, either directly or indirectly. At minimum we have uncovered no indication to date that the kinase domain acts to retard cell death signaling.

Through reconstitution studies we have determined that an essential domain for NF-κB activation is located between amino acids 391 and 422. Deletion of this element, which is enriched for positively and negatively charged amino acids, abrogates the ability of RIP to reconstitute the activity of the RIP-deficient clone. In other studies a version of RIP containing a larger deletion encompassing this element has been reported to be unable to induce NF-κB activity when overexpressed, and the intermediate domain of RIP is able to interact with TRAF2 (Hsu *et al.*, 1996b). If amino acids between residues 391 and 422 of RIP were able to bind TRAF2, the principal role of this region could be to recruit TRAF2 to the TNFR1 pathway. The larger domain encompassing the charge-enriched element is unable to restore a parental phenotype to the RIP deficient clone, or to provide constitutive NF-κB activation. However, when fused to the kinase domain (which is, in turn, devoid of activity when expressed in isolation), the two components give rise to constitutive activation. These findings suggest that the two domains normally function in concert and at least one action of the death domain is inhibitory.

In some respects the latter inference is reminiscent of findings on the action of FADD/MORT-1 which, like RIP, bears a C-terminal death domain and associates with CD95 in yeast. The death domain of FADD/MORT-1 acts as a dominant-negative regulatory element when expressed in isolation, and the N-terminal effector domain induces cell death when overexpressed in susceptible cells. However, when overexpressed in the cell lines used here (F.X.Pimentel-Muiños, data not shown), as well as in other cells (Stanger *et al.*, 1995), the RIP death domain is itself capable of inducing cell death. Because the death-promoting activity of both full-length RIP and the RIP death domain can be blocked by overexpression of the FADD/MORT-1 C-terminal death domain (F.X.Pimentel-Muiños, data not shown), it is likely that RIP acts

