The C-terminal basic tail of RhoG assists the guanine nucleotide exchange factor Trio in binding to phospholipids.

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RUNNING TITLE. Structural and Biochemical Characterization of the N-terminal DH/PH of Trio.

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SUMMARY

The multidomain protein Trio regulates among others neuronal outgrowth and axonal guidance in vertebrates and invertebrates. Trio contains two Dbl-homology/Pleckstrin-homology (DH/PH) tandem domains that activate several RhoGTPases. Here, we present the X-ray structure of the N-terminal DH/PH, hereafter TrioN, refined to 1.7Å resolution. We show that the relative orientations of the DH and PH domains of TrioN and free Dbs are similar. However, this relative orientation is dissimilar to Dbs in the Dbs/Cdc42 structure. In vitro nucleotide exchange experiments catalyzed by TrioN show that RhoG is ~3 times more efficiently exchanged than Rac, and support the conclusion that RhoG is likely the downstream target of TrioN. Residues 54 and 69, which are not conserved between the two GTPases are responsible for this specificity. Dot-blot assay reveals that the TrioN-PH domain does not detectably bind phosphatidylinositol-(3,4) bisphosphate, PtdIns(3,4)P₂, or other phospholipids. This finding is supported by our three-dimensional structure and affinity binding experiments. Interestingly, the presence of RhoG but not Rac or a C-terminally truncated RhoG mutant allows TrioN to bind PtdIns(3,4)P₂ with a µM affinity constant. We conclude the variable C-terminal basic tail of RhoG specifically assists the recruitment of the TrioN-PH domain to specific membrane-bound phospholipids. Our data suggest a role for the phosphoinositide 3-kinase, PI 3-kinase, in modulating the Trio/RhoG signaling pathway.

KEYWORDS: crystallography, GEF, PH domain, PtdIns(3,4)P₂, RhoG, Rac, Trio.
1- INTRODUCTION

Reorganization of the actin cytoskeleton is an essential step accompanying changes in cell shape, cell migration, cell-cell adhesion, cell transformation and tumorigenesis, and other cellular processes (reviewed in 1-4). More than any other cell type, neurons rely on changes in their actin cytoskeleton for growth, guidance and branching (reviewed in 5-8). It is now well established that members of the Rho family of small GTP-binding proteins, or RhoGTPases, are major regulators of the acto-myosin machinery in a large number of cell types. RhoGTPases (Rho, Rac, Cdc42 and their isoforms) function as protein switches in response to the activation of cell surface receptors to activate various cellular processes including gene transcription and the formation of actin stress fibers, membrane ruffling, and filopodia (reviewed in 3-4, 9).

One key step in the receptor/RhoGTPase signal pathway is the activation of the RhoGTPase. The activation step controls the intensity and the duration of the signal, and thus is subject to tight regulation. The activation step consists of switching the RhoGTPase from an inactive GDP-bound form to an active or GTP-bound form and is generally catalyzed by guanine nucleotide exchange factors (GEFs). Mammalian Rho specific GEFs, or RhoGEFs, form a family of approximately 47 multidomain proteins and often contain multiple protein-protein or protein-phospholipid binding domains suggesting regulation by inter- or intra-molecular interaction. RhoGEFs share a ~200 amino acid homology domain initially found in the proto-oncogene Dbl (diffuse B-cell lymphoma, 10) and referred to as the DH domain (reviewed in 11-14). A pleckstrin homology (PH) domain invariably follows the DH domain in all known RhoGEFs. PH domains are ~100 amino acid modules found in a variety of signaling proteins (reviewed in 15-17). The primary function of the PH domain is to bind phosphatidylinositolphosphates (PtdIns) thus localizing the target protein to the membrane. In some cases, they can serve as protein interacting domains (18-20). The DH/PH tandems of various RhoGEFs are sufficient for nucleotide exchange on RhoGTPases both in vivo and in vitro. While the DH domain is usually sufficient to activate the RhoGTPase, the PH domain is sometimes required.
The involvement of the PH domain in the exchange reaction is emphasized by several findings showing a modulation of this reaction by phospholipids. For example, the binding of PtdIns(3,4,5)P$_3$ to the PH domain of Sos or Vav1 allows Rac to be activated by disrupting an intramolecular interaction between the DH and PH domains (21-22). Alternatively, the PH domain of a subset of RhoGEFs will participate in the exchange reaction as is the case of Dbl big sister, Dbs (23). The three dimensional structure of various DH/PH tandems demonstrate the overall structures of the DH and PH domains are conserved, however the relative orientation of these two domains seems to be RhoGEF dependent (23-26). Thus, the PH domain of certain RhoGEFs could play a dual role by integrating cellular pathways involving generation of phospholipids to pathways involving activation of RhoGTPases.

Trio, a member of the Dbl family of proteins, plays an essential role in regulating the actin cytoskeleton during axonal guidance and branching (reviewed in 27). This multidomain protein was initially isolated as a binding partner of the cytoplasmic region of the leukocyte-antigen-related (LAR) receptor protein tyrosine phosphatase (RPTase) (28). LAR conveys attractive and repulsive cues from the extracellular medium to regulate the outgrowth of developing neurons. Several Trio-like proteins have been found in vertebrates including Trio, Duet/Duo/Kalirin (29-30), and in invertebrates including UNC-73 (Caenorhabditis elegans), and Dtrio (Drosophila). A Trio loss-of-function (trio-/-) mouse showed that Trio is essential for late embryonic development and that it functions in fetal skeletal muscle formation and in the proper organization of neuronal tissues (31). In PC12 cells, Trio induces neurite outgrowth (32) and mutations in the UNC-73 gene account for defects in axon guidance and cell motility (33). Genetic studies using Drosophila and C. elegans as model systems have positioned Trio at the center of signaling pathways that regulate axonal guidance and cell migration in the nervous system (33-37).

Unlike other RhoGEFs, which contain one DH/PH tandem domain, Kalirin, UNC-73, and Trio contain two DH/PH domains suggesting the possibility of activating at least two Rho-family members. In this respect, Trio and its homologues form a subfamily within the Dbl family of proteins. Besides the two DH/PH tandem domains, Trio contains multiple protein-protein
interacting domains and a Ser/Thr kinase domain (28). Additionally, a Src homology 3 (SH3) domain follows each DH/PH tandem domain in Trio suggesting a regulation by proline-rich containing proteins. This multidomain structure suggests that Trio is at the crossroads of multiple signaling pathways that regulate the actin cytoskeleton. In support of this view, the actin binding protein filamin and Tara both bind to Trio (38-39). In vitro exchange experiments have shown that the N-terminal DH/PH domain activates RhoG and Rac1 specifically but not the homologous Cdc42, while the C-terminal DH/PH domain is specific for RhoA (28, 40). Consistent with activating Rac, Trio has been functionally linked in Drosophila to the p21-activating kinase (PAK), a Rac downstream effector (35).

