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FAST is a BCL-X_L-associated mitochondrial protein

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Abstract

The TIA-1-interacting protein Fas-activated serine/threonine phosphoprotein (FAST) is a component of a signaling cascade that is initiated by ligation of the Fas receptor. Immunofluorescence microscopy using affinity-purified antibodies raised against recombinant FAST reveals that the endogenous protein associates with mitochondria. Subcellular fractionation confirms that FAST is a component of mitochondria. FAST is tethered to mitochondria by a lysine/arginine-rich domain at its carboxyl terminus that is structurally similar to the mitochondrial tethering motifs of monoamine oxidase B and cytochrome *b*5. At the mitochondrial membrane, FAST interacts with BCL-X_L. The BCL-X_L binding domain maps to a BCL-2-homology-3 (BH3)-related domain that is distinct from the mitochondrial-tethering domain (MTD). Although interactions between FAST and BCL-X_L require both the BH3-related domain and the MTD, the requirement for mitochondrial tethering can be conferred by a heterologous MTD. Our results suggest that FAST–BCL-X_L interactions are likely to regulate mitochondrial metabolism during Fas-induced apoptosis.

Keywords: FAST; BH3 domain; BCL-X_L; Fas; Mitochondria; TIA-1

In response to Fas ligation, caspase-8 is recruited to the cytoplasmic tail of the Fas receptor by the adaptor protein FADD/MORT1 [1,2]. The ensuing activation of caspase-8 results in the cleavage of BID. Truncated BID is translocated from the cytosol to the mitochondrial membrane where it interacts with anti-apoptotic proteins such as BCL-2 and BCL-X_L [3]. When the neutralizing capacity of BCL-2 and BCL-X_L is exceeded, tBID targets the pro-apoptotic proteins BAX and BAK, allowing the release of apoptotic effectors (e.g., cytochrome *c*, DIABLO, and AIF) from mitochondrial stores [4]. Thus, in some cell types, mitochondria play a critical role in the effector phase of Fas-induced apoptosis.

We previously described a Fas-activated serine/threonine phosphoprotein (FAST) that is a potential regulator of Fas-induced apoptosis. FAST was identified as a TIA-1-interacting protein in a yeast two-hybrid screen [5]. TIA-1 is an RNA-binding protein that serves as a downstream effector of the PKR/eIF2α translational control pathway [6]. In cells subjected to environmental stress, PKR-induced phosphorylation of the translation initiation factor eIF2α reduces the concentration of eIF2/GTP/$tRNAi^{Met}$, the ternary complex that loads the initator tRNA onto the small ribosomal subunit. Under these conditions, TIA-1 promotes the assembly of a non-canonical 48S pre-initiation complex that inhibits protein translation [7]. The PKR/eIF2/TIA-1 pathway functions as a regulatory checkpoint that controls both protein synthesis and cell survival. Overexpression of PKR [8], a phosphomimetic mutant of eIF2α [9] or TIA-1 [10,11], inhibits protein translation and promotes apoptotic cell death. In contrast, dominant negative mutants of PKR or a non-phosphorylatable mutant of eIF2α promote translation and inhibit apoptosis [12,13].

FAST is constitutively phosphorylated on serine and threonine residues in Jurkat cells. In response to Fas ligation, FAST is rapidly dephosphorylated and TIA-1 is concomitantly phosphorylated on serine residues [5]. These results suggested that FAST and TIA-1 might play a role in signaling Fas-induced apoptosis. Consistent with this possibility, we show that FAST is localized to the outer mitochondrial membrane. FAST is tethered

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to mitochondria by a lysine/arginine-rich domain at its carboxyl terminus. At the mitochondrial membrane, FAST interacts with BCL-X_L, suggesting that it may regulate Fas-induced apoptosis at the level of the mitochondrial membrane.

Materials and methods

Cells, antibodies, and reagents. COS7 cells and HeLa cells were obtained from the American Type Culture Collection. Cells were maintained in 10% FBS in DMEM. Anti-FAST-N is an affinity-purified rabbit polyclonal antibody that was obtained by immunizing a rabbit with a GST fusion protein encoding the first 100 amino acids of FAST (GST-FAST-N) [5]. The IgG fraction of the immune sera was affinity-purified in two steps: first, antibodies specific for GST were removed by passing the IgG fraction of the sera over a GST-Sepharose column. The eluate was then adsorbed to Sepharose-GST-FAST-N. Bound antibodies were eluted in 0.2 M glycine buffer (pH 3.0), the pH of the eluate was neutralized using unbuffered 1.5 M Tris, and the antibody fraction was dialyzed against phosphate-buffered saline (PBS). Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL).

