The Trio Guanine Nucleotide Exchange Factor Is a RhoA Target

Received for publication, May 3, 2000, and in revised form, July 3, 2000
Published, JBC Papers in Press, August 17, 2000, DOI 10.1074/jbc.M003775200

Quintus G. Medley‡§, Carles Serra-Pages‡¶, Elizabeth Iannotti‡, Katja Seipel‡§, May Tang‡, Stephen P. O’Brien‡, and Michel Streuli‡§**

From the ‡Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, Massachusetts 02115 and the Departments of ¶Pathology and §Medicine, Harvard Medical School, Boston, Massachusetts 02115

Trio is a complex protein containing two guanine nucleotide exchange factor domains each with associated pleckstrin homology domains, a serine/threonine kinase domain, two SH3 domains, an immunoglobulin-like domain, and spectrin-like repeats. Trio was originally identified as a LAR tyrosine phosphatase-binding protein and is involved in actin remodeling, cell migration, and cell growth. Herein we provide evidence that Trio not only activates RhoA but is also a RhoA target. The RhoA-binding site was mapped to the Trio immunoglobulin-like domain. RhoA isoprenylation is necessary for the RhoA-Trio interaction, because mutation of the RhoA carboxyl-terminal cysteine residue blocked binding. The existence of an intramolecular functional link between RhoA activation and RhoA binding is suggested by the finding that Trio exchange activity enhanced RhoA binding to Trio. Furthermore, immunofluorescence studies of HeLa cells showed that although ectopically expressed Trio was evenly distributed within the cell, co-expression of Trio with RhoA resulted in redistribution of Trio into punctate structures. Relocalization was not observed with Trio constructs lacking the immunoglobulin-like domain, indicating that RhoA acts to regulate Trio localization via binding to the immunoglobulin-like domain. We propose that Trio-mediated RhoA activation and subsequent RhoA-mediated relocation of Trio functions to modulate and coordinate Trio signaling.

Trio is an unusual member of the Dbl homology (DH) family of guanine nucleotide exchange factors (GEFs) because it contains two DH GEF domains in addition to a protein serine/threonine kinase, two SH3-like domains, an Ig-like domain, and at least four spectrin-like repeats (schematically shown in Fig. 1; Ref. 1). DH GEF family members function to activate Rho GTPases such as, RhoA, Rac1, and Cdc42 by promoting the exchange of GDP for GTP (2, 3). Once these molecular switches are activated, Rho GTPases function in diverse cellular processes including actin cytoskeleton organization, mitogen-activated protein kinase cascade signaling, and gene transcription (3–5). Targets for Rho GTPases include protein kinases, lipid kinases, and nonenzymatic proteins (2, 4, 6–8). During cell activation, Rho GTPases are released from a complex with GDP dissociation inhibitor (GDI) and then translocate from the cytoplasm to membranes (5, 9). Many DH GEF family members were isolated as oncogenes, indicating their importance to cell growth control (2), and genetic analysis has revealed that several DH GEF family members are important in development, among them Vav (10), the faciogenital dysplasia protein, FGD1 (11, 12), the Drosophila Still life (13) and DRhoGEF2 (14, 15) gene products, the Caenorhabditis elegans Trio-like unc-73 gene product (16), and Drosophila Trio (17–20). DH GEFs all contain a 260-amino acid DH domain immediately followed by a ~100-amino acid pleckstrin homology (PH) domain, and some DH GEFs such as Trio contain additional catalytic domains and auxiliary domains (2, 3). The regulation of DH GEFs is incompletely understood, but PH domain-mediated interactions with phospholipids or proteins, phosphorylation of DH GEF proteins, or interactions of DH GEFs with other GTPases may be involved (2, 21–26). The solution structure of the amino-terminal Trio-GEF/PH region revealed that the GEF and PH domains interact with one another, and mutational analysis demonstrated that the PH domain enhances Trio GEF activity for Rac1 in vitro (24).

Trio was originally isolated by virtue of its binding to the intracellular region of the LAR transmembrane protein-tyrosine phosphatase, and the Trio segment that binds the LAR PTP-D2 domain was shown to include the Ig-like and protein serine/threonine kinase domains (1). The Trio amino-terminal GEF domain (GEF-D1) displays Rac1 activity, and the carboxy-terminal GEF domain (GEF-D2) exhibits RhoA GEF activity in vitro (1). Trio GEF-D1 was also recently shown to activate RhoG in vitro and in cells (27). Expression of Trio GEF-D1 causes prominent membrane ruffling, whereas cells expressing Trio GEF-D2 have increased stress fibers and lamellae that terminate in miniruffles (28, 29). Furthermore, cells expressing Trio GEF-D1 display increased haptotactic migration and anchorage-independent growth, suggesting that Trio regulates matrix-induced signal transduction (28). Transient expression of full-length Trio alters actin cytoskeleton organization, as well as the distribution of focal contact sites (28). These findings support a role for Trio as a multifunctional protein involved in coordinating actin remodeling necessary for cell migration and growth.

