Tara, a novel F-actin binding protein, associates with the Trio guanine nucleotide exchange factor and regulates actin cytoskeletal organization

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Accepted 13 November 2000
Journal of Cell Science 114, 389-399 © The Company of Biologists Ltd

SUMMARY

Reorganization of the actin cytoskeleton is essential to numerous cellular processes including cell locomotion and cytokinesis. This actin remodeling is regulated in part by Rho family GTPases. Previous studies implicated Trio, a Dbl-homology guanine nucleotide exchange factor with two exchange factor domains, in regulating actin cytoskeleton reorganization, cell motility and cell growth via activation of Rho GTPases. Trio is essential for mouse embryonic development and Trio-deficiency is associated with abnormal skeletal muscle and neural tissue development. Furthermore, genetic analyses in Caenorhabditis elegans and Drosophila demonstrate a role for trio-like genes in cell migration and axon guidance. Herein we characterize a novel Trio-binding protein, Tara, that is comprised of an N-terminal pleckstrin homology domain and a C-terminal coiled-coil region. Trio and Tara associate as assessed by the yeast interaction-trap assays and mammalian co-immunoprecipitation studies. Ectopically expressed Tara localizes to F-actin in a periodic pattern that is highly similar to the pattern of myosin II. Furthermore, a direct interaction between Tara and F-actin is indicated by in vitro binding studies. Cells that transiently or stably overexpress Tara display an extensively flattened cell morphology with enhanced stress fibers and cortical F-actin. Tara expression does not alter the ability of the cell to attach or to initially spread, but rather increases cell spreading following these initial events. Tara stabilizes F-actin structures as indicated by the relative resistance of Tara-expressing cells to the F-actin destabilizer Latrunculin B. We propose that Tara regulates actin cytoskeletal organization by directly binding and stabilizing F-actin, and that the localized formation of Tara and Trio complexes functions to coordinate actin remodeling.

Key words: Rho, Rac, Dbl homology, Trio, Actin-binding protein, Pleckstrin homology, Coiled-coil

INTRODUCTION

The actin cytoskeleton is a dynamic structure that is extensively remodeled in migrating and proliferating cells. This actin remodeling, which involves the assembly and disassembly of filamentous (F)-actin, is regulated by diverse actin binding proteins, including proteins that function in F-actin formation, F-actin stabilization and destabilization, generation of supramolecular cytoskeletal structures, and regulation of cell contraction (Ayscough, 1998; Borisy and Svitkina, 2000; Cooper and Schafer, 2000). In migrating cells, actin polymerization drives the formation and extension of the lamellipodial leading edge, while the activation of myosin motors, particularly myosin II, is largely responsible for creating the traction force needed for cell movement (Condeelis, 1993; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). In non-muscle cells, myosin contractile activity is regulated mainly by serine/threonine phosphorylation. Phosphorylation of the regulatory myosin light chain increases myosin activity, as well as the association of myosin with F-actin, and leads to cell contraction, whereas phosphorylation of the non-muscle myosin II heavy chain is correlated with loss of cell contractility (Daniels and Bokoch, 1999; Sanders et al., 1999; van Leeuwen et al., 1999). Key upstream regulators of actomyosin-based contraction are members of the Rho family of GTPases, including Rho and Rac (Hall, 1998; Van Aelst and D’Souza-Schorey, 1997). Rac activation leads to membrane ruffling and cell spreading concomitant with loss of contractility and reduced cell adhesion to the substratum (Nobes and Hall, 1995; van Leeuwen et al., 1999), whereas Rho activation leads to bundling of F-actin and myosin II into stress fibers, formation of focal adhesions, and increased cell contractility (Amano et al., 1996; Chrzansowska-Wodnicka and Burridge, 1996; Kimura et al., 1996; Nobes and Hall, 1995). These processes occur in a spatial and temporal sequence in migrating cells, under the control of the coordinated activation of Rho GTPases (Hall, 1998; Van Aelst and D’Souza-Schorey, 1997).

Several Rho GTPase effector targets have been identified that regulate actomyosin-based contractility (Aspenstrom, 1999; Schoenwaelder and Burridge, 1999). Rho kinase increases myosin II activity and cell contraction by inhibiting phosphatase activity towards the myosin light chain (MLC) (Kimura et al., 1996). Rho kinase also increases myosin
activity by directly phosphorylating the MLC (Amano et al., 1996). Rho also mediates formation of stress fibers by activating Dia1, which may localize profilin-bound actin to sites of F-actin assembly (Watanabe et al., 1999). The target of Rac and Cdc42 GTPases, p21 activated kinase (Pak), negatively regulates MLC kinase (MLCK) activity (Daniels et al., 1998; Manser et al., 1997; Sells et al., 1997). Furthermore, Rac activation leads to myosin heavy chain (MHC) phosphorylation, perhaps mediated by Pak and Ca2+/calmodulin kinases (van Leeuwen et al., 1999). Thus, Rac activation leads to reduced MLC phosphorylation, increased MHC phosphorylation and loss of cell contractility, whereas Rho activation controls myosin activity by increasing MLC phosphorylation levels.