Antibodies obtained from commercial sources include anti-hemagglutinin (HA) (murine mAb clone 16B12, IgG1; Berkeley Antibody), anti-FLAG (FL) (murine mAb clone, Sigma), anti-BCL-X_L (rabbit polyclonal, Santa Cruz Biotechnology), anti-Myc (rabbit polyclonal, Santa Cruz Biotechnology), anti-BCL-X_L (mouse monoclonal, Santa Cruz Biotechnology), anti-cytochrome *c* (BD-Biosciences-PharMingen, San Diego, CA), human anti-mitochondria M2 (ImmunoVision, Inc, Springdale, AR), anti-AIF (affinity-purified goat polyclonal IgG, Santa Cruz Biotechnology), and anti-lamin B (mouse monoclonal IgG1; Calbiochem–Novabiochem International, Cambridge, MA). Isotype-specific secondary antibodies (ML grade) were obtained from Jackson ImmunoResearch Labs, West Grove, PA. Mito-Tracker Red CH-H_2XRos was obtained from Molecular Probes (Eugene, OR). Hoechst dye # 33258 and other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Plasmid constructions. Full length recombinant FAST was subcloned into the pMT2-HA vector as described previously [5]. The N-terminus of FAST (FASTN), the C-terminus of FAST (FASTC), and its deletion mutants (FASTCΔBH3—aa 427–549; FASTCΔBH3ΔTM—aa 449–549; FASTCΔC20—aa 368–529; and FASTCΔC20/XLC20) were also subcloned into the *Eco*RI site of pMT2-HA by PCR using pMT2-HA-FAST as template. The sequences of all constructs were verified before use. pcDNA3-FL-BCL-X_L construct was a gift from Dr. Tom Chittenden (ImmunoGen). GFP-MAOB construct was kindly provided by Dr. C.W. Distelhorst (Case Western Reserve University and University Hospitals of Cleveland, Cleveland, OH).

Western blot analysis. Recombinant proteins were boiled in SDS sample buffer, resolved on 4–20% polyacrylamide gradient gels (Invitrogen, Carlsbad, CA), transferred to nitrocellulose, and revealed by autoradiography. Whole cells were solubilized in SDS sample buffer, then boiled and sonicated to shear DNA. Whole cell extracts were then separated on 4–20% gradient gels, transferred to nitrocellulose, and probed with the indicated antibodies using previously described methods [5].

Transfections and immunoprecipitations. Cells were transfected using Superfect (Qiagen, Valencia, CA) according to the manufacturer's instructions. Cells cultured in six-well plates (2×10^5 cells/well were plated 20 h before transfection) were exposed to DNA complexes for 2–3 h, then trypsinized and replated into parallel plates for both immnofluorescence (24-well plates containing 11-mm coverslips) and Western blotting or immunoprecipitation (12-well plates). For immunoprecipitations, proteins expressed in vivo were immediately extracted by solubilizing cells in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 1 mM EDTA, and 50 mM Tris, pH 7.8). Lysates were precleared with protein A–Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) in 150 μl NP-40 lysis buffer for 1 h. Subsequently, precleared lysates were incubated with the antibody-protein A–Sepharose complex in 150 μl NP-40 lysis buffer for 1 h, washed three times, and then boiled in SDS-sample buffer for separation on a 4–20% polyacrylamide gradient gel (Invitrogen). Following transfer to nitrocellulose membranes, the resolved proteins were analyzed by Western blots.

Immunofluorescence. Cells were plated on 11-mm glass coverslips in 24-well plates. At various time points after transfection, the cells were fixed in 2–4% paraformaldehyde in PBS for 10 min, immersed in −20°C methanol for 10 min, rinsed in PBS, and incubated in blocking buffer (5% normal horse serum in PBS) for 1 h before addition of primary antibodies. Cells were incubated with the indicated antibodies (anti-HA ascites fluid was used at 1/2000, anti-FAST-N was used at 0.1 μg/ml, anti-cytochrome *c* was used at 1/200, and human anti-mitochondrial M2 autoantibody was used at 1:1000) for 1–12 h, washed several times in PBS, and incubated for 1 h with diluted isotype-specific secondary antibodies (1/200 for FITC-labeled secondary antibodies; 1/2000 for Texas red-labeled or Cy3-labeled secondary antibodies) in blocking solution supplemented with Hoechst dye. Cells were viewed using a Nikon Eclipse 800 microscope equipped with epifluorescence and appropriate filter sets optimized to detect the specific fluorochromes were used. Images were digitally captured using a CCD-SPOT RT digital camera and then merged and compiled using Adobe Photoshop software.

