SHORT REPORT

Murine Siva-1 and Siva-2, alternate splice forms of the mouse Siva gene, both bind to CD27 but differentially transduce apoptosis

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CD27, a member of the TNFR family known to provide essential co-stimulatory signals for T cell growth and B cell Ig synthesis, can also mediate cell death. Using the CD27 cytoplasmic tail as the bait in yeast two hybrid assay, we previously cloned human Siva, a pro-apoptotic molecule. Here we report the characterization of the mouse Siva gene as a 4 kb sequence containing 4 exons and 3 introns. RT–PCR has revealed the presence of two forms of mouse Siva mRNA, the longer full length form Siva-1 and the shorter Siva-2 lacking the sequence coded by exon 2. Immunoblotting with anti-Siva (human) antibodies clearly demonstrate the presence of both Siva-1 and Siva-2. Cotransfection experiments in 293T cells reveal that mouse CD27 receptor can interact with both forms of Siva. Although mouse Siva-1 can trigger apoptosis in Rat-1 cells and in some of the mouse cell lines in transient transfection experiments, similar to the observation made with human Siva, intriguingly its alternate splice form, Siva-2 appears to be much less toxic. It is therefore likely that Siva-2 could regulate the function of Siva-1.

Keywords: Siva; CD27; apoptosis; alternate splice forms; murine Siva gene; TNFR family

The Tumor Necrosis Factor Receptor (TNFR) super family members regulate such diverse biological functions as cell growth, differentiation and cell death. The members of this family include TNFRI and II, Fas (Apo/CD95), CD40, CD30, CD27, OX40, 4-1BB and the recently cloned members DR3, DR4 and GITR (reviewed by Smith et al., 1994; Baker and Reddy, 1996; Chinnaiyan et al., 1996; Kitson et al., 1996; Bodomer et al., 1997; Screaton et al., 1997; Pan et al., 1997; Nocentini et al., 1997). They are all type I transmembrane proteins containing the characteristic cysteine knot motif in their extracellular domains (McDonald and Hendrickson, 1993). The ligands for these receptors belong to the TNF family and are type II transmembrane proteins. The binding of the trimeric ligand to its specific receptor results in receptor oligomerization and a functional response wherein the receptor cytoplasmic tail appears to be critical for signal transduction. Several novel and important intracellular signaling molecules that associate with the cytoplasmic tails of various TNFR family members have been cloned and characterized. They can broadly be divided into three groups, the first comprising molecules containing death domain, the second having the TRAF (TNFR associated factor) domain and the third falling into neither of these categories.

The death domain (about 80 amino acids in length), originally identified in the cytoplasmic tails of Fas receptor and TNFR1 (Itoh and Nagata 1993; Tartaglia et al., 1993; Nagata, 1997), is indispensable for the induction of apoptosis through Fas, TNFR1 and the recently identified DR3 and DR4 (Chinnaiyan et al., 1996; Pan et al., 1997). Several intracellular molecules containing the death domain specifically bind to the receptor death domain. FADD interacts with Fas (Chinnaiyan et al., 1995) and TRADD and RIP associate with TNFR1 (Hsu et al., 1995, 1996a; Stanger et al., 1995). Despite the binding specificity, the death domains demonstrate functional heterogeneity. In the case of TRADD, its death domain is essential for binding to the death domain of TNFR1 and also for the induction of apoptosis (Hsu et al., 1995). The death domain of FADD, however, is only necessary for binding to Fas but not for the induction of apoptosis. It is the death effector domain present at the carboxy terminal region of FADD that associates with a caspase and brings about apoptosis. (Boldin et al., 1996; Muzio et al., 1996). Also, the death domains of TRADD and RIP play an important role in the TNFR1 induced NFkB activation (Hsu et al., 1995, 1996b; Ting et al., 1996; Kelliher et al., 1998). In a recent study, when a FADD mutant lacking the death domain was expressed in FADD knock out mice, the mutated form of FADD supported costimulation leading to T cell growth and development, suggesting that death domain containing molecules may play a dual role in cell death and its growth (Zhang et al., 1998; Walsh et al., 1998).