Rho GTPases are activated by Dbl-homology guanine nucleotide exchange factors (DH-GEFs) (Cerione and Zheng, 1996; Zohn et al., 1998). Among the DH-GEF family, Trio is unusual in that it contains two GEF domains, each specific for different Rho family GTPases both in vitro and in vivo (Bellanger et al., 1998; Blangy et al., 2000; Debat et al., 1996; Seipel et al., 1999). Trio regulates actin cytoskeletal reorganization and cell growth and migration, possibly by integrating diverse signals needed to coordinate actin cytoskeleton remodeling involved in cell migration and growth (Seipel et al., 1999). Absence of Trio causes mouse embryonic lethality associated with abnormal development of skeletal muscle and neural tissues (O’Brien et al., 2000). In C. elegans, the Trio-like unc-73 gene is necessary for both cell migration and axon guidance (Steven et al., 1998). Drosophila Trio was shown to regulate axon guidance, and is functionally linked to Rac, Pak, Abi protein tyrosine kinase, DLAR transmembrane protein tyrosine phosphatase, and the adaptor proteins Dock and Ena (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000). Mammalian Trio, but not C. elegans UNC-73 or Drosophila Trio, contains a kinase domain that is most closely related to Ca2+/calmodulin dependant kinases, but its substrates remain to be defined (Debat et al., 1996). A mammalian neuronal-specific Trio-like protein, termed Kalirin, may function in the secretion of bioactive peptides (Alam et al., 1997; Johnson et al., 2000; Mains et al., 1999).

To learn more about Trio function, we screened for proteins that interact with a portion of Trio that includes the N-terminal GEF-D1 domain. Herein, we describe and characterize the Trio-binding protein Tara, which consists of an N-terminal pleckstrin homology (PH) domain and a C-terminal coiled-coil region. Ectopically expressed Tara localizes in a periodic fashion to filamentous (F)-actin and enhances cell spreading, suggesting that Tara regulates cell morphology. HeLa cell lines stably expressing Tara are more resistant to Latrunculin B-mediated F-actin destabilization. In vitro binding studies indicate a direct interaction between F-actin and Tara. Based on these results, we propose that Tara regulates actin cytoskeletal organization by stabilizing F-actin.

**MATERIALS AND METHODS**

**Interaction-trap assay**

Plasmid DNAs and yeast strains used for the interaction trap assay were provided by Dr Roger Brent and colleagues and used essentially as described (Gyuris et al., 1993; Serra-Pages et al., 1995). The human fibroblast cell WI-38 (ATCC CCL 75) cDNA library used for the interaction-trap assay was kindly provided by Dr Claude Sardet. The extent of the Trio peptide (amino acids 1207-1715) fused to the LexA peptide is schematically shown in Fig. 1A. The yeast strain EGY48 (MATa trpl1 ura3 his3 LEU2::pLexAop6-LEU2), containing the plasmid pSH18-34, which includes the reporter lacZ gene under the control of a modified Gall promoter, was used as host for all interaction-trap assays.

**Plasmid constructions and DNA sequencing**

cDNA clones encoding Tara were isolated from a skeletal muscle λgt10 library (Clontech Laboratories, Palo Alto, CA) using standard techniques and sequenced using the diodeoxy method of sequencing. The amino acids of Tara or Trio encoded by various expression vectors or interaction-trap baits are given in Figs 1, 2. The following mammalian expression vectors were used: pMT.2, pMT.HA, pMT.GST, and pSP.SRα.2-HA. pMT.HA and pSP.SRα.2-HA encode initiator methionine codons followed by a HA epitope tag sequence immediately upstream of the cloning site (Serra-Pages et al., 1995), and pMT.GST encodes an initiator methionine codon followed by the GST coding sequence, followed by a cloning site. pMT.Tara encodes full-length Tara (amino acids 1-593), pMT.GST.H2 encodes a GST fusion protein containing Tara amino acids 13-385, pMT.GST.Tara encodes a GST fusion protein containing amino acids 1-593, pGEX.Tara encodes a GST fusion protein containing amino acids 13-385, pSP.SRα.Tara encodes full-length Tara (amino acids 1-593), and pSP.SRα.Tara.HA encodes full-length Tara (amino acids 1-593) with the HA epitope sequence located at the C-terminus. pMT2.HA.Trio encodes HA-tagged full-length Trio (amino acids 1-3038; Seipel et al., 1999). The human Tara cDNA sequence (GenBank Accession Number AF281030) is essentially identical to the previously deposited sequence, CAB42898.

