

## Fas-activated Serine/Threonine Kinase (FAST) Phosphorylates TIA-1 during Fas-mediated Apoptosis

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### Summary

We have identified a serine/threonine kinase that is rapidly activated during Fas-mediated apoptosis. Fas-activated serine/threonine kinase (FAST) is phosphorylated on serine and threonine residues in Jurkat cells. In response to Fas ligation, it is rapidly dephosphorylated and concomitantly activated to phosphorylate TIA-1, a nuclear RNA-binding protein that has been implicated as an effector of apoptosis. Phosphorylation of TIA-1 precedes the onset of DNA fragmentation, suggesting a role in signaling downstream events in the apoptotic program. Our results introduce FAST and TIA-1 as components of a molecular cascade involved in signaling Fas-mediated apoptosis.

Apoptotic cell death is required for the normal development and survival of multicellular organisms (1, 2). Just as cells induced to proliferate activate a programmed series of biochemical events leading to cell division (i.e., mitosis), cells induced to die activate a distinct program of biochemical events leading to cell death (i.e., apoptosis). Whereas the morphological changes accompanying both mitosis (3) and apoptosis (4) have been appreciated for many years, the biochemical events underlying these changes are only beginning to be understood. In the case of mitosis, genetic analysis of cell division cycle mutants in yeast has shown that the molecular effectors of cell division are a family of highly regulated cyclin-dependent kinases (5). Although some cyclin-dependent kinases are also activated during apoptosis (6, 7), specific molecular effectors of apoptosis have not yet been identified.

One of the most potent triggers of lymphocyte apoptosis is the Fas antigen, a tumor necrosis factor receptor-related cell surface molecule (8), whose expression is induced by lymphocyte activation (9, 10). Ligation of Fas antigen, either by Fas ligand or by specific mAbs, rapidly induces apoptosis in susceptible lymphocytes. Although the activation of a sphingomyelinase to produce ceramide may be involved in signaling Fas-mediated apoptosis (11), ceramide production also occurs in response to cytokines that do not trigger apoptosis (12, 13), indicating that additional effector molecules specific for apoptosis might be activated by ceramides. The ability of ceramides to activate selected protein kinases (14, 15) and phosphatases (16) suggests that these lipid mediators might regu-

late the phosphorylation of specific apoptotic effector molecules.

We have identified a family of RNA recognition motif (RRM)<sup>1</sup>-type RNA-binding proteins that are candidate effectors of apoptotic cell death. The first member of this family was identified using an mAb reactive with a 15-kD cytotoxic granule-associated protein (p15-TIA-1), the expression of which is restricted to cytotoxic lymphocytes (17–21). This antibody also recognizes two related RRM-type RNA-binding proteins designated p40-TIA-1 and p53-TIA-1 (based on their apparent molecular weights of 40,000 and 53,000) which are expressed in a variety of cell types (17, 22). Immunochemical analysis indicates that p15-TIA-1 is structurally related to the carboxyl terminus of p40-TIA-1 which encodes a glutamine-rich protein interaction domain (22). The ability of both p15-TIA-1 and p40-TIA-1 to trigger DNA fragmentation in permeabilized thymocytes has implicated these molecules as candidate effectors of CTL-mediated apoptosis (22). Although the molecular mechanism whereby the protein interaction domain of an RNA-binding protein might trigger apoptosis is unknown, we have proposed that p15-TIA-1 might affect the function of p40-TIA-1 in target cells by interacting with a common protein (22, 23). We therefore used the two-hybrid system to screen for proteins capable of interacting with the carboxyl terminus of p40-TIA-1. Here

<sup>1</sup> Abbreviations used in this paper: FAST, Fas-activated serine/threonine kinase; GST, glutathione-S-transferase; HA-FAST, hemagglutinin-tagged FAST; PVDF, polyvinylidene difluoride; RRM, RNA recognition motif.

we describe the characterization of Fas-activated serine/threonine kinase (FAST), a 60-kD serine/threonine kinase that binds to the carboxyl terminus of p40-TIA-1. FAST is a constitutively phosphorylated protein that is rapidly dephosphorylated after Fas ligation. Dephosphorylated FAST is activated to phosphorylate p40-TIA-1 before the onset of DNA fragmentation. Our results suggest that FAST activation may be involved in triggering downstream events in the apoptotic program.

## Materials and Methods

### Identification of TIA-1-binding Proteins

The cDNA encoding p40-TIA-1 was cloned into the multilinker of the pMA424 vector to produce a fusion protein consisting of the GAL4 DNA-binding domain (1-147) at the amino terminus and p40-TIA-1 at the carboxyl terminus. Transformation of yeast strain GGY1::171 with this recombinant plasmid followed by selection on synthetic culture-histidine plates resulted in the efficient expression of the fusion protein (data not shown). Because this fusion protein lacks the GAL4 activation domain, it was unable to induce  $\beta$ -galactosidase expression which in GGY1::171 is under control of the GAL4 promoter. The identification of cDNAs encoding TIA-1-binding proteins was then accomplished by cotransforming GGY1::171 cells with pMA424(TIA-1) and a cDNA library (B cell cDNA was cloned into the XhoI site of pSE1107) expressing fusion proteins consisting of the GAL4 activation domain (768-881) at the amino terminus and peptides encoded by individual cDNAs at the carboxyl terminus. Selection for double transformants expressing  $\beta$ -galactosidase (i.e., blue colonies on X-gal plates) identifies cDNAs encoding candidate TIA-1-binding proteins which juxtapose the GAL4 activation domain and the GAL4 DNA-binding domain as a consequence of the TIA-1:TIA-1 binding protein interaction. From  $1 \times 10^6$  double transformants screened, we identified four  $\beta$ -galactosidase-expressing transformants. The recombinant pSE1107 plasmid from one of these transformants contained a 1.5-kb insert which was used as a probe to isolate a 1.8-kb full-length cDNA. Dideoxy sequencing using Sequenase (U.S. Biochemical Corp., Cleveland, OH) was performed using oligonucleotide primers and alkali-denatured plasmid DNA as described by the suppliers.

**Construction of Fusion Proteins.** The cDNA encoding FAST was cloned into the EcoRI site of the polylinker region of pGEX-3X using oligonucleotide linkers. These constructs were designed to express FAST as a fusion protein with glutathione-S-transferase (GST). Individual fusion proteins were expressed in *Escherichia coli* (DH5), and purified from bacterial lysates using glutathione-Sepharose (Sigma Chemical Co., St. Louis, MO). The purified fusion proteins were analyzed on a 10% SDS polyacrylamide gel by staining with Coomassie blue. Fusion proteins were also analyzed by immunoblotting using rabbit polyclonal antisera raised against recombinant FAST.