The TRAF domain is about 230 amino acids and is present in all the six TRAF family members cloned to date. It is involved in the homo- and hetero-dimerization of the individual members and bridges the receptor with which it binds to the downstream targets resulting in the activation of NFκB and JNK. TNFR2, CD40, CD30, CD27 and LTFR are some of the members that signal through various TRAFs (Rothe et al., 1994, 1995; Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995; Song and Donner, 1995; Regnier et al., 1995; Gedrich et al., 1996; Nakano et al., 1996; Lee et al., 1996a,b; Cheng

and Baltimore, 1996; Akiba et al., 1998). The TRAFs also have a ring finger and several Zn finger domains, which also appear to play an important role in the effector functions of TRAFs (Rothe et al., 1995; Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995; Song and Donner, 1995; Regnier et al., 1995; Gedrich et al., 1996; Nakano et al., 1996).

Daxx belongs to the third group and has neither a death nor a TRAF domain. It associates with the death domain of Fas through a unique region in its carboxy terminal portion and a different region is necessary for the induction of apoptosis and activation of JNK (Yang et al., 1997). The Fas/Daxx mediated apoptotic pathway is inhibited by Bcl-2 and thus is distinct from the FADD induced death pathway. In our previous study, we described the cloning of a novel human intracellular pro-apoptotic molecule, Siva, that also can be grouped into this third category that is likely to play an important role in CD27 induced cell death (Prasad et al., 1997).

Our blast search of dbEST data base with human Siva cDNA identified two mouse clones with significant homology to human Siva (I.M.A.G.E. consortium clone 401367 and 579844), and were subsequently obtained from ATCC and sequenced using T3 and T7 primers. The clone 401367 contained the full length mouse Siva sequence as determined by the presence of an inframe stop codon upstream of the potential start codon and a 3' poly A tail after the stop codon. The nucleotides surrounding the start codon are characteristic of the consensus Kozak sequence, suggesting that the first ATG after the inframe stop codon is indeed the translation start site (data not shown). The predicted protein sequence of mouse Siva and its comparison with the reported human Siva is shown in Figure 1a. The identity between the two is 70% and the similarity is 80% and the predicted start site in case of mouse Siva matches with the second methionine in the human (amino acid residue 15), thereby making it slightly shorter than human Siva. As described in our

previous paper (Prasad et al., 1997), Siva has several interesting structural features such as a region that has weak but significant homology to the death domains of FADD and TRADD referred to as the death domain homology region (DDHR) and a cysteine rich carboxy terminal region that appears to resemble a box B like ring finger structure followed by a Zn finger-like domain. The amino terminal region and most of the cysteines appear to be highly conserved between mouse and human forms of Siva (Figure 1a), suggesting that these regions could be structurally and functionally important. Although the identity across the DDHR between mouse and human Siva is only 61%, the similarity is 72%, a continuous stretch of 13 amino acids is identical (residues 96–108 of mouse Siva, Figure 1a). Figure 1b depicts the tissue distribution of mouse Siva mRNA (upper panel) in comparison to the abundance of β-actin (lower panel). Maximum mRNA expression is seen in testis, muscle and heart with brain expressing the least. Although we tested a different panel of tissues for human Siva mRNA expression, the Siva mRNA levels also appeared to be relatively high in human testis (Prasad et al., 1997).

