Mechanisms of Guanine Nucleotide Exchange and Rac-mediated Signaling Revealed by a Dominant Negative Trio Mutant*

Received for publication, July 29, 2003, and in revised form, October 9, 2003
Published, JBC Papers in Press, November 3, 2003, DOI 10.1074/jbc.M308282200

Balazs Debreceni‡¶, Yuan Gao‡, Fukun Guo‡, Kejin Zhu‡, Baqing Jia‡, and Yi Zheng‡‡

From the ‡Division of Experimental Hematology, Children's Hospital Research Foundation, Cincinnati, Ohio 45229 and the ¶Department of Biochemistry and Medical Chemistry, University of Pecs, Faculty of Medicine, Szigeti ut 12, 7624 Pecs, Hungary

Rho family GTPases play important roles in a variety of cellular processes, including actin cytoskeleton reorganization, transcription activation, and DNA synthesis. Dominant negative mutants of Rho GTPases, such as T17NRac1, that block the endogenous Rho protein activation by sequestering upstream guanine nucleotide exchange factors (GEFs) have been widely used to implicate specific members of the Rho family in various signaling pathways. We show here that such an approach could produce potentially misleading results since many Rho GEFs can interact with multiple Rho proteins promiscuously, and overexpression of one dominant negative Rho protein mutant may affect the activity of other members of the Rho family. Based on the available structural information, we have identified the highly conserved amino acid pairing of Asn1406Trio-Asp1406Rac1 of the GEF-Rho GTPase interaction as the critical catalytic machinery required for the Rac1 GDP/GTP exchange reaction. The N1406A/D1407A mutant of Trio acted dominantly negative in vitro by retaining Rac1 binding activity but losing GEF catalytic activity and competitively inhibited Rac1 activation by wild type Trio. It readily blocked the platelet-derived growth factor (PDGF)-induced lamellipodia formation and inhibited the wild type Trio-induced serum response factor (SRF) activity. Moreover the mutant was able to selectively inhibit Dbl-induced Rac1 activation without affecting RhoA activity in cells. In contrast to the non-discriminatory inhibitory effect displayed by T17NRac1, the Trio mutant was ineffective in inhibiting PDGF-stimulated DNA synthesis and Dbl-induced transformation, revealing the Rac-independent functions of PDGF and Dbl. These studies identify a conserved pair of amino acid residues of the Trio-Rac interaction that is likely to be essential to the GEF catalysis of Rho family GTPases and demonstrate that a dominant negative mutant derived from a Rho GTPase regulator constitutes a new generation of specific inhibitors of Rho GTPase signaling pathways.

The Rho family proteins consist of about 20 mammalian members including RhoA, Rac1, and Cdc42 (1). These Ras-related GTPases act as intracellular molecular switches that transduce diverse signals by cycling between the active GTP-bound and the inactive GDP-bound states, leading to cellular responses such as actin cytoskeleton reorganization, transcription activation, and DNA synthesis (1–3). The Rho proteins become activated through interaction with a class of positive regulators, the Dbl family guanine nucleotide exchange factors (GEFs),1 which serve to absorb the incoming signals to stimulate GDP dissociation from and to facilitate GTP binding to Rho GTPases (4–6). They become inactivated when the bound GTP is hydrolyzed to GDP through their intrinsic GTPase activity that is further accelerated by a class of negative regulators, the Rho GTPase-activating proteins (7). In the GTP-bound state, Rho proteins are able to recognize specific effector targets and induce downstream molecular events (8). It has been increasingly appreciated that the spatial and temporal control of the GTP binding/GTP hydrolysis cycle of Rho GTPases is essential for their proper function in cells (9).

The Dbl family GEFs are large multidomain proteins that serve as signal-convergent or -divergent points of Rho GTPase-mediated signaling (4–6). It is estimated that over 80 Dbl-like molecules may exist in the human genome. The conserved Dbl homology (DH) domain in tandem with a Pleckstrin homology (PH) domain of this family of Rho GTPase regulators constitutes the minimum structural module capable of activating Rho proteins in cells. The DH domain is responsible for the catalytic GDP/GTP exchange activity, while the PH domain has a supporting role for DH targeting and may affect the DH-Rho protein interaction directly or indirectly (5). Some of the GEFs display specific activity toward one Rho GTPase (e.g. FGD1 for Cdc42, Tiam1 for Rac1, and p115RhoGEF for RhoA), while others can activate multiple Rho GTPases promiscuously (e.g. Dbl and Ect2 can activate RhoA, Rac1, and Cdc42, and Dbs activates both RhoA and Cdc42) (4–6). It is likely that under physiological conditions each GEF is involved in a specific aspect of the Rho GTPase-regulated signaling process. But despite extensive effort by cell biological, genetic, biochemical, and structural approaches (5, 6), detailed mechanisms of the GEF reaction, regulation, and involvement of most GEFs in specific Rho signaling pathways remain unclear.