In an effort to understand the regulation of this multi-RhoGTPase activator, we have initiated structural and biochemical studies on various domains of Trio. Here, we present the three-dimensional structure of the N-terminal DH/PH of Trio, TrioN, as solved by X-ray crystallography. We used site-directed mutagenesis to pinpoint RhoG residues responsible for its specificity toward TrioN. We show that TrioN does not bind phospholipids in vitro. However, the presence of RhoG significantly increases the binding affinity of TrioN to certain phospholipids, especially PtdIns(3,4)P₂. We show that the variable basic C-terminal tail of the RhoGTPase is responsible for this effect. We propose that the protein-protein interaction between the basic tail of RhoG and the TrioN-PH domain regulates the interaction and the localization of Trio to the plasma membrane.
2- EXPERIMENTAL PROCEDURES

*TrioN Purification and Crystallization:* Purification and crystallization of the N-terminal DH/PH domain of human Trio (residues 1225-1535), TrioN, are reported elsewhere (41). Briefly, His-tagged TrioN is purified from bacterial cell lysates on a Ni-NTA matrix (Qiagen) followed by an ion exchange Q-Sepharose column (Sigma) and a sizing column (Ultrogel Aca54, Biosepra). Rod-shaped crystals of TrioN were obtained by incubating the protein at ~60mg/ml on ice in (0.1M NaCl, 20mM HEPES pH = 7.5, 10mM dithioerytriol (DTE)) overnight.

*Data Collection and Structure Solution:* One TrioN crystal was transferred into a solution of 5% PEG6000 (Fluka), 20mM HEPES pH 7.5, 0.2M NaCl) to which glycerol was gradually added to a final concentration of 30% before freezing in liquid nitrogen (41). Data to 1.7Å resolution were collected at beamline A1 at the Cornell High Energy Synchrotron Source (CHESS) and processed with the HKL97 package (42). The crystal belongs to space group P3_1 2_1 with one TrioN molecule in the asymmetric unit and unit cell dimensions of (a = b = 99.5 Å, c = 98.3 Å). The TrioN-DH domain was located by molecular replacement in AMoRe (43) and CNS (44) using the DH domain of Dbs (23) as a search model. Similar searches with the DH/PH of Dbs or with only the DH domains of Sos (26) or Tiam1 (25) failed to yield a clear solution for the rotation and translation functions. The molecular replacement solution was subsequently input to the automatic refinement procedure implemented in the ARP/wARP program run for 100 cycles in the mode “warpNtrace” (45). The ARP derived model contained all the TrioN residues located in secondary structure elements, or ~90% of the sequence. Residues present in loops were not built in this procedure despite clear electron density. These loops were further built in the program O (46) and the model refined in CNS (44). The final TrioN model consisted of residues 1231 to 1535 and has crystallographic indicators R/R_free of 19.5/21.4%. Residues N-terminal to 1231 are disordered in the final electron density. Statistics on data collection and refinement are reported in Table 1. Coordinates of TrioN have been deposited in the protein data bank under accession number 1NTY.
**Rac1 and RhoG Purification:** (His)$_6$-Rac1 was cloned in the pET15b (Novagen) vector and overexpressed in *Escherichia coli* strain BL21. Cells were broken by passage in a French press in (50mM Na/K phosphate, 0.15M KCl, 0.1mM phenylmethylsulfonylic PMSF, pH 7.5). Clarified cell lysates were passed on a Ni-NTA matrix (Qiagen) and eluted in (20mM Tris-HCl, 0.5M NaCl, 0.25M imidazole pH = 8.0). The (His)$_6$-tag cleaved protein (thrombin, Sigma) was collected in the flowthrough of an ion-exchange Q-Sepharose column (Sigma), concentrated in 10kDa cutoff centricon (Amicon) and passed over a gel filtration column (Ultrogel Aca54, Biosepra). Rac (0.3M NaCl, 20mM Tris-HCl, 5mM β-mercaptoethanol pH = 8.0) was concentrated to 10mg/ml and stored at −80°C until needed. Purification of the GST-RhoG (cloned in the pGEX-2T vector, Pharmacia) proceeded in a similar way but the first step was a glutathione sepharose affinity column (Sigma).

**Generation and Purification of Rac1 mutants:** All Rac1 constructs were cloned into the NdeI and BamHI sites of pET15b and verified by automated DNA sequencing. The A3S, G30K, Q43N, G54N, D63E, P69T, (A3S/G54N), and (G54N/P69T) mutants of Rac were generated with the QuickChange XL™ site-directed mutagenesis kit (Stratagene). The mutants were overexpressed in *E. coli* BL21 Codon +™ cells (Stratagene) and purified as the wild type protein. The purified proteins were stored at −80°C in 10mg/ml aliquots until needed. The C-terminal truncation mutant of Rac (residues 1-184), RacΔ184, was cloned by PCR into the NdeI and BamHI sites of pET15b. The RacΔ184 construct was overexpressed and purified as wild type Rac1.

**Generation and purification of the RhoGΔ182:** The C-terminal truncated RhoG mutant, residues 1-182 (RhoGΔ182), was generated from (His)$_6$-RhoG cloned into pET15b using the primers 5'-CCCACGCGATCTAGTAAGGCGGTCTGC-3' and 5'-GCAGGACCGCCCTTACTAGATCGCGTGGG-3' with the QuickChange XL site-directed mutagenesis kit (Stratagene) and verified by DNA sequencing. Purification of (His)$_6$-RhoGΔ182 from *E. coli* BL21 proceeded as for Rac1.

**PH domain of PLC-δ1 purification:** The clone for the PH domain of PLC-δ1 was generously provided by Dr. Mario Rebecchi and the protein was purified according to (47).
**Phospholipid Dot-Blot:** PIP-strips were purchased from Echelon Biosciences (Utah). Dot-blot experiments were carried out according to the manufacturer’s protocol. Strips were incubated 30 minutes in TBST (0.15M NaCl, 10mM Tris-HCl, 0.05% Tween 20, pH = 8.0) with 5% non-fat milk at room temperature then transferred to the bacterially purified tagged protein solution at 0.5µg/ml in TBST overnight at 4°C. Each strip was then washed 3 times in TBST buffer before incubating in an anti-His antibody (Santa Cruz) solution and a secondary horseradish peroxidase conjugate antibody solution. Antibody binding was detected using ECL plus western blotting detection reagents (Amersham Biosciences) and visualized using a Kodak Image Station 440CF.

**Guanine Nucleotide Exchange Assays:** Decrease in N-methylanthraniloyl-GDP (mant-GDP, Molecular Probes) fluorescence (excitation at 360nm/emission at 440nm) was monitored using an LS-50B spectrophotometer (Perkin Elmer) at room temperature according to the protocol described by Ahmadian and colleagues (48). 1µM RhoGTPase (total volume of 0.12ml placed in a quartz cuvette) was exchanged with mant-GDP and the fluorescent probe was chased with the addition of the non-hydrolysable GTP-analogue GppNHp to 200µM in presence or absence of 0.2µM TrioN. The decrease in fluorescence was recorded every 2 seconds. Fluorescence data points were visualized and fitted to a single decaying exponential using the program Origin 5.0 (Microcal).