The hemagglutinin-tagged fusion protein was produced by cloning the cDNA-encoding FAST into the EcoRI site of pMT2-HA vector (provided by Dr. Michel Streuli, Dana-Farber Cancer Institute, Boston, MA). This recombinant plasmid was used to transfect COS cells in the transient assay described below.

**COS Cell Transfections.** COS cells were transfected with the pMT-2 plasmid containing the indicated insert DNA using the diethylaminoethyl dextran method as described (24). After 48 h of culture, transfected cells were solubilized with lysis buffer, and used in the immunoprecipitation and immunoblotting experiments described.

**Immunoblot Analysis.** Immunoblotting analysis was carried out as previously described (17). Immunoblots were developed using polyclonal or monoclonal antibodies reactive with FAST, followed by horseradish peroxidase-conjugated protein A/G. Blots were revealed using the enhanced chemiluminescence detection system (Renaissance; DuPont NEN Research Products, Boston, MA).

**Phosphoamino Acid Analysis.**  $^{32}$ P-labeled immunoprecipitates were separated on a 10% SDS polyacrylamide gel, transferred to polyvinylidene difluoride (PVDF) (Immobilon P; Millipore Corp., Bedford, MA), and visualized by autoradiography. The indicated bands were excised and digested with 5.7 M HCl at 110°C for 1 h before drying in a speed vac. Hydrolysates were then lyophilized and separated using two-dimensional electrophoresis/TLC as described (25).

**Kinase Assays.** For in vitro kinase assays, individual immunoprecipitates were washed three times with 1% digitonin lysis buffer, then once with TBS (150 mM NaCl, 20 mM Tris, pH 7.5) before resuspending in 30  $\mu$ l of kinase buffer (20 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 20  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP [Dupont NEN; 3,000 mCi/mmol]) for 20 min at 30°C. The reaction was terminated by the addition of SDS sample buffer. Proteins were separated on a 12% SDS polyacrylamide gel, and dried before autoradiographic exposure.

For renaturation kinase assays, immunoprecipitates were separated on a 10% SDS polyacrylamide gel and transferred to 0.45- $\mu$ m PVDF before incubating in 7 M guanidine, 50 mM Tris, 50 mM DTT, 2 mM EDTA, pH 8.3, for 1 h at room temperature. Immobilized proteins were then renatured by incubating in 100 mM NaCl, 50 mM Tris, 2 mM DTT, 2 mM EDTA, 1% BSA (Sigma Chemical Co., St. Louis, MO), 0.1% (wt/vol) NP-40, pH 7.5, for 12-18 h at 4°C. PVDF membranes were then incubated in 30 mM Tris, 10 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, pH 7.5, 30  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP (3,000-6,000 Ci/mmol; New England Nuclear, Boston, MA) for 30 min at room temperature, with gentle shaking. The kinase reaction was terminated by washing the membrane twice with 30 mM Tris, pH 7.5, and once with 1 M KOH for 5-10 min, before rinsing with water before autoradiographic exposure (26).

**$^{32}$ P-labeling and Cellular Activation.** Jurkat cells ( $2 \times 10^6$  cells/ml) were incubated in phosphate-free RPMI (GIBCO BRL, Gaithersburg, MD) containing 5% dialyzed FCS and 0.2 mCi/ml  $^{32}$ P<sub>0</sub> for 3-4 h at 37°C. After the addition of activating mAbs, cells were centrifuged at the indicated times and lysed in either digitonin lysis buffer (1% digitonin, 0.12% Triton-X 100, 150 mM NaCl, 1 mM EDTA, 20 mM triethanolamine, pH 7.8) or NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 7.8) containing freshly added protease inhibitors (1 mM PMSE, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 20 mM iodoacetamide). Immunoprecipitations using the indicated antibodies were performed as previously described (22).

**DNA Fragmentation.** Jurkat cells were treated with anti-Fas (7c11, IgM) for the indicated times, collected by centrifugation, and lysed in 20 mM Tris (pH 7.4), 0.4 mM EDTA, 0.4% Triton X-100. Solubilized cells were centrifuged at 14,000 rpm for 5 min. Supernatants were collected and adjusted to contain 0.5 M NaCl and an equal volume of isopropanol. After incubating overnight at -70°C, samples were thawed and centrifuged at 14,000 rpm for 10 min, washed once with 70% ethanol, and dried in a Speed-Vac (Savant, Farmingdale, NY). Pellets were resuspended in 20  $\mu$ l of Tris-EDTA buffer containing 0.1 mg/ml RNAase and incubated at 37°C for 30 min. After the addition of loading buffer, samples were separated on agarose gels (0.8%) and visualized under UV light.

**Antibodies.** mAbs reactive with FAST were prepared by immunizing a Balb/c mouse with affinity-purified GST-FAST (100

mg was administered intraperitoneally at 3-wk intervals for a total of four injections) in adjuvant before fusing immune splenocytes with the NS-1 hybridoma partner as previously described (17). Individual hybridomas were screened for production of antibodies reactive with FAST using a solid-phase ELISA as previously described (22). The isotype of the mAbs designated anti-2B5 and anti-2D5 are both IgG2a. Anti-2B5 was found to preferentially recognize recombinant FAST expressed in COS cells and was used to immunoprecipitate this protein for in vitro kinase assays. Anti-2D5 was found to preferentially recognize endogenous FAST, and was used to immunoprecipitate FAST from Jurkat cells.

Polyclonal antibodies reactive with FAST were prepared by immunizing rabbits with purified GST-FAST one time in CFA and subsequent times in incomplete Freund's adjuvant. Immune sera were depleted of antibodies reactive with GST using a Sepharose-GST column. Preimmune sera were used in control experiments using these antisera.

The production of mAbs reactive with TIA-1 and TIAR were described previously (22, 23), with the exception of ML29. This antibody was produced using recombinant p40-TIA-1 as immunogen. The isotype of ML29 is IgG1.

