Mechanisms of Guanine Nucleotide Exchange of

Rho GTPases by Dbl Family Proteins

Ph.D. dissertation

by

Balazs Debreceni

Department of Biochemistry and Medical Chemistry
Faculty of Medicine
University of Pecs

May 2004

Ph.D. program leader: Balazs Sumegi, D.Sc.

Table of Contents

List of abbreviations

BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine Serum Albumin
CR	Conserved Region
Dbl	Diffuse B-cell Lymphoma
DH	Dbl Homology
DMEM	Dulbecco's Modified Eagle's Medium
DTT	Dithiothreitol
FACS	Fluorescence Activated Cell Sorting
GAP	GTPase-Activating Protein
GDI	Guanine Nucleotide Dissociation Inhibitor
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GST	Glutathione S-Transferase
HA	Hemagglutinin
Mant-GDP	2 (3)-O-(Methylanthraniloyl)-GDP
PAK	$p21^{cdc42/rac}$-Activated Kinase
PBD	$p21^{cdc42/rac}$-Binding Domain
PBS	Phosphate Buffered Saline
PH	Pleckstrin Homology
PIP3	Phosphatidylinositol 3,4,5-Trisphosphate
PI-3K	Phosphatidyl Inositol 3-Kinase
PDGF	Platelet Derived Growth Factor
PKN	Protein Kinase N
Rho	Ras Homology
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SRF	Serum Response Factor
TBS	Tris Buffered Saline

CHAPTER 1
SCIENTIFIC BACKGROUND

The Dbl (Diffuse B-cell lymphoma) family proteins are guanine-nucleotide exchange factors catalyzing GDP/GTP exchange on the small molecular weight Rho (Ras Homology) GTPase proteins. As direct upstream activators of the Rho GTPases, they are involved in several physiological processes ranging from regulation of DNA synthesis to neuronal axis development. This chapter summarizes our current knowledge on this family of proteins.

1.1 The Rho GTPase family

The Rho family GTPases belong to the Ras superfamily of small molecular weight GTPases. Until today about twenty members of the Rho family have been identified, including Rho (A-E, H, G and 6-7), Rac (1-3), Cdc42, RhoBTB (1, 2), TC10, and Rif [Schmidt and Hall, 2002]. Their importance became clear about a decade ago when they were first shown to affect the actin cytoskeleton rearrangement in mouse fibroblasts. Since then it has become apparent that they are not only involved in organizing cytoskeletal changes in mammalian cells but also regulate many other processes, like G1 progression of the cell cycle, cell proliferation, DNA synthesis, transcription factor activation and activation of the ^{p67}NADPH-oxidase in neutrophil granulocytes, just to mention a few [Hall, 1998; Aspenstrom, 1999; Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002].

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 then bind GTP again. In the GTP-bound active state, Rho proteins adopt a different conformation in their effector binding loop, thereby becoming capable to

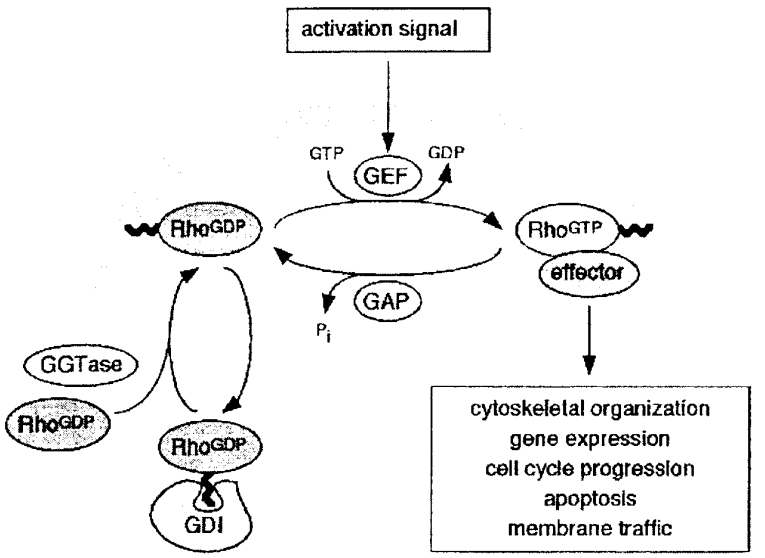

Fig. 1.1. The cycle of Rho family GTPases. (From [Schmidt and Hall, 2002])

recognize specific effector targets and inducing downstream molecular events [Bishop and Hall, 2000; Van Aelst and D'Souza-Schorey, 1997]. They posses GTPase activity which hydrolyzes GTP to GDP, returning the GTPase to the inactive state. The inactivation of the GTPase leads to the release and inactivation of the bound effectors (Fig 1.1). There are two groups of proteins that interact with the Rho family GTPases to regulate the GDP/GTP binding cyle: (i) the Dbl family proteins, that are Rho GTPase specific GEFs (guanine nucleotide exchange factors), and (ii) the Rho GAPs (GTPase activating proteins). 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. In addition to GEFs and GAPs, Rho GTPase proteins have a third group of regulators, the GDIs (GDP dissociation inhibitors). GDIs interact with the GDP-bound Rho proteins and keep them in the cytosol. When Rho GTPases are activated, they are released from GDI and translocated to the plasma membrane [Symons and Settleman, 2000].

The structure of Rho family GTPases consist of six β strands located in the middle of the protein and surrounding α helices connected with loops and 3_{10} helices. The P-loop (phosphate-binding loop) and the "effector loop" bind to the phosphate moiety, while other two loops to the guanine part of the bound nucleotide. By comparising the GTP-bound active state with the GDP-bound inactive state, two conformationally flexible regions surrounding the γ-phosphate of GTP were identified, named switch I and switch II. For strong GTP/GDP binding, Mg^{2+} is required to bring together the phosphate-binding regions, switch I and switch II [Hirshberg et al., 1997; Feltham et al., 1997; Wei et al., 1997; Ihara et al., 1998].

Previously Rho GTPases were believed to mainly function in the regulation of actin cytoskeletal reorganization in response to extracellular stimulation. For example, RhoA stimulates formation of stress fibers and focal adhesions [Ridley and Hall, 1992]. In contrast, Rac and Cdc42 proteins regulate lamellipodia and filopodia formation, respectively [Ridley et al., 1992; Nobes and Hall, 1995]. However, compiling evidence has revealed that Rho family proteins are also involved in diverse cellular events, such as gene expression, cell cycle progression, membrane trafficking, cytokinesis, and axon guidance and extension [Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002].

1.2 The Dbl protein family

1.2.1 History of the Dbl protein family

Dbl protein is the prototype of the Rho GEFs. It was described first as a potent proto-oncogene product, since overexpression of this B-cell lymphoma originated gene product leaded to malignant transformation of mouse fibroblasts [Eva and Aaronson, 1985]. Oncogenic activation occurs through an amino-terminal truncation of proto-Dbl. Subsequent sequence analysis of this gene product revealed a region with significant similarity to the yeast Cdc24 protein, which was known to be involved in the regulation of yeast bud site assembly and also in the activation of the Rho family GTPase Cdc42 [Johnson and Pringle, 1990; Ron et al., 1991]. Further investigations on Dbl and Cdc42 led to the biochemical demonstration that Dbl acts as an activator of Cdc42, by catalyzing GDP/GTP exchange on it *in vitro*. The conserved domain in Dbl and Cdc24 was found to be necessary for the nucleotide exchange, so this domain was named as DH (Dbl homology) domain [Hart et al., 1991]. In the past decade a large number of proteins were found with a tandem arrangement of DH and PH (pleckstrin homology) domains, making them possible candidates for Rho family GEF proteins. Many of these proteins were discovered by different cell transformation assays. For example, Vav, Lbc and Lfc can lead to cellular transformation in NIH 3T3 fibroblasts [Adams et al., 1992; Toksoz and Williams, 1994; Whitehead et al., 1995]. Tiam1 is involved in T-cell lymphoma invasion and metastasis [Habets et al., 1994], FGD1 and Bcr were found at the breakpoints of chromosomal rearrangements that are associated with faciogenital dysplasia (Aarskog-Scott syndrome) and chronic myelogenous and acute lymphocytic leukemias, respectively [Pasteris et al., 1994; Lifshitz et al., 1988]. Some Dbl proteins were identified as binding partners of other proteins, like Trio1 which binds to LAR transmembrane protein tyrosine phosphatase [Debant et al., 1996]. In the last few years DNA database searches for Dbl homologues led to the discovery of PDZ-RhoGEF

Table 1.1. Selected list of Dbl family members. (From [Zheng, 2001])

Dbl family member	GTPase substrates	Biological activities/unique distributions
FGD2	GEF for Cdc42	Embryonic development
Lbc/Brx	GEF for Rho	Proto-oncogene product; binds to nuclear hormone receptor
p115RhoGEF/Lsc	GEF for Rho	Proto-oncogene product; binds to Gα13 and Gα12
Net1	GEF for Rho	Proto-oncogene product
Ost/Dbs	GEF for Cdc42 and Rho; binds to Rac	Proto-oncogene product; at least three isoforms
p190RhoGEF	GEF for Rho	Inhibits neurite outgrowth; binds to microtubules
Kalirin/Duo	GEF for Rac and Rho	Two DH-PH modules; multiple isoforms as a result of alternative splicing
Asef	GEF for Rac	Enriched in brain; binding to tumor suppressor APC leads to activation
Pix/Cool	GEF for Rac	At least two isoforms; binds to Rac effector PAK
Frabin	GEF for Cdc42	Binds to actin
Vav3	GEF for Rho and Rac	Proto-oncogene product
hPEM-2	GEF for Cdc42	Predominantly expressed in brain
GEF-H1	GEF for Rac and Rho	Associates with microtubules
GTRAP48/KIAA0380/ PDZ-RhoGEF	GEF for Rho	Predominantly expressed in brain cerebellum; associates with neuronal glutamate transporter; binds to Gα13
LARG	GEF for RhoA	Fuses to MLL gene in acute myeloid leukemia; interacts with Gα12 and Gα13
Tiam2	GEF for Rac	Two isoforms; cerebellum and testis specific
Stef	GEF for Rac1	To be determined
p114RhoGEF	GEF for RhoA	To be determined
Ngef/ephexin	GEF for RhoA, Rac1, Cdc42	Proto-oncogene product; predominantly expressed in brain; mediates Eph regulation of growth cone
Collybistin	To be determined	Two splice variants, predominantly expressed in brain
Intersectin	GEF for Cdc42	Two splice variants; one is brain-specific

[Fukuhara et al., 1999] and p114-RhoGEF [Blomquist et al., 2000]. Today it is estimated that over 80 Dbl-like molecules may exist in the human genome [Zheng, 2001]. Table 1.1 shows a list of selected members of the Dbl protein family.