**Isothermal Titration Calorimetry (ITC) experiments:** All proteins used in ITC binding experiments were dialyzed overnight against ITC dialysis buffer (20mM HEPES, 0.15M NaCl, and 5mM ß-Mercaptoethanol, pH 7.5). After dialysis, the dialysis buffer is retained and used for making dilutions or rinsing the ITC cell. The ligands Inositol-(1,3,4)-Triphosphate (Cayman Chemical) and Inositol-(1,4,5)-Triphosphate (Echelon Biosciences) were re-suspended in double distilled water to a concentration of 100mM. Before the ITC experiment, the ligands were diluted to the working concentration using ITC dialysis buffer. All ITC experiments were performed on a VP-ITC (MicroCal) at 10°C. 400µM ligand was introduced into the cell containing 25-50µM protein in a total of 14 injections of 20µl each for the RhoG/TrioN complex or 19 injections of 15µl each for TrioN alone. The data was analyzed using Origin5.0 (MicroCal).
3- RESULTS

Bacterially purified TrioN catalyzes nucleotide exchange on RhoG and Rac. To determine if the purified N-terminal DH/PH domain of Trio, TrioN, is active, we conducted nucleotide exchange experiments using purified RhoG and Rac preloaded with the fluorescent GDP analogue, mant-GDP. By monitoring the decrease in fluorescence due to mant-GDP release, we found that TrioN accelerates nucleotide release on Rac and RhoG. Addition of TrioN to Rac increases its rate of nucleotide exchange by a factor of 10 from $1.6 \times 10^{-4}$ s$^{-1}$ to $1.6 \times 10^{-3}$ s$^{-1}$ (Figure 1, open and closed triangles). The intrinsic GDP release from RhoG is very slow ($0.4 \times 10^{-4}$ s$^{-1}$, Figure 1, open circles), but addition of TrioN resulted in a $\approx 100$ time increase in its rate of GDP release ($5.1 \times 10^{-3}$ s$^{-1}$, Figure 1, closed circles) confirming that TrioN is specific for RhoG (40).

Specificity of TrioN in Exchange. Previous mutagenesis work has shown that residues 53 to 70 determine Rac specificity toward the Dbl-family proteins (49). Whereas TrioN does not bind to Cdc42, it is capable of binding to and accelerating nucleotide exchange on a Cdc42 chimeric protein in which residues 53 to 70 are those of Rac. More specifically, mutating Phe56 of Cdc42 to Trp (Cdc42F56W) results in a mutant that is activated by Rac-specific GEFs including TrioN to a rate similar to the ones obtained with Rac1. Trp56 is a Rac specificity determinant and is conserved between RhoG and Rac; nevertheless, TrioN seems to favor RhoG over Rac. Inspection of the Tiam1/Rac and Dbs/Cdc42 interfaces (http://www.biochem.ucl.ac.uk/bsm/PP/server/index.html) implied that the GTPase residues 3, 30, 43, 54, 63, and 69, which are not conserved between Rac and RhoG might be responsible for TrioN specificity. Of these residues, 3 and 54 contributed the most to the interface, 1.4 and 0.8% of the total surface, respectively.

To test this hypothesis, we generated the A3S and G54N mutants of Rac and measured their rate of nucleotide exchange in the presence of TrioN. Figure 2 shows that changing Gly54 on Rac to Asn more than doubled the rate of nucleotide exchange catalyzed by TrioN ($3.5 \times 10^{-3}$ s$^{-1}$ versus $1.6 \times 10^{-3}$ s$^{-1}$ for wild-type Rac). On the other hand, the A3S mutation alone or in the G54N background had little effect on the rate of nucleotide exchange. The exchange rate of the RacG54N
mutant suggests that additional residues on RhoG contribute with Asn54 to its specificity for TrioN. We thus mutated positions 30, 43, 63, and 69 on Rac to their equivalent in RhoG in the Asn54 background. Figure 2 shows that TrioN exchanges the Rac double mutant (G54N, P69T) with a rate (5.0 \times 10^{-3} \text{ s}^{-1}) similar to the rate measured for RhoG (5.1 \times 10^{-3} \text{ s}^{-1}). The other mutations had little or negative effect on the RacG54N mutant. Thus, residues at positions 54 and 69 on RhoG are specificity determinants for nucleotide exchange by TrioN.

The structure of the Dbs/Cdc42 complex predicts that Ser3 and Asn54 of RhoG are to make hydrogen bonds with Asn1357 and Ser1358 of TrioN; the equivalent residues Ala3 and Gly54 of Rac cannot. We therefore mutated Asn1357 of TrioN to Leu arguing that the apolar side chain of a Leu at this position is likely to pack against the side chains of Ala3 and Gly54 on Rac rather than with Ser3 and Asn54 on RhoG. The equivalent residue in Dbs is a Leu (Leu759) and makes van der Waals interactions with Gly54 of Cdc42. Thus, the TrioN(N1357L) mutant should be more specific toward Rac than RhoG. Figure 2 shows that contrary to our prediction, TrioN(N1357L) has a higher rate of nucleotide exchange for RhoG (9.4 \times 10^{-3} \text{ s}^{-1}) than for Rac (0.85 \times 10^{-3} \text{ s}^{-1}) highlighting that important differences exist between the TrioN/RhoG and other GEF/RhoGTPase complexes.

Three-dimensional structure of the TrioN domain The structure of TrioN was refined to a final crystallographic residuals of $R = 19.7\%$ and $R_{\text{free}} = 21.5\%$ and excellent stereochemistry (Table 1). The final electron density was clear enough to build all the TrioN sequence in the map except for residues N-terminal to 1231. A ribbon representation of TrioN is illustrated in Figure 3. The DH-PH domains of TrioN form the traditional L-shaped structure typical of DH/PH domains of other RhoGEFs (23-26). Superposition of the DH-modules of TrioN, Sos (26), Tiam1 (25), Dbs (23), and Intersectin (24), demonstrates that the relative orientation of the DH and PH modules of TrioN is closer to Dbs than to any other DH/PH structure. Comparison of TrioN and Dbs in the structure of the Dbs/Cdc42 complex shows that the PH-module of TrioN is rotated by approximately 20° relative to the axis of the last helix of the DH domain ($\alpha 6$) away from the GTPase binding site (Figure 3B). Interestingly, TrioN superposes extremely well with
uncomplexed Dbs (J. Sondek, personal communication). The root mean square deviation, rmsd, calculated after superposing 264 Cα from both structures is 1.7 Å (Figure 3C). These structural observations predict that as for Dbs, the PH domain of TrioN is likely to rotate by ~20° in order to interact with the RhoGTPase upon binding to the latter.