Dominant negative mutants of Rho GTPases are widely in use to elucidate the role of individual Rho proteins in different signaling pathways. For example, microinjection of the dominant negative T17NRac1 (where Thr17 is replaced by Asn) into the cytoplasm of Swiss 3T3 fibroblasts inhibited membrane

1 The abbreviations used are: GEF, guanine nucleotide exchange factor; GST, glutathione S-transferase; DH, Dbl homology; PH, Pleckstrin homology; PDGF, platelet-derived growth factor; mantGDP, N-methylanthraniloyl GDP; GFF, green fluorescent protein; DME, Dulbecco’s modified Eagle’s medium; SRF, serum response factor; CR, conserved region; IRES, internal ribosome entry site; TRITC, tetramethylrhodamine isothiocyanate.

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc. This paper is available on line at http://www.jbc.org
A Dominant Negative Rho GEF Mutant

ruffling elicited by PDGF (10), whereas dominant negative RhoA, Rac1, and Cdc42 could all inhibit serum response factor activation and G_{2/M} phase transition of the cell cycle (11) and Ras-induced cell transformation (12–14). The proposed mechanism of the dominant negative mutant function is that it binds to its respective GEFs with high affinity and may sequester the endogenous Rho GEFs by forming a non-functional dominant negative Rho GEF complex (15, 16). It is often assumed by users of these mutants that a given mutant blocks one or the specific pathway(s) that is activated by its wild type counterpart. Given the recent realization that many Dbl family GEFs, particularly the core DH-PH module that is responsible for Rho GTPase recognition and catalysis, are promiscuous in nature (5, 6), it could be expected that overexpression of one dominant negative Rho GTPase (typically 2–3-fold over the endogenous protein to be effective, Ref. 15) might indiscriminately inhibit other Rho GTPase activities by sequestering multiple GEFs or one GEF that is capable of activating multiple Rho protein substrates, leading to incorrect conclusions.

In the present studies we further examined the specificity issue of dominant negative Rho proteins. We show that dominant negative Rac1 could indeed cause nonspecific inhibition of RhoA activity in an onco-Dbl-transfected cell system. In the course of examining the catalytic mechanism of the Dbl family GEF function and investigating whether certain substrate-specific Dbl family GEFs can be engineered as dominant negative mutants for the dissection of Rho GTPase-mediated signaling pathways, we constructed a DH domain mutant of the GEF for Rac, Trio N-terminal DH-PH domains (TrioN), based on the available information of the three-dimensional structures of Dbl family GEFs. We report here the identification of a conserved pair of amino acid residues, Asn^{1406}Trio-Asp^{1407}Rac1, of the TrioN-Rac1 complex that are essential for the GEF catalytic mechanism of Rac1 and present evidence that a TrioN mutant, N1406A/D1407A, behaves as a dominant negative to specifically block Rac1 activation in vitro and in vivo. Our results indicate that dominant negative mutants derived from a Rho GTPase regulator may constitute a new generation of specific inhibitors of Rho GTPase signaling pathways.

EXPERIMENTAL PROCEDURES
cDNA Constructs—The DH domain point mutants of TrioN (T1244A, N1406A, and N1406A/D1407A; residues 1225–1537 of Trio) were generated by oligonucleotide-directed mutagenesis of human Trio cDNA in pET15b vector by the polymerase chain reaction-based second extension amplification technique using the Phusion polymerase (Stratagene) with primers that contained the desired mutations (17). The sequences of mutagenized cDNA inserts were confirmed by automated DNA sequencing. The BamHI-EcoRI fragments encoding the DH-PH module of wild type or mutant TrioN were subsequently subcloned into the corresponding sites of pmX-IRES-GFP vector for recombinant retroviral production or pCEFL-GST vector for transient transfection (17, 18).