## Results

**Identification of a cDNA Encoding FAST.** We used a genetic screen in yeast (i.e., the two-hybrid system [27, 28]) to identify proteins capable of interacting with the carboxyl terminus of p40-TIA-1 (22). The deduced amino acid sequence of one such protein (designated FAST, calculated molecular weight of 62,900) which interacted strongly with TIA-1, but only weakly with the related RNA-binding protein TIAR (23), is shown in Fig. 1 A. Comparison of this amino acid sequence with proteins in the EMBL data library identified a region that was similar (23% identity over 240 amino acids using the FASTA algorithm, 12% identity over the 432 amino acid kinase domain using the alignment shown in Fig. 1 A) to a protein kinase domain encoded by the ICP10 gene of herpes simplex virus (29). Alignment of the amino acid sequence of FAST (amino acids 65–494) with the protein kinase domains of ICP10 and cAPK- $\alpha$ , reveals the presence of several of the "invariant residues" defined by protein kinase consensus motifs I-X (30) (*bold type*, Fig. 1 B). Both FAST and ICP10 also encode proline-rich, putative SH3-binding domains (underlined in the FAST sequence shown in Fig. 1 B; the ICP10 SH3-binding domain is encoded in the insert designated A in Fig. 1 B [29]). A single 1.8-kb FAST mRNA was found to be expressed in heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas by Northern analysis (data not shown).

**Analysis of Recombinant and Endogenous FAST.** Recombinant FAST possesses intrinsic protein kinase activity as shown in Fig. 2. In this experiment, lysates from COS transformants expressing a recombinant FAST fusion protein containing an NH<sub>2</sub>-terminal hemagglutinin tag (HA-FAST) were immunoprecipitated using mAbs reactive with the HA tag (anti-HA), FAST (anti-2B5, an IgG2a mAb produced as described in Materials and Methods), or an isotype-matched control

antibody, and immunoprecipitated proteins were incubated in kinase buffer containing [ $\gamma$ -<sup>32</sup>P]ATP (31). Fig. 2 A shows that precipitates prepared using anti-HA and anti-FAST, but not control mAb, include 65- and 60-kD phosphoproteins. The 65-kD phosphoprotein comigrates with recombinant HA-FAST in immunoblots (*arrow*, data not shown). The 65-kD phosphoprotein is also autophosphorylated in the renaturation kinase assay (Fig. 2 B, *arrow*). In this assay, electrophoretically separated proteins are transferred to PVDF filters, renatured, and incubated in the presence of [ $\gamma$ -<sup>32</sup>P]ATP (32). This result confirms that the 65-kD protein has intrinsic kinase activity. The origin of the 60-kD phosphoprotein identified in the in vitro kinase assay (Fig. 2 A) is not known, but it may be a degradation product derived from HA-FAST since it appears to be recognized by the anti-HA antibody. Phospho-amino acid analysis of the autophosphorylated kinase indicates that it is serine/threonine specific (Fig. 2 C).

An mAb reactive with recombinant FAST (anti-2D5) immunoprecipitates a 60-kD phosphoprotein from <sup>32</sup>P-labeled Jurkat cells (Fig. 3 A). In some experiments, two isoforms of endogenous FAST (centered at 60 kD) can be resolved after separation on SDS-polyacrylamide gels (*arrows*). Like the autophosphorylated recombinant protein, endogenous FAST is phosphorylated exclusively on serine and threonine residues (Fig. 3 B).

**Interaction between TIA-1 and FAST.** We used GST fusion proteins containing the carboxyl terminus of TIA-1 to demonstrate the intrinsic affinity between TIA-1 and FAST. Fig. 4 A shows that NP-40 lysates prepared from COS (HA-FAST) transformants (as well as immunoprecipitates prepared from these lysates using anti-FAST) contain a 65-kD protein that is recognized in immunoblots probed with an anti-FAST mAb. Affinity precipitates prepared from these same lysates using immobilized GST-p15-TIA-1 or GST-p40-TIA-1, but not GST alone, also include FAST (*arrow*; the 46-kD protein seen in the GST-p15-TIA-1 lane is the GST fusion protein itself, which is nonspecifically visualized in this assay). Because FAST contains a proline-rich domain (PPKPPPLQP) that may interact with SH3 domains (33), we also analyzed affinity precipitates prepared using immobilized GST fusion proteins containing the SH3 domain of the *fyn* tyrosine kinase. In data not shown, FAST was found to bind to *fyn*-SH3, but not *fyn*-SH2, indicating that FAST, like ICP10, can interact with proteins possessing SH3 domains.

The association between endogenous FAST and TIA-1 in mammalian cells was demonstrated in the coimmunoprecipitation experiments shown in Fig. 4 B. Jurkat cells metabolically labeled with either <sup>32</sup>PO<sub>4</sub> or [<sup>35</sup>S]methionine were solubilized in digitonin lysis buffer (the FAST:TIA-1 association was preserved in the presence of digitonin, but less efficiently in the presence of harsher detergents such as NP-40) and immunoprecipitated using mAbs reactive with either FAST (anti-2D5), TIA-1, or TIAR (anti-1H10 preferentially recognizes TIA-1, whereas anti-6E3 and anti-3E6 preferentially recognize TIAR [34]). mAbs reactive with TIA-1 and TIAR precipitate the 40-kD and 53-kD isoforms of their target RNA-binding proteins which are labeled with [<sup>35</sup>S]methionine

**A**

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MRRPRGEGP RAPRPTGAT CAGPGESWSP SPNSMLRVLL SAQTSPARLS 50
GLLLIPPVQP CCLGPSKWGD RVPVGGPSAG PVQGLQRLE QAKSPGELLR 100
WLGQNPskvr AHYSVALRR LGQLLGSRRP PPPVEQVTLQ DLSQLIIRNC 150
PSFDIHTIHV CLHLAVLLGF PSDGPLVCAL EQERRLRLLP KPPFPLQPLL 200
RGGQGLEAAL SCPRFLRYPR QHLISSLAEA RPEELTPHVM VLLAQHLARH 250
RLREPQLEEA IAHFLVQVET QLSKVVQKL VLPFGRNLNYL PLEQQFMPCL 300
ERILAREAGV APLATVNILM SLCQLRCLPF RALHFVFSFG FINYISGTPH 350
ALIVRRYLSL LDTAVELELP GYRGPRLPRR QQVPIFFQPL ITDRARCKYS 400
HKDIVAEGLR QLLGEEKYRQ DLTVPVPGYCT DFLLCASSG AVLFPVRTQDP 450
FLPYPPRSCP QGQAASSATT RDPAQRVVLV LRERWHFCRD GRVLLGSSRA 500
LRERHLGLMG YQLLPLPFEE LESQRGLPQL KSYLRQKLQA LGLRWGPEGG 550