The DH/PH interface Interactions between the DH and PH domains occur between Gln1322 and the end of helix α6 (residues 1403-1411) of the DH domain, and residues of helix αN (residue 1421), of strands β1 and β2 (residues 1426-1430, 1449, 1451, 1453) and Tyr1472 of the PH domain (Figure 3D). The interface consists primarily of apolar interactions (62%) and direct or water mediated hydrogen bonds (38%) between residues belonging to the two domains and buries a total surface area of 635 Å². Since the residues located at the interface of the two domains are conserved between TrioN, Kalirin and UNC-73 (Figure 3D), these proteins are expected to have a similar DH/PH configuration. These residues are also conserved in Dbs.

The TrioN-DH domain The DH-domain of Trio (residues 1231-1413) has the typical elongated α-helical bundle characteristic of other DH domains of known structure (26, 50-51). Superposition of the DH of Trio to available DH coordinates shows that the closest homologue is Dbs. The rmsd calculated after superposing 164 Cα of the two DH domains is 1.42 Å. The structural homology is strong in the α-helices and poor in the helix-connecting loops and in the last helix α6 (residues 1391-1412, Dbs’s secondary structure numbering is adopted hereafter). In TrioN, α6 contains a proline in its middle (P1402) that introduces a kink. The residues of TrioN equivalent to residues of Dbs that directly interact with Cdc42 in the Dbs/Cdc42 structure are all solvent exposed in TrioN and are positioned at one face of the DH domain; they are shown in red in Figure 3D.

The TrioN PH-module. The PH module of TrioN (residues 1424-1535) is very similar to the PH-module of the δ1-isoform of phospholipase C (PLC-δ1). It is formed by two perpendicular β-sheets capped by a C-terminal α-helix (residues 1516-1535). Superposition of the PH domains of PLC-δ1 and TrioN yields an rmsd of 1.7 Å in 75 Cα positions despite 13% sequence identity. As expected, the structural similarity is confined to amino acids in secondary structure elements but
not in connecting loops. **Figure 4A** shows a Cα superposition of the PLCδ1-PH domain (light grey) in complex with Ins(1,4,5)P₃ (53) and the TrioN-PH domain (dark grey). This figure shows that among others, loop β1/β2 and loop β3/β4 have different conformations between the two domains with important consequences for phospholipid binding and specificity. In the TrioN structure, loop β1/β2, which in the PLCδ1-PH domain contains basic residues that interact with the phosphate groups of the Ins(1,4,5)P₃ moiety (shown in gold ball-and-stick in **Figure 4A**) occludes the inositol-trisphosphate binding site. Loop β3/β4, which in the PLCδ1-PH domain contributes residues that interact with the 5-phosphate of the Ins(1,4,5)P₃, is moved towards the DH-module away from the inositol-trisphosphate binding site. The important basic residues that make direct contacts to phosphates 4 and 5 in the PLC-δ1/Ins(1,4,5)P₃ complex (Lys30, Lys32, and Lys57) are also not conserved in TrioN (found as Val1435, Pro1438, and Asp465, **Figure 4C**). As presented here, the structure of the TrioN-PH domain is predicted to be inhibitory for phosphoinositol binding as seen in the PLC-δ1-PH domain; unless the two loops β1/β2 and β3/β4 undergo severe structural rearrangements.

We then investigated the possibility that the TrioN-PH structure would stabilize a phosphate at position 3 on the inositol ring by comparing the structures of the TrioN and the Grp1 PH domains (54-55). Grp1 is an Arf exchange factor that specifically binds PtdIns(3,4,5)P₃ with high affinity. The two PH domains share only 10% sequence identity but superpose extremely well (calculated rmsd of 1.5 Å in 85 Cα positions, **Figure 4B**). Loop β3/β4 is longer in the TrioN-PH than in Grp1-PH domain, the opposite is true for loop β6/β7. Interestingly, two out of the three basic residues that directly interact with the phosphate at position 3 (Arg284 and Arg305) in Grp1 are conserved both in sequence and in location in TrioN (Arg1448 and Lys1475) while the third one (Lys273) is not (Val1435, see **Figure 4B & 4C**). There is no equivalent for Grp1 residue Lys343 in TrioN and Tyr295 is found as a phenylalanine (Phe1459) in TrioN. Lys343 and Tyr295 of Grp1 make strong interactions with phosphates P4 and P5 of the inositol ring. Thus, our structural data suggest that the TrioN-PH domain could stabilize a phosphate at position 3 but not at position 4 or 5 of an inositol ring (**Figure 4B**).
Phospholipid-binding to TrioN

It is well accepted that PH domains primarily function as protein anchors to the plasma membrane. To investigate its role in localizing TrioN to the membrane, we searched for phosphoinositols target of the TrioN-PH domain using the PIP-strip assay. This well-established qualitative assay has been successfully used to find phosphoinositols targets of the PH domains of several RhoGEFs (56). Purified His₆-tagged-TrioN (0.5µg/ml) was applied to a nitrocellulose filter that was spotted with various phospholipids (Figure 5J). Following extensive washing, an anti-His₆ antibody was applied to the nitrocellulose filter and revealed with a secondary horseradish peroxidase antibody (see Experimental Procedures). As a positive control, the purified PH-domain of PLC-δ1 was assayed in the same way. As expected (Figure 5I), PLC-δ1-PH domain binds strongly to PtdIns(3,4)P₂ and to PtdIns(4,5)P₂. Under the same conditions, we were unable to show any binding for the TrioN-PH domain to any phosphorylated lipid (Figure 5A). We increased the concentration of the TrioN-PH domain by two orders of magnitude (50µg/ml) and repeated the same experiments arguing that very likely it has a weak affinity for phospholipids. Figure 5B shows that even at this concentration, the levels of detected TrioN with this assay are within the background. To check the sensitivity of the antibody to the His-tag on TrioN, we blotted 0.1ng of His-TrioN on a nitrocellulose membrane and showed that the antibody under the same experimental conditions used for the PIP-strip experiments efficiently recognized the His-tagged TrioN (data not shown). Therefore, we conclude that within the sensitivity of the PIP-strip assay, the TrioN-PH does not bind to phosphoinositols, or if it does, its binding affinity is very weak.

Phospholipids-binding to TrioN in the presence of RhoG

By binding to negatively charged phosphoinositol head groups through a positively charged patch on their surface, PH-domains achieve not only high affinity but also specificity (16). Our data show that the binding of the TrioN-PH domain to phospholipids is weak if it exists at all. So how does the TrioN-PH domain target Trio to the membrane? One possibility is that the PH domain provides a weak binding site for a phosphoinositol, while another protein provides additional basic residues to increase the binding affinity. One potential candidate protein in the case of a PH-associated DH-domain is the
RhoGTPase itself. The conserved C-terminal CAAX box of RhoGTPases, the site of posttranslational modification for proper membrane targeting and function, is usually preceded by a cluster of basic residues believed to associate with the membrane bound phospholipids.