Cell fractionation. COS7 or HeLa cells in log phase were harvested by scraping, centrifuged, and resuspended in detergent-free buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, and 10 mM Hepes, pH 7.5) containing protease inhibitors (PMSF, leupeptin, aprotinin, and benzamidine) and 14.0 μM 2-mercaptoethanol. Cells were disrupted by shearing (30× using a 26-guage needle) until cell breakage was about 90% as assessed by phase contrast microscopy. Crude nuclei (CN) were pelleted by centrifugation at 3500 rpm in a refrigerated microfuge for 5 min. Heavy membrane fractions containing mitochondria (referred to as the P20 fraction) were prepared by centrifuging the resulting supernatants at 14,000 rpm (20,000*g*) for 20 min, and light membrane/polysomes were prepared by centrifuging the resulting supernatants at 100,000*g* for 1 h to obtain both a pellet (P100) and soluble fraction (S100). All fractions were solubilized in 1% SDS, sonicated to shear DNA, and concentrated by acetone (60%) precipitation. Sample aliquots that were loaded onto the gels were adjusted to represent equivalent volumes relative to the initial lysate. Proteins were resolved on a 4–12% NuPAGE gel (Invitrogen), transferred to nitrocellulose, and probed using the indicated antibodies.

Results and discussion

Subcellular localization of endogenous FAST

Rabbit polyclonal antibodies raised against the N-terminus of FAST (hereafter referred to as anti-FAST-N) (Fig. 1A) were affinity-purified using immobilized recombinant FASTN. The specificity of this antibody was confirmed by immunoblotting analysis. Cos cells expressing HA-tagged forms of full-length FAST (wt FAST), FASTN or FASTC were subjected to Western blotting analysis using anti-HA (Fig. 1B, left panel) or anti-FAST-N (Fig. 1B, right panel). This analysis demonstrates that anti-HA detects both wild type and mutant FAST. Anti-FAST-N

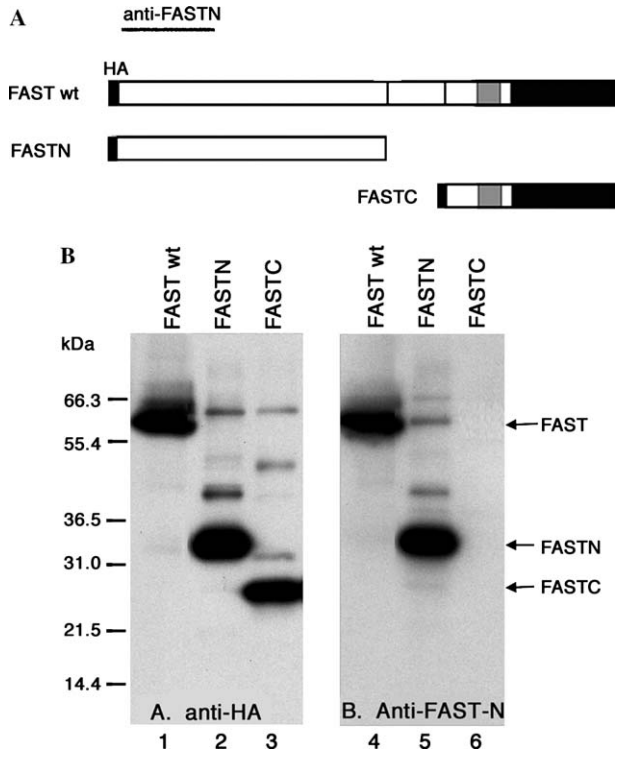

Fig. 1. Specificity of anti-FASTN. COS7 cells were transfected with vectors encoding HA-tagged full-length (FAST wt) or truncated (FASTN and FASTC) recombinant FAST. Cell lysates were subjected to Western blotting analysis with antibodies reactive with the HA tag (A) or FASTN (B). The relative migration of molecular size markers is shown at the left. The migration of full-length and truncated FAST is shown at the right.

detects wild type FAST and FASTN, but not FASTC. Although the concentration of endogenous FAST was too low to be detected in these lysates, polyclonal anti-FAST-N antibodies detect endogenous FAST in cellular fractions that are enriched for mitochondria (see Fig. 3).