The unusual configuration of tandem DH GEF domains, as well as a kinase domain, suggested the possibility that the
activity of one or more of the Trio domains could be regulated by the activity of another domain. To test this possibility, we initially examined whether Trio itself might be a target for Rho or Rac. Co-expression studies indicated that RhoA, but not Rac1 or Cdc42, bound Trio, and mapping studies identified the Trio Ig-like domain as the RhoA-binding site. Trio GEF-D2 activity increased the amount of RhoA bound to Trio, providing evidence for a direct link between Trio-mediated RhoA activation and RhoA binding to Trio. Because RhoA binding to Trio required the most carboxyl-terminal cysteine residue, which is the site of lipid modification, RhoA isoprenylation is required for binding to the Trio Ig-like domain. Furthermore, immunofluorescence studies of HeLa cells indicated that RhoA binding to the Trio Ig-like domain functions to regulate Trio localization.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Trio expression constructs were made using standard techniques and confirmed by restriction mapping and, in some cases, DNA sequencing. Trio cDNA constructs were cloned into the pMT.HA tag (30) or pMT.Myc tag vector expressions, which encode either a HA or Myc epitope tag sequence immediately upstream of the expression numbers UF091395). The HA-Trio.mD2 and HA-Trio.mGSIK constructs contain at their amino termini a HA tag sequence.

Structures contain the Rhotekin RhoA-binding domain (amino acids 7–89). The HA-Trio.mD2 and HA-Trio.mGSIK constructs have restricts used in this study.

For binding to the Trio Ig-like domain. Furthermore, immunofluorescence studies of HeLa cells indicated that RhoA binding to the Trio Ig-like domain functions to regulate Trio localization.

RESULTS

Trio Binds RhoA—The two Trio GEF domains have distinct substrate specificities: the amino-terminal GEF-D1 domain has Rac1 activity, whereas the carboxy-terminal GEF-D2 domain has RhoA activity in vitro (1). To assess whether Trio itself may be a target for RhoA, Rac1, or Cdc42, we performed a series of co-precipitation experiments to determine the ability of GST-tagged Rho GTPases to bind HA-tagged full-length Trio or various Trio deletion constructs (Fig. 1; the overall structure of Trio is schematically shown at the top and below). To this end, COS-7 cells were co-transfected with HA-Trio (amino acids 1–3038) together with GST-tagged Trio (HA-Trio, amino acids 1–3038) and, following transfection, proteins were recovered using Glutathione-Sepharose. As seen in Fig. 2, Trio bound GST-RhoA (Fig. 2, upper panel, lane 2), suggesting that Trio could indeed be a RhoA target. The binding appeared specific to RhoA because neither GST, GST-Rac1, -N17Rac1, nor -V12Cdc42 appreciably bound Trio, even though the expression levels of Trio and the various GST-tagged GTPases were comparable in the various co-transfections (Fig. 2 and data not shown). Equal expression levels of full-length HA-Trio in the presence of GST-V14RhoA and GST-N19RhoA were only possible at lower levels of GST-N19RhoA expression, perhaps because of cell toxicity. However, Trio con-
Trio Relocalization Mediated by RhoA Binding to Trio

**FIG. 2. Trio binds RhoA.** Shown is a SDS-PAGE analysis of [35S]methionine- and [35S]cysteine-labeled proteins isolated by co-precipitation with GST-tagged Rho family GTPases. COS-7 cells were transiently transfected with vectors encoding GST-tagged Rho family GTPases (see **top of the figure**) with either an expression vector encoding HA-tagged Trio (amino acids 1–3038) (lanes 1–6) or no vector (lanes 7–10). The GST-V14RhoA, -V12Rac1, and -V12Cdc42 constructs harbor mutations that block the GTPase activity, whereas the GST-N19RhoA and -N17Rac1 constructs bind GDP preferentially. Two days after transfection, cells were metabolically labeled, and then cell extracts were prepared, and co-precipitation analysis was performed using glutathione-Sepharose (lanes 1–10, upper panel). As control, 10% of the lysates were used to immunoprecipitate Trio using the anti-HA 12CA5 antibody (lanes 1–6, lower panel). Precipitated proteins were resolved by 6% SDS-PAGE and visualized by autoradiography. The position of Trio is indicated by an arrow, and the expected positions of HA-Trio are marked by asterisks located between the lanes. The 200,000 molecular mass standard in kDa is shown at the left of the figure.

Consistently bound more (2.5–6-fold) GST-V14RhoA than GST-N19RhoA, as determined by densitometric scanning of autoradiograms and correcting for the amount Trio present in the various co-transfections, suggesting that Trio preferentially binds activated RhoA (Fig. 2 and data not shown). Although GST-V12Rac1, -N17Rac1, and -V12Cdc42 did not appreciably bind Trio, they did bind endogenous COS-7 proteins, such as a protein that migrates somewhat slower than Trio and a 180-kDa protein (Fig. 2, upper panel, lanes 4–6, 9, and 10). Taken together, these findings indicate that Trio is a target for RhoA, but not for Rac1 or Cdc42, and that activated RhoA preferentially binds Trio. Attempts to precipitate endogenous Trio with the various GST-tagged GTPases were unsuccessful, probably because of the low level of endogenous Trio present in diverse cell lines (data not shown).