We investigated the possibility that RhoG increases the TrioN-PH domain’s affinity to phosphoinositols using the PIP-strip assay. Figure 5C shows that indeed in the presence of RhoG at 0.5µg/ml, TrioN associates strongly with PtdIns(3,4)P₂, phosphatidic acid (PA), phosphatidylinositol (PI), and to a lesser extent PtdIns(4,5)P₂. Incubating RhoG alone with a PIP-strip filter showed no binding to phospholipids (Figure 5E) indicating that TrioN and RhoG are both needed to achieve phospholipid binding. To evidence that the C-terminus of RhoG is in fact synergizing with TrioN for phospholipid binding, we generated a truncated form of RhoG lacking the C-terminal end (residues 1-182, RhoGΔ182). Nucleotide exchange assays using mant-GDP show that TrioN accelerates nucleotide exchange of this mutant at a rate similar to the one measured for wild type RhoG (data not shown). This result suggests that the C-terminal region of RhoG is not required for nucleotide exchange by TrioN. However, when incubated with TrioN on a nitrocellulose PIP-strip filter, RhoGΔ182 was unable to assist TrioN in phospholipid binding (Figure 5D). Therefore, we conclude that the C-terminal 10 residues of RhoG assist TrioN in phospholipid binding since their removal is enough to inhibit the binding of the GEF/RhoGTPase complex to phospholipids.

Since the sequence of the C-terminal residues of the RhoGTPases is variable, we checked whether the binding of Rac mirrors RhoG in stimulating the binding of phospholipids to TrioN. Figure 5F and 5G show that unlike RhoG, Rac or a C-terminal Rac deletion (RacΔ182) in complex with TrioN do not bind to phospholipids. A PIP-strip assay with Rac alone does not show interaction with phospholipids (Figure 5H). This result suggests that in addition to the specificity in protein-protein interactions, there is specificity concerning phosphoinositol binding of the TrioN complex with its target GTPase.

Titration Calorimetry experiments To confirm the PIP-strip results and to investigate the selectivity of TrioN in presence or absence of RhoG for the phosphoinositides, we measured the
heat generated upon titrating TrioN with the soluble head groups of PtdInsP$_2$, Ins(1,3,4)P$_3$ or Ins(1,4,5)P$_3$ using isothermal titration calorimetry techniques (ITC). As shown in Figure 6 and as reported for other RhoGEFs (56), the heat generated per injection is small. However, Figure 6 shows saturation in the titration curves at protein:InsP$_3$ molar ratios of 1:2 and higher. Fitting the titration curves to a one binding site model resulted in association constants ($k_{ass}$) that were consistent with the PIP-strip results. $k_{ass}$ calculated from the ITC experiments for TrioN alone (Figure 6A & 6C) are 4.9 $10^4$ and 5.9 $10^3$ M$^{-1}$ (corresponding to dissociation constant of 20.4 and 170µM) for Ins(1,3,4)P$_3$ and Ins(1,4,5)P$_3$, respectively, consistent with a low binding affinity. The ITC measured association constants also agree with sucrose loaded vesicle experiments made using $^{14}$C-labeled PC-vesicles with 2% PtdIns(4,5)P$_2$ binding to TrioN or the PH domain of PLC-δ1 done according to Arbuzova and colleagues (57, data not shown). A $k_d$ of 2µM and of 100µM were measured for the PtdIns(4,5)P$_2$ binding to the PH domains of PLC-δ1 and TrioN, respectively, using the sucrose loaded vesicle technique.

To quantify the binding affinity of TrioN to phospholipids in the presence of RhoG, we measured the heat generated by titrating Ins(1,3,4)P$_3$ and Ins(1,4,5)P$_3$ to a purified TrioN/RhoG complex in a microcalorimeter. Figure 6B & 6D and Table 2 show that in presence of RhoG the binding affinity of TrioN for Ins(1,3,4)P$_3$ increases by a factor of 22, from 4.9 $10^4$ M$^{-1}$ to 1.1 $10^6$ M$^{-1}$. An increase in the binding affinity by a factor of 3.5 from 5.9 $10^3$ M$^{-1}$ to 2.1 $10^4$ M$^{-1}$ is also observed for Ins(1,4,5)P$_3$ in the presence of RhoG. Therefore, the ITC and the PIP-strip data are consistent with an increase in binding affinity and specificity of TrioN toward phosphoinositols in the presence of RhoG and thus shows that the interaction between RhoG and TrioN can induce TrioN binding to phospholipids.

Exchange in the presence of InsP$_3$ Since we could show that TrioN and RhoG synergize to bind phospholipids, we wanted to check if this binding affects the reaction of nucleotide exchange. We thus compared the release of mant-GDP from RhoG in the presence or absence of TrioN and various phospholipid head groups. As shown in Figure 7, addition of 10 times excess (2µM) of Ins(1,3,4)P$_3$, Ins(1,4,5)P$_3$ or the soluble head group of PA over TrioN (0.2µM) had no effect on the
rate of nucleotide exchange. We thus conclude that binding of TrioN to RhoG to stimulate nucleotide exchange and TrioN binding to phosphorylated lipids facilitated by the C-terminus of RhoG are two separate events.
4- DISCUSSION

Trio is a multidomain protein that plays an essential role in signaling pathways that regulate axonal guidance and neuronal outgrowth in vertebrates and invertebrates. Trio is the representative member of a newly characterized family of RhoGEFs with two DH/PH tandem domains including Kalirin and UNC-73 (reviewed in 27). The N-terminal DH/PH domain, or TrioN, is specific for RhoG and Rac1 while the C-terminal DH/PH domain activates RhoA, suggesting that Trio can coordinate the simultaneous activation of at least three RhoGTPases signaling pathways in vivo. Activation of the RhoGTPases catalyzed by Dbl-family proteins takes place on the plasma membrane where the RhoGTPases are localized through a geranylgeranyl or farnesyl group that is posttranslationally attached to a C-terminal conserved cysteine. Recruitment of RhoGEFs to membrane domains where the RhoGTPases are localized is a key event in the activation of several signaling pathways. The invariable presence of a PH domain immediately following the DH domain and the binding of PH domains to phosphoinositols suggest this domain is directly involved in recruiting RhoGEFs to phosphoinositol rich membrane domains. While the PH domain of some RhoGEFs like Vav and Sos (21-22, 58) has high affinity for phosphorylated inositols, others show weaker binding affinity of ~5-10µM (56) suggesting that recruitment of RhoGEFs to membranes is subject to tight regulation.