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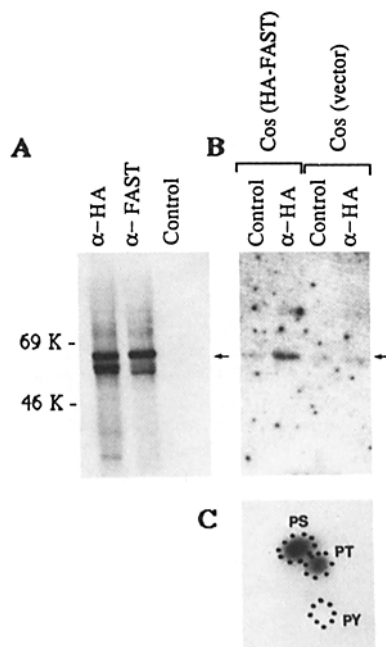
**B**

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FAST (64)   PSKWGDRPVGGPSAGPVQGLQRLLEQAKSPGELLRWLGQNPSKVRAHHYSVALRRLGQLLGSRRP PPPV (134)
ICP-10 (60) VAISNVAAGGDGRT--AVVALGGTSG---(50)---ARGGAEKDVGAESWSDG-----PSSDS
cAPK-α (40) DQFERIKTLGTGSF-GRVMLVKHME-----TGNYHAMKILDKQKVVKLK-----QIEHTLN
Consensus:   GGGV                         AK
                I                               II
                *                               **                               *                               * * *
FAST        EQVTLDLSQLIIRNCPSFDIHTIHVCLHLAVLLGFFSDGPLVCALEQERRRLR-----PPKPPPELQ (197)
ICP-10      ETEDSDSDEDTGSGSETLSRSSIWAAGATDDSDSDSRSDSVQPDVVVRRRWSGDGPAVAFPKPRR
cAPK-α      EKRIQAVNFPFLVKLEFSFKDNSNLYVMMEYVPGEMFSLRRIGRFSEPHARFYAAQIVLTFFEY----
Consensus:   E                         I
                III                       IV
                * * * * *
FAST        PLLRGGQGLEAALSCPRFLRYPRQHLLI-----SSLAEARPEELTPHVMVL (242)
ICP-10      PGDSPGNPGLGAGTGPGSATDPRASADSDSAAHAAAPQADVAPVLDVLDVQPTVGTDPGYPVPLLELTPEMAEA
cAPK-α      -----LHSLDLIYRDLKPENLLI
Consensus:   -----DLN
                VI
                * * * * *
FAST        LAQHLARHRLREPQLLEAIAHFLVQVETQLSKVV-----QKLVLVFPGRNLNYLPLEQQFMPCLERILAREA (310)
ICP-10      VARFLGDAVDREPALMLEYFCRCAREESKRVPRTFGSAPRLTDDFGLLNTA-LAEMRRLCLDLPVPPN
cAPK-α      DQQGY-----IQVIDFGFAKRVKGRIVILCGTP-----
Consensus:   -----DFG
                VII
                * * * * *
FAST        GVAPLATVNILMSLCQLRCLPFRALHFVFSFGFINYISGTPHALIVRRYLSL LDTAVELELPGYRGPRLP (380)
ICP-10      AYTP-----YHLREYATRLLVNGFKPL
cAPK-α      -----EYLAPEI-----
Consensus:   -----AE
                VIII
                * * * * *
FAST        RRQQVPIFFQPLITDRARCKYS HKDIVAEGLRQLLGEKEYRQDLTVPVPGYCTDFLLCASSSGAVLPVRTQ (450)
ICP-10      VRRSARLYRILGILVHLRIRRTREASFEWMSRKEVDLPGLTERLREHEAQLMILAQALNPYDCLIHSTP
cAPK-α      -----ILSKGYNKAVDWWALGVLIYEMAAGYPPFFADQPIQIYEVKIVSGKVRFPESH-----
Consensus:   -----D G
                IX
                * * * * *
FAST        DFFLPYPPRSCPQGQAASSATT RDPAQRVVLVLRERWHFCRDGRVL (496)
ICP-10      NTLVERGLQSALKYEEHYLKRHGGHYMESVHQMYTRIAGPLACRAT
cAPK-α      -----FSSDLKDLLRNLLQVDLTKRFG
Consensus:   -----L R
                X

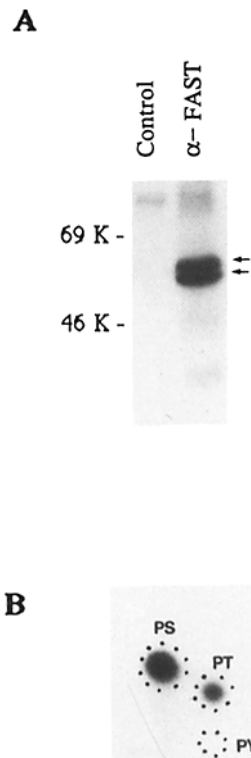
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**Figure 1.** (A) Deduced amino acid sequence of FAST. (B) Comparison of the kinase domains encoded by FAST, the HSV serine/threonine kinase ICP10, and the catalytic subunit of cAMP-dependent protein kinase (cAPK-α). Amino acid identity between FAST and ICP10 is indicated by asterisks. Residues conforming to the consensus kinase motifs (designated by roman numerals) are shown in bold type. Catalytic domain inserts identified by comparison with cAPK-α are overlined and labeled A-G. The proline-rich, putative SH3 domain-binding site is underlined. The EMBL database accession number for this nucleotide sequence is X86779.



**Figure 2.** HA-FAST is a serine/threonine kinase. (A) In vitro kinase assay performed using immunoprecipitates prepared from Cos (HA-FAST) lysates. Arrow points to the 65-kD autophosphorylated recombinant HA-FAST. (B) Renaturation kinase assay prepared using immunoprecipitates from COS (HA-FAST) or COS (vector) lysates. Proteins separated on a 10% SDS-polyacrylamide gel were transferred to nitrocellulose, and renatured before incubation with [ $\gamma$ - $^{32}$ P]ATP in kinase buffer. Arrow points to the 65-kD autophosphorylated protein precipitated from Cos (HA-FAST) transformants, but not COS (vector) transformants using anti-HA but not an isotype-matched control mAb. (C) Phosphoamino acid analysis of autophosphorylated HA-FAST. PS; phosphoserine; PT; phosphothreonine; PY; phosphotyrosine.