Trio Binds RhoA via Its Ig-like Domain—To map which region of Trio bound RhoA, a series of Trio amino-terminal truncation mutants (Fig. 1) were co-expressed together with GST-V14RhoA in COS-7 cells, and following metabolic labeling, cell extracts were prepared, and glutathione-Sepharose precipitation was performed (Fig. 3A). GST-V14RhoA bound HA-Trio.GSIK (lane 2), and HA-Trio.SIK (lane 3), but did not efficiently bind HA-Trio.GS (lane 4). Control anti-HA immunoprecipitations of cell extracts used for the glutathione-Sepharose precipitations showed that the expression levels of HA-Trio.GSIK, HA-Trio.SIK, and HA-Trio.GS were comparable (Fig. 3A, lanes 6–8). These results indicate that the Trio RhoA-binding site resides in the Trio carboxyl-terminal region (amino acids 2629–3038), which includes the Ig-like domain and the kinase domain.

To assess where RhoA bound within the Trio carboxyl-terminal region, HA-tagged Trio constructs encoding both the Ig-like and kinase domains (Trio.IK) or kinase domain alone (Trio.K), were co-expressed with GST-tagged Rho GTPases, and glutathione-Sepharose precipitations were performed (Fig. 3B). GST-V14RhoA bound Trio.IK but did not bind Trio.K, indicating that the Trio Ig-like domain was required for efficient V14RhoA binding (Fig. 3B, lane 2). Furthermore, binding studies using a construct encoding only the Trio Ig-like domain (Trio.I) confirmed that GST-V14RhoA bound the Ig-like domain (Fig. 3C, lane 2). Little if any binding of GST-N19RhoA, -V12Rac1, or -V12Cdc42 was observed with Trio.IK, Trio.I, and Trio.K (Fig. 3, B and C). The HA-tagged Trio.IK, Trio.K, and Trio.I constructs were all efficiently expressed as determined by anti-HA immunoblotting of total lysates (Fig. 3, B and C), as were the GST-Rho GTPases as determined by Coomassie staining (data not shown).

To establish whether the Ig-like domain was required for RhoA binding in context of full-length Trio, we created a Trio mutant, TrioΔig (amino acids 1–2626 and 2717–3038), that lacks only the Ig-like domain and TrioΔC (amino acids 1–2241) that lacks the carboxyl-terminal SH3, Ig-like, and kinase domain (Fig. 1). HA-tagged Trio, TrioΔC, or TrioΔig were co-expressed together with GST-V14RhoA or GST-N19RhoA in COS-7 cells, and following metabolic labeling, cell extracts were prepared, and glutathione-Sepharose precipitations were performed (Fig. 3D). GST-V14RhoA bound very little HA-TrioΔig compared with HA-Trio (Fig. 3D, lanes 3 and 7), consistent with the Ig-like-domain-binding RhoA. Control anti-HA immunoprecipitations using cell extracts showed that the expression levels of Trio, TrioΔC, and TrioΔig were comparable (Fig. 3D, lanes 9–16). GST-V14RhoA and -N19RhoA fusion proteins were also efficiently expressed (data not shown). Taken together, the mapping studies demonstrate that the Ig-like domain is the primary RhoA-binding site on Trio and that the Ig-like domain alone is sufficient to bind activated RhoA.

**RhoA Cysteine 190 Is Required for Trio Binding**—Our mapping studies demonstrated that the Trio Ig-like domain is required for efficient binding of RhoA to Trio. However, E. coli-derived V14RhoA did not bind Trio in vitro (data not shown), suggesting that a post-translation modification of RhoA, such as lipid modification, might be required for Trio binding. Consistent with this hypothesis, the binding of GST-V14RhoA to Trio was insensitive to the presence of high NaCl concentrations (up to 1 M NaCl) in the precipitate wash buffer (Fig. 4A), supporting a possible lipid-protein interaction. To test whether RhoA isoprenylation was required for Trio binding, a RhoA cysteine 190→serine mutant (34–36), GST-V14RhoA-C/S, was constructed and tested for its ability to bind Trio. To this end, GST-V14RhoA-C/S or GST-V14RhoA was co-expressed with HA-tagged Trio.GSIK or with Trio.SIK in COS-7 cells, and following metabolic labeling, cell extracts were prepared, and glutathione-Sepharose precipitations were performed (Fig. 4B). Whereas GST-V14RhoA efficiently bound HA-Trio.GSIK and HA-Trio.SIK (Fig. 4B, lanes 1 and 3, respectively), the GST-V14RhoA-C/S mutant did not bind HA-Trio.GSIK or Trio.SIK (Fig. 4B, lanes 2 and 4, respectively). The expression levels of GST-V14RhoA-C/S and GST-V14RhoA were comparable (Fig. 4B, lanes 1–4), and control anti-HA immunoprecipitations using total cell extracts showed that the expression levels of HA-Trio.GSIK and HA-Trio.SIK were also similar (Fig. 4B, lanes 5–8). Furthermore, binding experiments using the isolated Ig-like domain construct (Trio.I) with GST-V14RhoA-C/S and GST-V14RhoA also showed a requirement for RhoA isoprenylation (Fig. 4C). The finding that neither GST-V14RhoA-C/S nor E. coli-derived V14RhoA bound full-length Trio or the isolated Trio Ig-like domain strongly argues that RhoA isoprenylation is required for RhoA binding to Trio.
Trio GEF-D2 Activity Enhances RhoA Binding to Trio—