The subcellular localization of endogenous FAST was determined using two-color immunofluorescence microscopy with anti-FASTN, anti-cytochrome *c*, anti-mitochondrial antigen M2, or Mito-tracker dye (Fig. 2). Anti-FASTN detects endogenous FAST in both the nucleus and the cytoplasm (Figs. 2A and E: red; Fig. 2I: green). The cytoplasmic staining produced by anti-FASTN has the morphological features of mitochondria. Dual labeling with anti-cytochrome *c* (Fig. 2B, green), anti-mitochondrial antigen M2 (Fig. 2F, green) or Mito-Tracker dye (Fig. 2J, red) confirms that FAST is localized to mitochondria, as shown in the merged images (Figs. 2D, H, and L). The background staining produced by preimmune sera is shown in Fig. 2M. A similar staining pattern was observed in HeLa cells (Fig. 2O, anti-FASTN; Fig. 2P, anti-cytochrome *c*; Fig. 2Q, Hoechst; and Fig. 2R, merge). In addition, a chicken polyclonal antibody raised against full-length recombinant FAST produced a similar staining pattern, supporting the specificity of this reagent (data not shown).

To confirm the mitochondrial localization of endogenous FAST, COS cells were subjected to differential centrifugation to obtain fractions enriched for nuclei and mitochondria. Aliquots from each fraction were separated by SDS–PAGE, transferred to nitrocellulose, and probed with antibodies reactive with mitochondrial proteins (cytochrome *c* and AIF), nuclear proteins (lamin B), and FAST (anti-FASTN). As shown in Fig. 3, FAST is included in the crude nuclear (CN) fractions that contain both nuclear and mitochondrial proteins. FAST is also found in the post-nuclear supernatants (PNS) that contain mitochondrial markers (i.e., cytochrome *c* and AIF). FAST, cytochrome *c*, and AIF (but not lamin B) sediment in the 20,000*g* pellets (P20) that are enriched in mitochondria. FAST, cytochrome *c*, and AIF are not found in 100,000*g* pellets (P100) or their supernatants (S100). The lamin B found in the soluble fraction (S100) is probably derived from mitotic cells. Alternatively, a fraction of lamin B might be extracted from the nucleus under these conditions. This analysis is consistent with the results obtained by immunofluorescent microscopy and suggests that endogenous FAST is a nuclear and mitochondrial protein.

Identification of a mitochondrial targeting domain

Sequence analysis revealed three different motifs that might tether the carboxyl terminus of FAST to the mitochondrial membrane (Fig. 4A): (1) a hydrophobic putative transmembrane motif (PTM) located between amino acids 427 and 448 predicted according to the hydrophobicity analysis calculated from the amino acid sequence of FAST, (2) a BCL2-homology domain 3-(BH3)-related sequence that might bind to BCL-2 family members at the mitochondrial membrane (Fig. 4B), and (3) a lysine and arginine-rich domain at the carboxyl terminus (MTD: **K**SYL**R**Q**K**LQALGL**R** WGPEGG) which, in analogy to monoamine oxidase B (MAOB) and cytochrome *b*5 [14,15], might function as a mitochondrial tethering domain. A series of truncation and substitution mutants were designed to determine whether any of these motifs tether the carboxyl terminus of FAST to the mitochondrial membrane (Fig. 4C).

When overexpressed in COS7 cells, full-length recombinant FAST is distributed between the nucleus and the cytoplasm, but it is not strongly associated with mitochondria (Fig. 5S). In contrast, the isolated carboxyl terminus of FAST (FASTC, Fig. 4C) has a subcellular distribution that is similar to endogenous FAST (Fig. 5A). Thus, the carboxyl terminus of FAST has a mitochondrial tethering domain that may be blocked by