In order to determine murine Siva mRNA expression in some of the mouse cell lines, we isolated RNA from NIH3T3 and the murine pre-B 300-19 cells and subjected them to RT–PCR using the mouse Siva sense and anti-sense primers. In addition to the expected mouse Siva band (550 bp) (Figure 2a, lanes 2 and 3), we clearly observed a distinct lower band (350 bp) in pre-B 300-19 cells (lane 3). In order to further confirm the existence of this related form of Siva, we also carried out similar analysis from mRNA obtained from four mouse embryonic stem cell lines (Figure 2a, lanes 5–8) all of which clearly reveal the presence of two forms of Siva message. We isolated the two bands, cloned them into TA vector and sequenced them. As expected, the upper form corresponded to mouse Siva sequence and the lower form had an inframe deletion of 65 amino acids corresponding to residues 40–104 (Figure 2b), thereby
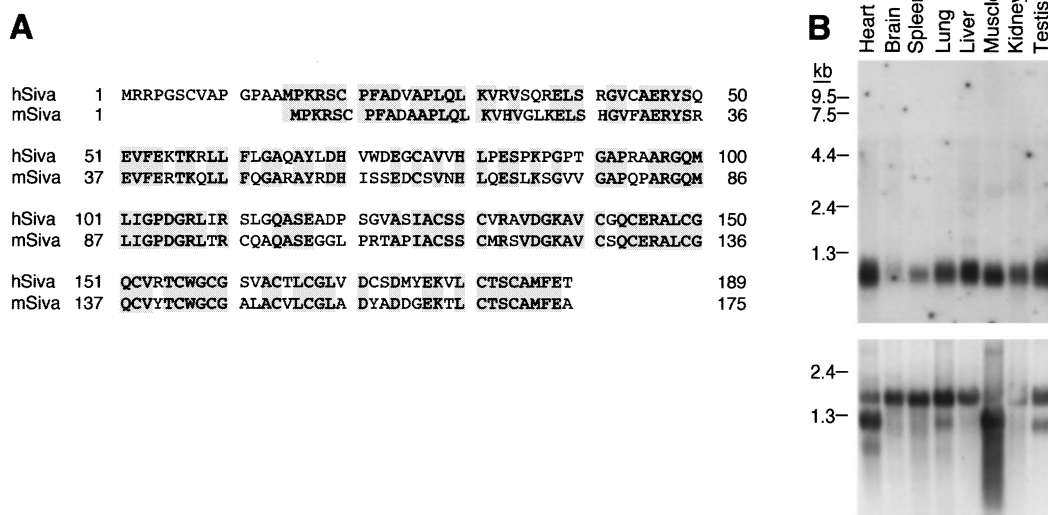

Figure 1 Murine homologue of Siva. (**a**) Comparison of human and mouse predicted amino acid sequence of Siva. The shaded boxed areas depict shared identical regions. The identity between the two is 70% and the similarity is 80%. (**b**) Multiple tissue Northern blot analysis of mouse Siva mRNA is shown in the upper panel and the lower panel represents the expression of β-actin mRNA in the same samples. The Siva transcript is about 600–700 bp. A mouse multiple tissue Northern blot (Clontech, Palo Alto, CA, USA) containing 2 μg of poly(A)$^+$ RNA per lane was probed with mouse Siva-1 insert labeled with [^{32}P]dCTP using T7 Quick Prime Kit (Pharmacia, Piscataway, NJ, USA)

making this a putative alternate splice form of Siva. Accordingly, we named the longer form Siva-1 and the shorter form Siva-2. Part of the amino terminal region and most of the DDHR including the conserved 13 amino acid stretch is missing in Siva-2, suggesting functional differences which we investigated below. The presence of the Siva protein in whole cell lysates was visualized by immunoblotting using affinity purified anti-Siva (human) antiserum raised in rabbits (Figure 2c). An anti-serum raised against bacterially expressed human Siva, clearly recognizes the GFP-human Siva (Figure 2c, lanes 5 and 6), GFP-mouse Siva-1 (Figure 2c, lane 7) but not GFP (data not shown). Two bands corresponding to the predicted molecular weights of the two forms of mouse Siva (18 and 13 kd) were seen in some of the mouse cell lines tested (Figure 2c; L12, lane 2; LICO, lane 3; LBB3.4.16, lane 4) but not in the mouse B cell hybridoma cell line AF3.G7 (Figure 2c, lane 1).