In this study, we have shown using a standard procedure involving a fluorescent-labeled nucleotide analogue (mant-GDP) that the bacterially expressed TrioN retains its exchange activity against Rac and RhoG. Whereas the intrinsic nucleotide release from RhoG is negligible, TrioN appreciably enhances its GTP to GDP exchange. According to our in vitro measurements, RhoG, which is highly homologous to Rac (73% sequence identity) is a better TrioN substrate than Rac since its observed rate of nucleotide exchange is ~3 times faster (Figure 1). Our results confirm earlier in vitro and in vivo reports that TrioN regulates RhoG activation (40). Activated RhoG can in turn stimulate Rac by binding to the ELMO/Dock180 complex (59).
We also solved the crystal structure of TrioN to 1.7Å resolution (Figure 3A). As expected, the Dbl-homology domain retains the α-helical bundle structure found in other DH domains, including the TrioN-DH solved by NMR (50). The conservation is more pronounced in the secondary structure elements (helices), less in the connecting loops, and breaks in the second half of helix α6 of the DH domains before entering the PH domain. Superposition of the coordinates of available DH/PH domains to TrioN shows that the divergence in the PH domain orientation starts at the residue corresponding to Pro1402 of α6 in TrioN. After this residue, the main chain diverges between the various GEFs leading to different relative orientations of the DH and PH domains. In this regard, TrioN is closer to the DH/PH domain of Dbs. As shown in Figure 3C, TrioN and the free form of Dbs superpose well. However, the PH domain of TrioN has to rotate by ~20° around the axis of helix α6 toward the GTPase binding site for TrioN to superpose on Dbs in the Dbs/Cdc42 complex (Figure 3B). The similarity between the DH/PH of Dbs and TrioN is striking, especially considering that the dissimilarity in the relative DH and PH orientation observed so far for RhoGEFs of known structures and because the two domains have different specificities regarding the RhoGTPase. This structural similarity further highlights the finding that the relative orientation of the DH and PH domains is independent of the RhoGEF specificity.

Because of the structural similarity between the apo-forms of Dbs and TrioN and because the residues of the PH domain of Dbs that interact with Cdc42 are conserved in TrioN, it is likely that the structure of the TrioN/RhoG complex resembles the structure of the Dbs/Cdc42 complex, implying a considerable rigid body movement of the PH domain for efficient exchange. However, recent truncation experiments showed that the TrioN-PH domain is not necessary for exchange (60), contradicting previous observations (50). To test whether the TrioN-PH domain is necessary for exchange, we mutated Tyr1472 to Phe. In Dbs, mutation of the equivalent PH-domain residue (Tyr889), which makes a direct hydrogen bond with Arg66 of Cdc42, strongly affects the rate of nucleotide exchange (23). We thus hypothesized that Tyr1472 should also make similar interactions with Arg66 of the GTPase and that the Y1472F mutation of TrioN should strongly reduce the rate of nucleotide exchange. To our surprise, the TrioN(Y1472F) mutant had a modest
effect on RhoG and Rac (2-fold decrease, Figure 2), suggesting that the TrioN-PH domain’s contribution to the reaction of exchange is not as dramatic as in the case of Dbs (60). This result, combined with the N1357L mutation suggests that despite structural homology between TrioN and apo-Dbs, our biochemical findings support the idea that important differences may distinguish their interfaces with the cognate GTPase.

The high-resolution crystal structure of TrioN can now be used as a basis to predict the residues that interact with RhoG/Rac and that determine its specificity. Based on the crystal structures of the Tiam1/Rac and Dbs/Cdc42 complexes and the mutagenesis work on TrioN (49-50), we can model the complex between TrioN and RhoG or Rac. The TrioN residues predicted to interact with RhoG/Rac are highlighted in Figure 3D. These residues, which comprise polar and apolar side chains are solvent accessible and surrounded by many water molecules visible in the electron density map. Thus, complex formation between TrioN and the RhoGTPase should be accompanied by a large change in entropy resulting from the expulsion of a large number of water molecules at the interface. Additionally, the TrioN structure predicts residues Asn1357 and Ser1358, located in the loop connecting helices α4 and α5, are specificity determinant for RhoG and Rac. These residues would make a hydrogen bond with Asn54 in RhoG but not with Gly54 in Rac1. Consistent with this hypothesis, the Rac(G54N) mutant results in an observed increase in the rate of nucleotide exchange, nevertheless alone the G54N mutation does not restore the rate of nucleotide exchange to the level measured for RhoG. Mutating Pro69 to Thr in the G54N background however mimics RhoG in nucleotide exchange assays catalyzed by TrioN (Figure 2). Thus positions 54 and 69 on RhoG are specificity determinant for TrioN. Position 54 is part of a polypeptide (residues 41-56) linking the switch regions that is not well conserved among RhoGTPases and that contains specificity determinants recognized by other RhoGEFs (49, 56, 61). Switch II residue 69, which is a Pro in all RhoGTPases except in RhoG where it is a Thr, contributes less than 0.2% to the interface of the Tiam1/Rac and Dbs/Cdc42 complexes.

In light of the TrioN structure, we can explain the rh40 mutant in C. elegans. This mutant carries a Ser to Phe mutation at position 1216 (S1216F) in UNC-73 reducing its ability to
hydrolyze Rac1 and to stimulate actin polymerization when expressed into Rat2 cells (33). The equivalent residue in TrioN, Thr1244, belongs to helix α1. The protein should easily accommodate the change of Thr1244 to a bulkier phenylalanine without major structural changes. However, introducing a phenylalanine at this position should significantly interfere with the binding of RhoGTPase to the DH module. Indeed, the equivalent residue in Dbs (Val643) is involved in tight van der Waals interactions with residues of the switch I region of Cdc42 including Thr35 and Val36.

Recently, a TrioN double mutant N1406A/D1407A has been described (52). This mutant binds to Rac1 but does not accelerate nucleotide exchange. Asn1406 and Asp1407 are two well-conserved residues in Dbl-family proteins located on helix α6 at the interface between the DH and PH domains (Figure 3D). In the TrioN three-dimensional structure, the side chain of Asn1406 is solvent exposed and makes one water mediated hydrogen bond with the side chain of the well conserved Gln1430 (β1) of the PH domain. The side chain of Asp1407 is buried at the interface with the PH domain and makes a strong hydrogen bond (3 Å) with Gln1430. The TrioN three-dimensional structure can explain the alanine mutagenesis results in two different ways. One explanation is that mutating these two residues to Ala should weaken the interaction of TrioN with the RhoGTPase since in the Dbs/Cdc42 structure, N810, the residue equivalent to N1406 of TrioN, makes strong hydrogen bonds with switch II residues 65 and 66 of the RhoGTPase. Alternatively, the double alanine mutant results in a relative DH/PH orientation that is inhibitory for exchange.