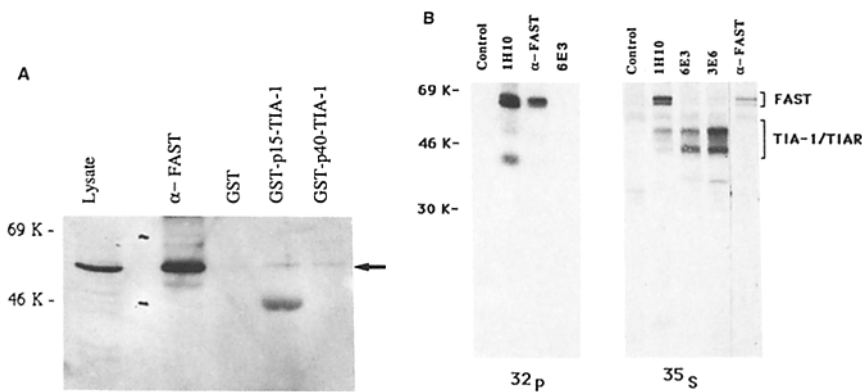
(right panel), but not with  $^{32}\text{PO}_4$  (left panel). The mAb reactive with TIA-1 (anti-1H10) coprecipitates a doublet centered at 60-kD which is labeled with both  $^{35}\text{S}$  (right panel) and  $^{32}\text{PO}_4$  (left panel). This 60-kD doublet comigrates with endogenous FAST precipitated with an mAb reactive with FAST (anti-2D5). The anti-FAST antibody does not coprecipitate TIA-1, suggesting that its epitope may be part of the FAST:TIA-1-binding site. The ability of anti-1H10 to precipitate p40-TIA-1 and p53-TIA-1 was surprising since a previously described mAb reactive with the carboxyl-terminus of TIA-1 (anti-2G9) did not precipitate the full-length RNA binding proteins from lysates prepared from noncytotoxic cells (34). Anti-ML29, an mAb reactive with a distinct amino-terminal TIA-1 epitope, also precipitates p40-TIA-1 and p53-TIA-1 from noncytotoxic cells (see below). These results suggest that the carboxyl terminus of TIA-1 is not accessible to anti-2G9 in cell lysates, possibly because it interacts with another protein. The detection of p40-TIA-1 protein in noncytotoxic cells is consistent with the expression of mRNA encoding p40-TIA-1 in a variety of cell types (22). The observation that FAST associates with TIA-1 but not TIAR in cell lysates is consistent with data obtained using the two hybrid system.



**Figure 3.** Characterization of endogenous FAST. (A) Jurkat cells metabolically labeled with  $^{32}\text{PO}_4$  were solubilized in NP-40 lysis buffer and immunoprecipitated using anti-FAST, or an isotype-matched control mAb. Arrows point to the FAST doublet centered around 60 kD. (B) Phosphoamino acid analysis of phosphorylated endogenous FAST. Both the upper and lower bands gave a similar phosphoamino acid analysis.

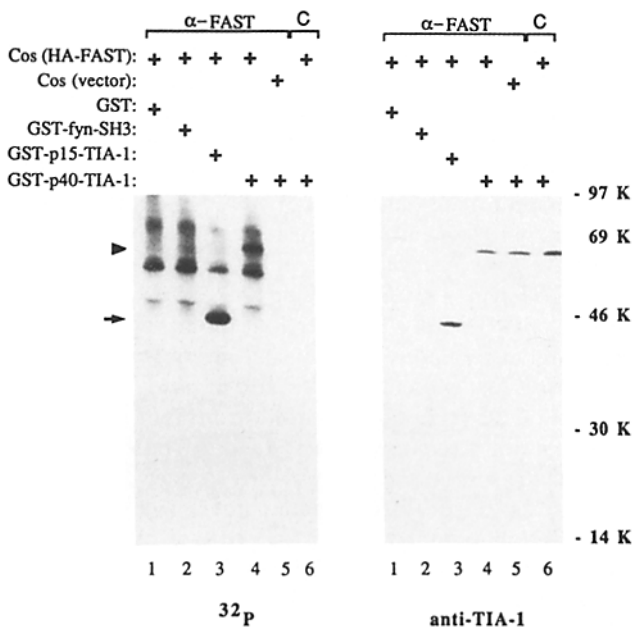
**TIA-1 Is a Substrate for FAST In Vitro** The ability of FAST to interact with the carboxyl terminus of TIA-1 suggests that it may phosphorylate TIA-1. We therefore tested the substrate specificity of FAST in the in vitro kinase assay shown in Fig. 5. Immunoprecipitates prepared from a COS (HA-FAST) transformant (lanes 1–5) or a COS (vector) transformant (lane 6) using anti-FAST (lanes 1–4 and 6) or a control mAb (lane 5) were incubated with [ $\gamma$ - $^{32}$ P]ATP in the presence of GST (lane 1), GST-fyn-SH3 (lane 2), GST-p15-TIA-1 (lane 3), or GST-p40-TIA-1 (lanes 4–6) before electrophoretic separation, nitrocellulose transfer, and autoradiography. The 65-kD HA-FAST protein was identified in anti-FAST precipitates from COS (HA-FAST) transformants (lanes 1–4), but not in control precipitates (lanes 5 and 6). Both GST-p15-TIA-1 (lane 3, arrow) and GST-p40-TIA-1 (lane 4, arrowhead) (identified in the anti-TIA-1 immunoblot shown in the right panel) were phosphorylated in the presence of HA-FAST, whereas GST (lane 1) and GST-fyn-SH3 (lane 2) were not. The observation that GST-fyn-SH3 is not an efficient substrate for FAST, in spite of the fact that it binds to FAST in vitro, indicates that TIA-1 may be a relatively specific substrate for FAST.