1.2.2 Function of Dbl GEFs

The Dbl family GEF proteins serve as signal convergent or divergent points of Rho GTPase-mediated signaling [Cerione and Zheng, 1996; Zheng, 2001; Schmidt and Hall 2002]. 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 in the DH domain targeting and may affect the DH-Rho protein interaction directly or indirectly [Zheng, 2001]. Some of the GEFs display specific activity toward one Rho GTPase (e.g. FGD1 for Cdc42, Tiam1 for Rac1 and

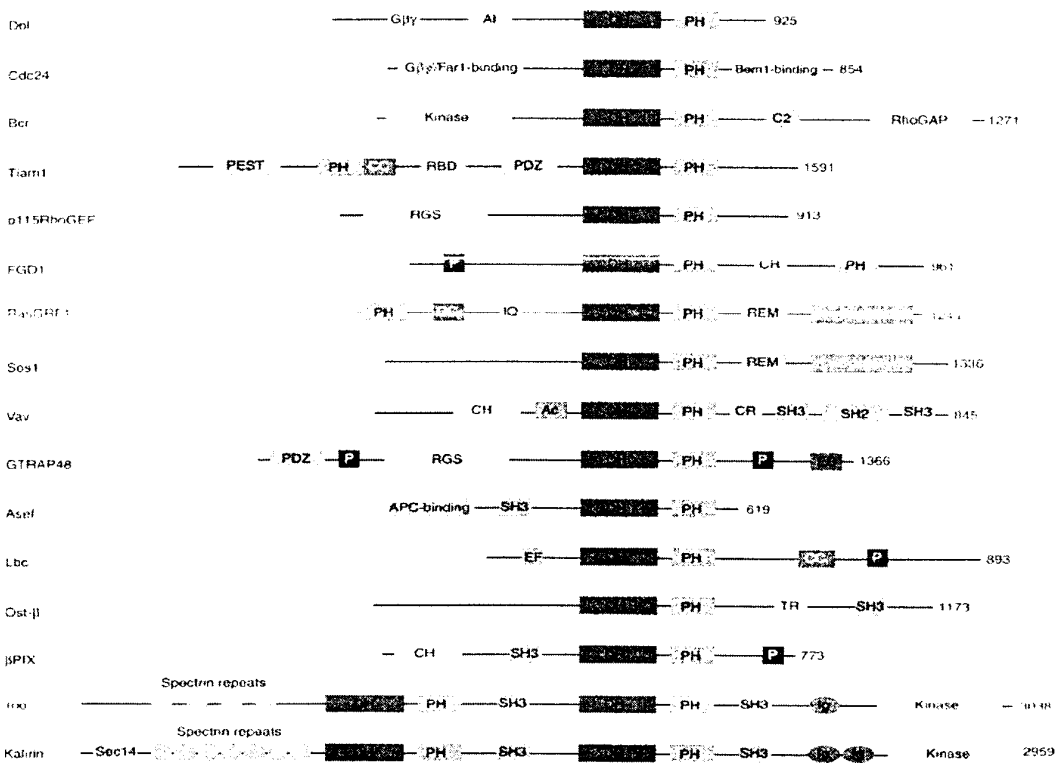

Fig. 1.2. Domain structures of representative Dbl family members. Abbreviations: Ac, acidic amino acid rich motif; AI, autoinhibitory domain; C2, calcium-dependent lipid binding; CC, coiled coil; Cdc25, RasGEF motif; CH, calponin homology; CR, cysteine-rich zinc butterfly motif; DH, Dbl homology; EB, EAAT4 binding; EF, EF hand calcium-binding motif; Gβγ, Gβγ binding domain; Ig, immunoglobulin-like; IQ, calmodulin binding motif; Kinase, serine/threonine kinase motif; P, proline-rich SH3-binding motif; PDZ, DHR or GLGF domain; PEST, amino acids P, E, S and T rich, degradation motif; PH, pleckstrin homology; RBD, Ras-binding domain; REM, Ras exchanger motif; RGS, regulator of G-protein signaling motif; RhoGAP, Rho GTPase-activating protein motif; Sec14, sec14-like; SH2, Src homology 2; SH3, Src homology 3; TR, Tat/RAG8-related. (From [Zheng, 2001])

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) [Cerione and Zheng, 1996; Zheng 2001; Schmidt and Hall, 2002]. It is likely that under physiological conditions each Dbl protein is involved in a specific aspect of Rho GTPase-regulated signaling process; but unfortunately, detailes on the mechanisms of the regulation and the involvement of most Dbl proteins in specific biochemical pathways remain unclear.

1.2.3 Domain structure of Dbl proteins

Dbl family members are large multidomain proteins. They all share a DH domain immediately followed by a PH domain. The DH-PH domain is about 180 amino acid long. Except for the DH-PH module, these proteins are very diverse in their stucture and size. They contain different domains in addition to the conserved PH-DH domain. These domains vary in the different Rho-GEFs (Fig. 1.3). Little is known about their exact role, but probaly they regulate the activity of the GEFs and provide connections to upstream activators. The DH-PH module is the minimal stuctural unit which is capable to cause cellular transformation.

The DH domain is highly conserved among the members of the family. The three dimensional stuctures of a few GEF DH domains have been resolved by NMR studies and X-ray crystallography. The DH domain contains nine alpha helices (α1-α9) and three conserved regions (CR1-CR3) [Aghazadeh et al., 1998; Liu et al., 1998; Soisson et al., 1998; Worthylake et al., 2000]. Outside the conserved regions the DH domains share little sequence homology [Whitehead et al., 1997, Schmidt and Hall, 2002].

The PH domain is a protein motif about 100 amino acids, which was first identified in pleckstrin [Whitehead et al., 1997]. The PH domain has been found in more than 100 proteins, including protein kinases, phospholipases, cytoskeletal proteins, Dbl family proteins and other regulators of small GTPases. The binding partners of PH domains have been reported to include phosphoinositides, βγ subunits of heterotrimeric G proteins and several isoforms of protein kinase C. The structures of several PH domains have been determined. Despite the low homology among PH domains, their three-dimensional structures are very similar. The core structure of PH domains consists of a β sandwich formed by two sets of antiparallel β strands. The β sandwich structure is capped at one end by a C-terminal α helix. The positive charged amino

acids at the other end are implicated in binding phosphoinositides [Hoffman and Cerione, 2002].

1.2.4 Mechanism of the DH domain-mediated guanine nucleotide exchange

The function of Dbl GEFs is to catalyse the exchange of bound GDP for GTP on Rho family GTPases. The three-dimensional structure of the Tiam1-Rac1 complex has been resolved [Worthylake et al., 2000], which remarkably helps us to improve our understanding of the potential mechanism of the GTP/GDP exchange. The main function of the exchange factor is to facilitate the release of bound GDP from the Rho protein, which - following a temporary nucleotide-free state of the complex - results in binding of GTP. The affinity of Rho proteins to GTP and GDP is similar, however the concentration of GTP is about 10 fold higher in the cells than the concentration of GDP, which explains the binding of GTP to the nucleotide-free complex. In the case of the Tiam1-Rac1 complex interactions are mediated primarily by CR1, CR3 and the C-terminus of α9 of Tiam1, and by switches I and II of Rac1. It seems that Tiam1 first interacts with the conformationally rigid β2-β3 strands and switch II residues 65 to 74 of Rac1. This leads to conformational changes of switch I and the remaining part of switch II, resulting in destabilization of the nucleotide binding. One of the most important residue in the complex formation is the conserved Glu1047 in CR1 which participates in three hydrogen bonds with Tyr32, Thr35 and Val36 of Rac1. These interactions support the movement of Thr35 ~6 Å relative to its position in Rac1/GMPPNP and preventing its ability to bind Mg^{2+}. The interaction between Tiam1 and Rac1 also results in shifting of switch I 2.7 Å up along the nucleotide binding cleft and moving Ile33 near to the nucleotide binding site, destabilizing GDP binding. Furthermore, shifting of Ile33 abolishes the hydrogen bond between the nucleotide and Cys18, and diminishes the interaction between Phe28 and the guanine base. The complex formation also causes

conformational changes in switch II between residues 59 and 64, leadig to the destabilization of nucleotide binding by sterically hindering Lys16 of the P-loop (phosphate-binding loop) and the Mg^{2+} binding site. In summary, the interaction between Tiam1 and Rac1 stimulates the GDP/GTP exchange by destabilizing the GDP binding and facilitating the dissociation of GDP from the complex. According to mutagenesis studies similar mechanism of nucleotide exchange may be used by other GEFs [Zhu et al., 2000; Debreceni et al., 2004].

1.2.5 Types of Dbl family protein regulation

Since Dbl family proteins may exert potent protooncogenic effects, their activity must be strictly regulated in cells under normal conditions. Despite the fact that our understanding on the detailed mechanisms of how this regulation is maintained is still poor, there are a few examples of controlling mechanisms, which can be regarded as the main types of different regulation mechanisms.

Before stimulation, Dbl proteins are in an inactivated or partially activated state. The maintainance of these proteins in inactive form can be attributed to four main different mechanisms. The first is the intermolecular interaction between the DH and the PH domains, like in the case of Vav and Sos1. Binding of PIP3 (phosphatidylinositol-3,4,5-trisphosphate) by the PH domains of these GEFs results in inhibition of the DH domain [Han et al., 1998; Nimnual et al., 1998]. Another inhibitory mechanism might be exerted through molecular interactions between a regulatory domain of the Dbl protein and the PH or DH domain, which can mask the Dbl protein from their activators. There is evidence for this type of regulation in the case of Dbl and Vav [Aghazadeh et al., 2000; Bi et al., 2001]. Other results suggest that oligomerisation of DH domains could be a third way of maintaining Dbl proteins in an inactive state [Anborgh et al., 1999; Zhu et al., 2001]. Finally, inhibitory factors

specific for different Dbl proteins may bind to them and keep them in a minimum activity state. An example of this category includes the interaction of tumor metastasis suppressor nm23H1 with Tiam1, which results in a decrease in GEF activity of Tiam1 towards Rac1 [Otsuki et al., 2001].

Various upstream signals can attenuate the abovementioned negative regulatory modes resulting in stimulation of the GEF catalytic activity. These activating signals mainly originate from heterotrimeric G proteins, protein kinases and phosphoinositol kinases. It was shown that Dbl protein can bind to α_{13} and $\beta\gamma$ subunits of heterotrimeric G proteins through its N-terminal regulatory sequences [Nishida et al., 1999; Jin and Exton, 2000]. $G\alpha_{13}$ also stimulates p115RhoGEF [Hart et al., 1998]. An example of protein kinase activation is Vav, which can be phosphorylated by Src family tyrosine kinases at Tyr174, which results in the loss of inhibition of the DH domain by the N-terminal regulatory sequence [Aghazadeh et al., 2000; Bustelo, 2001]. Vav and Sos1 also can be activated by PIP3 binding to the PH domain. Furthermore the PI-3K (Phosphatidyl-Inositol 3-Kinase) activator wortmannin can block signal transduction from growth factor receptors toward Rac1 [Das et al., 2000; Hall, 1998; Van Aelst and D'Souza-Schorey, 1997].