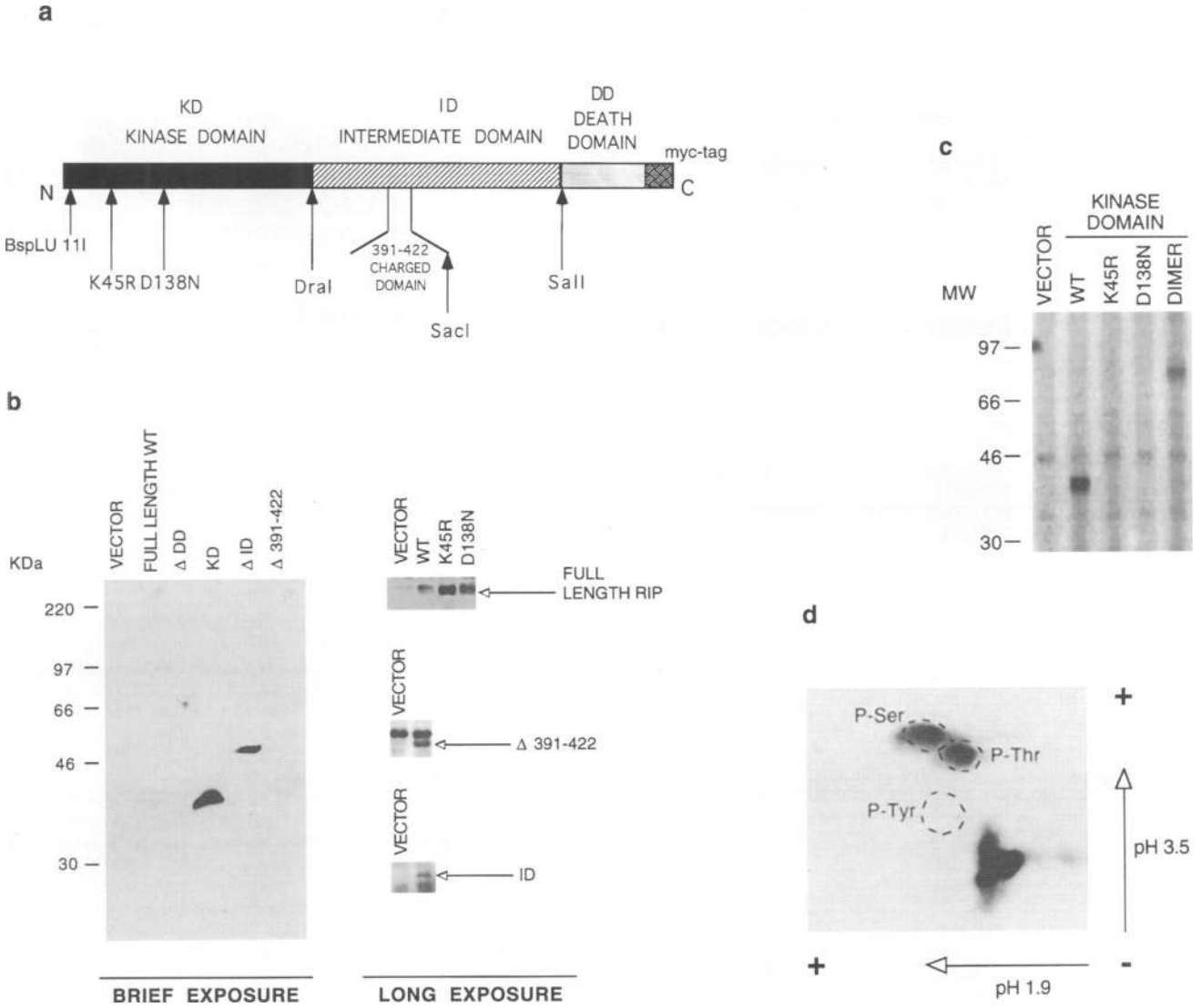


**Fig. 5.** (**a**) Schematic representation of murine RIP showing the position of the restriction sites used in the construction of the deletions used in Figures 6–8. Lys45 or Asp138 was replaced by Arg or Asn, respectively, to abrogate the kinase activity of RIP. (**b**) Anti-*myc* Western blot assay of total lysates from COS cells transfected with the indicated versions of RIP. Some of the forms were detected after short exposures of the blots (5 s, left panel), whereas others required longer exposures (~15 min, right panel). (**c**) *In vitro* kinase assay of immunoprecipitated RIP kinase domain expressed in COS cells, showing that the mutations K45R and D138N block its kinase activity. A dimer version of the kinase domain of RIP was used to show that RIP itself is a substrate, not a coprecipitating protein. (**d**) Phosphoamino acid analysis of autophosphorylated RIP.

through FADD/MORT-1, perhaps by titrating it away from naturally occurring intracellular inhibitors.

The observation that TNF acts antagonistically on cells constitutively activated by the expression of truncated RIP lacking the death domain is explicable when one considers that the TNF response pathway appears to require the assembly of a multipeptide complex. Assembly of a fraction of that complex in the presence of death domain-deleted RIP may result in the consumption of RIP which might otherwise be free to exert an effect on downstream elements. Alternatively, it may result in association with the membrane complex of a significant fraction of downstream targets for RIP, thereby removing them from the action of the untethered RIP.

The ability of RIP to interact with elements of both the cell death and NF-κB induction pathways exemplifies an important feature of signal transduction within the TNFR, CD40 and Fas/APO-1 receptors: that there is substantial crosstalk between their signal relay mechanisms and that, as a result, the precise role played by one or more participants may not be obvious until the appropriate genetic tools are employed.