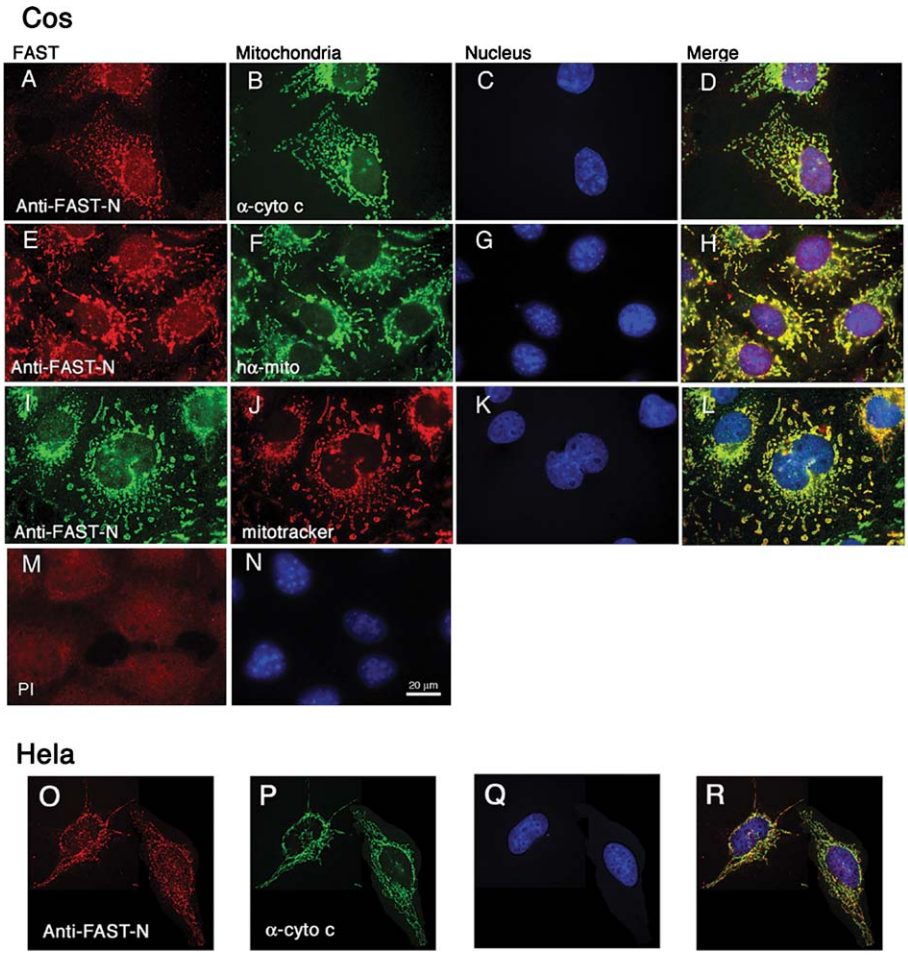

Fig. 2. Subcellular localization of endogenous FAST determined by immunofluorescence microscopy. Upper panel: COS7 cells were stained with affinity-purified anti-FAST-N (A and E: red; I: green) and counter-stained using mitochondrial markers (B: anti-cytochrome *c*, green; F: anti-mitochondrial M2, green; and J: mitotracker dye, red). The location of nuclei is revealed by Hoechst staining (C, G, K, and N) and the merged views are shown in panels D, H, and L. The background staining produced by preimmune sera is shown in panel M. Lower panel: HeLa cells were stained with anti-FAST-N (O: red) and counter-stained with anti-cytochrome *c* (P: green) and Hoechst (Q: blue). The merged view is shown in R. Size bar represents 20 μm.

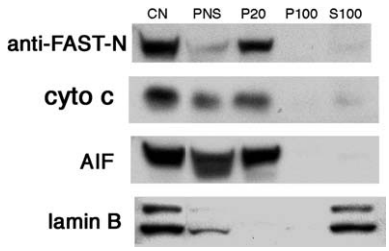

Fig. 3. Distribution of endogenous FAST in fractionated cell lysates. COS7 cells in log phase were lysed without detergent and fractionated using differential centrifugation. Fractions containing crude nuclei (CN), post nuclear supernatants (PNS), heavy membrane (P20), light membrane (P100), and soluble proteins (S100) were subjected to 4–12% NuPAGE and the proteins were transferred onto a nitrocellulose membrane. The membrane was then blotted with anti-FAST-N antibody, anti-cytochrome *c* antibody, anti-AIF antibody, and anti-lamin B antibody.

intramolecular interactions with the amino terminus of FAST. The mitochondrial localization of HA-FASTC was confirmed by dual immunofluorescence microscopy using anti-HA (Fig. 5A; green) and anti-human mitochondrial antibody (Fig. 5B; red). Both antibodies produce a punctate cytoplasmic fluorescence that partially overlaps in the merged view (Fig. 5C; yellow). As a positive control for mitochondrial localization, COS7 cells were transfected with GFP fused to the lysine/arginine-rich mitochondrial targeting motif of monoamine oxidase B (MAOB) [15] (Figs. 5D–F). Truncation mutants lacking the BH3-related domain alone (FASTCΔBH3: Figs. 5G–I) or the BH3-related domain together with the hydrophobic, putative transmembrane domain (FASTCΔBH3ΔTM: Figs. 5J–L) retained their mitochondrial association. In contrast, a FASTC truncation mutant lacking the last 20 amino acids is no longer tethered to mitochondria (FASTCΔC20: Figs. 5M–O), indicating that this lysine and arginine-rich domain is required for mitochondrial tethering. When the mitochondrial tethering domain of BCL-X_L is appended to the carboxyl terminus of FASTCΔC20, mitochondrial localization is restored (Figs. 5P–R).