**Cell labeling, protein analysis and protein purification**

Cell proteins were metabolically labeled with [35S]methionine and [35S]cysteine (DuPont NEN, Boston, MA) in L-methionine- and L-cysteine-free RPMI media (Life Technologies, Gaithersburg, MD) containing 2% dialyzed fetal calf serum (FCS), 2 mM Hepes (pH 7.2) for 4 hours at 37°C. Following labeling, cells were washed in PBS, and lysed in NP-40 lysis buffer (1% Nonidet P-40 (NP-40), 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin and 10 μg/ml aprotinin. Insoluble material was removed from the lysates by centrifugation in a microcentrifuge. Cell lysates were then precleared with 25 μl Protein G-Sepharose slurry (Pharmacia) for 1 hour. For GST pull-downs, 0.5 ml of the lysates were incubated with 25 μl glutathione-Sepharose beads for 1 hour at 4°C. Following washing of the beads five times with PBS-0.1% NP-40, samples were separated by SDS/PAGE. For immunoprecipitations, ~1 μg anti-Tara.397 mAb, ~1 μg control isotype-matched mAb or 1 μl of ascites fluid of anti-hemagglutinin mAb 12CA5 and 25 μl Protein G-agarose slurry were added per 0.5 ml precleared lysate for 1 hour. Immunoprecipitates were then washed with buffer containing 0.1% NP-40, 0.05% SDS, 150 mM NaCl and 50 mM Tris-HCl (pH 8.0). Immunoprecipitated proteins were analyzed by using SDS-PAGE analysis with reducing conditions, followed by autoradiography. Immunoblotting analysis was carried out using standard techniques. Proteins from cell lysates or immunoprecipitated proteins from non-labeled cells were resolved by SDS-PAGE, then transferred to nitrocellulose and immunoblots were probed with the anti-Tara.27 mAb, the anti-HA mAb HA 11, or the anti-GST mAb 112. Immunoblots were developed with Immunopure Protein A/G-horseradish peroxidase (Pierce, Rockford, IL) and the chemiluminescence reagent, luminol, essentially as described by the supplier (DuPont NEN). For F-actin co-sedimentation assays, GST-Tara protein was produced in COS7 cells using transient transfections. Cells were lysed in 1% NP40 buffer, and then extracts were incubated.
with glutathione-Sepharose beads for 1 hour at 4°C. Following the incubation, glutathione-Sepharose beads were recovered, washed six times with PBS-0.1% NP-40. Tara protein was released by thrombin protease digestion for 1 hour at room temperature, and beads were removed by centrifugation. GST-Tara.13-385 fusion protein was produced in Escherichia coli, and the fusion protein was recovered by glutathione-Sepharose chromatography. Tara.13-385 protein was released by thrombin digestion using conditions similar to those described above. The amount of purified Tara.13-385 used for the F-actin co-sedimentation assay (~0.5 μg) was approximated using bovine serum albumin (BSA) standards. The full-length Tara protein was highly susceptible to protease degradation in E. coli and could not be produced in sufficient quantities.

Northern blot analysis

Northern blot analysis was carried out using a human multiple tissue northern blot (Clontech) and a multiple cell line blot (Clontech), both of which contain 2 μg of poly(A)⁺ selected RNA from different human tissues or cell lines per lane, and were hybridized with a random primed [³²P]-α-dCTP labeled Tara cDNA probe (nucleotides 222-1779) according to the manufacturer’s instructions.

Monoclonal antibodies

The following mAbs were used for immunoprecipitations, immunoblotting and immunofluorescence: anti-Tara.397 and anti-Tara.27 mAbs (see below), anti-Trio.18 mAB (see below), anti-HA 12CA5 (Boeringer Mannheim, Indianapolis, IN), anti-HA HA 11 (BabCO, Richmond, CA), rabbit anti-non-musyo II heavy chain BT561 (Biomedical Technologies, Stoughton, MA), anti-GST 112 (Immunogen Inc., Cambridge, MA), anti-actin (Sigma, St. Louis, MO), anti-vimentin LN-6 (Sigma), anti-tubulin DM1A (Sigma) or anti-α-actinin BM75.2 (Sigma). To generate the anti-Tara mAbs, mice were immunized with E. coli-derived GST-Tara.13-385 fusion protein. To this end, E. coli NMS22 cells were transfected with the pGEX-Tara.13-385 expression plasmid, and GST-Tara.13-385 fusion protein was purified from bacterial lysates using Glutathione Sepharose 4B (Pharmacia) chromatography. The resulting bead-protein complexes were used to immunize BALB/c mice. Splenocytes from immunized mice were fused to NS-1 myeloma cells and HAT-resistant hybridomas were initially selected using enzyme-linked immunosorbant assay (ELISA). The anti-Trio.18 mAB was generated in a similar manner using as antigen GST-Trio.1848-2438 (Trio amino acids 1848-2438).

F-actin co-sedimentation assays

F-actin co-sedimentation assays were done essentially as described by the manufacturer (Cytoskeleton, Denver, CO). Briefly, protein preparations were incubated with 40 μg freshly polymerized actin (F-actin) for 1 hour at RT. Following incubation, the protein plus F-actin solution was subjected to centrifugation (160,000 g) to pellet F-actin and protein bound to F-actin. After solubilization of the pellet fraction in a volume equal to the initial incubation volume, equal volumes of the pellet and supernatant fractions were analyzed by SDS-PAGE.

GenBank Accession Number

The GenBank Accession Number for Tara is AF281030.