Expression of Recombinant Proteins—Expression and purification of GST fusion small GTP-binding proteins or the His_{6}-tagged TrioN proteins from the pGEX vector or pET15b vector-transformed Escherichia coli were carried out as described previously (19, 20). The concentration and integrity of purified proteins were estimated by the Bradford assay and Coomassie Blue-stained SDS-PAGE.

In Vitro GDP/GTP Exchange Assay—The time courses for GDP/GTP exchange of Rac1 or D68A-Rac1 in the presence or absence of TrioN or TrioN mutants were determined by using the [3H]GDP binding nucleotide cellulose filtration method or by monitoring the fluorescence changes caused by nucleotide analog mantGDP dissociation from Rac1 (19, 20). The GEF reaction buffer contained [3H]GDP- or mantGDP-loaded Rac1 protein with 20 mm Tris-HCl, pH 7.6, 100 mm NaCl, 10 mm mantGDP, 0.5 mM GTP, and 1 mM dihydrothreitol supplemented with TrioN or TrioN mutants.

Complex Formation Assay—Complex formation between His_{6}-tagged TrioN or TrioN mutants and GST-fused dominant negative Cdc42 (T17Nc42) or Rac1 (T17N Rac1) were carried out similarly to that described previously (16). Approximately 5 μg of each GST-fused small GTPase oncogene were immobilized on agarose-glutathione beads, washed three times in a buffer containing 20 mM Tris-HCl (pH 7.6), 100 mM NaCl, and 2 mM EDTA, and then mixed with 2 μg of purified TrioN proteins in the presence of bovine serum albumin (~30 μg of total protein) for 1 h. The washed precipitates from the mixtures were subjected to 10% SDS-PAGE and transferred to nitrocellulose for Western blot analysis using anti-His_{6} antibody (Roche Applied Science). The immune complexes were visualized by using chemiluminescence reagents (Amersham Biosciences).

Retroviral Expression of TrioN and Dominant Rac1 and Cdc42 Mutants in Cells—Wild type, N1406A/D1407A, and T1244A of TrioN, T17N Rac1, and T17N Cdc42 were expressed in NIH 3T3 or Swiss 3T3 cells by using the pMX-IRES-GFP vector, which contains a fluorophore (GFP) as a bicistronic mRNA (17, 21). Retroviral packaging and infection were carried out according to the described methods (22). Briefly, ectopicPhoenix cells were transfected with individual retroviral vector, and the retroviruses were harvested 48 h post-transfection. The fibroblasts were infected with the retroviruses, and cells were harvested 72 h postinfection. GFP-positive cells were used for further analysis.

In Vivo Rac1 and RhoA GTPase Activation Assay—The onco-Dbl-expressing cells were generated previously (17, 18). The glutathione-agarose-immobilized GST-PAK1, which contains the p21-binding domain of human PAK1 (residues 51–140), and the GST-Rhotekin, which contains the site required for RhoA-GTP recognition of Rhotekin residues (18–20), were expressed and purified in E. coli by using the pGEX-KG vector described previously (23, 24). NIH 3T3 cells were grown to ~90% confluence in DMEM containing 10% calf serum before being washed with ice-cold phosphate-buffered saline once and lysed on the dish in a buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM mantGDP, 1 mM dithiothreitol, 1% Triton X-100, 0.1% SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were clarified by centrifugation at 13,000 × g at 4 °C for 15 min. Lysates containing equal amounts of total proteins were incubated with GST, GST-PAK1, or GST-Rhotekin (10 μg/sample) for 40 min at 4 °C under constant agitation. The lysate-incubated beads were then washed three times with the lysis buffer, and the bound Rac1 or RhoA was detected by antibody against Rac1 or RhoA (Santa Cruz Biotechnology).

Fluorescence Microscopy—Log phase growing fibroblasts were seeded at a density of 1 × 10^{4} cells/12-mm round coverslips (Fisher Scientific) overnight before fixation in phosphate-buffered saline containing 4% paraformaldehyde for 10 min at room temperature. The cells were permeabilized in Tris-buffered saline (pH 7.4) containing 0.2% Triton X-100 for 5 min and stained for F-actin using rhodamine-phalloidin (Molecular Probes). Coverslips were mounted onto slides in 50% glycerol, Tris-buffered saline. Stained cells were analyzed by using a Leica confocal fluorescence microscope (17).