In order to confirm that Siva-1 and -2 are really alternate splice forms and not artifacts of RT–PCR and also to understand the organization of the mouse Siva gene, we cloned the mouse Siva gene by genomic PCR using the 5' sense and 3' anti-sense primers of Siva. We obtained a 4 kb band and the sequence analysis revealed the presence of 4 exons and 3 introns. Siva-1 is coded by all the 4 exons and in Siva-2, exon 2 is completely missing, which encodes a major portion of the DDHR.

The difference in the molecular weight between S1 and S2 can be accounted for by the size of exon 2. Based on the splice junction analysis of the gene sequence, it is clear that Siva-2 is an alternate splice form of the Siva gene. The exon/intron organization of the mouse Siva gene is shown in Figure 2d.

In order to determine whether the two forms of mouse Siva associate with mouse CD27, we co-expressed murine CD27 wild type (CD27WT) and cytoplasmic tail less (CD27ΔCT) receptor forms along with GFP-Siva-1 and -2. The cell surface expression of the receptors in 293T cells was confirmed by flow cytometry procedures. The surface expression of CD27WT was slightly higher than CD27ΔCT (data not shown). In contrast, the percentage of cells transfected with GFP was always higher than GFP-S1 or GFP-S2, however GFP-S1 and GFP-S2 were comparable (data not shown). The cells were lysed and anti-CD27 immunoprecipitates were performed, the proteins were separated by SDS–PAGE and immuno-blotted with anti-GFP rabbit serum. As shown in Figure 3, despite the fact that the level of GFP was very much higher than either GFP-S1 or GFP-S2 fusion proteins (Figure 3, compare lane 1 to 3 and 4), it failed to co-precipitate with either CD27WT or CD27ΔCT receptors (Figure 3, lanes 4 and 7). A distinct band corresponding to GFP-S1 and GFP-S2
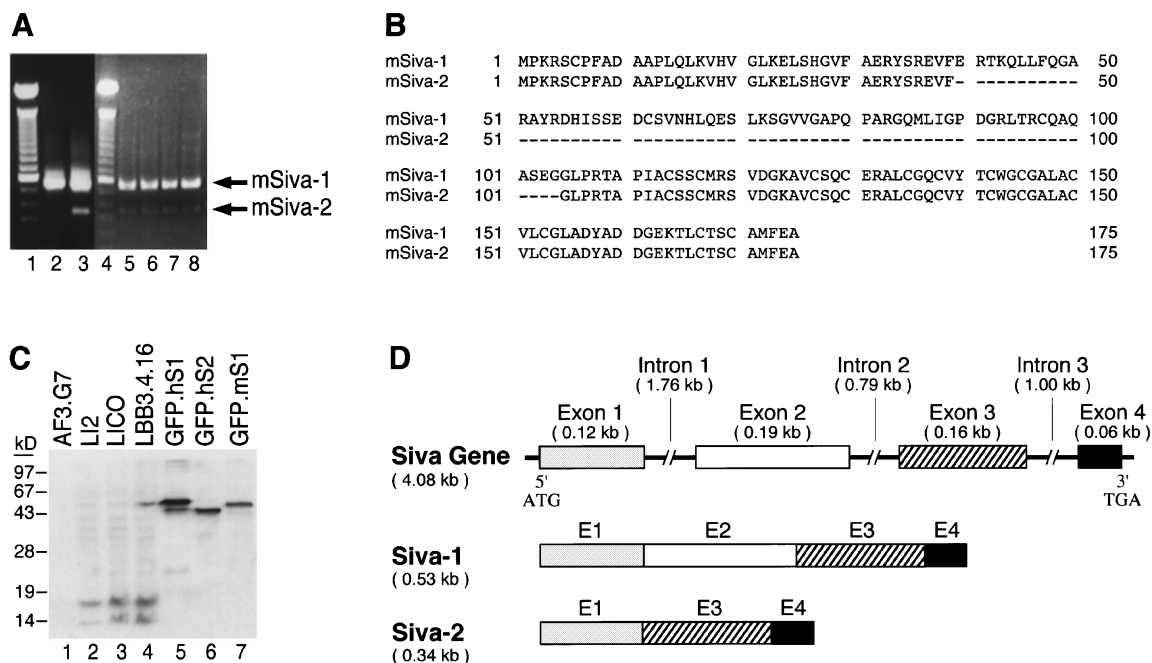