RESULTS

Trio interacts with Tara, a broadly expressed PH domain-containing coiled-coil protein

Trio is a complex protein containing two DH-GEF domains, a PSK domain, and multiple auxiliary domains (Fig. 1A) (Debant et al., 1996). To learn more about the function of Trio, we performed an interaction-trap screen to identify Trio-binding proteins using a portion of Trio, termed TGD1, containing amino acids 1207-1715 (Fig. 1A) as bait to screen GenBank. This region of Trio was chosen as bait because ectopic expression of TGD1, which includes the GEF-D1, PH-D1 and SH3-D1 domains, regulates actin cytoskeletal organization, cell growth and cell migration (Seipel et al., 1999). A cDNA designated H2 was isolated, which encodes a peptide that efficiently bound the Trio.1207-1715 bait, but did not bind the Trio.1848-2438 bait (see Fig. 1A). To determine whether the H2 peptide also bound Trio in mammalian cells, co-precipitation studies were performed using extracts prepared from [³⁵S]methionine and [³⁵S]cysteine metabolically labeled cells (Fig. 1B). To this end, a glutathione S-transferase (GST)-tagged H2 peptide (GST-H2) was transiently expressed in COS7 cells, together with various hemagglutinin (HA)-tagged Trio deletion constructs (see Fig. 1B). GST-H2 was then recovered using glutathione
Fig. 1. Identification of a Trio interacting peptide, H2. (A) Isolation of the Trio-binding peptide H2 using the interaction-trap assay. The structure of the 3038 amino acid Trio protein is shown schematically at the top of the figure and below is shown the Trio.1207-1715 interaction bait, as well as the control Trio.1848-2438 bait. The interaction-trap assay for protein-protein interaction was as described in the Materials and Methods. SP, spectrin like domain; GEF-D, guanine nucleotide exchange factor domain; PH, pleckstrin homology domain; SH3-D, src-homology 3 domain; Ig, immunoglobulin-like domain; PSK, serine/threonine kinase. (B) Co-precipitation of Trio and the Trio interaction-trap assay positive clone, H2, from mammalian cells. The GST-H2 fusion construct and various HA-tagged Trio deletion constructs (the extent of the Trio sequences contained in the constructs is given at the top of the figure) were co-expressed in COS7 cells. Following cell labeling with [35S]methionine and [35S]cysteine, cell extracts were prepared, GST-H2 was recovered, and associated labeled proteins were analyzed by SDS-PAGE and autoradiography (lanes 1-5). Control anti-HA immunoprecipitated proteins from whole cell lysates are shown in lanes 6-10. The expected position of the various Trio deletion constructs are indicated by asterisks. Molecular mass standards in kDa are shown on the left of the figure, and the position of the GST-H2 fusion protein is indicated by an arrow.

To gain insight into its possible functions, Tara was ectopically expressed (Fig. 1B, lanes 6-10). Based on the interaction-trap assay and the co-precipitation studies, Trio amino acids 1207-1551, comprising the GEF-D1 and PH-D1 domains, are sufficient for the H2 and Trio interaction. Taken together, these results indicate that the H2 peptide binds Trio in mammalian cells, as well as in the interaction-trap assay.

The primary structure of the 593 amino acid H2 gene product was deduced following sequence determination of overlapping cDNA clones (Fig. 2A). This full-length gene product was designated Tara for Trio-associated repeat on actin (see below). The original interaction-trap H2 peptide contains Tara amino acids 13-385. A search of protein databases (GenBank, PIR, Swiss-Prot and EMBL) revealed that the Tara sequence was unique, but had a high degree of similarity with p116RIP (46% overall amino acid identity; Gebbink et al., 1997), and a lower level of sequence identity with a number of proteins that either contain pleckstrin homology (PH) domains or coiled-coil regions (Lupas, 1996; Musacchio et al., 1993; Shaw, 1997). The Tara C-terminal half was predicted to form a coiled-coil structure using an algorithm to detect coiled-coil regions (Fig. 2B; Lupas et al., 1991). Indeed, co-precipitation studies using tagged versions of Tara demonstrated that Tara amino acids 333-416, which are part of the coiled-coil region, are required for dimerization (data not shown). The Tara PH domain had the highest sequence similarity to the PH domains of p116RIP (Gebbink et al., 1997), the Etk/Bmx protein tyrosine kinase (Qiu et al., 1998) and bovine myosin X (Yonezawa et al., 2000; Fig. 2C). p116RIP was shown to inhibit RhoA-stimulated contractility and to promote neurite outgrowth in N1E-115 cells, and was isolated by virtue of its binding to RhoA (Gebbink et al., 1997). The overall predicted structure of Tara, with its N-terminal PH domain and C-terminal coiled-coil region, is shown schematically in Fig. 2B.

To characterize Tara protein, anti-Tara monoclonal antibodies, termed anti-Tara.27 and anti-Tara.397, were developed using GST-Tara.13-385 fusion protein as antigen. Immunoblotting experiments using the anti-Tara.27 mAb and extracts prepared from HeLa cells demonstrated that endogenous Tara has an apparent molecular mass of about 80,000 even though the expected molecular mass of Tara is only 68,000 (Fig. 2E, lane 2). Because endogenous Tara co-migrated with COS cell-derived recombinant full-length Tara (Fig. 2E, lane 3), this discrepancy was due to the electrophoretic properties of Tara, and not the absence of additional coding sequence. Attempts to co-immunoprecipitate endogenous Trio and Tara were unsuccessful, possibly because of the low level of Tara and Trio expression in diverse cell types (data not shown). However, co-precipitation analysis of HeLa cells ectopically expressing HA-tagged full-length Tara (HA-Tara) and HA-Trio.1118-1919 demonstrated that full-length Tara, like the H2 peptide, bound Trio (Fig. 2F). Tara neither modulated Trio GEF-D1 activity in in vitro GEF assays nor bound RhoA or Rac1 (data not shown).