1.3 Rho GTPases, Dbl GEFs and diseases

Threre is a growing list of mutations in genes that can lead to disesase development and are related to Rho GTPase signaling pathways. In contrast to the Ras proteins that are frequently mutated in human cancers, only one example of Rho-specific mutation is known to date that is associated with cancer development, a fusion of RhoH and BCL3 coding genes that results in pathogenic progression of certain non-Hodgkin lymphomas. On the other hand, increased activity or overexpression of different Rho GTPases have been demonstrated in various tumor cells (Table 1.2). For

Table 1.2. Aberrant regulation of Rho proteins in cancer. (From [Sahai and Marshall, 2002])

Target	Mechanism of deregulation	Tumor types
RhoA	Overexpression	Colon, breast, lung, testicular germ cell, head and neck squamous-cell carcinoma
RhoC	Overexpression	Inflammatory breast cancer, pancreatic ductal adenocarcinoma
RhoH	Rearrangement	Non-Hodgkin's lymphoma, multiple myeloma
	Point mutation	Diffuse large B-cell lymphomas
Rac1	Overexpression	Breast
Rac1B	Alternative splicing	Colon, breast
Rac2	Overexpression	Head and neck squamous-cell carcinoma
Cdc42	Overexpression	Breast

example RhoC was found to be overexpressed in human pancreatic adenocarcinomas, and the expression rate correlated with the metastatic activity and poorer prognosis of the tumors [Suwa et al., 1998]. RhoA, Rac1 and Cdc42 were also shown to be expressed at elevated levels in human lung, colon and breast cancer biopsies [Fritz et al., 1999]. It seems that the continous cycling between the GTP- and GDP-bound states of these proteins is necessary for their transforming activity. One evidence for this that the "fast cycling" mutant of Cdc42, F28LCdc42 has greater transforming potential than its GTPase defective (constitutively active) G12V mutant [Lin et al., 1997]. Moreover, fibroblasts expressing one of the "fast cycling" mutants of RhoA, Rac1 or Cdc42, showed signs of cellular tranformation, and these cells were tumorigenic when injected into nude mice [Lin et al., 1999]. Another evidence for the importance of GTP/GDP cylcing is that the activators of Rho GTPases, Dbl family GEFs, are potent oncogens. In fact, many of them were discovered by their transforming acitivity [Schmidt and Hall, 2002; Sahai and Marshall, 2002]. Despite of the large number of Rho GEFs found to be oncogenic by fibroblast transforming assays, only a few of them were shown to be involved in human cancer development or progression. Engers and coworkers found that four of the five investigated renal-cell carcinoma cell lines showed elevated levels

of the Rac1 specific Tiam1, and the expression level correlated inversely with the *in vitro* invasiveness. Moreover, mutational analysis revealed different point mutations of the Tiam1 gene, of which one was located in the DH domain which is necessary for proper function of Tiam1 [Engers et al., 2000]. Another example is LARG (Leukemia-Associated Rho GEF) that was indentified as a fusion partner of the mixed-lineage leukemia (MLL) gene in a patient with acute myeloid leukemia [Kourlas et al., 2000]. However it is not clear how this fusion affects Rho activity in acute myeloid leukemia. Bcr (the product of Breakpoint Cluster Region gene) is another GEF involved in cancer development. The N-terminal part of Bcr is fused to the non-receptor tyrosine kinase Abl in Philadelphia chromosome positive leukemias [Laurent et al., 2001].

Rho signaling parthways are also involved in other disesases. X-linked mental retardation is a neurological disorder associated with an abnormal morphology of synaptic spines on neuronal cell dendrites. In this disease mutations have been found in three genes that are linked to Rho protein signaling: the Rho GAP oligophrenin1 [Biluart et al., 1998], the Rho effector PAK3 [Allen et al., 1998] and the Rac2 specific Rho GEF, ARHGEF [Kutsche et al., 2000]. FGD1 is a Cdc42 specific GEF which is required for normal skeletal formation. Mutations in its gene lead to faciogenital dysplasia, a disease with skeletal and urogenital anomalies [Pasteris et al., 1994].

With the accumulating evidence of Dbl family GEFs and Rho GTPases involved in pathological conditions, drug development aimed at interfering with the interactions between Dbl family GEFs and their Rho GTPase substrate would be a worthy effort.

Chapter 2
Mapping of Rho GTPase-dependent Sites in the DH Domain of Dbl and Trio1 that are Required for GDP/GTP Exchange

2.1 Introduction

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 recently by x-ray crystallography or NMR spectroscopy [Aghazadeh et al., 1998; Liu et al., 1998; Soisson et al., 1998]. 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. In particular, a highly conserved serine or threonine residue in the CR1 region (Ser1216 in UNC-73), a stretch of hydrophobic leucine residues in the CR3 region (LLLKELL in Dbl), and the C-terminal Gln-Glu residues of the last α-helix of DH domain (Asn673- Asp674 in Dbl), have been proposed to be important determinants involved in the GTPase interaction, because mutations of these residues in UNC-73, Trio, or Dbl have led to an apparent loss of GEF activity toward Rac1 or Cdc42 [Aghazadeh et al., 1998; Liu et al., 1998]. Other conserved residues in the vicinity of these sites have also been speculated to play roles in the GTPase interaction. However, what biochemical function these residues exert in the nucleotide exchange reaction (GEF catalysis *versus* substrate binding) and to what extent these sites contribute to the GEF interaction have not been assessed. To identify specific sites of DH domain contributing to the catalytic 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 DH domain in the GEF reaction and binding activity.

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 T17N Rac1 (where Thr17 is replaced by Asn) into the cytoplasm of Swiss 3T3 fibroblasts inhibited membrane ruffling elicited by PDGF [Ridley et al., 1992], and dominant negative RhoA, Rac1 and Cdc42 could all inhibit serum response factor activation and G1/S phase transition of the cell cycle [Olson et al., 1995] and Ras-induced cell transformation [Qiu et al., 1995a, 1995b, 1997]. 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-GEF complexes [Feig, 1999; Hart et al., 1994]. It is often assumed by users of these mutants that a given mutant blocks only the specific pathway(s) that is (are) 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 catalysis, are promiscuous in nature [Zheng, 2001; Schmidt and Hall, 2002], it could be expected that overexpression of one dominant negative Rho GTPase, (typically two to three folds over the endogenous protein to be effective [Feig, 1999]), might indiscriminatively 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 our experiments we have 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.

Based on our findings with the Dbl mutants and the available information of a few three-dimensional structures of Dbl family GEFs, we have constructed a DH domain mutant of the GEF for Rac, Trio N-terminal DH-PH module (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 catalytic mechanism 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.

2.2 Experimental Procedures

2.2.1 Generation of mutant Dbl and Trio cDNA constructs

The DH domain point mutants of Dbl and TrioN were generated by oligonucleotide-directed mutagenesis of human oncogenic Dbl and Trio cDNA in pBluescript and pET15b vectors, respectively, by the polymerase chain reaction-based second extension amplification technique using the Pfu polymerase (Stratagene), with primers that contained the desired mutations. The BamHI fragments encoding the DH-PH module of onco-Dbl mutants were subsequently subcloned into the BglII and BamHI sites of pVL1392 vector together with the cDNAs encoding the glutathione S-transferase (GST) for insect cell expression [Zheng et al., 1996b] or into the BamHI site of the mammalian pZipneo vector for transfection into NIH 3T3 cells [Khosravi-Far et al., 1994] The same BamHI fragments of the wild type and mutant Dbl were also cloned into the pKH3 vector, which contains a triple hemagglutinin (HA) tag at the N

terminus for transient expression in COS-7 cells [Mattingly et al., 1994]. The BamHI-EcoRI fragments encoding the DH-PH module of wild type or mutant TrioN (encoding residues 1225 to 1537) were subsequently subcloned into the corresponding sites of pMX-IRES-GFP retroviral vector for recombinant retroviral production or pCEFL-GST vector for transient transfection into NIH 3T3 cells. The sequences of mutagenized cDNA inserts were confirmed by automated DNA sequencing.

2.2.2 Expression of recombinant proteins

Expression and purification of GST-fusion small GTP-binding proteins or the $(His)_6$-tagged TrioN proteins from the pGEX or pET15b vector-transformed E. coli were carried out as described previously [Zhang et al., 1998]. Production and purification of the Sf9 insect cell expressed GST-Dbl and DH mutants were performed similarly as described [Zheng et al, 1996b]. 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.

2.2.3 *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 [Li and Zheng, 1997; Zhang et al., 2000]. The GEF reaction buffer contained [^{3}H]GDP- or mantGDP-loaded Rho protein (Rac1 or Cdc42) with 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM

GTP, and 1 mM DTT supplemented with GST, GST-Dbl or Dbl mutants, TrioN or TrioN mutants.

2.2.4 Complex formation assay

Complex formation between HA-Dbl, Dbl DH mutants, $(His)_6$-TrioN or TrioN mutants and GST-fused dominant negative Rho GTPases (T17NCdc42, T17NRac1 or T19NRhoA) were carried out as follows: Approximately 5 μg of each GST-fused small G-proteins were immobolized on agarose-glutathione beads, washed three times in a buffer containing 20 mM TrisHCl (pH 7.6), 100 mM NaCl, and 2 mM EDTA, then mixed with 2 μg purified Dbl protein in the presence of BSA (~50 μg total protein) for 1 hr. 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. The immuncomplexes were visualized by using chemiluminescence reagents (Amersham Pharmacia).

2.2.5 Retroviral expression of TrioN and dominant negative Rac1 and Cdc42 mutants in cells

Wild type, N1406A/1407A and T1244A of TrioN, T17NRac1 and T17NCdc42 were expressed in NIH 3T3 or Swiss 3T3 cells by using the pMX-IRES-GFP vector which expresses the green fluorescent protein (GFP) as a bicistronic mRNA. Retroviral packaging and infection were carried out according to described methods [Liu et al., 2000]. Briefly, 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.

2.2.6 *In vivo* GTPase activation assay

The glutathione agarose-immobilized GST-PAK1 which contains the p21-binding domain (PBD) of human PAK1 (residues 51-149) and the GST-Rhotekin which contains the site required for RhoA-GTP recognition of Rhotekin (residues 7-89) were expressed and purified in E. coli by using the pGEX-KG vector as previously described [Ren et al., 1999]. NIH 3T3 cells were grown to ~90 % confluence in DMEM medium containing 10 % calf serum before being washed with ice-cold PBS once and lysis on the dish in a buffer containing 50 mM TrisHCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 % Triton X-100, 0.1 % SDS, 10 μg/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were clarified by centrifugation at 13,000 x g at 4 °C for 15 min. Lysates containing equal amount 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 were detected by anti-Rac1 or RhoA antibody (Santa Cruz Biotechnology).