Figure 2 Alternate splice forms of mouse Siva. (**a**) RT–PCR demonstrates the presence of mouse Siva-1 and Siva-2. Lanes 1 and 4 represent 1 kb ladder DNA standards. Two bands (upper 550 bp and lower 350 bp) are seen in pre-B 300-19 (lane 3) and in the four mouse embryonic stem cell line mRNA samples (lanes 5–8 corresponding to Ba11, Bc1, Bh3 and Ba6 respectively, all originating originally from 129sv mice). The primers used for the PCR were Siva 5' sense primer (ACA ACC GAA TTC CAT GCC CAA GCG GAG CTG CCC GTT) and 3' anti-sense primer (AGA CAC GGT ACC TCA GGC TTC AAA CAT AGC ACA G). (**b**) Alignment of the predicted amino acid sequence of mouse Siva-1 and mouse Siva-2. An inframe deletion of 65 amino acids (residues 39–104) is seen in Siva-2. (**c**) Detection of the naturally occurring Siva proteins in various mouse cell lines by immunoblotting with anti-Siva (human) rabbit antiserum. Whole cell lysates were separated on a 13.5% SDS–PAGE, blocked and probed with affinity purified anti-Siva anti-serum and HRP conjugated anti-rabbit antibodies and the protein bands were visualized by ECL. Histidine tagged human Siva protein was used to immunize rabbits and affinity purification was carried out using GST-Siva cross linked to affigel matrix (Biorad). Cell lysates containing GFP-Siva-1 (human) (lane 5), GFP-Siva-2 (human) (lane 6) and GFP-Siva-1 (mouse) (lane 7) served as controls. Protein bands corresponding to mouse Siva-1 (18 kd) and Siva-2 (13 kd) can be seen in cell lysates of L12, LICO, LBB3.4.16 (lanes 2–4). (**d**) Organization of the mouse Siva gene. As shown, Siva gene is about 4.08 kb made up of 4 exons and 3 introns. Full length Siva-1 is coded by all the four exons and Siva-2 lacks the exon 2. Genomic PCR was carried out using the same mouse Siva 5' sense and 3' anti-sense primers used in RT–PCR and genomic DNA derived from NIH3T3 cell line as the template. The 4 kb PCR product which was then cloned into TA vector (Invitrogen) and sequenced

was apparent in the CD27WT immunoprecipitates (Figure 3, lanes 5 and 6) but not in lanes corresponding to CD27ΔCT immunoprecipitates (Figure 3, lanes 8 and 9) clearly suggesting that both S1 and S2 can associate with the cytoplasmic tail of the CD27 receptor.

In order to assess the function of the two forms of Siva, we carried out transient transfection assays in Rat-1 (transformed with *myc*) cells and in several mouse cell lines (AF3.G7, L12 and LICO). After about 40 h of transfection, the cells were harvested, lysed and the DNA was precipitated and separated on a 2% agarose gel. As shown in Figure 4a, transfection with pEGFP-Siva-1 resulted in significant apoptosis in Rat-1 cells, (lane 2) but not when vector alone (lane 1) or