Northern blot analysis demonstrated that Tara mRNA is broadly expressed among human tissues and cell lines, with the highest relative expression levels seen in heart and placenta (Fig. 3). Of the cell lines tested, HeLa, SW-480 and G361 cells contained the largest amount of Tara mRNA. The most abundant Tara mRNA species in all of the samples had a length of about 2.8 kb, whereas minor mRNA species between 2.0 to 4.4 kb were present in several of the mRNA samples (Fig. 3).

Ectopically expressed Tara enhances cell spreading and partially co-localizes with Trio

To gain insight into its possible functions, Tara was ectopically
Tara binds F-actin and enhances cell spreading

As seen in Fig. 4, Tara expression (red, part A) enhanced spreading with cells appearing larger compared to control HeLa cells that were transfected with a plasmid encoding green fluorescent protein (gfp; green, part B), suggesting that Tara regulates cytoskeletal organization and cell contraction. The ability of Tara to enhance cell spreading was also seen in other cell types.

Fig. 2. Characterization of the Trio-binding protein Tara. (A) Deduced 593 amino acid sequence of the human Tara protein based on the nucleotide sequence of Tara/H2 cDNA clones (GenBank Accession Number AF281030). The standard one letter amino acid code is used. (B) The C-terminal region of Tara is predicted to form a coiled-coil structure. Coiled-coil probabilities (x-axis) were calculated for each residue (y-axis) using a window of 28 amino acids (Lupas et al., 1991). (C) The N-terminal region of Tara contains a PH domain. Shown is an alignment of the Tara PH domain sequence with the PH domain sequences of p116RIP (amino acids 381-481; Gebbink et al., 1997), Etk/Bmx (amino acids 28-133, Qiu et al., 1998) and bovine myosin X (amino acids 1201-1343; GenBank Accession number T18519). At the bottom of the alignment are indicated consensus amino acid residues, with residues conserved in three out of four sequences shown in lower case, and absolutely conserved residues shown in upper case. (D) The overall structure of Tara is schematically shown with the relative length of the N-terminal PH domain and the C-terminal coiled coil region. (E) Tara has an apparent molecular mass of 80 kDa. Shown is an immunoblot analysis of endogenous Tara immunoprecipitated from HeLa cells (lane 2) or of recombinant Tara immunoprecipitated from COS7 cells (lane 3). A control Ig immunoprecipitate is shown in lane 1. Molecular mass standards in kDa are shown on the left of the figure, and the positions of the HA-Tara and Ig proteins are indicated on the right of the panel. (F) Tara binds Trio.1118-1919 in mammalian cells. Shown is a SDS-PAGE analysis demonstrating co-immunoprecipitation of HA-Tara and HA-Trio.1118-1919. COS7 cells were transfected with vectors encoding either HA-Tara and HA-Trio.1118-1919 (lanes 1 and 3) or HA and HA-Trio.1118-1919 (lanes 2 and 4). Following cell labeling with [35S]methionine and [35S]cysteine, cell extracts were prepared, and anti-Trio mAb (lanes 1 and 2) or control anti-HA mAb (lanes 3 and 4) precipitations were performed. The 97 kDa molecular mass standard is shown on the left of the figure, and the positions of the HA-Trio.1118-1919 and HA-Tara proteins are indicated by arrows.
cell types, including MCF7 and N1E-115 cells (data not shown). The Tara protein itself was observed in a filamentous pattern, as well as in vesicular structures (Fig. 4A). A similar staining pattern was observed in other cell types (data not shown). Because the size and number of Tara-positive vesicular structures correlated with Tara expression levels, the vesicular localization of Tara might have been an overexpression artifact. Attempts to visualize endogenous Tara in HeLa cells and a number of other cell types was unsuccessful, possibly due to limited expression levels of endogenous Tara.

The association between Tara and Trio was also tested by immunofluorescence co-localization studies using HeLa cells ectopically expressing full-length Trio (amino acids 1-3038) and Tara (Fig. 4). Trio expression (green) alone caused the cells to spread with a circular, pancake-like morphology (Fig. 4C) (Seipel et al., 1999). Trio was present throughout the cell and concentrated at membrane ruffles (Fig. 4C). In cells expressing both Trio (red, Fig. 4D) and Tara (green, Fig. 4E), there was partial co-localization of Trio and Tara, particularly at membrane ruffles (yellow/orange, Fig. 4F). Cells expressing both Trio and Tara had the Trio-mediated pancake-like appearance, with Tara localization seen in the filamentous pattern as well as in vesicular structures. Possibly, Tara and Trio interact at specific sites such as membrane ruffles to regulate actin cytoskeletal reorganization. However, given the limited co-localization of ectopically expressed Tara and Trio, a more detailed study is required to elucidate the physiological relevance of this interaction.