2.2.7 Fluorescence microscopy

Log phase growing fibroblasts were seeded at a density of $3x10^4$ cells/12-mm round coverslips (Fisher Scientific) overnight before fixation in PBS containing 4 % paraformaldehyde for 10 min at room temperature. The cells were permeabilized in Tris-buffered saline (TBS) (pH 7.4) containing 0.2 % Triton X-100 for 5 min, and were stained for F-actin using rhodamine conjugated phalloidin (Molecular Probes).

Coverslips were mounted onto slides in 50 % glycerol-TBS. Stained cells were analyzed by using a Zeiss confocal fluorescence microscope.

2.2.8 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 or absence of the wild type TrioN cDNA in pKH3 vector. 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 NIH3T3 cells was carried out by using a luciferase assay kit from Promega.

2.2.9 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. The cells were cultured and synchronized as previously described. [Olson et al., 1995]. Release from S-phase synchrony was achieved by washing the cells once with PBS followed by two washes with DMEM medium for 5 min. BrdU (10 μM) was added to the medium upon release with 10 % serum or PDGF (10 nM), and labeling was carried out for 16 hrs before the cells were fixed and stained with anti-BrdU antibody (BD Biosciences).

2.2.10 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 as described [Lin et al., 1997]. Briefly, $2x10^4$ cells were suspended in DMEM medium 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 DMEM 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.

2.3 Results

2.3.1 Identification of conserved amino acids in the DH domain of Dbl that are critical for GEF catalysis

The DH domains of β-PIX, Sos, and the N terminal GEF domain of Trio (TrioN) share a similar tertiary structure that is composed of 10 α helices folded into a flattened, elongated bundle [Aghazadeh et al., 1998; Liu et al., 1998; Soisson et al., 1998]. Amino acid sequence alignment predicts a surface formed by two conserved helices near the center of one face of the DH domain that may be involved in the Rho GTPase

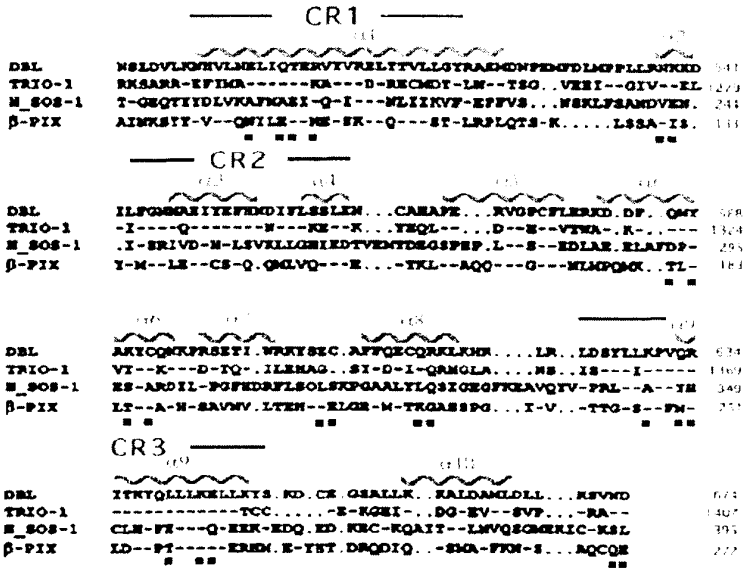

Fig 2.1. Amino acid sequence alignment of the DH domains of Dbl, Trio1, H-Sos and β-Pix. The three conserved regions, CR1, CR2, and CR3, are indicated.

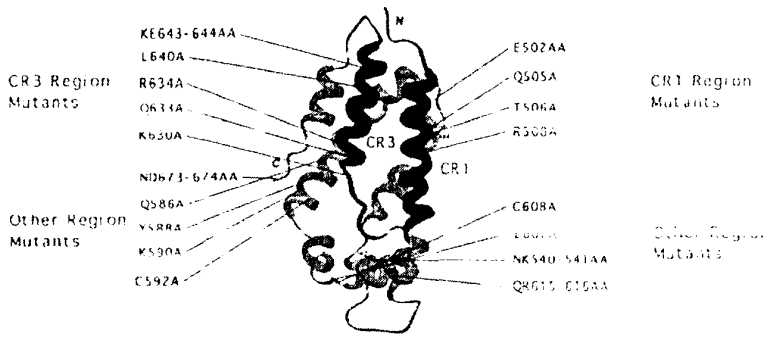

Fig. 2.2. Location of mutation sites in the DH domain of Dbl. The ribbon depiction of the three-dimensional structure of Dbl's DH domain shows the sites of the Ala point mutations. The CR1 and CR3 regions are highlited by darker shading.

interaction (Fig. 2.1). These two helices, α1 and α9, correspond to CR1 and CR3 of the primary sequences, respectively, whereas the conserved region 2 (CR2) of DH domain, which folds into α3 and α4 helices, is located at the opposite side of the helix bundle. 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 DH domain at CR1, CR3, and surrounding regions in the DH-PH backbone of oncogenic Dbl based upon the structural predictions (Fig. 2.2). Previous studies have shown that the DH-PH module of Dbl (residues 498-825) constitutes the minimum structural unit retaining wild type GEF activity and cell transformation capability [Hart et al., 1994]; the use of this module in

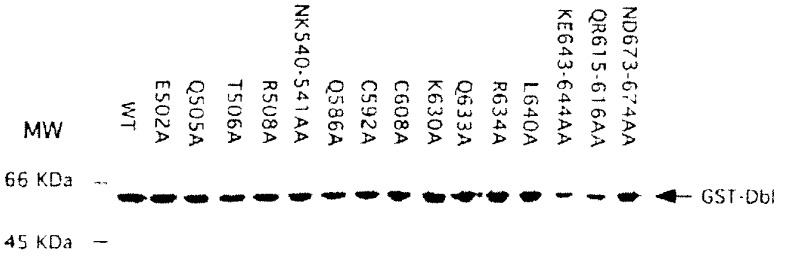

Fig. 2.3. SDS-PAGE of representative Dbl mutants. Coomassie blue-stained SDS-PAGE of representative insect cell expressed, glutathione-agarose affinity purified, GST-DH-PH mutants. 2 μg of GST fusion proteins were loaded on each lane.

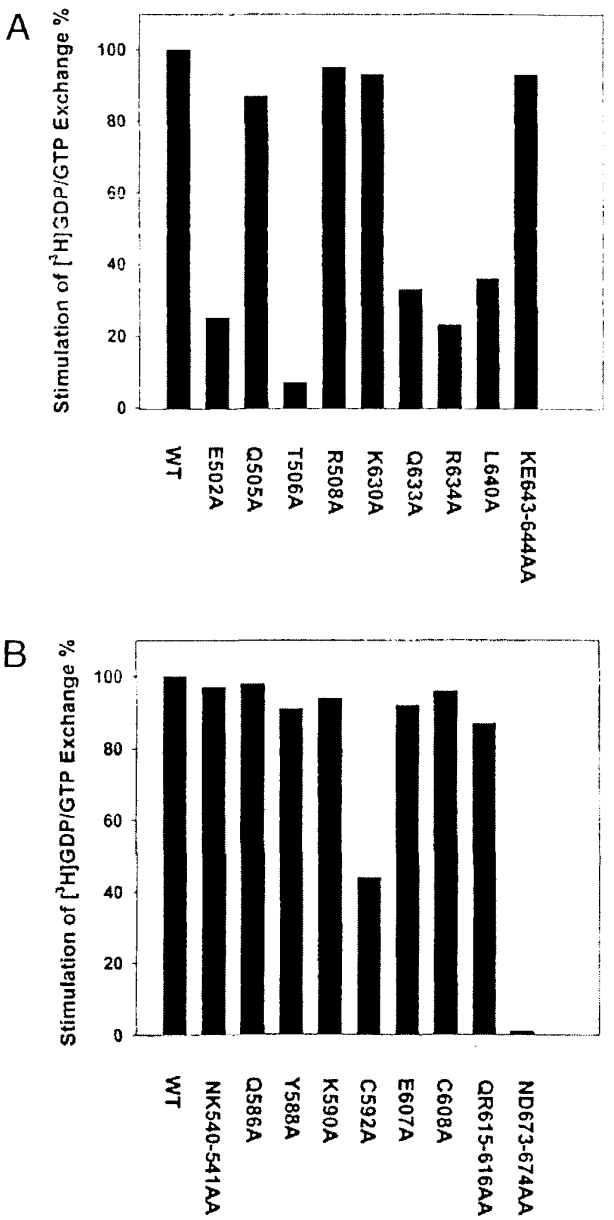

Fig. 2.4. Effects of DH mutations on the GEF activity of the DH-PH module of Dbl toward Cdc42. A, GEF activites of the purified CR1 and CR3 region mutants assayed on Cdc42. B, Effect of mutations in the DH domain outside the CR1 and CR3 regions on the GEF activity toward Cdc42.

the structure-function study, therefore, warrants extrapolation of the identified sites to the situation of full-length Dbl molecule.

To test the *in vitro* GEF activity, each DH mutant was expressed in Sf9 insect cells as GST fusion and was purified to homogeneity by glutathione affinity

chromatography (Fig. 2.3). Of the four CR1 region mutants and five CR3 region mutants examined, five displayed significant loss of GEF activity toward Cdc42 (Fig. 2.4). The T506A mutation of CR1, which corresponds to T1244A of Trio and S1216A of UNC-73 [Aghazadeh et al, 1998; Liu et al., 1998], caused the most severe effect, whereas L640A, where the leucine resides in the N terminus of hydrophobic LLLKLL 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 the 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 (Fig. 2.4, panel A). Additional mutations made in other regions of the DH domain located at the CR1/CR3 side of the surface, including those in the α2 (NK540-541AA), α6 (Q586A, Y588A, K590A), loop 7 (E607A, C608A), and α8 QR615-616AA) regions, were without effect on the GEF activity. However, Cys592 of the α6 region and 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 (Fig. 2.4, panel B) The loss of GEF activity of the mutants L640A, C592A and ND673-674AA were further verified by the examination of the time courses of [^{3}H]GDP/GTP exchange reaction catalyzed by the same amount of DH mutants (Fig. 2.5). Together with those mutations that do not affect the exchange capability on Cdc42, which help exclude the involvement of parts of α2, α6, L7, 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. These mutagenesis data on the DH domain of Dbl are consistent with the NMR mapping results of the DH domain of Trio derived from Trio-Rac1 binding

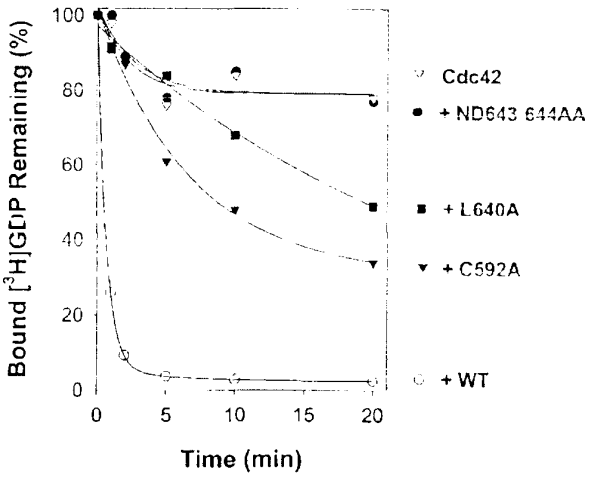

Fig. 2.5. Comparison of the time courses of the DH mutant-stimulated GDP dissociation from Cdc42. 1 mg of [^{3}H]GDP loaded Cdc42 was incubated with 0.2 mg of GST, GST-Dbl (WT) or the DH mutants in the GEF reaction buffer containing 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.5 mM GTP. At the indicated time points, aliquots of samples were withdrawn for quantification by passing through nitrocellulose filters. The radioactive counts of Cdc42-[^{3}H]GDP at 0 min were taken as 100 %.

interaction [Liu et al., 1998], also indicating that the Dbl family members utilize the conserved structural surface to interact with Rho GTPases.

