Mechanisms of Guanine Nucleotide Exchange of Rho GTPases by Dbl Family Proteins

Ph.D. theses

Balázs Debreceni

Department of Biochemistry and Medical Chemistry
University of Pécs, Faculty of Medicine

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Program leader: Balázs Sümegi, D.Sc.
Mentor: Yi Zheng, Ph.D.

Introduction

The Rho family GTPases are small molecular weight gunanine nucleotide-binding proteins that belong to the Ras superfamily of GTPases. Their importance became clear about a decade ago when they were first shown to regulate actin cytoskeleton rearrangements in mouse fibroblasts. Since then about twenty members of the Rho family have been identified, with RhoA, Rac1 and Cdc42 being the best characterized. RhoA induces the formation of actin stress fibers and focal adhesions, Rac and Cdc42 proteins regulate lamellipodia and filopodia formation, respectively. In our days it is apparent that they are not only involved in organizing cytoskeletal changes in cells but also involved in the regulation of many other processes, like G_1 progression of the cell cycle, cell proliferation, DNA synthesis, transcription factor activation and activation of the p^{67}NADPH-oxidase in neutrophil granulocytes, just to mention a few. Rho GTPase proteins are highly conserved molecules, sharing about 50-90 % sequence similarity within the family and about 30-50 % similarity to other Ras GTPase proteins. Similarly to Ras proteins, Rho GTPases are post-translationally modified at their C-terminus (CAAX sequence), acquiring an isoprenoid lipid tail which is important in directing them to the plasma membrane.

Rho GTPase proteins exist either in an active GTP-bound or in an inactive GDP-bound state. After stimulation by upstream signals, GDP-bound Rho GTPases release GDP and bind GTP. In the GTP-bound active state Rho proteins recognize and activate their specific effector targets and induce downstream molecular events. They posses GTPase activity which hydrolyzes GTP to GDP, returning the GTPase to the inactive state. The inactivation of GTPases leads to the release and inactivation of the bound effectors. There are two groups of proteins that interact with Rho family GTPases to regulate the GDP/GTP binding cyle: (i) the Dbl family proteins that are Rho GTPase specific guanine nucleotide exchange factors (Rho GEFs), and (ii) the Rho GTPase activating proteins (Rho GAPs). The former greatly enhance the GDP/GTP exchange rate by stimulating GDP dissociation from and facilitating GTP binding to Rho GTPases, the latter accelerate the GTPase activity. It has been increasingly accepted that the spatial and temporal control of the GTP-binding/GTP-hydrolysis cycle of Rho GTPases is essential for their proper function in cells.

The Dbl family GEFs are large multidomain proteins that contain at least one DH-PH domain unit, where the PH domain is invariably located immediately C-terminal to

the DH domain. While the DH domain alone is able to facilitate the exchange of bound GDP for GTP *in vitro* on Rho proteins, the DH-PH unit is necessary for their activity *in vivo*. In the past few years a large number of Dbl family members have been discovered. Except for the DH-PH module, these proteins are very diverse in their stucture and size. In addition to the conserved PH-DH domain they contain different domains that vary in the different Rho-GEFs. Little is known about their exact role, but probaly they regulate the activity of the GEFs and provide connections to upstream activators. Since many Dbl family proteins may exert potent protooncogenic effects, their activity must be strictly regulated in cells under normal conditions. Before stimulation the inactivity of Dbl proteins is maintained by different mechanisms in cells: By intramolecular interactions between the PH and the DH domains, or by interactions between the DH or PH domain and a regulatory domain. Oligomerisation through the DH domains and binding to specific inhibitory factors also helps to keep them in a minimum activity state. Numerous upstream signals can attenuate the negative regulation and stimulate the GEF activity. These include signals originating from G protein coupled receptors, cytokine receptors, growth factor receptors etc. Activated Ras protein, α_{13} and $\beta\gamma$ subunits of heterotrimeric G proteins and phosphatidylinositol 3,4,5-trisphosphate were shown to bind directly to certain Dbl proteins and increase their activity. However the the detailed mechanisms of the activation of Dbl proteins and how they facilitate the GDP/GTP exchange on Rho proteins is still not clear.