The TrioN-PH domain adopts a fold common to all known PH domains (reviewed by 16). The classical PH fold (residues 1426 to 1535) is preceded by a β-strand (βN, residues 1413 to 1415) and a $3_1$-helix (αN, residues 1421 to 1424) that immediately follow the DH domain. Since βN runs antiparallel to β4, it should be considered as part of the TrioN-PH domain (Figure 3A). Overall, the structure of the TrioN-PH domain is more similar to Grp1 (54-55) than to PLC-δ1 (53), especially in loops β1/β2 and β3/β4. The TrioN-PH domain loop β1/β2 occludes the site where Ins(1,4,5)P$_3$ binds in the PLC-δ1-PH domain and loop β3/β4, which contributes to the binding of the Ins(1,4,5)P$_3$ in PLC-δ1, is displaced toward the DH domain (Figure 4A). Sequence
alignment of the PH domains of TrioN, PLC-δ1 and Grp1 shows that important basic side chain residues, which interact with the phosphates at position 4 and 5 on the inositol ring (like Lys30, Lys32, Lys57 in PLC-δ1 and Lys273, Tyr295, and Lys343 in Grp1, Figure 4C) are not conserved in the TrioN-PH domain. Thus, structural and primary sequence comparisons predict that binding of phosphates at positions 4 or 5 of an inositol ring to the PH domain of TrioN is not favorable. These predictions are in total agreement with our PIP-strip and ITC experiments which estimate a dissociation constant of Ins(1,4,5)P$_3$ of ~100µM.

Interestingly, the structure of TrioN predicts that Arg1448 and Lys1475 of the PH domain can stabilize a phosphate at position 3 on the inositol ring despite the lack in the TrioN-PH domain sequence of a signature motif found in PH domains known to bind 3-phosphoinositides with high affinity (55). The PIP-strip assay done with TrioN alone did not show any interaction with any of the 3-phosphoinositides lipids, suggesting that Arg1448 and Lys1475 are not enough to stabilize a phosphate at this position (Figure 4D). Alternatively, additional basic residues are provided by another protein to the TrioN-PH domain for it to bind 3-phosphoinositides. Strong evidence in favor of the latter hypothesis is provided by a PIP-strip blot in which equimolar concentrations of TrioN and RhoG showed strong preferential binding to PtdIns(3,4)P$_2$, PtdIns, and PA. Figures 5A and 5C show that the affinity of the TrioN/RhoG complex for PtdIns(3,4)P$_2$ has dramatically increased in presence of RhoG. The PIP-strip experiments show that this affinity is probably in the same range of binding affinity of the PLC-δ1-PH domain to PtdIns(4,5)P$_2$, or ~1µM (62). Titration calorimetry experiments confirm this observation and suggest a binding affinity of ~1µM for Ins(1,3,4)P$_3$ when RhoG is bound to TrioN (Table 2). Since RhoG alone is unable to bind to any phospholipid (Figure 5E), we conclude that the complex provides a binding site for the PtdIns(3,4)P$_2$. Interestingly, this effect is specific to RhoG but not Rac, which has a different C-terminal sequence.

Deleting the C-terminal $^{183}$IKRGSCILL$^{192}$ sequence of RhoG resulted in a mutant (RhoGΔ182) that is indistinguishable from the full-length RhoG regarding exchange by TrioN. However, this mutant is unable to assist TrioN in binding to phosphoinositols in vitro (Figure 5D).
This result thus suggests the basic C-terminal loop of RhoG is not required for the exchange reaction but that it synergizes with TrioN to bind specific phospholipids. Our data do not show where on TrioN the PtdIns(3,4)P₂ binds but it is very likely that it is the PH domain. The ten C-terminal residues of RhoG could synergize with TrioN in binding to phospholipids in two ways. Either they provide basic residues that directly interact with the phosphoinositols or they could indirectly alter the structure of TrioN to enable it to bind phosphoinositols. The involvement of the C-terminal basic residues of RhoG in recruiting the GEF Trio to membranes becomes more interesting when envisioned in the context of another RhoGTPase regulator, the guanine dissociation inhibitor, RhoGDI. RhoGDI competes with RhoGEF activation of RhoGTPases by blocking the GDP-dissociation of the RhoGTPase and by releasing it from cellular membranes. The structure of prenylated Cdc42 in complex with RhoGDI (63) shows that the GDI not only binds to the switch regions on the RhoGTPase but also to its C-terminal basic tail. In light of our data, the GDI would not only destabilize the RhoGEF binding to the RhoGTPase but more importantly by interacting with the C-terminal basic tail of the GTPase, the GDI could prevent proper recruitment of the RhoGEF to where the RhoGTPase is localized.

The inability of TrioN to bind phospholipids in the absence of RhoG has important consequences regarding its recruitment to cellular membranes and its regulation. Our data suggest that the presence of phospholipids in itself is not enough to recruit TrioN to phospholipid bound membranes. However, the interaction with RhoG is required for TrioN recruitment to specific phospholipid rich domains. Particularly, the variable C-terminal sequence of RhoG provides specificity determinants for TrioN association with PtdIns(3,4)P₂. The combination of a faster exchange dictated at the protein-protein association level and specificity in protein-phospholipid interaction provided by RhoG C-terminus could explain the in vivo preference of TrioN for RhoG. This synergy in binding to phospholipids proposes that Trio ties the activation of RhoG signaling pathways with signaling pathways that generate the specific phospholipid, either PI, PA, or PtdIns(3,4)P₂.
Finally, the finding that the TrioN/RhoG binding to phosphoinositides is specific to PtdIns(3,4)P₂ suggests a role for the phosphoinositide 3-kinase (PI 3-kinase) in the activation of Trio and RhoG. So far, a direct involvement of PI 3-kinase in the activation of Trio or RhoG that leads to the regulation of the actin cytoskeleton has not been reported. A direct role for PI 3-kinase in Rac signaling through the activation of Rac specific GEFs is well established. PtdIns(3,4,5)P₃, a PI 3-kinase product, directly activates the RacGEFs P-Rex1 (64), SWAP-70 (65), Vav1 (21-22), and Sos (58). Rac also binds in a GTP-dependent manner to the RhoGAP homology on PI 3-kinase (66). In COS-7 cells, binding of Rac or RhoG to PI 3-kinase activates an anti-apoptotic pathway that prevents UV-induced cell death (67). A role for PI 3-kinase in RhoG regulation has yet to be shown.
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References


FOOTNOTES

Abbreviations used in the text: Dbs, Dbl big sister; DH, Dbl homology; GEF, guanine nucleotide exchange factor; GST, glutathione S-transferase; Ins(1,4,5)P$_3$, inositol 1,4,5-triphosphate; Ins(1,3,4)P$_3$, inositol 1,3,4-triphosphate; ITC, isothermal titration calorimetry; LPA, lysophosphatidic acid; LPC, lysophosphacholine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PH, Pleckstrin homology; PS, phosphatidylserine; PtdIns(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4)P$_2$, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P$_3$, phosphatidylinositol 3,4,5-trisphosphate; PI, phosphoinositide; PI 3-kinase, phosphoinositide 3-kinase; S1P, sphingosine-1-phosphate.
**Figure Legends**

**Figure 1:** Nucleotide exchange experiments catalyzed by TrioN. 1µM Rac (△) or RhoG (O) was loaded with mant-GDP, which was chased with excess GppNHp in presence or absence of 0.2µM TrioN. Decrease in fluorescence was monitored every 2 seconds on a spectrometer. Data points were visualized and fitted to a decaying exponential using the software Origin 5.0 to yield an observed rate of nucleotide exchange. See Experimental Procedures for more details. Intrinsic rates, open symbols: Rac (△, 1.6 $10^{-4}$ s$^{-1}$), RhoG (O, 0.4 $10^{-4}$ s$^{-1}$). Extrinsic rates, closed symbols: Rac (▲, 1.1 $10^{-3}$ s$^{-1}$), RhoG (●, 5.1 $10^{-3}$ s$^{-1}$).