2.3.2 Interactions between the DH-mutants of Dbl and its effecors

To directly test the effect of the mutations on the binding activity to the small G-protein substrates, we employed a GST-agarose affinity pull-down assay using the immobilized GST-N17Cdc42 or GST-N19-RhoA, which binds to wild type Dbl tightly in a dominant-negative fashion [Hart et al., 1994], and COS-7 cell lysates overexpressing the HA-tagged DH mutants. As shown in Fig. 2.6, among the five mutants made in the CR1 and CR3 regions, which suffer partial or complete loss of GEF activity, 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.

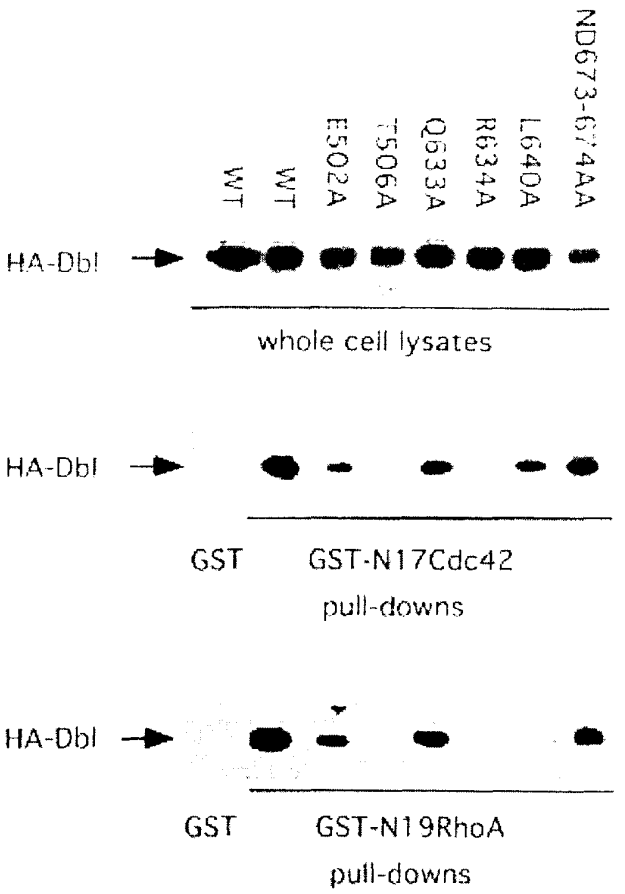

Fig. 2.6. Interaction of the DH mutants with dominant-negative Cdc42 and RhoA. Wild type (WT) Dbl and various DH mutants were expressed as HA-tagged proteins in COS-7 cells by transient transfection. The glutathione-agarose immobilized GST, GST-N17Cdc42, or GST-N19RhoA (5 mg/sample) were incubated with the respective COS-7 cell lysates for 1 h followed by centrifugation and washes. The expression of respective HA-DH mutants in the cell lysates and their coprecipitation patterns with GST-N17Cdc42 and GST-N19RhoA were detected by anti-HA Western blotting.

L640A could still bind to Cdc42 but failed to interact with RhoA at a detectable level. The N673A/D674A mutant, which is made at the C-terminal end of DH domain and is catalytically inactive, 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 catalytic aspect of the GEF reaction, whereas residues Thr506 and Arg634 may be responsible for the binding interaction with the

GTPases, although we cannot rule out the possibility that mutations at these sites have resulted in a disruption of global folding of the DH domain. Residue Leu640, which is highly conserved among DH domains, appears to contribute in part to the specificity of RhoA binding.

To correlate the *in vitro* GEF activity with the ability of the DH mutants to activate Rho GTPases in cells, we cotransfected the wild type and mutant DH constructs with HA-tagged Cdc42 or RhoA into COS-7 cells. The expression of HA-Dbl and HA-Cdc42 or HA-RhoA were visualized by anti-HA Western blotting of the cell lysates (Fig. 2.7), and the relative level of the active GTPase, Cdc42-GTP or RhoA-GTP, was detected by using the activation state-specific probe, the PBD of PAK1 or PKN, the effector domain that recognize specifically Cdc42-GTP or RhoA-GTP [Li et al., 1999]. Immobilized GST-PBDs of PAK1 and PKN were able to pull down significantly more Cdc42-GTP and RhoA-GTP in wild type Dbl coexpressing cells, respectively, than when Cdc42 or RhoA alone was expressed in the cells (Fig. 2.7), further indicating that the effector pull-down assay is effective in detecting the Rho GTPase-activating potential of Dbl in these cells. The five CR1 and CR3 mutants as well as the PH junction mutant ND673-674AA that have suffered partial or complete loss of *in vitro* GEF activity were mostly ineffective in Cdc42 or RhoA activation when coexpressed with the respective HA-GTPases (Fig. 2.7), indicating that the activating potentials of the DH mutants in cells correlate with the *in vitro* GEF activities. Notably, the catalytically impaired mutants E502A, Q633A, and ND673-674AA, which remained capable of binding to Cdc42 and RhoA were unable to activate the small G-proteins in cells, suggesting that the GEF catalytic activity of Dbl is required for Rho GTPase activation *in vivo*.

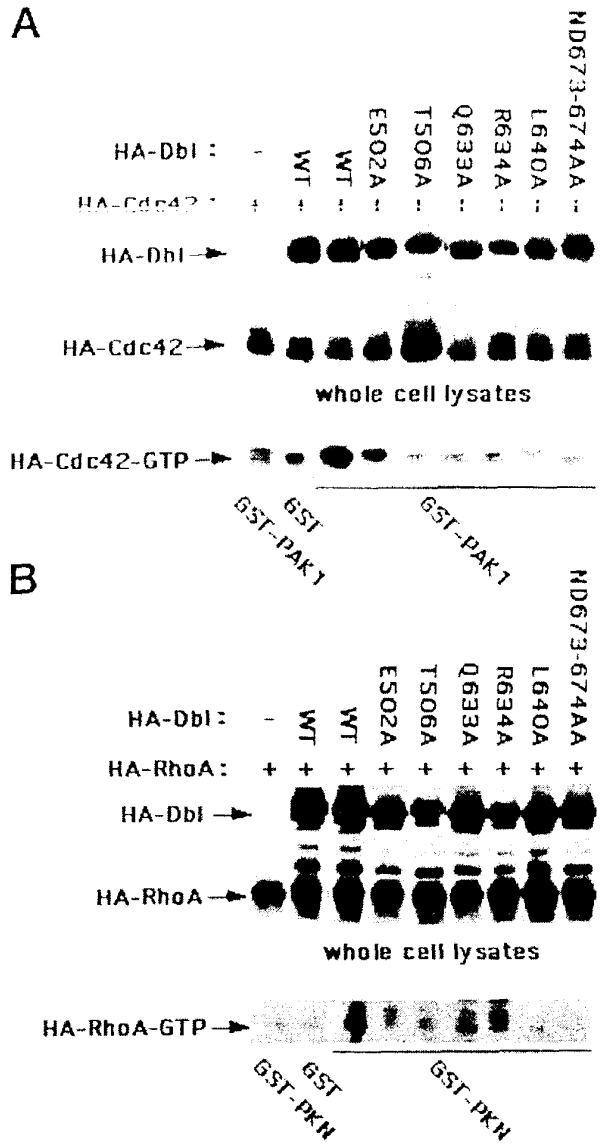

Fig. 2.7. Cdc42- and RhoA-activation potential of the DH mutants in cells. The HA-tagged wild type (WT) Dbl or the DH mutants were co-transfected with HA-tagged Cdc42 or RhoA into COS-7 cells. 48 h post-transfection, cell lysates were subjected to GST-PAK1 or GST-PKN affinity precipitation. The co-precipitated, GTP-bound small GTPases were detected by anti-HA Western blotting. 10 % of the amount of whole cell lysates used for GST-PAK1 or GST-PKN incubations were also subjected to anti-HA blotting in parallel.

2.3.3 Administration of dominant negative Rac1 could non-specifically 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 [Feig, 1999]. These mutants were shown to bind to the Dbl-like GEFs with high affinity *in vitro* [Hart et al., 1994] and to inhibit Rac-mediated lamellipodia formation and membrane ruffling or Cdc42-mediated filopodia induction *in vivo* [Ridley et al., 1992; Nobes and Hall, 1995; Kozma

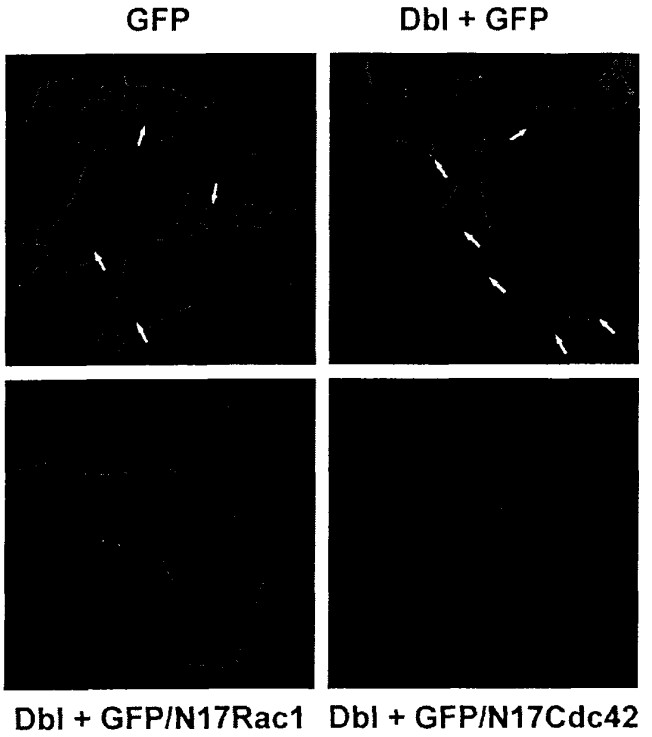

Fig. 2.8. The effect of dominant negative Rac1 or Cdc42 on the actin cytoskeleton structure of oncogenic Dbl-transformed NIH 3T3 cells. GFP alone or GFP together with T17NRac1 or T17NCdc42 was expressed in wild type-or oncogenic Dbl-expressing NIH 3T3 cells by retroviral infection, and the GFP-positive cells were isolated by FACS. The actin structures were visualized by rhodamine-conjugated phalloidin staining 16 h after serum withdraw. Arrows point to the membrane ruffles and stress fibers of the cells.

et al., 1995]. 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 [Zheng, 2001; Schmidt and Hall, 2002], 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. 2.8, 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 [Hart et al., 1994; Olson et al., 1996]. 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. 2.9). When T17NRac1 or T17NCdc42 was coexpressed in the Dbl expressing cells, it appears that either protein could inhibit the lamellipodia and actin stress fiber formation (Fig. 2.8). Moreover, they effectively suppressed both endogenous Rac1 and RhoA activities that were upregulated by onco-Dbl (Fig. 2.9). 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.