pEGFP-Siva-2 (lane 3) was transfected. Relative level of the expressed proteins are shown in the anti-GFP immunoblot of whole cell lysates (Figure 4b). The expression of GFP protein was higher by several orders of magnitude (lane 1) in comparison with GFP-Siva-2 (lane 3) or GFP-Siva-1 (lane 2). We have extended the functional data to some of the mouse cell lines we routinely use in our lab (Figure 4c). The representative results are – AF3. G7 (lanes 1–4), L12 (lanes 5–7) and LICO (lanes 8–10). Lipofectamine, the vehicle used for transfection (Figure 4c, lane 1) or the GFP vector alone did not result in the generation of a significant DNA fragmentation ladder (Figure 4c, lanes 2, 6 and 8). Expression of GFP-mS1 however did result in significant apoptosis as evidenced by the presence of
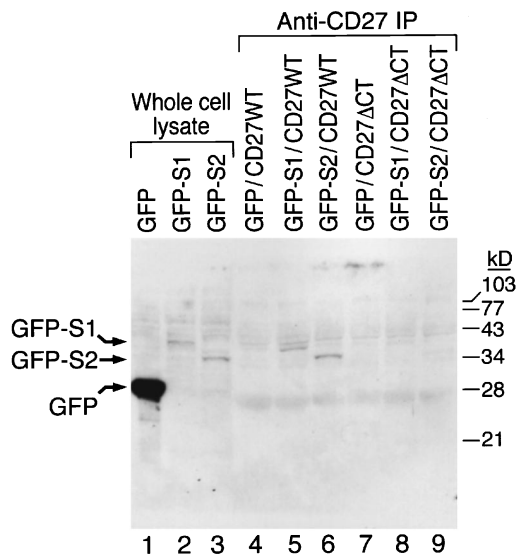

Figure 3 CD27 binds to both Siva-1 and -2 in 293T cells. The mouse CD27WT and CD27ΔCT DNA was transiently co-expressed with either GFP, or GFP-Siva-1 or GFP-Siva-2. CD27WT and mutated receptor as well as Siva-1 and Siva-2 expression were comparable (data not shown). The CD27 immunoprecipitates were separated and immunoblotted with anti-GFP antiserum. Prestained protein standards are shown on the left hand side. Lanes 1–3 represent whole cell lysate samples from CD27WT/GFP, CD27WT/GFP-Siva-1 and CD27WT/GFP-Siva-2 samples and similar results were obtained with CD27ΔCT transfectants (data not shown). GFP does not coprecipitate with CD27WT or CD27ΔCT form of the receptor (lanes 4 and 7). Siva-1 and Siva-2 do coprecipitate with the full length receptor (lanes 5 and 6) but not with the cytoplasmic tail deleted form of the receptor (lanes 8 and 9). Mouse spleen cDNA was subjected to PCR using the murine CD27 sense primer (ACA CAC AAG CTT AGT CAC CAT GGC ATG GCC ACC TCC CTA CTG G) in combination with the 3′ anti-sense primer spanning the 3′ end of CD27 (ACA CAC CTC GAG CGG TCA AGG GTA GAA AGC AGG CTC) or the region of CD27 corresponding to the carboxy terminus of the transmembrane region (ACA CAC CTC GAG TCA TCT TCT TTG ATG GAA GAA CAA GAT TGC) so as to obtain mCD27 full length (CD27WT) and a deletion mutant missing the cytoplasmic tail (CD27ΔCT, lacking residues 266–309) respectively and cloned into the pcDNA vector using the *Hind*III and *Xho*I sites. 293T cells were transiently transfected with pCDNA-mCD27WT or pCDNA-mCD27ΔCT in combination with pEGFPN1 or pEGFP-mS1 or pEGFP-mS2 using the lipofectamine procedure. Cells were harvested and an aliquot was used to assess CD27 receptors by flow cytometry. Remaining cells were lysed in 1% Triton X-100 lysis buffer. CD27 receptor complexes were immunoprecipitated using anti-mouse CD27, anti-hamster mouse monoclonal and anti-mouse IgG beads. The samples were then subjected to SDS–PAGE electrophoresis (12% gel) and immunoblotted with anti-GFP rabbit antiserum (Clontech)
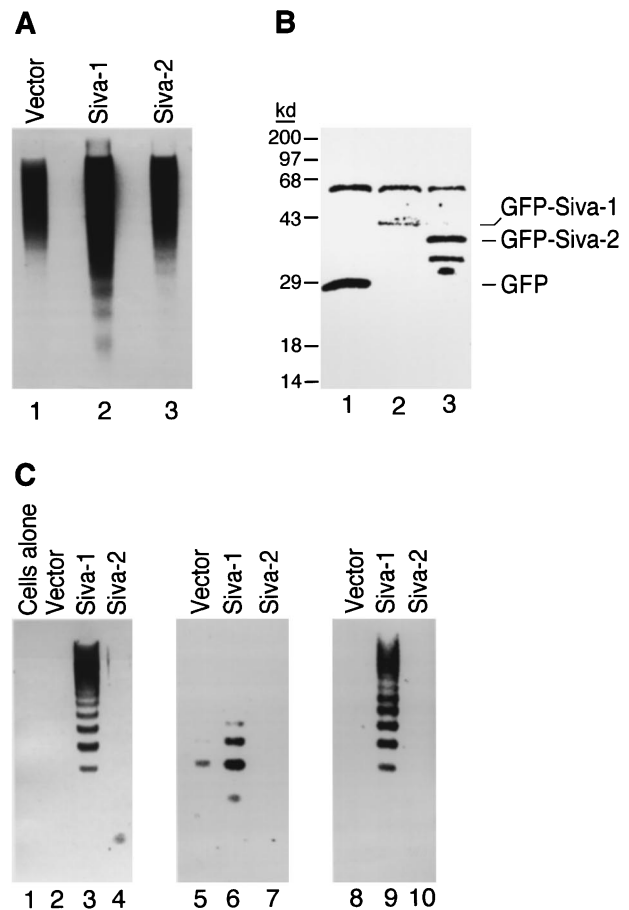