## Materials and methods

### *Generation of mutant cell lines*

The Jurkat subline J77 was transfected with linearized NF-κB reporter construct, SV40κB14, and selected with 5 μg/ml mycophenolic acid in the presence of 250 μg/ml xanthine and 100 μM hypoxanthine. The TNF-responsive clone, SVT35, was obtained after sorting stably transfected cells for TNF-induced CD14 expression and single cell cloning by limiting dilution. Mutagenesis with the alkylating agent ICR191 (Sigma, St Louis, MO) was performed essentially as described previously (McKendry *et al.*, 1991). SVT35 cells were treated with 3 μg/ml ICR191 in complete culture media for 5 h, conditions that routinely kill >90%

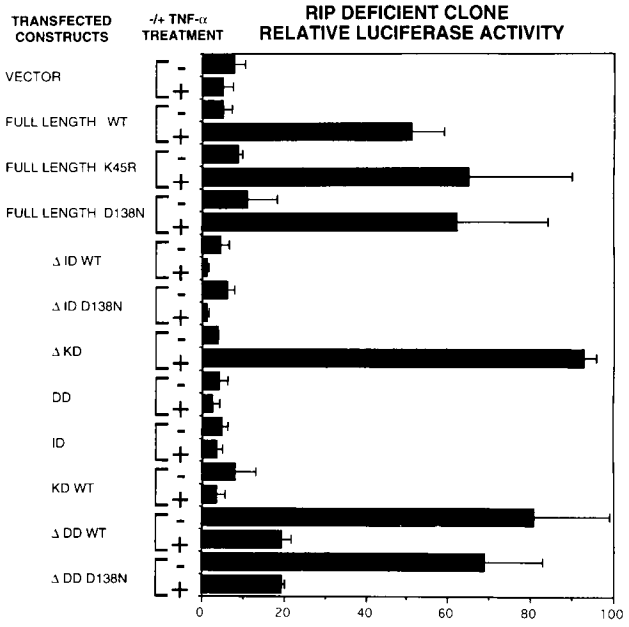


**Fig. 6.** Mutagenic and deletional analysis of RIP. The indicated versions of RIP were transfected into the RIP-deficient clone and their abilities to restore the TNF-α-dependent NF-κB activation phenotype were analyzed. Procedures are described in the legend to Figure 4, and the different deletions used are shown schematically in Figure 5a.

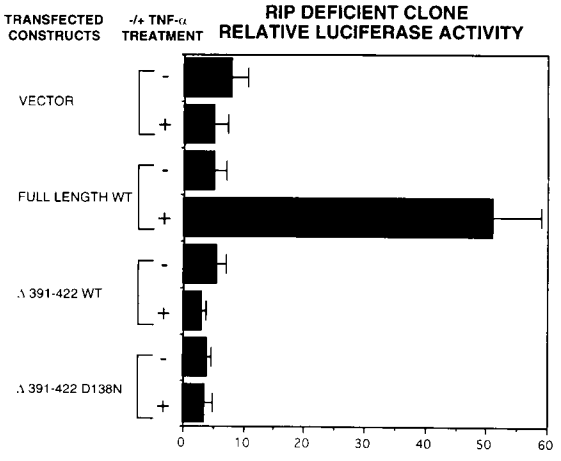


**Fig. 7.** A charge-rich domain between amino acids 391 and 422 of RIP is essential for NF-κB activation by TNF-α. The indicated versions of RIP were transfected into the deficient clone and their abilities to restore the TNF-α-dependent NF-κB activation phenotype were analyzed. Procedures are described in the legend to Figure 4, and the deletion used is shown schematically in Figure 5a.

of the cells. The remaining cells were allowed to recover before two additional rounds of mutagenesis were carried out. Mutagenized cells deficient in TNF-α signaling were enriched by six successive rounds of flow cytometric sorting for CD14-negative cells after 12 h of stimulation with 10 ng/ml TNF-α (Sigma). The enriched population was plated onto microtiter plates by limiting dilution, and the resulting clones were screened by stimulation with either 10 ng/ml TNF-α or 10 ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma) plus 1 μM ionomycin (Calbiochem, San Diego, CA). Surface expression of CD14 was detected by staining with a phycoerythrin (PE)-conjugated CD14 monoclonal antibody (Sigma), followed by analyses on a Coulter Epics XL flow cytometer. Screening for cells that were TNF non-responsive but PMA/ionomycin responsive allowed mutants with lesions in the earlier steps