2.3.4 Identification of the conserved Asn1406-Asp65 pairing of TrioN-Rac1 as the critical determinants for GEF catalysis

The Dbl-like GEFs outnumber Rho substrates by at least an 4 to 1 ratio [Zheng, 2001; Schmidt and Hall, 2002], and there is evidence that certain GEFs may play an important role in specifying Rho protein mediated pathways [Daniels et al., 1999]. 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

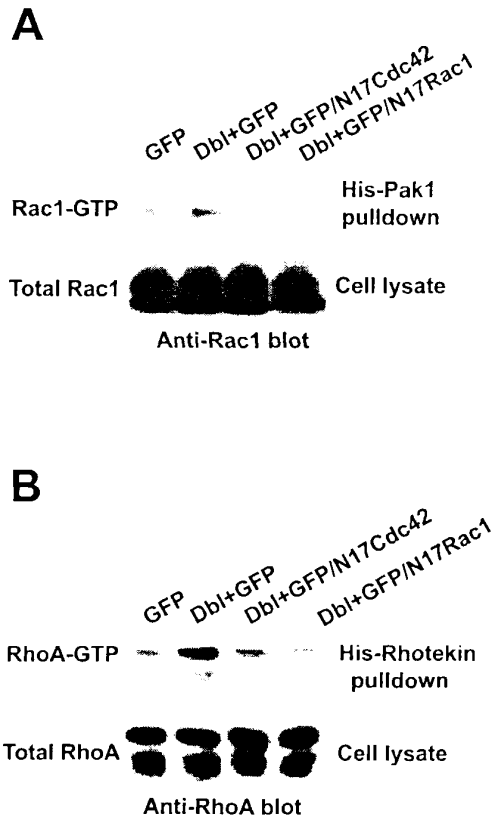

Fig 2.9. Dominant negative Rac1 or Cdc42 mutant indiscriminately inhibited Dbl-induced RhoA activity. Lysates of the cells were incubated with Ni^{2+}-agarose bead-immobilized His_6-tagged PAK1 or Rhotekin. Total Rac1 or RhoA in the lysates and the effector domain-bound, active Rac1 or RhoA were detected by Western blot analysis using anti-Rac1 or anti-RhoA antibody. Data are representative of three independent experiments.

dominant negatively in inhibiting individual Rho GTPase substrate and could be explored to specifically downregulate Rho GTPase-mediated signaling.

Based on our results with the point mutants of Dbl, and crystallographic and structural mapping data [Aghazadeh et al., 1998; Liu et al., 1998; Worthylake et al., 2000; Rossman et al., 2002; Snyder et al., 2002], we have focused in the present studies on the TrioN structural module that contains the N-terminal DH and PH domains of Trio and is specific for Rac1 [Debant et al., 1996; Bellanger et al., 1998], hoping to derive a TrioN mutant that could act dominant negatively on Rac1. We chose to examine two DH domain residues of TrioN in detail. One is Asn1406 at the C-terminal end of DH domain, another is Thr1244 located in the CR1 region. These residues correspond to the Asn1232 and Thr1051 of Tiam1, respectively, and both are highly conserved among Dbl family members [Whitehead et al., 1997]. Asn1232 of Tiam1 forms a hydrogen bond with the conserved switch II residue Asp65 of Rac1 in the Tiam1-Rac1 complex (Fig. 2.10), and such a pairing appears to be highly conserved in all the available structures of GEF-Rho protein complexes. Thr1051 of Tiam1 is involved in van der Waal's interaction with the switch I residue Val36 of Rac1 in the Tiam1-Rac1 complex; such a pairing is also conserved in the available GEF-Rho GTPase structures [Worthylake et al., 2000; Rossman et al., 2002; Snyder et al., 2002]. We produced N1406A/D1407A and T1244A mutants of TrioN as well as wild type TrioN as $(His)_6$-tagged fusion proteins and tested their GEF catalytic and substrate binding activities on Rac1 by reconstituting the purified components. As shown in Fig. 2.11, panel A, the T1244A mutant remained partially active in stimulating [^{3}H]GDP dissociation from Rac1, while N1406A/D1407A had lost the GEF catalytic activity completely. The latter mutant could actually further slow intrinsic [^{3}H]GDP dissociation from Rac1, suggesting it retained the ability to interact with Rac1. 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. 2.11, panel B). A single point mutant of TrioN, N1406A, that contains mutation at the critical Asn1406 position, behaved similarly to the N1406A/D1407A double mutant (data not shown). These observations prompted us to further test whether the pairing residue of the substrate, Asp65 of Rac1,

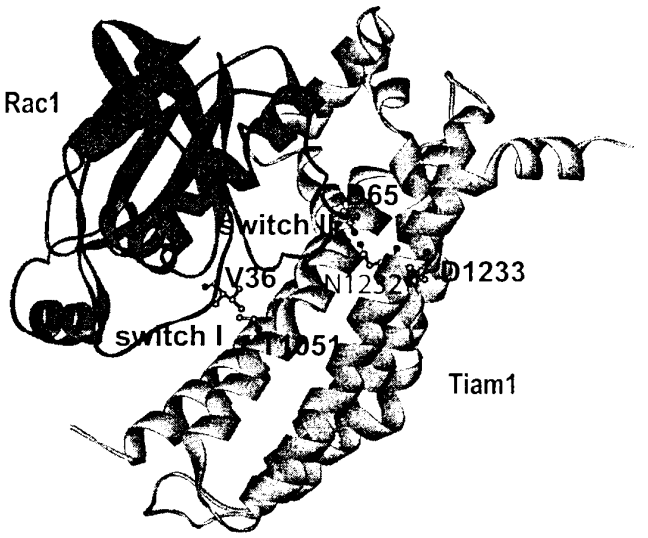

Fig. 2.10. Ribbon depiction of the three-dimensional structure of the Tiam1 DH domain in complex with Rac1. A highly conserved pairing of Asn1232 of Tiam1 (or other GEFs) and Asp65 of Rac (or other Rho proteins) forms a specific hydrogen bond in the core of the DH-Rho protein complex. The pairings of Asp65 of Rac1 with Asn1232 of Tiam1 and Val36 of Rac1 with Thr1051 of Tiam1 are depicted. Asp1233 of the DH domain is not involved in Rac1 interaction. Residues Thr1051, Asn1232, and Asp1233 of Tiam1 correspond to Thr1244, Asn1406, and Asp1407 of Trio, respectively.

might also play a similar role in GEF catalysis. As shown in Fig. 2.12, 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 critical structural determinants responsible for the GEF

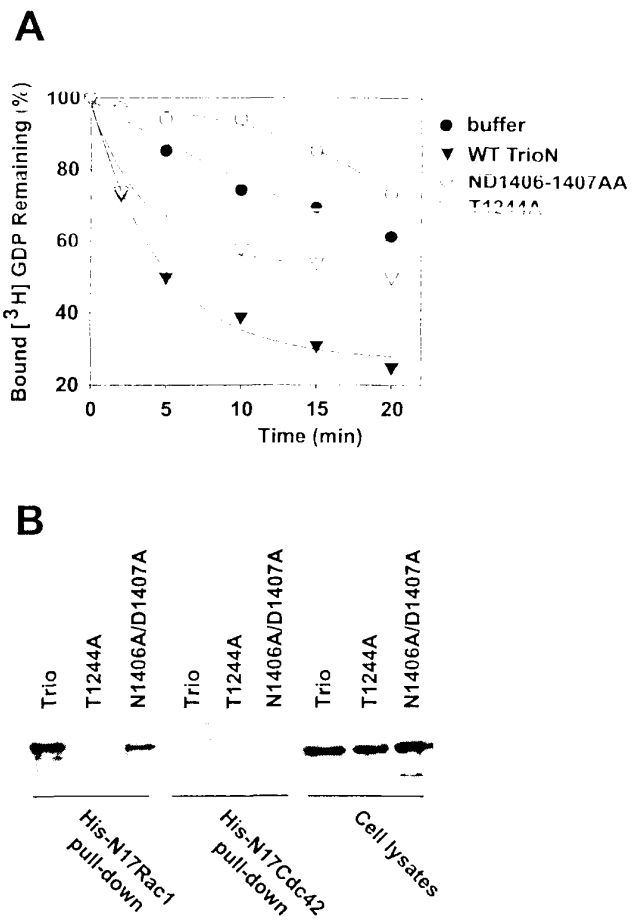

Fig 2.11. The N1406A/D1407A TrioN mutant selectively inhibits GEF catalysis without affecting GEF-Rho binding. A, the time courses of Rac1 GEF reactions under the catalysis of control buffer, wild type TrioN, N1406A/D1407A of TrioN, and T1244A of TrioN were determined in a GEF reaction buffer containing 1 μM [^{3}H]GDP-loaded Rac1 and a 0.4 μM concentration of the respective TrioN proteins. The GEF reactions were terminated at the indicated time points by the filtration method. The counts of the [^{3}H]GDP-Rac1 at 0 min were taken as 100 %. B, complex formation analysis of TrioN and its mutants with Rac1. Purified His_6-tagged wild type (WT) TrioN, T1244A, or N1406A/D1407A was incubated with the agarose-immobilized GST-tagged T17NRac1 or T17NCdc42, and the co-precipitates were subjected to anti-His_6 Western blotting.

catalysis, consistent with previous structural prediction from the Tiam1-Rac1 complex [Worthylake et al., 2000]. 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 of a glutamic acid of Sec7 domain for the ARF GTPase activation

[Beraud-Dufour et al., 1998] or a critical arginine residue termed "arginine finger" found in the RhoGAP catalytic reactions [Nassar et al., 1998].