**Tara is associated with F-actin**

The filamentous expression pattern of Tara suggested that Tara may localize to F-actin. To test this possibility, HeLa cells ectopically expressing Tara, or control HeLa cells, were stained for F-actin using rhodamin-conjugated phalloidin, as well as for Trio using the anti-Tara.397 mAb. As seen in Fig. 5, the F-actin staining pattern (red, part A) and the Trio staining pattern (green, part B) were largely overlapping (orange-yellow, part C). Indeed, higher magnification images of the F-actin and Trio staining (Fig. 5D-F) highlighted the co-localization of F-actin and Trio. As observed in Fig. 4, ectopic Trio expression caused the HeLa cells to display enhanced spread morphology compared with the mock transfected HeLa cells. A similar filamentous staining pattern was observed in other cell types (data not shown), as well as in HeLa cells expressing HA-tagged Trio and stained with anti-HA mAb instead of the anti-Tara mAb (Fig. 5G-I). There was no significant co-localization of Trio with either vimentin or tubulin, indicating that Trio does not localize to intermediate filaments or microtubules (data not shown). Taken together, these results strongly argue that Trio is associated with F-actin.

The periodicity of Tara localization on F-actin, which occurred at about 350 nm intervals, was reminiscent of the staining pattern of myosin II (Verkhovsky et al., 1995). Indeed, co-staining of ectopically expressed Trio (red, Fig. 6A) and endogenous non-muscle myosin II heavy chain (green, Fig. 6B) demonstrated that these two proteins had a nearly identical localization in HeLa cells (Fig. 6C; inset shows a fivefold
Tara binds F-actin and enhances cell spreading

Fig. 5. Ectopically expressed Tara localizes to F-actin in HeLa cells. Shown are immunofluorescence images of HeLa cells transiently transfected with Tara (A-F) or with Tara-HA (G-I) and stained for (B,E) Tara (green), using anti-Tara mAb, (H) Tara (green), using anti-HA mAb, or (A,D,G) F-actin, using rhodamin-labeled phalloidin (red). Merged images of A,B, D,E or G,H are shown in panels C,F,I, respectively, with coincidence of red and green appearing yellow/orange. D-F are 2.5-fold magnified images of A-C. Ectopic expression of both Tara and Tara-HA caused enhanced cell spreading, and both Tara and Tara-HA predominantly localize to F-actin in a periodic fashion. HeLa cell transfections and immunofluorescence were as described in Materials and Methods. The intensely red staining cells in A are non-Tara expressing (i.e., non-transfected) HeLa cells. In order to obtain a reasonable F-actin signal in the well-spread, Tara-expressing cells, images were overexposed which resulted in intense phalloidin (red) staining of less spread and thicker, non-transfected cells. Scale bar: 50 μm for A-C,G-I; 20 μm for D-F.

enlargement). Furthermore, co-staining of ectopically expressed Tara (red, Fig. 6D) and endogenous α-actinin (green, Fig. 6E) revealed that α-actinin and Tara alternate along actin fibers particularly at the leading edge of the cell (Fig. 6F, inset shows a fivefold enlargement). α-actinin, but not Tara, was observed at focal contacts located at the edges of cells (see Fig. 6E,F). The co-localization of Tara with the actin-binding proteins myosin and α-actinin further supported the idea that Tara is a F-actin associated protein. To test whether Tara directly bound myosin II or α-actinin, co-immunoprecipitation experiments were performed, but failed to demonstrate a direct interaction between myosin and Tara or between α-actinin and Tara (data not shown).