Because the Trio Ig-like domain preferentially bound activated RhoA and the Trio GEF-D2 domain activates RhoA (1), it was hypothesized that Trio GEF-D2 activity could modulate RhoA binding to Trio. To test this possibility, we compared the ability of wild-type GST-RhoA or constitutively activated GST-V14RhoA to bind Trio using either Trio.GSIK, which has the ability to activate RhoA, or a Trio GEF mutant, Trio.mGSIK, which lacks detectable GEF activity in vitro (data not shown). To initially confirm that Trio GEF-D2 did indeed increase GTP-bound RhoA levels in cells, rhotekin pull-down experiments were performed (32). To this end, HA-tagged wild-type RhoA was co-expressed with either HA-Trio.GSIK or HA-Trio.mGSIK in COS-7 cells, and the amount of GTP-bound RhoA was measured. The results showed that Trio GEF-D2 activity did indeed increase GTP-bound RhoA levels in cells, as indicated by the increased band intensity in the co-precipitation experiments. The position of the Trio mutants are indicated by arrows. The 200,000 molecular mass standard in kDa is shown at the left of the figure. B, the Trio Ig-like domain binds RhoA. Shown are immunoblot analysis of proteins binding to GST-tagged Rho GTPases. The Trio constructs HA-Trio.IK or HA-Trio.K (see Fig. 1) were co-expressed in COS-7 cells with GST or GST-tagged Rho GTPases (see top of the figure). Two days after transfection, cell extracts were prepared, and co-precipitation analysis was performed using glutathione-Sepharose (lanes 1–6). As control, 5% of the total lysate was directly applied to the gels (lanes 7–12). Following electrophoresis and transfer of proteins to Immobilon-P, the blot was probed with the anti-HA mAb HA 11. The position of the Trio proteins are indicated by arrows. Molecular mass standards in kDa are shown at the right of the figure. C, isolated Trio Ig-like domain binds RhoA. Binding studies were done as described in the legend to Fig. 3B except the TrioC construct was used, and GST-N17Rac1 was omitted. D, Trio lacking the Ig-like domain inefficiently binds RhoA. Shown is a SDS-PAGE analysis of [35S]methionine- and [35S]cysteine-labeled proteins isolated by co-precipitation with GST-V14RhoA or GST-N19RhoA. COS-7 cells were transiently transfected with vectors encoding either GST-V14RhoA or GST-N19RhoA. Two days after transfection, cells were labeled, cell extracts were prepared, and co-precipitation analysis was performed using glutathione-Sepharose (lanes 1–8) or anti-HA 12CA5 antibody (lanes 9–16) as described above.

Fig. 3. Mapping of the RhoA binding site to the Trio Ig-like domain. A, Trio constructs containing the Ig-like and kinase domains bind V14RhoA. Shown is a SDS-PAGE analysis of [35S]methionine- and [35S]cysteine-labeled proteins isolated by co-precipitation with GST-V14RhoA. COS-7 cells were transiently transfected with vectors encoding HA-Trio.GSIK, HA-Trio.SIK, or HA-Trio.GS (see Fig. 1) together with a vector encoding GST-V14RhoA. Two days after transfection, cells were labeled, cell extracts were prepared, and co-precipitation analysis was performed using glutathione-Sepharose (lanes 1–4) or anti-HA 12CA5 antibody (lanes 5–8) as described in the Fig. 2 legend. The position of the Trio mutants are indicated by arrows. The 200,000 molecular mass standard in kDa is shown at the left of the figure. In the Fig. 2 legend. The position of the Trio mutants are indicated by arrows. The 200,000 molecular mass standard in kDa is shown at the left of the figure.