Transient Luciferase Reporter Assay—For determination of serum response factor-dependent gene expression, the SRF-luciferase reporter plasmid (Stratagene) that contains the promoter response elements of SRF was used in transient co-transfections with the pCEFL vector or pCEFL-N1406A/D1407A in the presence of absence of the wild type TrioN cDNA (in pKH3 vector, Ref. 17). Transfection into NIH 3T3 cells was performed using LipofectAMINE reagents (Invitrogen) according to the manufacturer’s protocols. Analysis of luciferase expression in the co-transfected NIH 3T3 cells was carried out by using a luciferase assay kit from Promega. Transfection efficiencies were routinely corrected by obtaining the ratio of the luciferase and the β-galactosidase activities observed in the same sample. To assure that co-expression of wild type TrioN (hemagglutinin-tagged) was not affected by N1406A/D1407A, TrioN mutants were analyzed by anti-hemagglutinin Western blotting in parallel.

DNA Synthesis Assay—DNA synthesis was monitored by measuring incorporation of the artificial thymidine analogue 5-bromo-2’-deoxyuridine (BrdUrd) (Sigma) into newly synthesized DNA. The cells were cultured and synchronized as described previously (11). Release from cell cycle quiescence was achieved by washing cells with phosphate-buffered saline followed by two washes with DMEM for 5 min. BrdUrd (10 μM) was added to the medium upon release with 10% serum or PDGF (10 μM), and labeling was carried out for 16 h before the cells were fixed and stained with anti-BrdUrd antibody (BD Biosciences).

Soft Agar Growth Assay—NIH 3T3 cells expressing GFP, onco-Dbl together with GFP, onco-Dbl with GFP and TrioN mutant N1406A/D1407A, T17NRac1, or T17N Cdc42 were assayed for their capability to grow in soft agar as described previously (17, 25). Briefly, 2 × 10^{3} cells were suspended in DMEM supplemented with 10% calf serum and 0.3%
agarose and plated on top of a solidified DMEM with 10% calf serum and 0.5% agarose. Cells were fed weekly by the addition of 1 ml of DMEM supplemented with 10% calf serum and 0.3% agarose. Two and a half weeks postplating, colonies larger than 50 μm were scored under a microscope.

RESULTS

Administration of Dominant Negative Rac1 Could Nonspecifically Affect RhoA Activity in Cells—The dominant negative mutants of Rac1 and Cdc42, T17NRac1 and T17NCdc42, are widely used to inhibit Rac- or Cdc42-regulated signaling pathways. They were derived based on the corresponding mutant of Ras, which is capable of sequestering Ras-specific GEFs and blocks endogenous Ras activity (15). These mutants were shown to bind to the Dbl-like GEFs with high affinity in vitro (16) and to inhibit Rac-mediated lamellipodia formation and membrane ruffling or Cdc42-mediated filopodia induction in vivo (10, 26, 27) Given the apparent existence of a large number of Dbl family GEFs in mammalian cells and the realization that many of them can bind to and activate multiple Rho proteins (5), we were concerned that non-discriminative application of these dominant negative Rho mutants in cells where abundant GEFs are expressed may produce misleading results. To address the possibility that expression of one type of dominant negative Rho protein may unbalance the activity of distinct Rho family members by sequestering a commonly shared GEF, we transduced T17NRac1 or T17NCdc42 into the NIH 3T3 cells expressing oncogenic Dbl protein and examined the effect on cell actin structure and endogenous Rac1 and RhoA activities. As shown in Fig. 1A, the cells expressing Dbl showed a more compact cell shape. Filament actin staining revealed extensive lamellipodia along the cell edges and the retaining stress fibers in the cells in accordance with the previous findings that Dbl can bind to and activate multiple Rho proteins including Rac1 and RhoA (16, 28). Effector domain pull-down assays showed that the levels of the GTP-bound Rac1 and RhoA were significantly enhanced in the Dbl-expressing cells compared with the parental cells (Fig. 1B). When T17NRac1 or T17NCdc42 was co-expressed in the Dbl-expressing cells, it appears that either protein could inhibit the lamellipodia and actin stress fiber formation (Fig. 1A). Moreover they effectively suppressed both endogenous Rac1 and RhoA activities that were up-regulated by onco-Dbl (Fig. 1B). These results indicate that the conventional dominant negative mutants of Rho GTPases may function nonspecifically to inhibit distinct Rho GTPase activities by sequestering Dbl-like GEFs that are promiscuous in substrate recognition.