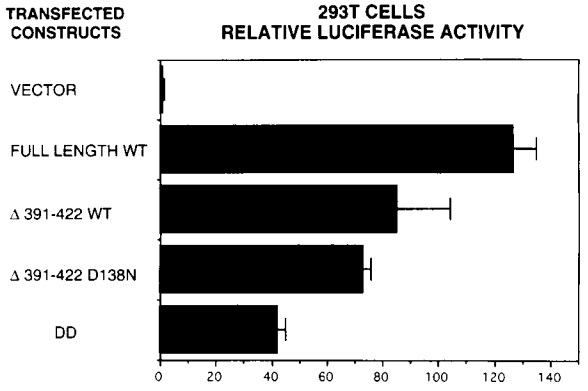


**Fig. 8.** Different domains of RIP are required for NF-κB induction by overexpression in 293T cells. Cells were transfected with a mixture of 5 μg each of SV40κB–luc and the indicated plasmids. At 24 h after transfection, cells were harvested and their luciferase activities measured. Shown are relative inductions of luciferase activity with respect to the control (vector only), obtained in at least two independent experiments.

of the pathway for NF-κB activation to be detected. The flow cytometry profiles shown in Figure 1 were obtained from 10 000 cells of the individual cell lines that were either unstimulated (control) or stimulated for 8 h with 10 ng/ml TNF-α, 10 μg/ml anti-human TNFR1 goat antisera (R & D Systems, Minneapolis, MN) or 10 ng/ml PMA plus 1 μM ionomycin.

### *Immunoblotting*

*TNFR and anti-myc immunoblotting.* Equivalent amounts of detergent-soluble proteins from the indicated cell lines were resolved by SDS–PAGE, electrophoretically transferred onto Immobilon membrane and probed with goat antisera against human TNFR1 or TNFR2 (R & D Systems) or purified anti-*myc* monoclonal antibody (clone 9E10; Oncogene Science, Uniondale, NY). Immunoreactive proteins were detected with peroxidase-conjugated rabbit anti-goat or anti-mouse IgG (Dako, Carpinteria, CA) and chemiluminescence (DuPont NEN, Boston, MA).

*RIP immunoblotting.* Triton X-100-soluble lysates from $7.5\times10^5$ of each of the indicated cell lines were resolved by 8.5% SDS–PAGE and subsequently electrophoretically transferred onto Immobilon membrane. Immunoreactive proteins were detected with a mouse monoclonal antibody directed against a GST–human RIP (residues 412–671) fusion protein (Pharmingen, San Diego, CA) and a secondary peroxidase-conjugated goat anti-mouse antibody (Dako), followed by visualization with chemiluminescence. To demonstrate equivalent loading of the lanes, the membrane was stripped with 7 M guanidium–HCl and reprobed with a phospholipase C (PLC)-γ1 antiserum (Ting *et al.*, 1992).

### *Apoptosis assays*

Parental and RIP-deficient mutant cells were incubated with an agonistic anti-Fas/APO-1 antibody (IgM clone CH-11; UBI, Lake Placid, NY). Cells were stained with 1 μg/ml annexin V-FITC (Endogen, Cambridge, MA) and 5 μg/ml propidium iodide, and analyzed on the flow cytometer as described previously (Martin *et al.*, 1995; Vermes *et al.*, 1995). The DNA fragmentation assay was performed essentially as described by Herrmann *et al.* (1994). Briefly, cells were lysed in a buffer containing 1% NP-40, 20 mM EDTA and 50 mM Tris–HCl, pH 7.5. After a 5′ spin to remove large debris and high molecular weight DNA, supernatant was digested with RNaseA and proteinase K. Low molecular weight DNA was then precipitated and resolved on a 1.2% agarose gel.