B, the Trio Ig-like domain binds RhoA. Shown are immunoblot analysis of proteins binding to GST-tagged Rho GTPases. The Trio constructs HA-Trio.IK or HA-Trio.K (see Fig. 1) were co-expressed in COS-7 cells with GST or GST-tagged Rho GTPases (see top of the figure). Two days after transfection, cell extracts were prepared, and co-precipitation analysis was performed using glutathione-Sepharose (lanes 1–6). As control, 5% of the total lysate was directly applied to the gels (lanes 7–12). Following electrophoresis and transfer of proteins to Immobilon-P, the blot was probed with the anti-HA mAb HA 11. The position of the Trio proteins are indicated by arrows. Molecular mass standards in kDa are shown at the right of the figure. C, isolated Trio Ig-like domain binds RhoA. Binding studies were done as described in the legend to Fig. 3B except the TrioC construct was used, and GST-N17Rac1 was omitted. D, Trio lacking the Ig-like domain inefficiently binds RhoA. Shown is a SDS-PAGE analysis of [35S]methionine- and [35S]cysteine-labeled proteins isolated by co-precipitation with GST-V14RhoA or GST-N19RhoA. COS-7 cells were transiently transfected with vectors encoding HA-Trio, HA-TrioΔC, or HA-TrioΔIg (see Fig. 1) together with a vectors encoding either GST-V14RhoA or GST-N19RhoA. Two days after transfection, cells were labeled, cell extracts were prepared, and co-precipitation analysis was performed using glutathione-Sepharose (lanes 1–8) or anti-HA 12CA5 antibody (lanes 9–16) as described above.

Trio Relocalization Mediated by RhoA Binding to Trio—Because the Trio Ig-like domain preferentially bound activated RhoA and the Trio GEF-D2 domain activates RhoA (1), it was hypothesized that Trio GEF-D2 activity could modulate RhoA binding to Trio. To test this possibility, we compared the ability of wild-type GST-RhoA or constitutively activated GST-V14RhoA to bind Trio using either Trio.GSIK, which has the ability to activate RhoA, or a Trio GEF mutant, Trio.mGSIK, which lacks detectable GEF activity in vitro (data not shown). To initially confirm that Trio GEF-D2 did indeed increase GTP-bound RhoA levels in cells, rhotekin pull-down experiments were performed (32). To this end, HA-tagged wild-type RhoA was co-expressed with either HA-Trio.GSIK or HA-Trio.mGSIK in COS-7 cells, and the amount of GTP-bound
RhoA was estimated by precipitation using GST-rhotekin, which binds GTP-bound RhoA but not GDP-bound RhoA. Cells transfected with Trio.GSIK had readily detectable levels of GTP-bound RhoA (Fig. 5A, lane 2), whereas Trio.mGSIK transfected cells had little GTP-bound RhoA (lane 3), indicating that RhoA nucleotide exchange occurs with Trio.GSIK but not with Trio.mGSIK. To then assess the ability of HA-Trio.GSIK and HA-Trio.mGSIK to bind GST-RhoA, glutathione-Sepharose precipitations were performed using extracts prepared from metabolically labeled cells (Fig. 5B). The constitutively activated form of RhoA, GST-V14RhoA, bound about equally well to HA-Trio.GSIK and HA-Trio.mGSIK (Fig. 5A, lanes 1 and 3, respectively). However, the amount of wild-type GST-RhoA bound to HA-Trio.GSIK was consistently ~2.0-fold higher than the amount of GST-RhoA bound to HA-Trio.mGSIK, as determined by densitometric scanning of the autoradiograms. This finding argued that the prior activation of RhoA by the Trio GEF-D2 domain increased the amount of RhoA bound to Trio.

**RhoA Expression Alters Trio Localization**—To assess whether RhoA binding to Trio could function to regulate Trio localization within cells, Trio and RhoA were expressed either alone (Fig. 6, A–D) or together (E–J) in HeLa cells, and then Trio (green) and RhoA (red) localization were determined by immunofluorescence staining. The expression patterns of HA-Trio alone (Fig. 6B), HA-Trio.Ig alone (Fig. 6C), and HA-Trio.mD2 alone (Fig. 6D; a full-length Trio construct containing the GEF-D2 inactivating mutation) were similar and showed an even distribution throughout the cell with a slight concentration at the edges. In contrast, the expression pattern of GST-RhoA was largely patchy and punctate (Fig. 6A). Ectopic expression of the Trio and Rho constructs also displayed perinuclear staining. Analysis of cells co-expressing HA-Trio (green) and GST-RhoA (red) showed the cells to be more rounded and both proteins to have similar punctate and patchy distributions that partially co-localize (see arrows in Fig. 6, E and F). This finding indicated that HA-Trio and GST-RhoA associated in cells and that RhoA expression altered Trio localization from a fairly even distribution into punctate structures. Consistent with a role for RhoA mediating Trio relocalization was the observation that GST-RhoA co-expressed with HA-Trio.mGSIK (which lacks RhoA binding activity, had little if any effect on Trio localization (Fig. 6, G and H). Additionally, co-expression of GST-RhoA and HA-Trio.mD2, which lacks RhoA activating activity, also displayed reduced localization of Trio.
into punctate structures (Fig. 6, I and J). These results are thus consistent with an intramolecular functional link between Trio-mediated RhoA activation and RhoA-mediated relocalization of Trio via RhoA binding to the Trio-Ig-like domain.