Objectives

The three-dimensional structures of the DH domain of the Dbl family members β-PIX, Sos and the N-terminus of Trio have been resolved. The tertiary structures are remarkably similar among the three DH domains, all depicting a flattened, elongated α-helix bundle in which two of the three conserved regions, conserved region 1 (CR1) and conserved region 3 (CR3) are exposed near the center of one surface. Sequence analysis and limited mutagenesis studies suggest that several conserved residues in these regions may be involved in the formation of a Rho GTPase-interacting pocket. However, what biochemical function these residues exert in the nucleotide exchange reaction (nucleotide exchange *versus* Rho protein binding) and to what extent these sites contribute to the GEF-Rho protein interaction have not been assessed. To identify specific sites of the DH domain contributing to the nucleotide exchange or binding

activity for Rho GTPases, we have characterized a large panel of DH mutants of oncogenic Dbl *in vitro*. The results help define specific roles of individual residues of the DH domain in the GEF reaction and binding activity.

Dominant negative mutants of Rho GTPases (such as T17NRac1 or T17NCdc42) are widely in use to elucidate the role of individual Rho proteins in different signaling pathways. The proposed mechanism of the dominant negative mutant function is that they bind to their respective GEFs with high affinity and may sequester the endogenous Rho GEFs by forming non-functional dominant negative Rho protein-GEF complexes. It is often assumed by users of these mutants that a given mutant blocks only the specific pathway that is activated by its wild type counterpart. Given the recent realization that many Dbl family GEFs, particularly the core DH-PH module which is responsible for Rho GTPase recognition and nucleotide exchange, are promiscuous in nature, it could be expected that overexpression of one dominant negative Rho GTPase, (typically two to three folds over the endogenous protein to be effective), might indiscriminatively inhibit other Rho GTPase activities by sequestering multiple GEFs or one GEF that is capable of activating multiple Rho proteins, leading to incorrect conclusions. In our experiments we have examined the specificity issue of dominant negative Rho proteins. We show that dominant negative Rac1 (T17NRac1) could indeed cause nonspecific inhibition of RhoA activity in an onco-Dbl transfected cell system.

Based on our findings with the Dbl mutant N673A/D674A and T17NRac1 we have constructed a DH domain mutant of the Rac specific GEF, the N-terminal DH-PH module of Trio (TrioN), to examine the catalytic mechanism of Dbl family GEFs, and investigate whether mutants of certain substrate-specific Dbl-family GEFs can be used to specifically block their corresponding Rho GTPase-mediated signaling pathways. We show, that a conserved pair of amino acid residues, Asn1406Trio-Asp65Rac1 of the TrioN-Rac1 complex is essential for the GEF elicited nucleotide exchange of Rac1, and present evidence that a TrioN mutant, N1406A/D1407A, behaves as a dominant negative inhibitor to specifically block Rac1 activation *in vitro* and *in vivo*. Our results indicate that dominant negative mutant derived from a Rho GTPase regulator constitutes a new generation of specific inhibitor of Rho GTPase signaling pathways.

Materials and Methods

Generation of mutant Rho GEF cDNA constructs

The DH domain point mutants of Dbl and TrioN were generated by polymerase chain reaction (PCR) using primers that contained the desired mutations. The DNA fragments encoding the mutants were subsequently subcloned into pVL1392, pZipneo, pKH3, pMX-IRES-GFP or pCEFL-GST vectors. The sequences of mutagenized cDNA inserts were confirmed by automated DNA sequencing.

Expression of recombinant proteins

GST-fused small GTP-binding proteins or the $(His)_6$-tagged TrioN proteins were expressed in and purified from pGEX or pET15b vector-transformed *E. coli*. GST-Dbl and DH mutants were produced in and purified from Sf9 insect cells. The concentration and integrity of purified proteins were estimated by the Bradford assay and Coomassie Blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using serum albumin as a standard.