Figure 4 Siva-1 but not Siva-2 induces apoptosis. (**a**) Rat-1 cells were transiently transfected with pEGFPC1 (lane 1), pEGFP-Siva-1 (lane 2) and pEGFP-Siva-2 (lane 3). After 40 h, the cells were lysed and assayed for fragmented DNA. As evidenced by the presence of DNA ladder, Siva-1 (lane 2) but not Siva-2 (lane 3) induces significant apoptosis. Vector alone transfection was comparable to Siva 2 (lane 1 vs lane 3). (**b**) In the same representative experiment as shown in (**a**), cell lysates prepared after 10 h of transfection were separated by SDS–PAGE (12%), transferred and immunoblotted with anti-GFP rabbit polyclonal antibody. The relative levels of the expression of GFP (lane 1), GFP-Siva-1 (lane 2) and GFP-Siva-2 (lane 3) are shown. (**c**) DNA fragmentation assays, were also performed in the mouse cell lines AF3.G7 (lanes 1–4), L12.10 (lanes 5–7), LICO.H11 (lanes 8–10). Lipofectamine used for transfection (lane 1) or vector alone (lanes 2, 5 and 8) did not give rise to significant DNA ladder. In comparison, transfection with pEGFP-Siva-1 (lanes 3, 6 and 9) but not pEGFP-Siva-2 (lanes 4, 7 and 10) resulted in the generation of significant DNA ladder. DNA fragmentation assay has been described (Prasad *et al.*, 1997)

DNA ladder in Figure 4c, lanes 3, 6 and 9. In comparison, the expression of GFP-mS2 which lacks the exon 2 did not yield significant cell death (Figure 4c, lanes 4, 7 and 10).

CD27, a member of the TNFR family is known to provide important co-stimulatory signals for T cell growth and B cell Ig secretion (Kobata et al., 1994, 1995; Agematsu et al., 1994; Hintzen et al., 1995; Jacquot et al., 1997). Recently, we have shown that ligation of CD27 can also result in apoptosis (Prasad et al., 1997) despite the fact that its cytoplasmic tail is relatively short, and lacks a death domain (Camerini et al., 1991; Gravestein et al., 1993). Accordingly, to determine whether or not CD27 uses a bridging molecule to transduce the apoptotic signal, we used the cytoplasmic tail of CD27 as bait in the yeast two hybrid system and cloned a novel intracellular molecule, Siva, that binds to CD27 and is apoptotic (Prasad et al., 1997).