To characterize the association of Trio and RhoA in more detail, HeLa cells coexpressing HA-Trio and GST-RhoA, or as control HA-TrioΔIg and GST-RhoA, were analyzed by scanning confocal microscopy (Fig. 7). Two horizontal slices taken from either the lower half of the cell (Fig. 7, A and B) or the upper half of the cell (Fig. 7, C and D) of cells co-expressing either HA-Trio and GST-RhoA or HA-TrioΔIg indicated that there was a high degree of co-localization of GST-RhoA and HA-Trio in punctate bodies at the edge of the cell (Fig. 7A, arrows) and over the nucleus at the top of the cell (Fig. 7B). These examples, as well as the other cell layers (data not shown), are consistent with the Trio and RhoA association occurring mainly at the plasma membrane. The coefficient of co-localization of HA-Trio with GST-RhoA was 0.93, indicating that approximately 93% of the HA-Trio was complexed with GST-RhoA, whereas the HA-TrioΔIg had a coefficient of only 0.30 and co-localization appeared mainly in the endoplasmic reticulum (Fig. 7, B and D). As seen in Fig. 6 (E and F) and Fig. 7 (A and C), the HA-Trio and GST-RhoA expressing HeLa cells appeared less well spread and thicker than the HA-TrioΔIg and GST-RhoA cells, suggesting that the binding of RhoA to Trio has striking effects...
on cell morphology possibly through regulation of one or more of the enzymatic activities of Trio.

**DISCUSSION**

The DH GEF family member Trio is of particular interest because it is a multifunctional protein that contains three enzymatic domains (two GEF domains and a protein kinase serine/threonine protein kinase domain) and is implicated in coordinating actin cytoskeletal reorganization and cell growth regulation (1, 28, 29). Herein we demonstrate that in addition to activating RhoA, Trio is itself a RhoA target, and mapping studies identified the Trio Ig-like domain as the RhoA-binding site. Furthermore, we show that RhoA binding to Trio requires RhoA lipid modification. We also provide evidence for the existence of an intramolecular cascade whereby the Trio GEF-D2 domain activates RhoA and then activated RhoA binds the Trio Ig-like domain. Finally, immunofluorescence studies using HeLa cells ectopically expressing Trio and RhoA indicate that RhoA binding regulates Trio cellular localization.

The preferential binding of GST-RhoA to both full-length Trio and the isolated Trio Ig-like domain, suggests that Trio binds GTP-bound RhoA. It is thus possible that conformational changes in RhoA that accompany GTP binding expose a Trio-binding site on RhoA in addition to the isoprenyl group. However, we have been unable to study the nucleotide dependence of RhoA binding to Trio using purified proteins in vitro, perhaps because of the strict requirement of the RhoA isoprenyl group for binding, as well as possible deleterious effects on this moiety of the detergents used to solubilize the protein. We also cannot rule out the possibility that the interaction between RhoA and Trio may not be direct but instead is mediated by an adapter protein. However, co-precipitation analysis using [35S]methionine and [35S]cysteine metabolically labeled proteins did not reveal significant amounts of a third protein in the RhoA and Trio complexes (see Figs. 3 and 4 and data not shown), thus arguing that the interaction is direct. Furthermore, RhoGDI was shown to bind the isoprenyl group of Cdc42 directly via two β-sheets that form an Ig-like fold (37–39). RhoA isoprenylation was also shown necessary for binding to protein kinase C (40, 41) and phospholipase D (42, 43), and binding of Ha-Ras to Raf-1 requires Ha-Ras post-translational modification (44). In these cases, however, it is unclear whether the GTPase post-translational modification is necessary to localize the GTPase to the membrane for binding to ensue or whether the modification is directly involved in binding the target protein as appears to be the case with Trio.

RhoA binding to Trio may have consequences on the in vivo function of both proteins. RhoGDI regulates the activation status of Rho GTPases by inhibiting intrinsic or GTPase-activating protein-stimulated GTPase activity (3, 5). It is therefore possible that after binding to Trio, RhoA-GTPase activity is altered. Furthermore, the proximity of the Ig-like domain to the Trio GEF-D2 domain could ensure efficient delivery and binding of activated RhoA to Trio. With respect to Trio function, the resulting relocalization of Trio after binding RhoA may affect interactions between the GEF-D1, GEF-D2 or the kinase domain with their respective substrates. Additionally, the relocalization of Trio-mediated by RhoA may also reflect a conformational change that uncovers latent protein or membrane-binding sites within the Trio protein. Finally, the dissociation of the RhoA and Trio complex may require interaction(s) with another isoprenyl-binding entity such as RhoGDI, the plasma membrane, or lipid second messengers. In the case of the Rac and RhoGDI complex, it was shown that a number of lipids including arachidonic acid, phosphatidic acid, and phosphatidylinositol could disrupt the Rac and RhoGDI complex (45).