Tara binds F-actin in vitro

To test whether Tara bound directly to F-actin, co-sedimentation assays were performed using F-actin and COS cell-derived Tara (Fig. 7A). In the absence of F-actin, Tara was located exclusively in the soluble fraction (Fig. 7A, lane 1). However, in the presence of F-actin, about half of the Tara protein was located in the pellet fraction, as determined by immunoblot analysis. α-Actinin was used as positive control for the F-actin sedimentation assay and BSA as negative control. As seen in Fig. 7A, the known actin-binding protein α-actinin almost exclusively localized to the F-actin pellet fraction (lane 10), whereas <5% of the BSA localized to the F-actin pellet fraction (lane 12), as determined by Coomassie Blue staining of gels. Purified COS cell-derived Tara (see Materials and Methods) also bound F-actin as determined using the F-actin sedimentation assay (Fig. 7B). The presence of misfolded protein could account for the finding that only about half of the Tara preparations bound F-actin. Co-immunoprecipitation experiments did not demonstrate any interaction between Tara and G-actin, thus indicating that Tara binds specifically to F-actin and not G-actin (data not shown). Furthermore, purified, E. coli-derived Tara.13-385 protein (Tara amino acids 13-385; see Materials and Methods) efficiently
Fig. 7. Tara binds F-actin in vitro. (A) COS cell-derived Tara binds F-actin using a co-sedimentation assay. Shown is an anti-Tara immunoblot (lanes 1-4) or Coomassie Blue-stained gels (lanes 5-12) of the F-actin co-sedimentation assay soluble (S) and pellet (P) fractions. F-actin co-sedimentation assays were as described in Materials and Methods using COS7 cell-derived recombinant Tara in the form of a whole-cell lysate (lanes 1-8), purified α-actinin (lanes 9-10) or purified BSA (lanes 11-12). For the assay, recombinant Tara, the known actin-binding protein α-actinin, or control BSA were incubated in the presence (lanes 3, 4, 7-12) or absence (lanes 1, 2, 5, 6) of F-actin, and following incubation, F-actin was pelleted by centrifugation. Pelleted proteins were solubilized in a volume equal to the assay volume and then equal volumes of the pellet and soluble fraction were analyzed by anti-Tara immunoblotting or by Coomassie Blue staining. The positions of Tara and G-actin are indicated at the left and the positions of α-actinin, BSA, G-actin are shown on the right. (B) Partially purified recombinant Tara binds F-actin. Shown is an anti-Tara immunoblot (lanes 1-4) of the F-actin co-sedimentation assay soluble (S) and pellet (P) fractions using partially purified Tara protein. COS7 cell-derived GST-Tara fusion protein was isolated by Glutathione-Sepharose chromatography, and Tara was isolated following incubation with thrombin as described in the Material and Methods. The position of Tara is shown on the left. (C) E. coli-derived Tara.13-385 binds F-actin. Shown is an anti-Tara immunoblot (lanes 1-4) of the F-actin co-sedimentation assay soluble (S) and pellet (P) fractions using ~0.5 μg E. coli-derived, purified Tara.13-385 (Tara amino acids 13-385) protein. E. coli-derived GST-Tara.13-385 fusion protein was isolated by glutathione-sepharose chromatography, and Tara.13-385 was isolated following incubation with thrombin as described in Material and Methods. The position of Tara.13-385 is shown on the right. (D) A Coomassie Blue stained gel of E. coli-derived, Tara.13-385 protein used for F-actin co-sedimentation assays. In addition to Tara.13-385, there was a ~80 kDa contaminating protein present in the preparations. The identity of the Tara.13-385 protein was confirmed by immunoblotting with an anti-Tara mAb (Fig. 7C, and data not shown). Molecular mass standards in kDa are shown on the left of the panel and the position of Tara.13-385 is shown on the right.

bound F-actin (Fig. 7C,D). However, it was not possible to determine the stoichiometry of the Tara.13-385 and F-actin interaction, because the low yields of Tara.13-385 protein precluded performing the F-actin co-sedimentation assays with saturating amounts of Tara.13-385. Taken together, the immunofluorescence localization of Tara to F-actin, the co-localization of Tara with myosin II and the in vitro co-sedimentation of Tara with F-actin strongly argue that the N-terminal region of Tara (i.e., amino acids 13-385) directly binds F-actin.

HeLa cells stably overexpressing Tara exhibit altered cell morphology and redistributed F-actin

To gain additional insight into the possible function of Tara, HeLa cell lines stably expressing C-terminally HA-tagged Tara (Tara-HA) were established and characterized. These cell lines, referred to as HeLa(+Tara) cells, express ~fivefold more Tara-HA than endogenous Tara as determined by immunoblotting and densitometric scanning (data not shown). Compared to control HeLa cells, the HeLa(+Tara) displayed enhanced F-actin staining (Fig. 8). Altered F-actin staining was evident in HeLa(+Tara) cells compared with control HeLa cells in serum-free media (Fig. 8A,B), in cells induced for 30 minutes by factors present in FCS (Fig. 8C,D) or in cells treated with the G-actin sequestering drug Latrunculin B (Lat B) (Fig. 8E,F). Under all three culture conditions the HeLa(+Tara) cells had more prominent F-actin staining, particularly cortical F-actin staining. LatB treatment of control HeLa cells resulted in nearly complete disappearance of F-actin with only punctate structures remaining (Fig. 8E). In contrast, LatB-treated HeLa(+Tara) cells retained cortical F-actin staining, although the overall amount of F-actin was reduced. These results suggest that Tara functions to stabilize F-actin. Furthermore, the HeLa(+Tara) cells displayed a flattened morphology compared with the control HeLa cells. Thus, both transient and stable expression of Tara alters the actin cytoskeletal organization and confers an enhanced spread morphology.

To establish whether Tara expression altered cell attachment or the rate of cell spreading, HeLa(+Tara) and control cells were stained for F-actin at various times following cell plating (Fig. 9). HeLa(+Tara) and control HeLa cells attached at a similar rate (Fig. 9A,B) and there was little difference in the extent of cell spreading during the first 8 hours after plating (Fig. 9A-F). However, by 24 hours about half of the HeLa(+Tara) cells were more spread than control HeLa cells (Fig. 9G,H). Enhanced F-actin in stress fibers was already evident in the HeLa(+Tara) cells by 4 hours, and by 24 hours HeLa(+Tara) cells contained prominent stress fibers. Enhanced cortical F-actin was also noticeable 4 and 8 hours after plating in the HeLa(+Tara) cells, but by 24 hours the level of cortical F-actin was relatively normal (Fig. 9C-H). The finding that the HeLa(+Tara) cells initially attached and spread in a manner similar to the control HeLa cells suggests that ectopic Tara expression does not significantly affect the rate of cell
attachment or initial spreading, but rather that Tara overexpression alters the actin cytoskeleton to promote enhanced cell spreading.