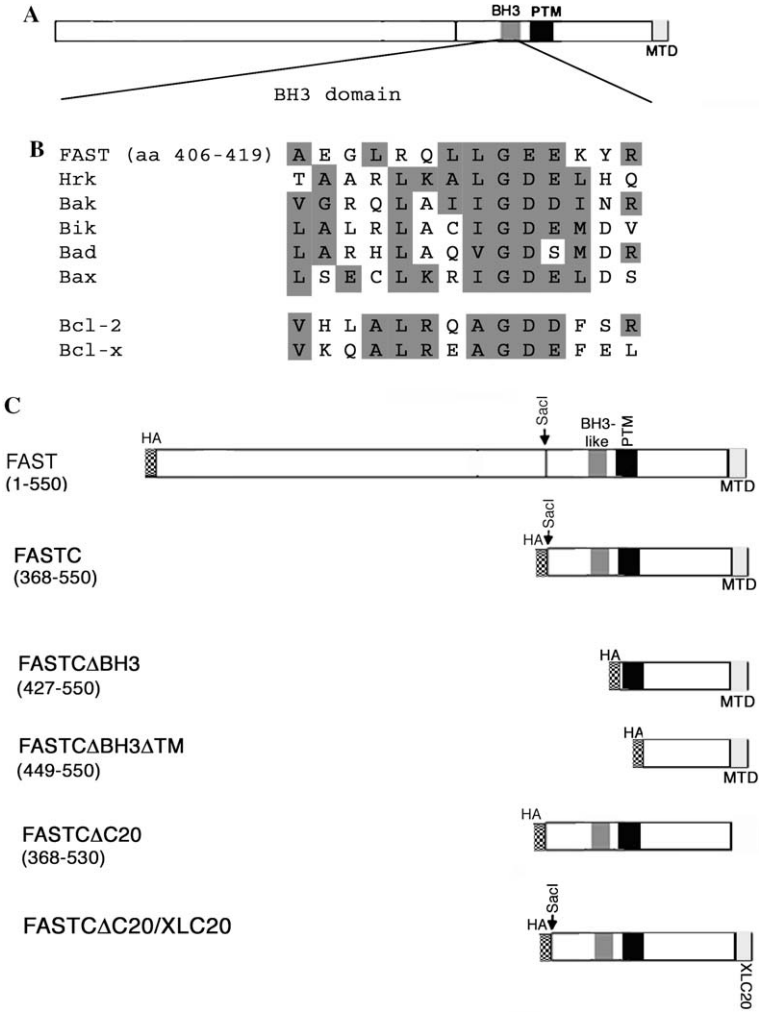

Fig. 4. Structural features of FAST. (A) Schematic depiction of the human FAST protein. The relative location of the putative transmembrane domain (PTM), the BH3-related domain, and the arginine/lysine-rich mitochondrial tethering domain (MTD) is indicated. (B) Alignment of amino-acid residues in the BH3-homology regions of BCL-2 family members and human FAST. Human FAST, GenBank Accession No. AF168682; human HRK, U76376; human BAK, U23765; human BIK, U34584; human BAD, AF021792; human BAX, U23765; human BCL-2, M14745; and human BCL-X, Z23115. Amino acids in human FAST that match with those in other BCL-2 family members are shaded. (C) Schematic depiction of the human FAST protein and deletion mutants used in this study.

FAST interacts with BCL-X_L

We used co-immunoprecipitation analysis to determine if the BH3-like motif binds to BCL-X_L. COS cells were co-transfected with the indicated HA-FAST variants together with FLAG-BCL-X_L. The expression of the HA-FAST variants and FLAG-BCL-X_L was confirmed by immunoblotting with anti-HA and anti-FLAG antibodies (Fig. 6A, lanes 1–6). Cell lysates were immunoprecipitated using anti-HA, separated on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with rabbit anti-BCL-X_L. This analysis reveals that BCL-X_L binds to recombinant HA-FAST (Fig. 6B, lane 2) and recombinant HA-FASTC (Fig. 6B, lane 3). A FASTC truncation mutant lacking amino acids 368–426 (including the BH3-related motif: amino acids 406–419) does not bind to BCL-X_L (Fig. 6B, lane 4). HA-FASTCΔC20, a mutant that is not tethered to mitochondria, also does not bind to BCL-X_L (Fig. 6B, lane 5), suggesting that BCL-X_L binds to the carboxyl terminus of FAST only when tethered to mitochondria. Consistent with this conclusion, HA-FASTCΔC20/X_LC20, the mutant that substitutes the last 20 amino acids of BCL-X_L (i.e., its mitochondrial tethering motif) for the last 20 amino acids of FAST, regains BCL-X_L binding (Fig. 6B, lane 6).