2.3.5 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 dominant negatively *in vitro*, we subjected the mutant to a competition assay in a Rac1 GEF

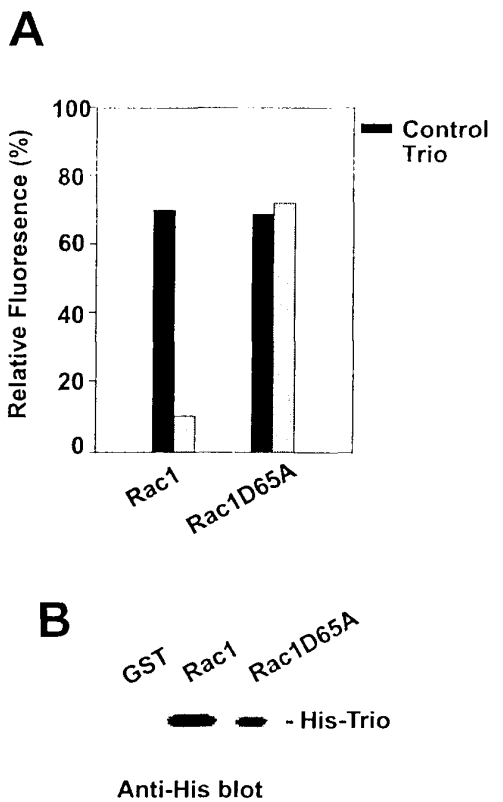

Fig. 2.12. Asp65 of Rac1 is essential to GEF catalysis activated byTrioN. A, the responsiveness of Rac1 and its mutant D65ARac1 to TrioN stimulation was measured in a GEF reaction assay using fluorescent labeled mantGDP. 1 μM Rac1 or D65ARac1 preloaded with mantGDP was incubated with 0.4 μM TrioN in a standard GEF reaction buffer for 5 min. The fluorescence change resulting from mantGDP dissociation from Rac1 was traced with a fluorometer. B, the direct binding interaction of Rac1 or D65ARac1 with TrioN was measured by a complex formation assay using purified His_6-TrioN and immobilized GST-Rac1 or GST-D65ARac1. The glutathione-agarose-co-precipitated His_6-TrioN was detected by anti-His_6 blotting.

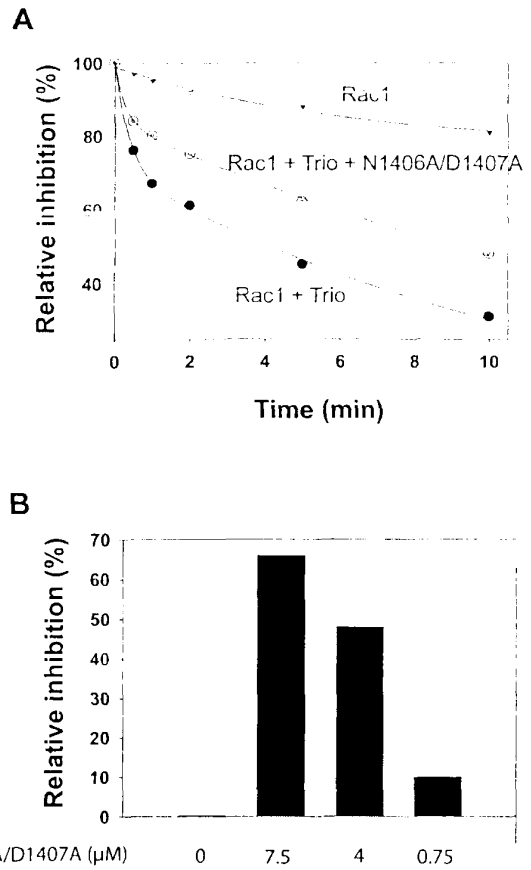

Fig. 2.13. Dominant negative effect of the Trio mutant N1406A/D1407A on Rac1 activation in vitro. A, [^{3}H]GDP-loaded Rac1 (1 μM) was incubated with 0.5 μM wild type Trio, 0.5 μM wild type Trio together with 2 μM N1406A/D1407A, or the control buffer. [^{3}H]GDP dissociation from Rac1 was measured by terminating the GEF reactions in an ice-cold stop buffer and filtering the reaction mixtures through nitrocellulose filters. The relative Rac1-bound [^{3}H]GDP was normalized to 100 % at time 0. B, the relative inhibition of wild type Trio-stimulated [^{3}H]GDP dissociation from Rac1 by increasing concentrations of the N1406A/D1407A mutant was determined at 2 minute time point of the respective GEF reactions. 100 % inhibition of wild type Trio represents the intrinsic [^{3}H]GDP dissociation by Rac1.

reaction catalyzed by wild type TrioN. As shown in Fig. 2.13, N1406A/D1407A was able to inhibit the wild type TrioN-stimulated [^{3}H]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 [Ridley et al., 1992], while RhoA activity is essential for actin stress fiber formation [Ridley and Hall, 1992]. 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 (Fig. 2.14). 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 [Liu et al., 2000]. 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. 2.15). 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. 2.15). These results indicate that N1406A/D1407A can act dominant 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 if N1406A/D1407A could affect wild type TrioN stimulated serum response factor activity. As shown in Fig. 2.16, 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 affected wild type TrioN expression in the transient expression system (data not shown).

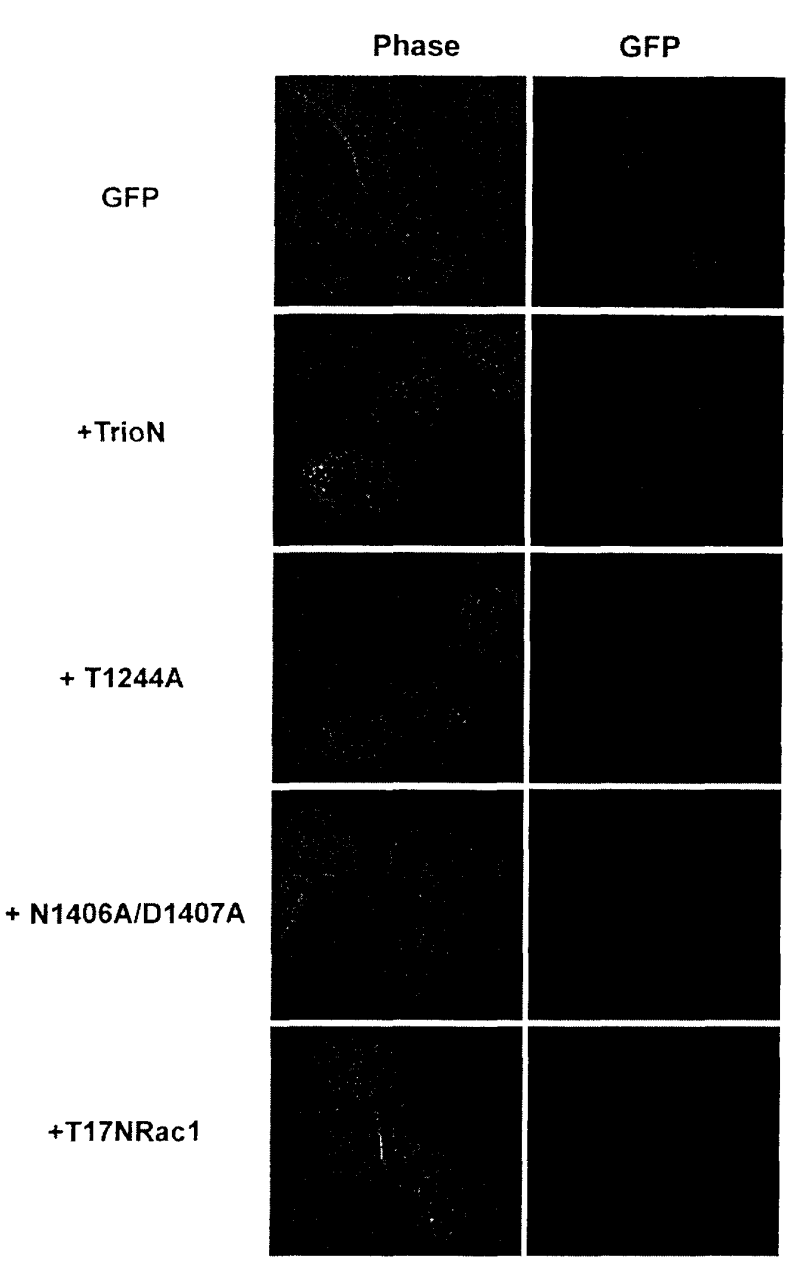

Fig. 2.14. Morphology of cells expressing WT Trio or Trio mutants. GFP alone, GFP with wild type, T1244A, or N1406A/D1407A mutant of Trio, or GFP with N17Rac1 were stably expressed in Swiss 3T3 fibroblasts by retroviral infection. The cell morphology was visualized by phase contrast microscopy (left column), while the GFP and the correlating Trio mutant or N17Rac1 expression was visualized by fluorescent microscopy (right column).

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.

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

We have shown that overexpression of T17NRac1 causes non-discriminative inhibition of RhoA activity in Dbl cells (Figs. 2.8 and 2.9). 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. 2.17), suggesting that this mutant can distinguish Rac1 from RhoA activity. Further effector pulldown assays show that N1406A/D1407A specifically inhibited Rac1 activity without affecting RhoA in the Dbl-cells (Fig. 2.18), 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 [Olson et al., 1995] and was shown to be required for Dbl-induced cell transformation by the administration of T17NRac1 [Lin et al., 1999]. 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. 2.19). 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 (Fig. 2.19) despite of its significant impact on PDGF-induced actin structure of the cells (Fig. 2.15). Consistent with the notion that multiple Rho GTPases

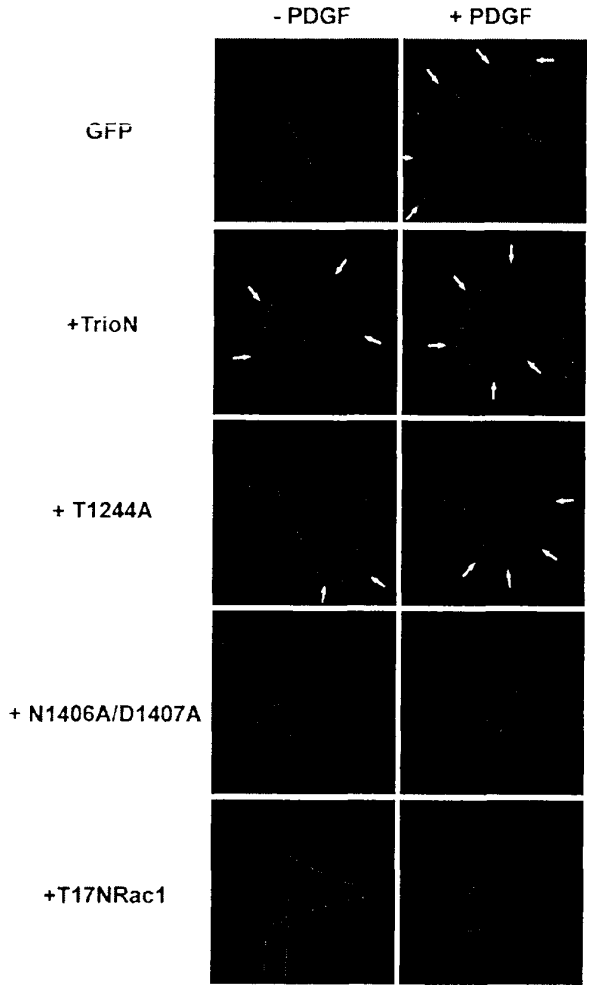

Fig. 2.15. Effect of Trio mutants on the actin structure induced by PDGF in Swiss 3T3 cells. GFP or GFP together with Trio mutant or N17Rac1 expressing cells were serum starved overnight and treated with PDGF (10 nM) or control buffer for 5 min. The cells were fixed and stained for F-actin filaments with rhodamine labelled phalloidin and visualized by confocal fluorescence microscopy.