Our results clearly demonstrate that the murine Siva-1 and the recently reported rat Siva (Padanilam et al., 1998) are both very similar to that of the human Siva. The predicted starting methionine of the mouse and rat Siva transcripts appear to correspond to the second methionine of human Siva, thereby making them both shorter by about 15 amino acids when compared to their human counterpart. In case of human Siva, we also obtained few clones whose sequence corresponded to the second methionine as the starting codon (data not shown in our previous communication). They, however, lacked an upstream, inframe stop codon. It is therefore likely that the true starting point may correspond to the second methionine in the reported human Siva sequence; a puzzle that can only be resolved by amino-terminal protein sequence analysis. Although the homology of Siva across the three species is about 70%, the murine and rat forms of Siva are much more closely related.

The cysteines which comprise the ring finger-like domain and the Zn finger-like domain of human Siva are also present in the murine and rat homologues. A stretch of 13 amino acids identical between the murine and human Siva (residues 82–94 of murine Siva in Figure 1a) is also seen in the rat sequence (Padanilam et al., 1998), suggesting that this region, a part of the originally described DDHR is likely to be very important for the function of Siva. The naturally occurring alternate splice form of Siva-1, Siva-2, lacks this region (a part of exon 2) and is not as apoptotic as the full length Siva. Accordingly, we believe that the cell death effector region is in the exon 2.

The original yeast Siva clone (H2) that we isolated from yeast using human CD27 cytoplasmic tail as the bait, coded for most of the cysteine rich carboxy terminal region suggesting that this region mediates the binding to the CD27 receptor (Prasad et al., 1997). In agreement with this murine Siva-1 and Siva-2, both have the cysteine rich region and associate with the full length CD27 receptor (Figure 3). It is therefore likely that the cells may regulate the function of the CD27 receptor by manipulating the endogenous levels of the Siva proteins. Whether or not Siva-2 behaves like dominant negative inhibitor of Siva-1 induced apoptosis needs further experimentation.

Our results demonstrate that murine Siva-2 is much less cytotoxic than Siva-1. Similar results have also been obtained with respect to human Siva-1 and Siva-2 (manuscript under preparation). The relative levels of protein expression were usually in the following order – GFP>>>> GFP-mSiva-2 >>>GFP-mSiva-1, and thus inversely corresponded to the degree of cytotoxicity exhibited by these three proteins. This observation underscores the ability of Siva-1 to elicit apoptotic response in susceptible cells. In some cell lines tested, apoptosis could not be detected, despite relatively high levels of protein expression of murine Siva-1 fusion protein (data not shown) suggesting that these cells may harbor efficient neutralizing mechanisms to counteract the pro-apoptotic nature of the Siva-1. It is also possible that the levels of the downstream targets of Siva may vary from cell type to cell type and thus may influence the ability of a particular cell type to undergo apoptosis. In the case of rat Siva, although its apototic properties have not been established, the transcripts for both Siva and CD27 receptor appear to be upregulated and co-localized in rat kidneys undergoing apoptosis due to ischemia (Padanilam et al., 1998).

As mentioned earlier, the Siva protein appears to have some similarity to the TRAFs and also to the death domain containing TRADD and FADD intracellular signaling molecules. It, however, appears to have nothing in common with DAXX – a protein that interacts with Fas (Cheng et al., 1995). Unlike DAXX, overexpression of Siva-1 alone can induce apoptosis. To the best of our knowledge, alternate splice forms for any of the death or TRAF domain containing intracellular signaling molecules have not yet been reported. Some of the BCL-2 family members, however, are expressed as long and short forms. The BCL-XL has the BCL-2 homology region (NWGRIV) and is anti-apoptotic and surprisingly BCL-XS which lacks this region is apoptotic, and depending on the relative levels of both forms, the cell may live or die (Yang and Korsemeyer, 1996). Siva lacks any BCL2 homology region, but it does harbor limited homology to the pre-BCL2 homology region defined as a part of the S2 region by Cory, (1995) and deletion of exon 2 also removes most of this region (data not shown). We are currently mapping the apoptosis inducing region of Siva-1 and investigating the mechanisms underlying differences between the susceptibilities of cell types to Siva induced apoptosis.

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