Identification of the Conserved Asn1406-Asp65 Pairing of TrioN-Rac1 as the Critical Determinant for GEF Catalysis—The Dbl-like GEFs outnumber Rho substrates by at least a 4 to 1 ratio (5, 6), and there is evidence that certain GEFs may play an important role in specifying Rho protein-mediated pathways (29). We rationalized that if the substrate-specific GEF mutants that preserve specific Rho GTPase binding but are catalytically compromised could be engineered, they might act dominant negatively in inhibiting individual Rho GTPase binding but are catalytically compromised could be engineered, they might act dominant negatively in inhibiting individual Rho GTPase substrate and could be explored to specifically down-regulate Rho GTPase-mediated signaling. Previously we have characterized a large panel of point mutants of oncogenic Dbl made in the DH domain and mapped conserved region 1 (CR1), conserved region 3 (CR3), and a part of α6 of the DH domain as well as the DH-PH junction site that are exposed near the center of one side of the molecule as the important sites involved in the
Thr1051 of Tiam1 is involved in van der Waals interaction with the switch I residue Val36 of Rac1 in the Tiam1-Rac1 complex (32). In particular, the highly conserved Asn1406 of TrioN appears to stimulate GDP dissociation from Rac1 by engaging Asp65 of Rac1 in a role that might be analogous to a glutamic acid of Sec7 domain for the ADP-ribosylation factor GTPase catalytic finger (38). Asp1233 of the DH domain is conserved among Dbl family members (Fig. 2, A and B), and such a pairing appears to be highly conserved in all the available structures of GEF-Rho protein complexes (32–34). We produced the purified components. As shown in Fig. 3, C and D, the D65A mutant of Rac1 has completely lost the responsiveness to TrioN stimulation in a fluorescence-based GEF activity assay while still retaining the ability to bind to TrioN in complex formation. These results indicate that the Asn1406–Asp65 pairing of TrioN and Rac1 interaction serves as a critical structural determinant responsible for the GEF catalysis, consistent with the previous structural prediction from the Tiam1-Rac1 complex (32).

Indeed a direct complex formation assay demonstrated that while T1244A only weakly interacted with Rac1, the N1406A/D1407A mutant mostly preserved the substrate binding activity under the assay conditions (Fig. 3B). A single point mutant of TrioN, N1406A, that contains mutation at the critical Asn1406, behaved similarly to the N1406A/D1407A double mutant (Fig. 2B and data not shown). These observations prompted us to further test whether the pairing residue of the substrate, Asp65 of Rac1, might also play a similar role in GEF catalysis. As shown in Fig. 3, A and B, the D65A mutant of Rac1 has completely lost the responsiveness to TrioN stimulation in a fluorescence-based GEF activity assay while still retaining the ability to bind to TrioN in complex formation. These results indicate that the Asn1406–Asp65 pairing of TrioN and Rac1 interaction serves as a critical structural determinant responsible for the GEF catalysis, consistent with the previous structural prediction from the Tiam1-Rac1 complex (32). In particular, the highly conserved Asn1406 of TrioN appears to stimulate GDP dissociation from Rac1 by engaging Asp65 of Rac1 in a role that might be analogous to a glutamic acid of Sec7 domain for the ADP-ribosylation factor GTPase activation (38) or a critical arginine residue termed “arginine finger” found in the Rho GTPase-activating protein catalytic reactions (39). The N1406A/D1407A Mutant of TrioN Acts Dominant Negatively for Rac1 Activation in Vitro and in Vivo—To examine whether the N1406A/D1407A mutant of TrioN could act dominantly negatively in vitro, we subjected the mutant to a competition assay in a Rac1 GEF reaction catalyzed by wild type Tiam1. As shown in Fig. 4, N1406A/D1407A was able to inhibit the wild type TrioN-stimulated [3H]GDP release from Rac1, and this inhibitory effect was dose-dependent, indicating that the mutant can indeed inhibit Rac1 activation albeit effective only at concentrations higher than wild type TrioN. To analyze the in vivo behavior of the mutant, next we tested its effects on cell morphology and actin cytoskeleton in Swiss 3T3 cells. Previously it has been established that Rac1 activation is a critical step in membrane ruffling and lamellipodia formation in these cells (10), while RhoA activity is essential for actin stress fiber formation (40). The GFP marker alone or GFP together with wild type TrioN, N1406A/D1407A, or T1244A was expressed in the cells by retroviral induction, and the GFP-positive cells (>90%) were analyzed. The GFP expression level by using the pMX-IRES-GFP vector has been demonstrated previously to quantitatively correlate with the IRES-regulated bicistronic gene expression (21). F-actin staining of these cells revealed that under the serum-free conditions both wild type TrioN and T1244A induced peripheral lamellipodia structures, whereas N1406A/D1407A caused a change of actin structure similar to T17NRac1 (Fig. 5). Upon PDGF stimulation, the GFP-expressing cells readily produced lamellipodia extensions, and the TrioN- and T1244A-expressing cells displayed further enhancement of their lamellipodia structures. In contrast, N1406A/D1407A readily inhibited the PDGF induction of lamellipodia but retained actin stress fibers of the cells, whereas T17NRac1 yielded a similar effect on lamellipodia but also eliminated actin stress fibers simultaneously (Fig. 5). These results indicate that N1406A/D1407A can act dominantly negatively to inhibit Rac1-mediated actin reorganization.