The binding of Rho GTPases to Ig-like domains as seen for RhoGDI and Trio may indicate that this type of interaction occurs in other proteins related to Trio such as UNC-73 (16), Kalirin-12 (46), and Duet (47), as well as other cytoskeletal proteins that have Ig-like domains such as Titin (48), Twitchin (49, 50), and myosin light chain kinase (51, 52). Multiple isoforms of Kalirin are generated by alternative splicing, and the largest isoform, Kalirin-12, which is most similar to Trio and contains two Ig-like domains, localizes largely to cell bodies, whereas shorter Kalirin isoforms, which lack the Ig-like domains, localize to punctate structures distributed along neuronal processes, as well as to cell bodies (46, 53). It is thus possible that the Kalirin Ig-like domains may play a similar role as the Trio Ig-like domain to localize these GEFs to specific sites within the cell. Furthermore, binding of isoprenylated GTPases to Ig-like domains may also be involved in regulating the activity of these proteins. The ability of Trio to bind post-translationally modified RhoA suggests a potentially vital role for isoprenylation not only for localization of RhoA to the plasma membrane but also in determining RhoA’s ability to bind proteins. Indeed, binding of Rho family GTPases via their isoprenyl group to target proteins may result in specific and stable complexes that are important in the regulation of downstream signaling pathways.

Acknowledgments—We thank Dr. H. Saito for critical review of the manuscript, Drs. John Blenis and Margaret Chou for plasmids encoding GST-tagged V12Rac1 and GST-tagged V14RhoA, Dr. Walter Blattler for the anti-GST mAb, and Michelle Lowe for expert technical assistance.

**REFERENCES**

1. Debat, A., Serra-Pages, C., Seipel, K., O’Brien, S., Tang, M., Park, S. H., and Streuli, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5466–5471
2. Cerione, R. A., and Zheng, Y. (1996) Curr. Opin. Cell Biol. 8, 216–222
3. Zohn, I. M., Campbell, S. L., Khosravi-Far, R., Rossman, K. L., and Der, C. J.
Trio Relocalization Mediated by RhoA Binding to Trio