**TIA-1 Is a Substrate for FAST In Vivo** The identification of p40-TIA-1 as a FAST substrate in vitro suggested that it might also be a physiological FAST substrate. Although TIA-1 is not constitutively phosphorylated (our unpublished observations), its ability to trigger DNA fragmentation in permeabilized thymocytes suggests that it might play a role in signaling apoptosis (22). We therefore monitored the phosphorylation of TIA-1 in  $^{32}\text{PO}_4$ -labeled Jurkat cells triggered



**Figure 4.** Interaction between TIA-1 and FAST. (A) In vitro. Lysates prepared from COS (HA-FAST) transformants were precipitated with anti-FAST or the indicated affinity ligand, (GST: glutathione-S-transferase; GST-p15-TIA-1: fusion protein encoding the carboxyl terminus of TIA-1; GST-p40-TIA-1: fusion protein encoding the full length TIA-1 RNA-binding protein), separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed using an mAb reactive with FAST (anti-2B5). Arrow points to the 65-kD HA-FAST protein. The relative migration of molecular size markers is indicated at the left. (B) In vivo. Jurkat cells metabolically labeled with either  $^{32}\text{PO}_4$  (left panel) or  $^{35}\text{S}$  methionine (right panel) were solubilized in digitonin lysis buffer and immunoprecipitated using mAbs reactive with: TIA-1 (1H10), TIAR (6E3 and 3E6), FAST (anti-2D5). The relative migration of molecular size markers is shown at the left.

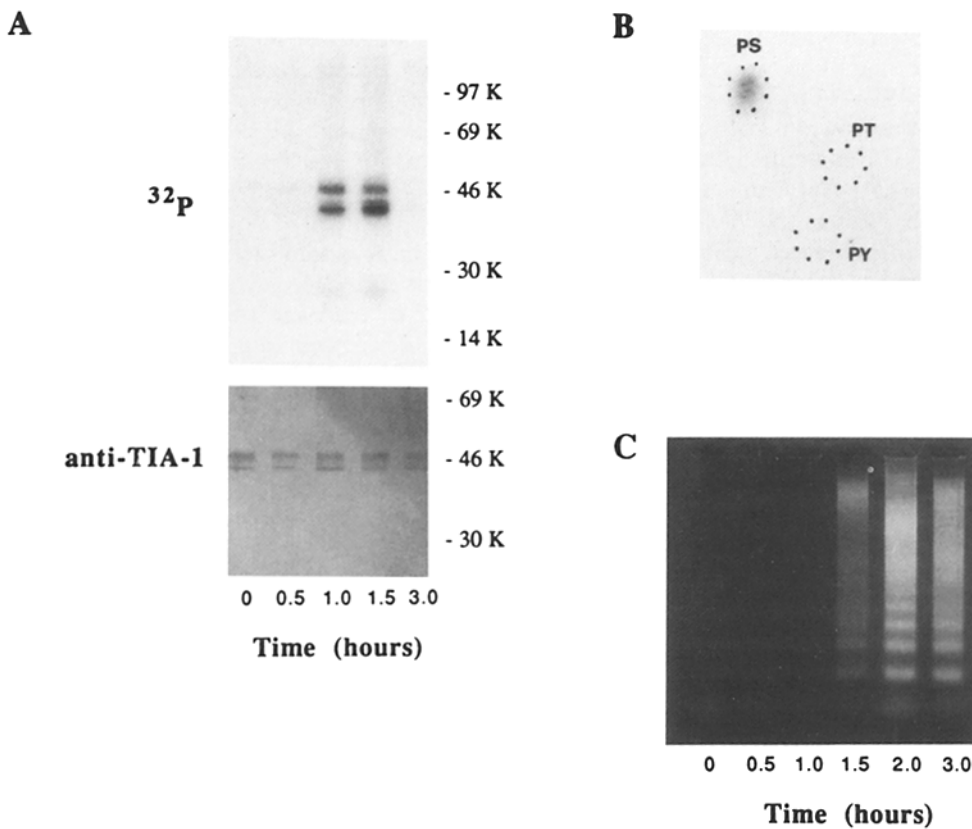
to undergo apoptosis by antibodies reactive with Fas. Immunoprecipitates prepared using an mAb reactive with TIA-1 (anti-ML29) showed that both TIA-1 isoforms (p40-TIA-1 and p53-TIA-1) are transiently phosphorylated (Fig. 6 A, upper



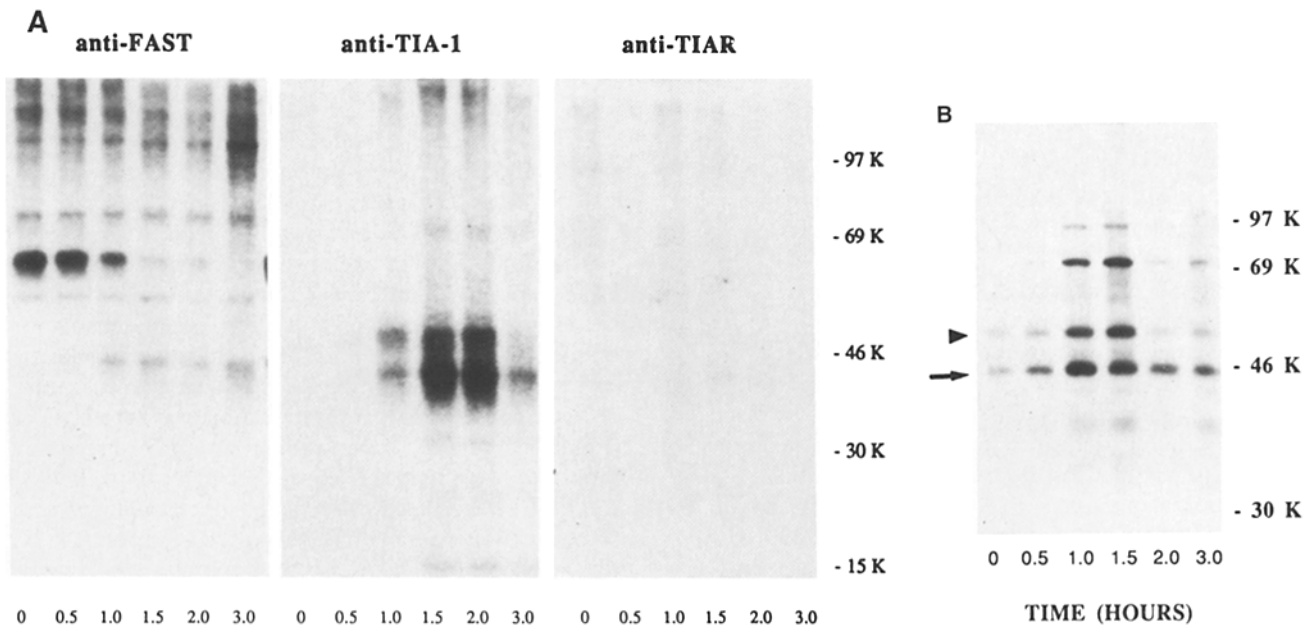
**Figure 5.** TIA-1 is a substrate for FAST in vitro. (Left) Analysis of in vitro kinase assays prepared using anti-FAST immunoprecipitates from COS (HA-FAST) lysates (lanes 1–4) or from COS (vector) lysates (lane 5). Analysis of control immunoprecipitates prepared from COS (HA-FAST) lysates are shown in lane 6. Kinase assays were performed in the presence of the following purified, exogenous proteins added in equimolar amounts (1  $\mu\text{M}$ ): GST (lane 1), GST-fyn-SH3 (lane 2), GST-p15-TIA-1 (lane 3), GST-p40-TIA-1 (lanes 4–6). The relative migration of molecular size markers is shown at the right. Arrow points to phosphorylated p15-TIA-1 and arrowhead points to phosphorylated p40-TIA-1 which were identified by immunoblotting using an mAb reactive with TIA-1 (anti-2G9) as shown in the right panel.