### *Reporter constructs and mRIP expression plasmids*

The NF-κB reporter SV40κB14 was constructed by placing eight NF-κB sites with a synthetic basal TATA element 5′ of the coding sequence for the surface antigen, CD14. The NF-κB binding sites consist of four concatemers of the synthetic oligonucleotide 5′-CTAG**TGGGGACTTT-CCACCTGGGGACTTTCCAC**CT-3′, each of which contains two NF-κB binding sites derived from the SV40 virus. In addition, SV40κB14 contains the guanine phosphoribosyl transferase (gpt) gene allowing for

selection with mycophenolic acid in the presence of xanthine and hypoxanthine. The SV40κB–luc reporter was constructed in an analogous manner to SV40κB14 with the firefly luciferase coding sequence replacing that of CD14. However, SV40κB–luc does not possess any drug selection marker. The different forms of murine RIP were *myc*-tagged at the C-terminus and expressed under the control of the spleen focus forming virus LTR in Jurkat cells or the cytomegalovirus promoter in COS and 293T cells. K45R and D138N mutations were introduced by site-directed mutagenesis according to Ausubel *et al.* (1989). All the deletions were created with adapters between the unique sites *Bsp*LU11I (N-terminal end of the kinase domain), *Dra*I (C-terminal end of the kinase domain), *Sal*I (C-terminal end of the intermediate domain) and *Not*I (C-terminal insertion site), except the Δ391–422 and the dimer kinase domain constructs, where a PCR amplification step was introduced. All of the constructs have the same translational initiation consensus and the same five N-terminal residues. The dimer kinase domain version contains one wild-type and one K45R mutated kinase domain monomer. All constructs were checked for expression in COS cells by either anti-*myc* Western blotting of the whole-cell extracts or introducing an anti-*myc* immunoprecipitation step prior to the Western blot.

### *Cell transfections and luciferase assays*

Jurkat cells were electroporated at 310 V and 960 μF in a 4 mm gap cuvette using 15 μg of a mixture of DNAs containing a 3:1 ratio of RIP plasmid versus the SV40κB–luc reporter. COS cells were transfected with 5 μg DNA for 4 h, using the DEAE–dextran/chloroquine method, and harvested 48 h after transfection. 293T cells were transfected using the calcium phosphate precipitation method, as described previously (Ausubel *et al.*, 1989). Luciferase assays were performed using the assay system purchased from Promega (Madison, WI) as per the manufacturer's instructions. Luciferase activity was quantitated with a Monolight 2010 luminometer.

### *Kinase assay and phosphoamino acid analysis*

Transfected COS cells were lysed in a buffer containing 1% Triton X-100, 0.15 M NaCl, 0.02 M HEPES, pH 7.3, 5 mM EDTA, 5 mM NaF, 0.2 mM $NaVO_3$ (ortho), 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride, and immunoprecipitated for 3 h using the monoclonal anti-*myc* antibody 9E10 (Oncogene Science). Immunoprecipitates were washed three times with lysis buffer and twice with 20 mM HEPES, pH 7.3. The kinase assay was performed in 10 μl kinase buffer containing 10 μCi [$\gamma$-$^{32}$P]ATP, 10 μM cold ATP, 20 mM HEPES, pH 7.3, 10 mM $MnCl_2$ and 10 mM $MgCl_2$ for 20 min at room temperature, and resolved in a 12% protein gel. For phosphoamino acid analysis, the kinase gels were transferred to polyvinylidine difluoride membranes (Millipore, Bedford, MA) and the autophosphorylated bands excised. The membrane was subjected to acid hydrolysis in 6 N HCl for 1 h at 100°C. The resulting supernatant was dried out and a 2-D electrophoresis run onto a cellulose thin-layer chromatography plate at two different pHs: 1.9 in the first dimension and 3.5 in the second (Coligan *et al.*, 1991).

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