36123

(1998) Oncogene 17, 1415–1438
4. Hall, A. (1998) Science 279, 509–514
5. Van Aelst, L., and D’Souza-Schorey, C. (1997) Genes Dev. 11, 2295–2322
6. Aspenstrom, P. (1999) Curr. Opin. Cell Biol. 11, 95–102
7. Lim, L., Manser, E., Leung, T., and Hall, C. (1996) Eur. J. Biochem. 242, 171–185
8. Lamarque, N., and Hall, A. (1994) Trends Genet. 10, 436–440
9. Takai, Y., Sasaki, T., Tanaka, K., and Nakashima, H. (1995) Trends Biochem. Sci. 20, 227–231
10. Zouzidzinas, A., Fischer, K. D., Lira, S. A., Forrester, L., Bryant, S., Bernstein, A., and Barbadic, M. (1995) EMBO J. 14, 1–11
11. Olsen, M. F., Pasteris, N. G., Gorski, J. L., and Hall, A. (1996) Curr. Biol. 6, 1628–1633
12. Pasteris, N. G., Cadle, A., Logie, L. J., Porteous, M. E., Schwartz, C. E., Stevenson, R. E., Glover, T. W., Wilroy, R. S., and Gorski, J. L. (1994) Cell 79, 669–678
13. Sone, M., Hoshino, M., Suzuki, E., Kuroki, K., Nakagoshi, H., Saigo, K., Nabeshima, Y., and Hama, C. (1997) Science 275, 543–547
14. Barrett, K., Leptin, M., and Settleman, J. (1997) Cell 91, 905–915
15. Hacker, U., and Perrimon, N. (1998) Genes Dev. 12, 274–284
16. Steven, R., Kubiszeki, T. J., Zheng, H., Kulkarni, S., Mancillas, J., Ruiz Morales, A., Hogue, C. W., Pawson, T., and Culotti, J. (1998) Cell 92, 765–786
17. Newsome, T. P., Schmidt, S., Dietzl, G., Kelemen, K., Asling, B., Debant, A., and Dickson, B. J. (2000) Cell 101, 283–294
18. Bateman, J., Chuang, T. H., and Van Vactor, D. (2000) Neuron 26, 93–106
19. Liebl, E. C., Fordhoedel, D. J., Franco, L. S., Sample, S. H., Hess, J. E., Cowger, J. A., Chandler, M. P., Shupert, A. M., and Seeger, M. A. (2000) Neuron 26, 107–118
20. Abugov, T., Saito, M., Sone, M., Suzuki, E., Ito, K., and Hama, C. (2000) Neuron 26, 119–131
21. Gulbins, E., Coggeshall, K. M., Baier, G., Katasz, S., Burn, P., and Altman, A. (1993) Science 260, 822–825
22. Han, J., Das, B., Wei, W., Van Aelst, L., Mostoller, R. D., Khosravi-Far, R., Westwick, J. K., Der, C. J., and Broek, D. (1997) Mol. Cell. Biol. 17, 1346–1353
23. Rivas, V., Becker, J. F., Sakaguchi, K., Tachibana, M., and Miki, T. (1994) EMBO J. 13, 4776–4786
24. Liu, X., Wang, H., Eberstdt, M., Schnuchel, A., Oleniczak, E. T., Meadows, R. P., Schkeryantz, J. M., Janowick, D. A., Harlan, J. E., Harris, E. A., Staunton, D. E., and Feuk, S. W. (1998) Cell 93, 269–277
25. Yron, I., Deckert, M., Reff, M. E., Mushni, A., Schwartz, M. A., and Altman, A. (1999) Cell Adhes. Commun. 7, 1–11
26. Kariya, K., Elliott, C. M., Buchanan, F. G., Downes, C. P., and Exton, J. H. (1999) J. Biol. Chem. 274, 12753–12758
27. Blangy, A., Vignal, E., Schmidt, S., Debant, A., Gautheier-Rouvire, C., and Fort, P. (2000) J. Cell Sci. 113, 729–739
28. Seipel, K., Medley, Q. G., Kedersha, N. L., Zhang, X. A., O’Brien, S. P., Serra-Pages, C., Hemler, M. E., and Streuli, M. (1999) J. Cell Sci. 112, 1825–1834
29. Bellanger, J. M., Lazaro, J. B., Dirieng, S., Fernandez, A., Lamb, N., and Debant, A. (1998) Oncogene 16, 147–152
30. Serra-Pages, C., Kedersha, N. L., Fazikas, L., Medley, Q., Debant, A., and Streuli, M. (1998) EMBO J. 14, 2827–2838
31. Chou, M. M., and Blenis, J. (1996) Cell 85, 573–583
32. Ren, X. D., Kiosses, W. B., and Schwartz, M. A. (1999) EMBO J. 18, 578–585
33. Serra-Pages, C., Medley, Q. G., Tang, M., Hart, A., and Streuli, M. (1998) J. Biol. Chem. 273, 15611–15620
34. Mosmaw, J. F., and Case, P. J. (1992) J. Biol. Chem. 267, 17438–17443
35. Ziman, M., Preuss, D., Mulholland, J., O’Brien, J. M., Botstein, D., and Johnson, D. I. (1993) Mol. Biol. Cell 4, 1397–1316
36. Davis, C. R., Richman, T. J., Deliduka, S. B., Blaisdell, J. O., Collins, C. C., and Means, A. R., and Kataoka, T. (1995) Nature 379, 614–619
37. Keep, N. H., Barnes, M., Barsukov, I., Badii, R., Lian, L. Y., Segal, A. W., Moody, P. C., and Roberts, G. C. (1997) Structure 5, 623–633
38. Hoffman, G. R., Nasser, N., and Cerione, R. A. (2000) Cell 100, 345–356
39. Chang, J. H., Pratt, J. C., Sawasdikosol, S., Kapeller, R., and Burakoff, S. J. (1998) Mol. Cell. Biol. 18, 4986–4993
40. Kamada, Y., Qadota, H., Python, C. P., Anraku, Y., Ohy, Y., and Levin, D. E. (1996) J. Biol. Chem. 271, 9193–9196
41. Yamazaki, M., Zhang, Y., Watanabe, H., Yokozeki, T., Ohno, S., Kaibuchi, K., Shihata, H., Mukai, H., Ono, Y., Frohman, M. A., and Kanaho, Y. (1999) J. Biol. Chem. 274, 6055–6058
42. Kuribara, H., Tago, K., Yokozeki, T., Sasaki, T., Takai, Y., Morii, N., Narumiya, S., Katada, T., and Kanaho, Y. (1995) J. Biol. Chem. 270, 25613–25671
43. Chuang, T. H., Bohl, B. P., and Bokoch, G. M. (1993) J. Biol. Chem. 268, 26206–26211
44. Johnson, R. C., Penzes, P., Eipper, B. A., and Mains, R. E. (2000) J. Biol. Chem. 275, 19324–19333
45. Kariya, K., Tachibana, M., Akasaka, K., Shirouzu, M., Yokoyama, S., and Katoaka, T. (1995) J. Biol. Chem. 270, 30274–30277
46. Labeit, S., and Kolmerer, B. (1995) Science 267, 293–296
47. Moerman, D. G., Benian, G. M., Barstead, R. J., Schriefer, L. A., and Waterston, R. H. (1998) Genes Dev. 12, 93–105
48. Benian, G. M., Kiff, J. E., Neckelmann, N., Moerman, D. G., and Waterston, R. H. (1988) Nature 334, 45–50
49. Cohn, N. J., Kroon, B., Needleman, D. S., Hurwitz, M. Y., Kemp, B. E., and Means, A. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2284–2288
50. Potier, M. C., Chelot, E., Bekarsky, Y., Gardiner, K., Rossier, J., and Turnell, W. G. (1995) Genomics 29, 562–570
51. Pfeffer, P., Johnson, R. C., Alam, M. R., Kamhampati, V., Mains, R. E., and Eipper, B. A. (2000) J. Biol. Chem. 275, 6395–6403