To evaluate the effect of N1406A/D1407A on Rac1-mediated signaling to the nucleus, we carried out a set of experiments to examine whether N1406A/D1407A could affect wild type
TrioN-stimulated serum response factor activity. As shown in Fig. 6, transfection of the cDNA of TrioN into the cells elicited a ~5-fold increase in the SRF reporter activity, and this effect was readily reversed by co-expression of the N1406A/D1407A mutant in a dose-dependent manner. Western blot analysis of the co-transfectants indicated that increased expression of N1406A/D1407A does not affect wild type TrioN expression in the transient expression system (Fig. 6). Combined with the above described in vitro inhibitory effect on Rac1 activation and in vivo inhibitory effect on Rac1-mediated actin reorganization, these results demonstrate that the N1406A/D1407A mutant of TrioN constitutes a dominant negative inhibitor of Rac-mediated signaling.

The N1406A/D1407A Mutant of TrioN Can Distinguishing Rac1 from RhoA in Cells—We have shown that overexpression of T17NRac1 causes non-discriminative inhibition of RhoA activity in Dbl cells (Fig. 1). Upon examination of the N1406A/D1407A-expressing Dbl cells, we found that in contrast to T17NRac1, expression of this TrioN mutant maintained the enhanced actin stress fibers but induced a contracted cell body and disappearance of lamellipodia (Fig. 7A), suggesting that this mutant can distinguish Rac1 from RhoA activity. Further effector pull-down assays show that N1406A/D1407A specifically inhibited Rac1 activity without affecting RhoA in the Dbl cells (Fig. 7B), establishing its specific nature in a cellular environment.

To demonstrate the advantage of the TrioN mutant over the dominant negative T17NRac1 mutant, we next compared the effects of these mutants in two cellular functions: the PDGF-induced DNA synthesis and the Dbl-induced cell transformation. Rac1, as well as other Rho proteins, has previously been implicated in serum-induced cell cycle progression through G1 phase (11) and was shown to be required for Dbl-induced cell transformation by the administration of T17NRac1 (44). We observed that in the GFP-expressing control cells serum starvation led to a low basal DNA synthesis activity that could be significantly stimulated by PDGF, serum, wild type TrioN, or the T1244A mutant of TrioN (Fig. 8A). Whereas T17NRac1...
effectively blocked the PDGF-elicited DNA synthesis but had no effect on the serum-induced activity, N1406A/D1407A did not alter the PDGF-induced BrdUrd incorporation (Fig. 8A) despite its significant impact on PDGF-induced actin structure of the cells (Fig. 5). Consistent with the notion that multiple Rho GTPases contribute to Dbl-mediated cell transformation (28, 44), the N1406A/D1407A mutant that inhibited endogenous Rac1 activity (Fig. 7B) did not affect anchorage-independent growth of the Dbl cells, contrary to the marked inhibitory effect of T17NRac1 or T17NCdc42 (Fig. 8B). These results further highlight potential complications of the use of conventional dominant negative Rho protein mutants and suggest that a dominant negative mutant derived from regulators of specific Rho family members such as Trio is better suited for the dissection of specific Rho GTPase signaling pathways.