**DISCUSSION**

We report the identification of a novel F-actin and Trio GEF-binding protein termed Tara, which consists of a PH domain and a coiled-coil region. Ectopic expression of Tara alters actin cytoskeletal organization, promotes cell spreading, and increases F-actin stability, suggesting that Tara regulates actin cytoskeletal organization. F-actin-binding proteins perform many functions, including actin bundling, crosslinking, stiffening, contracting, capping and fragmenting. Our data suggest that Tara likely mediates F-actin bundling, crosslinking and/or stiffening, as ectopically expressed Tara enhanced and stabilized F-actin structures. The relative resistance of Tara expressing cells to Latrunculin B-mediated F-actin destabilization argues that Tara stabilizes F-actin structures. Alternatively, or in addition, Tara may serve as a linker protein to recruit proteins required for F-actin formation and turnover.

Tara belongs to a subgroup of actin-binding proteins that lack conventional actin-binding domains, and in some of these proteins actin-binding activity was mapped to coiled-coil regions (Sobue and Sellers, 1991) or PH domains (Yao et al., 1999). Consistent with previously identified F-actin bundling and crosslinking proteins, which contain either multiple actin-binding domains or are able to dimerize (Ayscough, 1998), Tara contains at least two actin-binding sites per coiled-coil dimer. Tara and myosin II co-localize along F-actin in a periodic fashion. A similar pattern was previously observed for myosin II, but the basis for this particular spacing is unknown (Kolega, 1998; Verkhovsky et al., 1995). The co-localization of Tara and myosin II suggests that Tara and myosin II directly interact, but we did not detect a physical interaction between these two proteins.

Tara is most similar in overall structure and amino acid identity to p116RIP, a RhoA-binding protein that regulates actin remodeling in neurites by inhibiting RhoA-induced contractility (Gebbink et al., 1997). However, we did not observe Rho family GTPase binding to Tara (data not shown), suggesting that although Tara and p116RIP are structurally similar they may have different modes of action. Based on the characterization of several PH domains (Shaw, 1996), the Tara PH domain may constitute a phospholipid binding domain that mediates binding of Tara to the plasma membrane. Tara may thus link the plasma membrane with the actin cytoskeleton. As the activity of a number of proteins, including actin binding proteins, is modulated by phospholipid binding (Gilmore and Burridge, 1996; Rameh and Cantley, 1999; Steimle et al., 1999; Yao et al., 1999), it is also possible that the PH domain regulates the Tara F-actin binding activity. Alternatively, the Tara PH domain could mediate protein-protein interactions, including the direct binding to actin, as was reported for some PH domains. The coiled-coil region of Tara was shown to dimerize and thus enables Tara dimerization or formation of higher order structures. Coiled-coils can form rod-like structures that may be important for specific function and/or facilitate binding to other proteins (Lupas, 1996). Indeed, a number of actin binding proteins are coiled-coil proteins, including myosin II, caldesmon and α-actinin.

Tara was isolated by virtue of its binding to a subregion of Trio that includes GEF-D1. This region of Trio is of particular interest as previous studies demonstrated that Trio GEF-D1 activates Rac1 and RhoG in vitro and in vivo (Bellanger et al., 1998; Blangy et al., 2000; Debant et al., 1996), and that ectopically expressed Trio GEF-D1 promotes cell spreading, membrane ruffling and cell migration (Seipel et al., 1999). Furthermore, activation of either Rac or Rho by the first or second Trio GEF domain may coordinate actin cytoskeletal

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**Fig. 8.** Altered F-actin in HeLa cells stably expressing Tara. Immunofluorescence images of (A,C,E) control HeLa cells or (B,D,F) HeLa(+Tara) cells, which stably express recombinant Tara-HA at levels ~ fivefold above endogenous Tara levels, stained for F-actin using rhodamin-labeled phalloidin. Cells were cultured in serum-free medium for 1 day before induction and then incubated for 30 minutes with (A,B) serum-free media as control, (C,D) media containing 10% FCS or (E,F) serum-free media containing 200 nM Latrunculin B. Results are representative of at least three independent HeLa(+Tara) and control HeLa cell lines. All images are of identical exposure times and magnification. Scale bar: 50 μm.
Altered spreading of HeLa cells stably expressing Tara. Shown are immunofluorescence images of (A,C,E,G) control HeLa cells or (B,D,F,H) HeLa(+Tara) cells (A,B) 2 hours, (C,D) 4 hours, (E,F) 8 hours and (G,H) 24 hours after plating stained for F-actin using rhodamin-labeled phalloidin. All images are of identical scale bar: 25 μm.

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We thank Drs Nancy Kedersha and Haruo Saito for critical review of the manuscript; and Dr Roger Brent and colleagues for plasmid DNAs and yeast strains used for the interaction-trap assay. This work was supported by grants CA55547 and CA75091 from the National Institutes of Health. M. S. is a Leukemia and Lymphoma Society Scholar.