To confirm the physiological significance of the interaction between FAST and BCL-X_L, we used co-immunoprecipitation analysis to show that endogenous FAST interacts with endogenous BCL-X_L. HeLa cell lysates were subjected to differential centrifugation to obtain P20 fractions that are enriched in mitochondria (Fig. 3). The P20 fractions were immunoprecipitated with either rabbit anti-BCL-X_L antibody or a matched

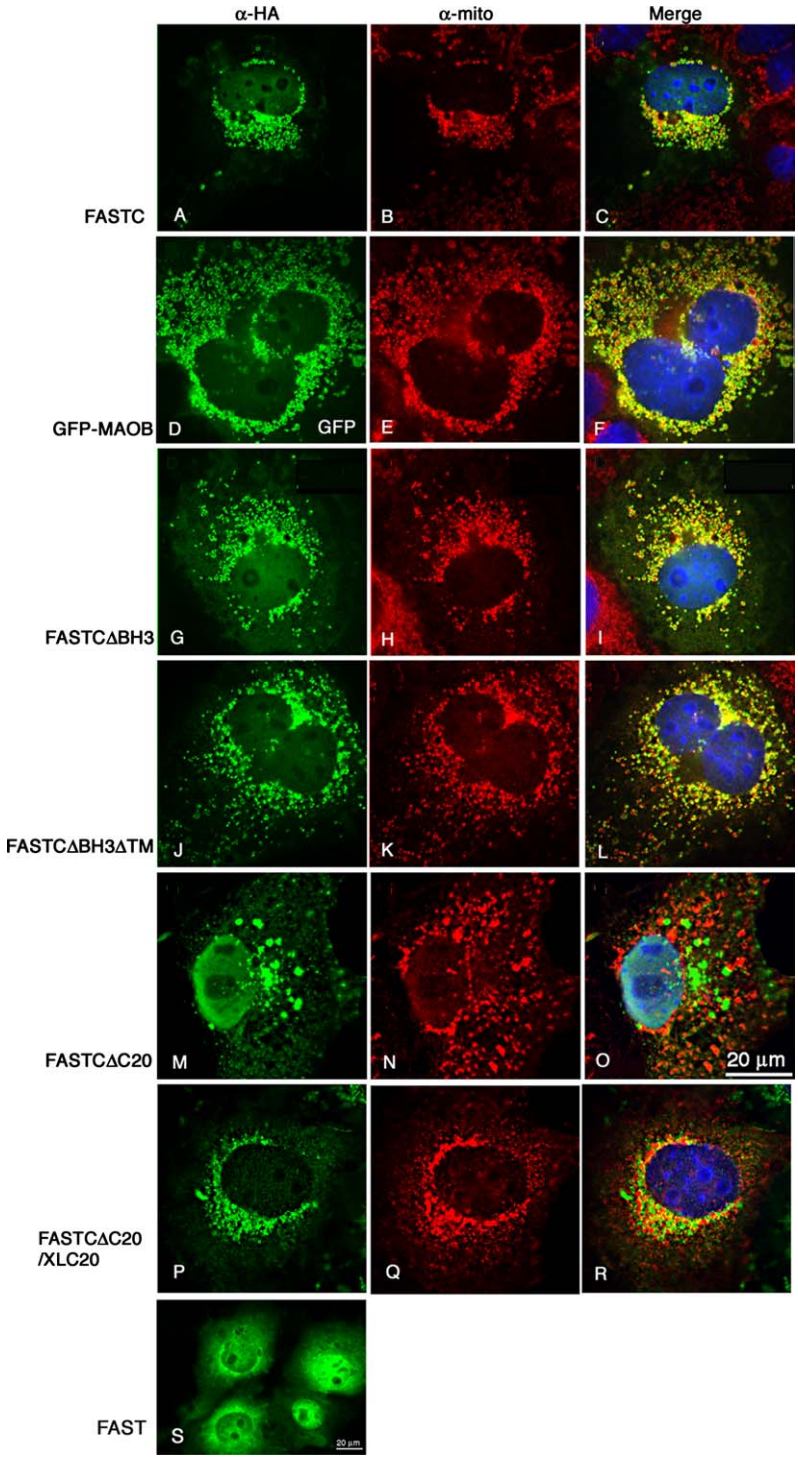

Fig. 5. Identification of a mitochondrial tethering domain. The indicated COS transfectants were fixed and processed for immunofluorescence microscopy using anti-HA (panels A, G, J, M, P, and S, green) and anti-mitochondria (middle panels, red). GFP autofluorescence is shown in panel D (green). The merged views are depicted at the far right. Size bar represents 20 μm.

control antibody (rabbit anti-Myc). Immunoprecipitates were then subjected to Western blotting analysis with anti-FAST-N and mouse anti-BCL-X_L. Immunoprecipitates prepared using anti-BCL-X_L, but not anti-myc, include both BCL-X_L (Fig. 6C, lower panel, lane 2) and FAST (Fig. 6C, upper panel, lane 2). This experiment reveals that endogenous FAST interacts with endogenous BCL-X_L in subcellular fractions enriched for mitochondria.