**DISCUSSION**

Dominant negative mutants of Rho GTPases, such as T19NRhoA, T17NRac1, and T17NCdc42, have been widely in use to elucidate signaling pathways in different cell biological systems. They were derived based on the biochemical principle of a similar point mutant of Ras, which was discovered to be inhibitory for Ras signaling by random mutagenesis screening (41). Our current understanding of these dominant negative mutants predicts that they compete with the endogenous small GTPase counterparts for high affinity binding to their GEFs to form activation-defective GTPase-GEF complexes (15). Indeed previous biochemical analysis of dominant negative Rho proteins have confirmed their high affinity binding to Dbl-like GEFs when they are in a nucleotide-depleted state (16). Although it seems logical to expect that the dominant negative Rho proteins may have a nonspecific effect when applied to sophisticated cellular systems by overexpression because many Dbl-like GEFs show promiscuity in binding activity toward multiple Rho GTPases (4–6), it remains to be demonstrated, let alone to be cautioned, that such a potential may lead to erroneous conclusions in certain cases.

To provide direct experimental evidence that such speculation may bear truth, we used the Dbl-expressing cells that contain elevated Rac1 and RhoA activities as a model system in the present studies. The rational is that if dominant negative Rac1 could interfere with Dbl-mediated RhoA activation in the commonly shared GEF-expressing system, the nonspecific effect of dominant negative mutants of Rho proteins may well be manifested in more physiological relevant cellular contexts where GEF expressions are at a lower level, and the GEF activation of Rho proteins should occur more subtly. Our results clearly demonstrate that T17NRac1, as well as...
T17NCdc42, can block the actin reorganization and biochemical activities of both Rac1 and RhoA in Dbl cells (Fig. 1). The effects are consistent with the explanation that these mutants may titrate out the Rho protein activator Dbl by sequestering it in an activation-deficient dominant negative Rho GTPase-Dbl complex as suggested by previous in vitro biochemical studies (16). Therefore it is necessary to raise concerns to a body of literature in which the non-selective use of dominant negative Rho protein mutants was the basis for implicating the involvement of specific Rho family members in complex cell signaling networks.

Our laboratory has been interested in the structure-function relationship of regulators of Rho GTPases, including the Dbl family GEFs (5). The Dbl-like GEFs for Rho GTPases share a common structure unit, the DH-PH module, that constitutes the minimal structural domains required for activation of Rho substrates in cells. Previous biochemical studies from a number of laboratories including ours have found that certain Dbl family members can work specifically toward one or one subset of Rho protein substrate, while others may function quite promiscuously toward multiple Rho substrates (4–6). In scanning mutagenesis studies of the DH domain function, we have identified multiple residues located in the CR1, CR3, and the DH-PH junction site that are exposed near the center of one side of the molecule as the important sites involved in the formation of a Rho GTPase interactive pocket (17). Based on the structural information derived from the complexes of Rac1-Tiam1, Cdc42-Dbs, Cdc42-Intersectin, and RhoA-Dbs, Sondek...
and coworkers (32–34) have also analyzed a number of amino acid pairings between Rho proteins and GEFs that are essential for GEF catalysis and identified a few residues of the DH domain that appear to be responsible for substrate selection. Although the residues of the DH-PH module identified so far, including Gly1368, Met1369, and Leu1376 of Intersectin and Lys278, Leu279, and Leu280 of Dbs, appear to contribute to Cdc42 or RhoA specificity to a different extent (34, 42), the key residue(s) that might play a critical role in the generalized Rho protein GEF catalysis similar to a glutamic acid of Sec7 domain for the ADP-ribosylation factor GTPase activation (38) or an arginine residue of Rho GTPase-activating proteins that is essential for accelerating the GTPase activity of Rho proteins (39) remains to be identified.