In vitro GDP/GTP exchange assay

The time courses for [^{3}H]GDP/GTP exchange of Rho family GTPases (Cdc42, Rac1 or D65ARac1) in the presence or absence of the exchange factors (Dbl, Dbl mutants, TrioN or TrioN mutants) were determined by using the [^{3}H]GDP-binding nitrocellulose filtration method or by monitoring the fluorescence changes caused by nucleotide analog mant-GDP dissociation from Rac1.

Complex formation assay

HA-Dbl, Dbl DH mutants, $(His)_6$-tagged TrioN or TrioN mutants were incubated with agarose-immobilized GST-fused dominant negative Rho GTPases (T17NCdc42, T17NRac1 or T19NRhoA). The washed precipitates from the mixtures were subjected to 10 % SDS-PAGE and transferred to nitrocellulose for Western blot analysis using anti-$(His)_6$ (Boehringer-Mannheim) or anti-HA (Roche Molecular Biochemicals) antibody.

Expression of TrioN and dominant negative Rac1 and Cdc42 mutants in cells

Wild type, N1406A/D1407A and T1244A of TrioN, T17NRac1 and T17NCdc42 were expressed in NIH 3T3 or Swiss 3T3 cells by using the pMX-IRES-GFP vector. Retroviral packaging and infection were carried out as follows: Ecotropic Phoenix cells were transfected with individual retroviral vector and the retroviruses were harvested 48 hrs post-transfection. The fibroblasts were infected with the retroviruses and cells were harvested 72 hrs post infection. GFP positive cells were sorted by fluorescence activated cell sorting (FACS) and were used for analysis immediately after sorting.

***In vivo* GTPase activation assay**

Glutathione agarose-immobilized GST-PAK1 and GST-Rhotekin were expressed and purified in *E. coli* by using the pGEX-KG vector. Lysates of NIH 3T3 cells containing equal amount of total proteins were incubated with GST, GST-PAK1, or GST-Rhotekin. The lysate-incubated beads were washed, and the bound Rac1 or RhoA were detected by anti-Rac1 or RhoA antibody (Santa Cruz Biotechnology).

Fluorescence microscopy

Log phase growing fibroblasts were seeded on round coverslips (Fisher Scientific) overnight before fixation and permeabilization. The cells were stained for F-actin using rhodamine conjugated phalloidin (Molecular Probes). Stained cells were analyzed by using a Zeiss confocal fluorescence microscope.

Transient luciferase reporter assay

For determination of serum response factor (SRF)-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 or absence of the wild type TrioN cDNA in pKH3 vector. Analysis of luciferase expression in the co-transfected NIH3T3 cells was carried out by using a luciferase assay kit from Promega.

DNA synthesis assay

DNA synthesis was monitored by measuring incorporation of the artificial thymidine nucleotide analog 5-bromo-2'-deoxyuridine (BrdU) (Sigma) into newly synthesized DNA. BrdU (10 μM) was added to the medium with 10 % serum or PDGF (10 nM), and 16 hrs later the cells were fixed and stained with anti-BrdU 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 T17NCdc42, were assayed for their capability to grow in soft agar. Cells were suspended in DMEM medium supplemented with 10 % calf serum and 0.3 % agarose. Two and a half week post-plating, colonies larger than 50 μm were scored under a microscope.

Results

Analysis of the DH domain point mutants of Dbl

To examine the biochemical roles of the residues in the DH domain and to assess the contribution of individual residues to DH function in a biological setting, we have generated 19 alanine substitution mutations of the DH domain at CR1, CR3, and surrounding regions in the DH-PH backbone of oncogenic Dbl, based upon the structural predictions. Of the four CR1 and five CR3 region mutants examined, five displayed significant loss of GEF activity toward Cdc42 in the in vitro GDP/GTP exchange assay. The T506A mutation of CR1 caused the most severe effect, whereas L640A, where the leucine resides in the N terminus of hydrophobic LLLKELL sequences of CR3, appeared to retain only a fraction of the Cdc42 activation potential. Mutation of the charged residues Glu502, Gln633, and Arg634 also led to partial but significant loss of GEF activity, whereas the neighboring charged residues Gln505, Arg508, Lys630, or Lys643-Glu644 did not seem to directly participate in the GEF reaction because mutations of these residues had no detectable effect. Asn673-Asp674 of the junction site with the PH domain appeared to be important because alanine substitution at these residues resulted in partial or complete loss of GEF function.