panel) on serine residues (Fig. 6 B) in response to Fas ligation. Because immunoblotting analysis showed that the amount of TIA-1 protein is not significantly affected by Fas ligation (Fig. 6 A, lower panel), its transient phosphorylation is likely to result from its sequential modification by a serine/threonine kinase and a serine/threonine phosphatase. The kinetics of DNA fragmentation induced by Fas ligation are shown in Fig. 6 C. In this representative experiment, phosphorylation of TIA-1 precedes the onset of DNA fragmentation by at least 30 min.

Although FAST and TIA-1 form an intermolecular complex in Jurkat lysates (Fig. 4 B), TIA-1 is only phosphorylated after Fas ligation. This result suggests that FAST is activated in response to this stimulus. We therefore monitored the phosphorylation of FAST and TIA-1 in Jurkat cells treated with anti-Fas. As shown in Fig. 7 A (left panel), phosphorylated FAST rapidly disappears from Jurkat cells triggered with anti-Fas. Although immunoblotting analysis was not sufficiently sensitive to detect the endogenous FAST protein in this experiment, parallel experiments using  $^{35}\text{S}$ -labeled Jurkat cells showed that the amount of FAST protein does not significantly change during the first 3 h of Fas-mediated apoptosis (data not shown). Therefore, the disappearance of phosphorylated FAST probably results from its specific dephosphorylation. Dephosphorylation of FAST is usually detected 30–60 min after the addition of anti-Fas, and the dephosphorylation of FAST coincides with the phosphorylation of TIA-1 (Fig. 7 A, middle panel). The specificity control in this experiment is an mAb reactive with TIAR, a TIA-1-related RNA-binding protein (23) that is not phosphorylated after Fas ligation (Fig. 7 A, right panel). The simplest interpretation of these results is that dephosphorylation activates FAST to phosphorylate TIA-1. This possibility is supported by the observation that Fas ligation activates the autophosphorylation and transphosphorylation activities of FAST in vitro (Fig. 7 B). In this experiment, Jurkat cells were treated with anti-Fas for the indicated times, solubilized in NP-40 lysis buffer, and immunoprecipitated using an mAb reactive with FAST (anti-



**Figure 6.** Phosphorylation of TIA-1 during Fas-mediated apoptosis. (A) (Upper panel)  $^{32}\text{P}$ -labeled Jurkat cells were treated with anti-Fas for the indicated times, solubilized in NP-40 lysis buffer, and immunoprecipitated using an mAb reactive with TIA-1 (anti-ML29). After separation on a 10% SDS polyacrylamide gel, and transfer to nitrocellulose,  $^{32}\text{P}$ -labeled TIA-1 was revealed by autoradiography. The relative migration of molecular size markers is shown at the right. (Lower panel) The same nitrocellulose filter used to produce the autoradiogram shown in the upper panel was subjected to immunoblotting analysis using an mAb reactive with TIA-1 (anti-ML29). The relative migration of molecular size markers is shown at the right. (B) Phosphoamino acid analysis of p40-TIA-1. PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine. (C) Kinetics of DNA fragmentation induced by Fas ligation. Jurkat cells treated with antibodies reactive with Fas in parallel with the experiment shown in A were analyzed for DNA fragmentation as described in Materials and Methods.



**Figure 7.** FAST is dephosphorylated and concomitantly activated in response to Fas ligation. (A)  $^{32}\text{P}$ -labeled Jurkat cells were treated with anti-Fas for the indicated times, solubilized in NP-40 lysis buffer, and immunoprecipitated using mAbs reactive with FAST (anti-2D5, left panel), TIA-1 (anti-ML29, middle panel), or TIAR (anti-6E3, right panel). After separation on a 10% SDS-polyacrylamide gel,  $^{32}\text{P}$ -labeled proteins were identified using autoradiography. The relative migration of molecular size markers is shown at the right. (B) Activation of FAST during Fas-mediated apoptosis. Jurkat cells ( $5 \times 10^6$ ) treated with anti-Fas for the indicated times were solubilized in NP-40 lysis buffer and immunoprecipitated using an mAb reactive with FAST (anti-2D5). Immunoprecipitates were subjected to the in vitro kinase assay in the presence of the FAST substrate GST-p15-TIA-1 ( $1 \mu\text{M}$ ), separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose and exposed for autoradiography. (Arrowhead) Autophosphorylated FAST. (Arrow) Phosphorylated GST-p15-TIA-1 that was localized by immunoblotting using an mAb reactive with TIA-1 (anti-2G9). The relative migration of molecular size markers is shown at the right.

2D5). Precipitates containing endogenous FAST were further incubated in kinase buffer supplemented with [ $\gamma$ - $^{32}$ P]-ATP, and GST-p15-TIA-1. Both autophosphorylation of FAST (*arrowhead*) and transphosphorylation of GST-p15-TIA-1 (*arrow*, identified by immunoblotting analysis, not shown) increased after Fas ligation. The kinetics of FAST activation paralleled the dephosphorylation of FAST and the phosphorylation of TIA-1 observed in Fig. 7 A. Phosphorylation of FAST-associated proteins (pp70 and pp95) was also induced by Fas ligation. The identity of these candidate FAST substrates is not known.