In this study we examined the role of the two most conserved residues of the TrioN GEF module, Thr1244 that is located in the CR1 and Asn1406 that is located close to the C terminus of the DH domain, in detail. The corresponding mutation of T1244A was first described in the Caenorhabditis elegans UNC-73 gene, which resulted in a loss of function (30, 43), whereas a mutant of Dbl corresponding to N1406A/D1407A of TrioN was found to lack transforming activity (17). The crystal structures of four pairs of Rho-GEF complexes all show a highly conserved mode of interaction of Thr1244 and Asn1406 with the former engaging in a van der Waals interaction with the switch I residue Val130 of Rho proteins and the latter involved in hydrogen bonding to the switch II Asp135 of Rho proteins (Fig. 2 and Refs. 32–34). Interestingly T1244A was found to be partially inactive in the GEF activity toward Rac1, correlating with its loss of binding activity to the substrate, whereas N1406A/D1407A and N1406A were found to be completely inactive in the GEF assays but retained the substrate binding activity (Fig. 3). N1406A/D1407A was not only inactive in stimulating GDP dissociation of Rac1 but also inhibited the intrinsic GDP/GTP exchange activity of Rac1 (Fig. 3). The reciprocal mutant of D65ARac1 behaved toward TrioN in a manner similar to N1406A toward Rac1, confirming the importance of the Asn1406-Asp65 pairing between TrioN and Rac1 in the GEF catalytic mechanism. In cells, overexpression of T1244A led to a partial stimulation of membrane ruffling compared with wild type TrioN, while N1406A/D1407A failed to elicit such an effect (Fig. 5). Taken together, these results implicate Asn1406 of TrioN as the critical determinant in the GEF catalytic reaction of Rac1. Similar biochemical observations were also made for the corresponding Asn-Asp residues in Lbc-RhoA, FGD1-Cdc42, and Dbl-Cdc42 pairings of the GEF-Rho protein reactions. We propose that the highly conserved asparagine residue found in all Dbl family GEFs plays a parallel role in catalyzing Rho GTPase exchange as the famed arginine finger found in the Rho GTPase-activating protein catalytic reactions (39) or the glutamic acid residue found in the Sec7 domain in catalyzing the guanine nucleotide exchange of ADP-ribosylation factor family small G-proteins (38).

The discovery of the key mechanistic role of Asn1406 of TrioN provided a valuable clue that a mutant generated from this residue might behave in a dominant negative manner in inhibiting Rac-mediated signaling pathways. Indeed we found that the mutant N1406A/D1407A of TrioN could inhibit Rac1 activation by wild type TrioN in vitro and effectively block the Rac1-mediated membrane ruffling and lamellipodia formation induced by PDGF in cells (Figs. 4 and 5). Furthermore N1406A/D1407A effectively blocked the TrioN-stimulated SRF transcriptional activity (Fig. 6). These combined results indicate that N1406A/D1407A constitutes a dominant negative inhibitor of Rac signaling pathways. However, the N1406A/D1407A mutant did not seem very effective in competing with wild type TrioN for inhibiting Rac1 exchange in vitro possibly because the effectiveness of the competitive inhibition partly depends on the concentrations of Rac1 and wild type Trio in addition to the concentration of the Trio mutant present. Examination of the kinetics of TrioN-catalyzed Rac1 exchange in the presence of increasing concentrations of N1406A/D1407A (Fig. 4) shows that N1406A/D1407A can interfere with the initial reaction rate of Rac1 exchange competitively and may compete for binding to rather than stabilizing a subpopulation of Rac1 in the

2 B. Debreceni and Y. Zheng, unpublished observations.
unlike T17N Rac1, N1406A/D1407A did not affect Dbl-induced growth of the cells in soft agar despite its inhibitory effect on the Dbl-induced membrane ruffling and endogenous Rac1 activity. These results strongly suggest that N1406A/D1407A acts more specifically in blocking Rac-mediated signaling events than does T17N Rac1.

In summary, in the current work we present evidence that non-discriminative administration of a dominant negative mutant of Rho proteins such as T17N Rac1 may cause a nonspecific effect on multiple Rho GTPase-regulated signaling pathways. We have identified a conserved pair of amino acid residues of the Trio-Rac complex that is essential to the GEF catalysis of Rho GTPases, providing insights into the catalytic mechanism of Rho GTPase activation reaction. We offer an alternative to dominant negative Rho GTPases, the dominant negative regulator of Rho proteins such as N1406A TrioN, as a new generation of Rho GTPase pathway-specific inhibitors. We expect that the future design of a dominant negative mutant similar to N1406A of TrioN made in the full-length backbone of the Dbl family GEFs may provide more valuable pathway-specific inhibitors that are useful for the dissection of sophisticated signal transmission through individual Rho proteins in the complex cell signaling paradigms.

REFERENCES