Together with those mutations that do not affect the exchange capability on Cdc42, which help exclude the involvement of parts of α2, α6, loop 7, and α8, the results of the loss of function mutations point to a GTPase interaction surface along the shallow groove formed by the N-terminus of α1 and the middle section of α9, extending to part of α6 and the DH-PH junction. In the binding activity test E502A and Q633A retained most of their binding activities to both Cdc42 and RhoA, whereas T506A and R634A were inactive in binding to either GTPase. The functionally inactive N673A/D674A mutant retained almost the wild type binding activity to both Cdc42 and RhoA. We conclude from these data that residues Glu502, Gln633, and Asn673-Asp674 are mostly involved in the GDP/GTP exchange of the GEF reaction, whereas residues Thr506 and Arg634 may be responsible for the binding interaction with the GTPases. Furthermore the mutants E502A, Q633A, and N673A/D674A that showed no GDP/GTP exchange activity but remained capable of binding to Cdc42 and RhoA were unable to activate the small G-proteins in cells, suggesting that the guanine nucleotide exchange activity of Dbl is required for Rho GTPase activation *in vivo*.

T17NRac1 non-specifically inhibits the Rho pathway

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 NIH 3T3 cells expressing oncogenic Dbl protein (Dbl cells) and examined the effects on the cells' actin structure and endogenous Rac1 and RhoA activities. The cells expressing Dbl showed a more compact cell shape, and filament actin staining revealed extensive lamellipodia formation 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. 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. When T17NRac1 or T17NCdc42 was coexpressed in the Dbl expressing cells, it appeared that either protein could inhibit the lamellipodia and actin stress fiber formation. Moreover, they effectively suppressed both endogenous Rac1 and RhoA activities that were upregulated by onco-Dbl. These results indicate that the conventional dominant negative mutants of Rho GTPases may function

non-specifically to inhibit distinct Rho GTPase activities by sequestering Dbl-like GEFs that are promiscuous in substrate recognition.

Asn1406-Asp65 pairing of TrioN-Rac1 is critical for GDP/GTP exchange

We rationalized that if substrate-specific GEF mutants that preserve specific Rho GTPase binding without nucleotide exchange activity could be engineered, they might act dominant negatively by inhibiting individual Rho GTPases and could be explored to specifically downregulate Rho GTPase-mediated signaling. Therefore, based on our results with the N673A/D674A mutant of Dbl and crystallographic data, we have constructed point mutants of the Rac1 specific N-terminal DH-PH module of Trio1 (TrioN), hoping to derive a TrioN mutant that could act dominant negatively on Rac1. We produced N1406A/D1407A and T1244A mutants of TrioN as well as wild type TrioN as $(His)_6$-tagged fusion proteins and tested their GDP/GTP exchange and Rho protein binding activities on Rac1 by reconstituting the purified components. The T1244A mutant remained partially active in stimulating GDP dissociation from Rac1, while N1406A/D1407A had lost the exchange activity completely *in vitro*. Mutation of the pairing Asp65 residue of Rac1 to Ala also lead to the loss of responsiveness of Rac1 to TrioN stimulation, indicating that the Asn1406-Asp65 pairing of TrioN and Rac1 interaction serves as critical structural determinant responsible for the GDP/GTP exchange. Furthermore, N1406A/D1407A was also able to inhibit the wild type TrioN-stimulated GDP release from Rac1; 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.