**Figure 2:** Specificity in exchange. Rac and TrioN mutants were generated and tested for nucleotide exchange. Rates are plotted as average of at least three experiments as percentage of RhoG’s rate. Rate values in $10^{-3}$ s$^{-1}$ are as follow: RhoG = 5.1, Rac = 1.6, A3S = 1.1, G54N = 3.5, A3/G54N = 3.8, G30K/G54N = 0.9, Q43N/G54N = 1.8, G54N/D63E = 1.9, G54N/P69T = 5.0, TrioN(N1357L)/Rac = 0.85, TrioN(N1357)/RhoG = 9.4, TrioN(Y1472F)/Rac = 0.63, TrioN(Y1472F)/RhoG = 2.8

**Figure 3:** **A-** Ribbon diagram of TrioN. The DH domain is in blue and the PH domain is in hot pink. Secondary structure elements are labeled. **B-** Superposition of TrioN and the DH/PH domain of Dbs in the Dbs/Cdc42 structure (PDB accession number 1KZ7). The DH domains are in blue, the PH domain of Dbs in light pink, Cdc42 in yellow, and the switch domains are in green. Generated with Molscript (68) and Raster3D (69). **C-** Superposition of the structure of TrioN and the DH/PH domain of free Dbs. **B-** and **C-** have the same orientation. **D-** Sequence alignment of the DH/PH domains of Trio (N- and C-terminal, TrioN and TrioC), Kalirin/Duo/Duet, UNC-73.
(N- and C-terminal), and Dbs. Conserved residues are highlighted in yellow. Residues at the interface with the RhoGTPase in the Dbs/Cdc42 structure are in red. Residues at the interface between the DH and PH domains of TrioN are highlighted with a (*).

**Figure 4:** A- Superposition of the PH domains of PLC-δ1 (light grey) and TrioN (dark grey). The residues of PLC-δ1 involved in the binding to the Ins(1,4,5)P₃ moiety are shown in blue ball-and-stick. The corresponding residues in TrioN are shown in pink ball-and-stick and labeled. B- Superposition of the PH domains of Grp1 (light grey) and TrioN (dark grey). The residues of Grp1 involved in the binding of Ins(1,3,4,5)P₄ are shown in blue ball-and stick. The corresponding residues in TrioN are shown in pink ball-and-stick and labeled. C- Sequence alignment of the PH domains of TrioN, PLC-δ1, and Grp1 deduced from the superposition of their three dimensional structures done with O (47). Secondary structure elements deduced from the TrioN-PH domain structure are also shown. Residues that interact with the phosphates of the inositol ring at position 3, 4, and 5 are shown in red, green , and blue, respectively.

**Figure 5:** TrioN binds strongly to phospholipids only in the presence of RhoG. His₆-tagged bacterially purified TrioN was applied to strips spotted with various phospholipids (Echelon) and revealed with an anti-His or an anti-GST antibody as detailed under Experimental Procedures. All strips were revealed under the same conditions. Each experiments was repeated at least three times of which one representative is shown. A- His₆-TrioN at 0.5μg/ml. B- His₆-TrioN at 50μg/ml. C- His₆-TrioN/RhoG complex at 0.5μg/ml. D- A RhoG deletion mutant lacking the C-terminal basic residues, RhoGΔ182, in complex with His₆-TrioN at 0.5μg/ml. E- GST-RhoG at 0.5μg/ml. F- His₆-TrioN/Rac complex at 0.5μg/ml. G- A Rac deletion mutant lacking the C-terminal basic residues, RacΔ184, in complex with His₆-TrioN at 0.5μg/ml. H- His₆-Rac at 0.5μg/ml. I- Bacterially expressed PLC-δ1-PH domain at 0.5μg/ml. This strip serves as a positive control and
shows that our experimental procedure is reliable. J- Nomenclature of the different phospholipids spotted on the nitrocellulose strip. Comparison of C- and I- shows that the binding affinity of TrioN/RhoG to PtdIns(3,4)P₂, PA, or PtdIns is similar to PLC-δ1 binding to PtdIns(4,5)P₂, which is ~1µM (62).

**Figure 6:** Calorimetric titration of TrioN or Trio/RhoG experiments with InsP₃, 50µM TrioN in the cell at 10°C in 20mM HEPES pH 7.5, 0.1M NaCl was titrated with A- 400µM Ins(1,4,5)P₃ or C- 400µM Ins(1,3,4)P₃ solubilized in the same buffer. Similarly, 30µM TrioN/RhoG in the cell was titrated with B- 400µM Ins(1,4,5)P₃ or D- 400µM Ins(1,3,4)P₃. The fitted theoretical curves to the integrated data are shown in red and yield Kₐ values of A- 170µM, B- 20.4µM, C- 47µM, and D- 0.9µM. **Table 2** lists the thermodynamic parameters deduced from the fitting.

**Figure 7:** Phosphorylated lipids do not affect the exchange activity of TrioN on RhoG. Change in relative fluorescence due to mant-GDP release was recorded after adding 2µM of Ins(1,4,5)P₃, Ins(1,3,4)P₃ or 1,2-Dihexanoyl-sn-glycero-3-phosphate, a soluble PA analog, as indicated to 1µM RhoG and 0.2µM TrioN. Data were fitted to a decaying exponentials to yield Kₐ values, which are indistinguishable from the control done in the absence of soluble phospholipids. RhoG alone or in presence of 0.2µM TrioN are shown as controls.
Table 1: Statistics on data collection and model refinement.

Values for the highest resolution shell are given in parenthesis.

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R_{merge} (I) = \sum_h \sum_i |I_i(h)| - <I(h)> | / \sum_h \sum_i I_i(h), where h is a vector of the reciprocal space, I_i is the i^{th} measurement of the intensity I(h) and <I(h)> is the weighted mean of all measurements of I(h). 

<I/σ(I)> indicates the overall average of the intensity divided by its standard deviation. 

R_{work} = \sum_h [|F_o(h)| - |F_c(h)|] / \sum_h |F_o(h)| where F_o and F_c are the observed and calculated structure factor amplitudes calculated for the working set of data. 

R_{free} same as R_{work} for 10% of the data randomly omitted from refinement. Ramachandran statistics are for residues in most favored regions, additional allowed regions, and in generously allowed regions.
Table 2: Summary of titration calorimetry data for TrioN:

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Figure 1
Figure 2
Figure 3A
Figure 3B
Figure 3C
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Figure 4C
Figure 5
Figure 6
Figure 7
The C-terminal basic tail of RhoG assists the guanine nucleotide exchange factor Trio in binding to phospholipids
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