To determine whether the dephosphorylation of FAST is specific for Fas-mediated apoptosis, we similarly measured the phosphorylation of FAST in Jurkat cells activated by an antibody reactive with the CD3:TCR complex. Whereas activation via CD3:TCR was a potent stimulus for the production of IL-2, we observed no change in the phosphorylation of FAST or TIA-1 in response to anti-CD3 (data not shown). These experiments were done in parallel with the experiments shown in Fig. 7, and included time points of 1, 2, and 3 h. Recent results have shown that Jurkat cells treated with anti-CD3 are induced to express Fas-ligand, and that activation-induced death is dependent upon Fas ligation (35). Because this process is much slower than Fas-mediated apoptosis, it is still possible that anti-CD3 might induce the phosphorylation of TIA-1 at later times of incubation. We were unable to test this possibility because of technical limitations of the metabolic labeling assay. Since activation via the CD3:TCR complex is a potent stimulus for the secretion of IL-2 in these cells, the observation that FAST remains phosphorylated and catalytically inactive at early times suggests that FAST may be selectively activated during Fas-mediated apoptosis.

## Discussion

We have identified a serine/threonine kinase, designated FAST, that binds to the carboxyl terminus of the RRM-type RNA-binding protein TIA-1. The deduced amino acid sequence of FAST reveals a weak similarity to a serine/threonine kinase domain found at the amino terminus of the large subunit of herpes virus ribonucleotide reductase (this gene is designated ICP6 [36] and ICP10 [29] in HSV-1 and HSV-2, respectively). In HSV-2, ICP10 is essential for virus replication (37), and its overexpression induces cellular transformation. The ICP10 kinase domain is unusual in that it only partly conforms to the consensus motifs comprising the catalytic core of eukaryotic protein kinases (30, 38, 39). ICP10 is also unusual in that mutation of the invariant lysine residue in motif II does not affect its activity (40). Thus, although ICP10 possesses kinase activity, its phylogenetic relationship to other known protein kinases is relatively distant. FAST is more closely related to ICP10 than it is to other eukaryotic protein kinases. It also lacks several "invariant" residues defined by kinase consensus motifs. These include the alanine residue in motif II, both the aspartic acid and asparagine residues in motif VI, and the aspartic acid residue in motif VII. Because the aspartic acid residue in motif VII

normally interacts with the asparagine residue in motif VI (39), the absence of both of these normally invariant amino acids suggests that the catalytic core of FAST may differ significantly from that of most protein kinases. In addition, there is an amino acid insertion that disrupts the spacing of motif I. Both FAST and ICP10 also possess several inserts (labeled A-F in Fig. 1 B) which could interrupt the catalytic core of the kinase domain. Each of these inserts is found in a position that could be accommodated without disrupting the conserved catalytic core (39), however, suggesting that they may contribute to substrate specificity. FAST and ICP10 both encode a proline-rich domain that is a potential binding site for SH3 domains (33). ICP10 interacts with proteins containing SH3 domains (e.g., son-of-sevenless and Grb-2) in cells. FAST also interacts with proteins possessing SH3 domains *in vitro* (unpublished results), but the relevance of this property to the function of FAST is unknown.

Our results indicate that FAST can efficiently bind and phosphorylate the carboxyl terminus of TIA-1 *in vitro*. The observation that FAST and TIA-1 form a complex in cell lysates supports the possibility that FAST might also phosphorylate TIA-1 *in vivo*. We have shown that TIA-1 is rapidly phosphorylated on serine residues in response to antibody-mediated Fas ligation. Phosphorylated TIA-1 is detected in cell lysates prepared between 1 and 2 h after Fas ligation. Because TIA-1 is no longer phosphorylated in cell lysates prepared 3 h after Fas ligation, its phosphorylation may be regulated by a constitutively active phosphatase. This result implies that the kinase responsible for phosphorylating TIA-1 is activated within 1 h, and inactivated within 3 h, of Fas ligation. The kinase activity of FAST precipitated from Jurkat lysates is transiently increased after Fas ligation, with kinetics that parallel the phosphorylation of TIA-1. Because dephosphorylation of FAST is also observed 1 h after Fas ligation, it may be the target of a Fas-activated Ser/Thr phosphatase. Taken together, our results strongly suggest that FAST is responsible for the phosphorylation of TIA-1 during Fas-mediated apoptosis.

Fas ligation has been shown to activate sphingomyelin hydrolysis, resulting in the production of ceramide (11). Ceramide, in turn, has been shown to directly trigger apoptosis in selected Fas-sensitive cell lines (11). Although ceramide may be an important component of the Fas signaling mechanism, it is clearly not sufficient for the induction of apoptosis, since ceramide is produced after ligation of a variety of receptors that do not trigger apoptosis (e.g., receptors for IL-1 $\beta$ , IFN- $\gamma$ , and TNF- $\alpha$ ) (12, 13). On the other hand, ceramide has been shown to activate several intracellular protein kinases (14, 15) and phosphatases (16). Thus, the presence or absence of specific kinases or phosphatases might influence a cell's susceptibility to Fas-mediated apoptosis. It is possible, for example, that ceramide activates the Ser/Thr protein phosphatase responsible for FAST activation. It will be important to correlate the expression of FAST, and its putative activating phosphatase with the relative susceptibility to Fas-mediated apoptosis in various cell lines.

Our results introduce FAST and TIA-1 as components of a molecular cascade that is activated during Fas-mediated apop-



tosis. It remains to be determined whether activation of FAST or phosphorylation of TIA-1 is required for signaling apoptosis. By determining the effect of functionally inactive FAST mutants on Fas-mediated apoptosis, it may be possible to answer this important question. It also remains to be determined whether p15-TIA-1 expressed in the granules of cytotoxic lymphocytes can cooperate with the Fas pathway to activate the FAST/TIA-1 cascade. Because CTLs can trigger apoptosis via two independent mechanisms (i.e., granule exocytosis and Fas) (41–44), identifying a role for p15-TIA-1 in this process will require the production of CTLs which lack p15-TIA-1. If TIA-1 is required for signaling apoptosis, how

might the phosphorylation of a nuclear RNA-binding protein signal apoptotic cell death? One possibility is that TIA-1 regulates the processing of an RNA transcript encoding a regulator of apoptosis. Alternative splicing of ICE (45), Ich-1 (46), and bcl-x (47–49) can produce mRNAs encoding both positive and negative regulators of apoptosis. Thus, if TIA-1 were to regulate the splicing of a proteins such as ICE, Ich-1, or bcl-x, its phosphorylation could result in the preferential selection of isoforms that promote apoptosis. By determining the RNA sequence recognized by the RRM motifs of TIA-1, we might be able to identify a specific RNA transcript whose translation is affected by TIA-1.

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