The N1406A/D1407A mutant of TrioN acts dominant negatively for Rac1 activation *in vivo*

To analyze the *in vivo* behavior of the mutant, we tested its effects on cell morphology and actin cytoskeleton in Swiss 3T3 cells. F-actin staining of the cells expressing wild type TrioN, one of the TrioN mutants or T17NRac1 revealed that under 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. Upon stimulation with PDGF, an effective Rac1 pathway

activator, the control 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 formation of lamellipodia induced by PDGF but retained actin stress fibers of the cells, whereas T17NRac1 yielded a similar effect on lamellipodia but also eliminated actin stress fibers simultaneously. These results indicate that N1406A/D1407A can act dominant negatively to inhibit Rac1-mediated actin reorganization. Furthermore transfection of cDNA of the N1406A/D1407A mutant into the cells inhibited the activation of the serum response factor elicited by wild type TrioN. 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, suggesting that this mutant can distinguish Rac1 from RhoA activity. Further effector pulldown assays showed that N1406A/D1407A specifically inhibited Rac1 activity without affecting RhoA in the Dbl-cells.

The N1406A/D1407A mutant of TrioN can distinguish Rac1 from RhoA in cells

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: in PDGF-induced DNA synthesis and Dbl-induced cell transformation. We observed that in the 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. 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 BrdU incorporation despite of its significant impact on PDGF-induced actin structure of the cells. The N1406A/D1407A mutant that inhibited endogenous Rac1 activity did not affect anchorage-independent growth of the Dbl cells, contrary to the marked inhibitory effect of T17NRac1 or T17NCdc42. These results further highlight potential complications of the use of conventional dominant negative Rho protein mutants, and suggest that 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.

Conclusions

The Dbl related GEFs for Rho GTPases form a fast expanding cell growth regulatory protein family that is expected to play an important role in Rho GTPase regulation in diverse physiological systems. A large body of biochemical data has pointed to the conserved structural motif of this family, the DH domain, as the primary interactive site with Rho GTPases. In the present studies we have attempted to identify specific residues of DH domain that are critical for defined biochemical activities, i.e. the nucleotide exchange or the binding activities for Rho GTPases. The systematic alanine substitution approach in the postulated GTPase interactive surface of DH domain has allowed us to identify a panel of DH mutants made in the α1, α6, and α9 regions and the PH junction site that suffer complete or partial loss of GEF activity toward Cdc42 and RhoA, suggesting that the mutation sites are required for the GEF function. The lack of effect by mutations in parts of α2, α6, loop 7, and α8, on the other hand, helped exclude the involvement of these regions. Our results provide strong support of a GTPase interaction surface of DH domain along the shallow groove formed by the N-terminus of α1 and the middle section of α9, extending to a part of α6 and the DH-PH junction.

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. Since many Dbl family GEFs shows promiscuity in binding activity towards multiple Rho GTPases it seems logical to expect that dominant negative Rho proteins may have non-specific effects when applied to sophisticated cellular systems by overexpression. Indeed, or 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. 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. Therefore it is necessary to raise concerns to a body of literature in which non-selective use of dominant negative Rho protein mutants was solely dependent upon to implicate the involvement of specific Rho family members in complex cell signaling networks.

We have also examined the role of two most conserved residues of the TrioN GEF module, Thr1244 that is located in the CR1 region and Asn1406 that is located close to the C-terminus of the DH domain. The results implicate Asn1406 of TrioN as the

critical determinant in the GEF catalytic reaction of Rac1. 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 GAP catalytic reactions or the glutamic acid residue found in the Sec7 domain in catalyzing the guanine nucleotide exchange of ARF family small G-proteins.

The discovery of the key mechanistic role of Asn1406 of TrioN provided a valuable clue that mutant generated from this residue might behave in a dominant negative manner in inhibiting Rac-mediated signaling pathways. Indeed, our results indicate that N1406A/D1407A mutant of TrioN constitutes a dominant negative inhibitor of Rac-signaling pathways. Furthermore the results strongly suggest that N1406A/D1407A acts more specifically in blocking Rac-mediated signaling events than T17NRac1. Our results indicate that a dominant negative Rho GEF such as N1406ATrioN can serve as an alternative to dominant negative Rho GTPases for the dissection of sophisticated signal transmitting pathways through individual Rho proteins.

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