Our results show that FAST is tethered to mitochondria by its lysine and arginine-rich carboxyl terminus. At the mitochondrial membrane, FAST interacts

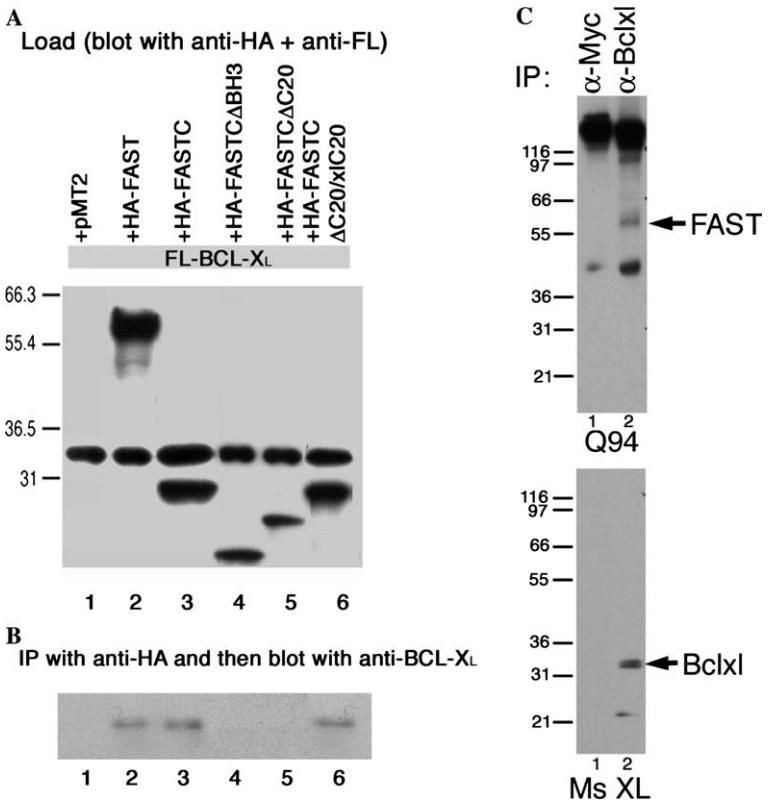

Fig. 6. Co-immunoprecipitation analysis of FAST and BCL-X_L. (A) COS7 cells were co-transfected with FLAG-BCL-X_L together with either vector control (lane 1), or the indicated FAST truncation mutants (see Fig. 4C) (lanes 2–6). Cell lysates were solubilized, separated on a 4–20% SDS–polyacrylamide gel, transferred to nitrocellulose, and simultaneously probed with anti-FLAG and anti-HA to assess the expression of the input proteins. (B) Cell lysates corresponding to panel A were immunoprecipitated with anti-HA, separated by SDS–PAGE, transferred to nitrocellulose, and probed with anti-BCL-X_L. (C) P20 fractions from Hela cells were immunoprecipitated with rabbit anti-BCL-X_L antibody, separated by SDS–PAGE, transferred to nitrocellulose, and probed with anti-FAST-N antibody (upper panel). The same blot was stripped and re-probed with mouse anti-BCL-X_L antibody. Molecular size markers are shown on the left side of the gel.

with BCL-X_L. The BCL-X_L binding site includes a sequence motif that is related to the BH3 domain, an amphipathic α helix found in a family of proteins that promote apoptotic cell death [16]. The BH3-only proteins have been proposed to function as sensors of environmental stress [17]. Stress-induced modifications (e.g., phosphorylation and proteolytic cleavage) direct these proteins to the mitochondrial membrane, where they interact with BCL-2 family members to bring about a dissipation of mitochondrial membrane potential and release of apoptotic effector proteins (e.g., cytochrome *c*, AIF, and SMACK/DIABLO) [18]. Unlike the BH3-only proteins, FAST does not promote apoptosis, suggesting that it interacts with BCL-2 family members in a fundamentally different way.

The ability of FAST to interact with BCL-X_L at the mitochondrial membrane suggests that it may participate in the regulation of Fas-induced apoptosis. Interestingly, FAST is overexpressed in patients with a variety of immune mediated inflammatory diseases, including rheumatoid arthritis, systemic lupus erythematosus, and diabetes [19]. Overexpression of FAST could contribute to these autoimmune syndromes by regulating Fas induced apoptosis, a prelude to autoimmune disease in several experimental systems [20].

Acknowledgments

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