contribute to Dbl-mediated cell transformation [Olson et al., 1996; Lin et al., 1999], the N1406A/D1407A mutant that inhibited endogenous Rac1 activity (Fig. 2.20) did not affect anchorage-independent growth of the Dbl cells, contrary to the marked inhibitory effect of T17NRac1 or T17NCdc42 (Fig. 2.20). 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

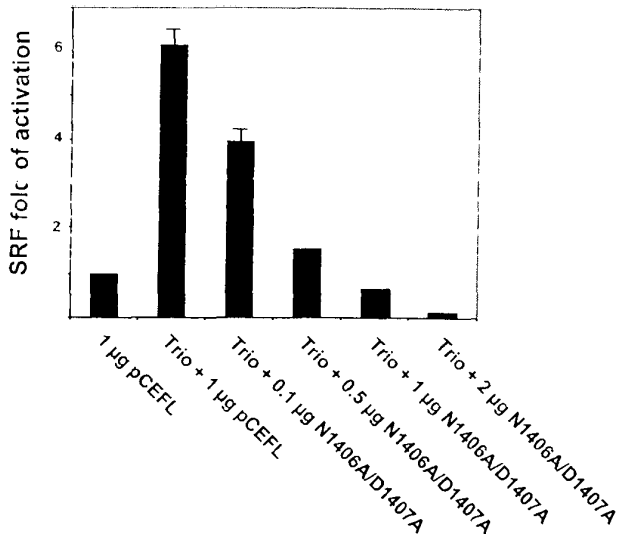

Fig. 2.16. Effect of the Trio mutant N1406A/D1407A on serum response factor activation by wild type Trio. 0.1 µg of WT Trio cDNA in pCEFL was cotransfected with the indicated amount of the Trio mutant N1406A/D1407A (0, 0.1, 0.5, 1, 2 µg, respectively) together with the SRF-luciferase reporter construct (1 µg) into cultured NIH 3T3 cells. The transfected cells were allowed to recover for 30 hrs, and were starved in DMEM supplemented with 0.5 % calf serum for 16 hrs prior to lysate preparation. The luciferase activities were expressed as the fold of activation relative to the activity induced by the pCEFL vector alone.

members such as Trio is better suited for the dissection of specific Rho GTPase signaling pathways.

2.4 Discussion

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 [Cerione and Zheng, 1996; Whitehead et al., 1997; Zheng, 2001; Schmidt and Hall, 2002]. 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. DH domain does not share significant sequence

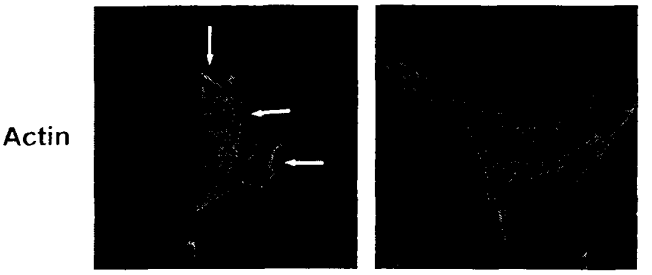

Fig. 2.17. The Trio mutant N1406A/D1407A acts as a Rac1-specific inhibitor in oncogenic Dbl transformed cells. The actin structure of the Trio mutant N1406A/D1407A expressing, oncogenic Dbl transformed cells shows reversal of the lamellopodia and membrane ruffle formation without affecting stress fiber formation. The cells were visualized similarly as in Fig. 2.8. Arrows point to the membrane ruffles of the cells.

homology with other subtypes of small GTPase activators such as the Cdc25 domain and Sec7 domain that are specific to Ras or ARF [Sprang and Coleman, 1998; Cherfils and Chardin, 1999], indicating that the DH-Rho protein interaction employs a distinct mechanism. Deletions or mutations within the DH domain have been reported to result in loss of cellular functions by the GEFs [Ron et al, 1991; Hart et al., 1994; Crespo et al., 1996; Qian et al., 1998], suggesting that an intact DH domain, likely its Rho GTPase-interactive ability, is essential for the cellular effects of Dbl family members. However, a few important issues, e.g. how the mutations have affected the biochemical properties of the proteins and/or whether substitutions of given amino acids may have led to disruption of the DH folding, were not properly addressed in previous mutagenesis studies of Dbl family members.

The recently available three-dimensional structure of DH domain has provided a frame work to further examine these structure-function issues [Aghazadeh et al., 1998; Liu et al., 1998; Soisson et al., 1998]. The amino acid sequence analysis and limited mutagenesis studies based on the structural information have helped laying out the groundwork with the postulation that one side of the α-helical bundle of DH domain,

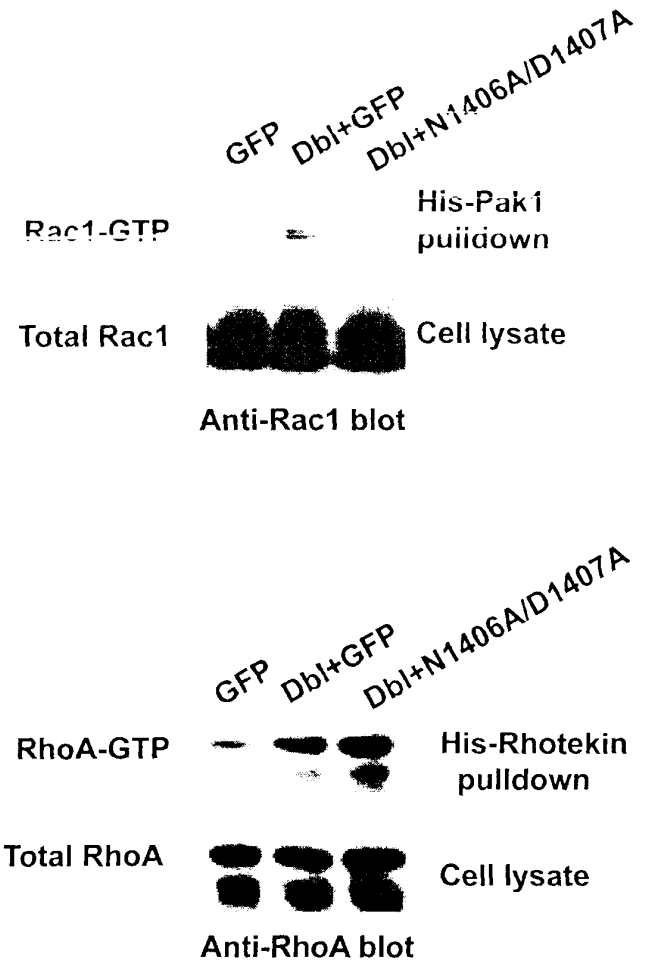

Fig. 2.18. N1406A/D1407A selectively inhibits Rac1 activation by Dbl in cells. Cell lysates derived from NIH 3T3 cells expressing GFP, Dbl and GFP, or Dbl, GFP and N1406A/D1407A, were incubated with immobilized His-tagged Pak1 or Rhotekin. Total Rac1 or RhoA in the lysates and the effector-domain bound, active Rac1 or RhoA were revealed by Western blot analysis using anti-Rac1 or anti-RhoA antibody.

including CR1 and CR3 regions, is the possible site involved in the recognition of Rho GTPases [Aghazadeh et al., 1998; Liu et al., 1998; Soisson et al., 1998]. By taking one step further along these lines, we have attempted in the present studies to achieve two goals: to identify specific residues of DH domain that are critical for defined biochemical activities, i.e. the catalytic or the binding activities for Rho GTPases, and to establish a causal relationship between the GEF catalytic activity and cellular transforming function of Dbl [Zhu et al., 2000]. The systematic alanine substitution

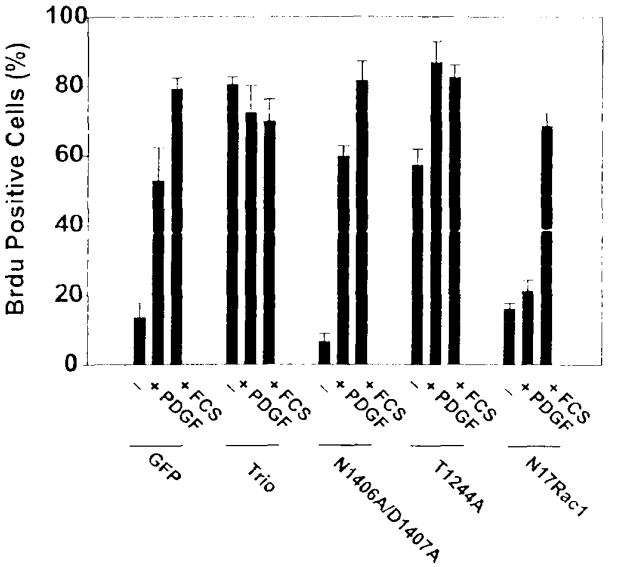

Fig. 2.19. Comparison of the effects of dominant negative Trio and Rac1 mutants on the PDGF-stimulated DNA synthesis. NIH 3T3 cells expressing GFP alone or GFP with wild type Trio, Trio mutant T1244A, N1406A/D1407A, or N17Rac1 were serum starved overnight and treated with PDGF (10 nM) or 10 % calf serum in the presence of BrdU (10 μg/ml) for 14 hrs. The cells were fixed and stained with monoclonal anti-BrdU followed by TRITC-conjugated anti-IgG. The total and BrdU positive cell numbers were counted under a phase contrast or fluorescent microscope. Data are representative of three independent experiments.

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, L7, and α8, on the other hand, helped exclude the involvement of these regions [Zhu et al., 2000]. Kinetic and binding analysis further revealed that although most GEF-deficient mutants displayed decreased catalytic activities in the GEF reaction, T506A and R634A suffered major loss in G-protein binding activity. The E502A, Q633A, and N673A/D674A mutants, in particular, have retained the binding ability to the Rho GTPases but were partially or completely inactive in GEF catalysis. Taken together, our results provide strong support of a GTPase

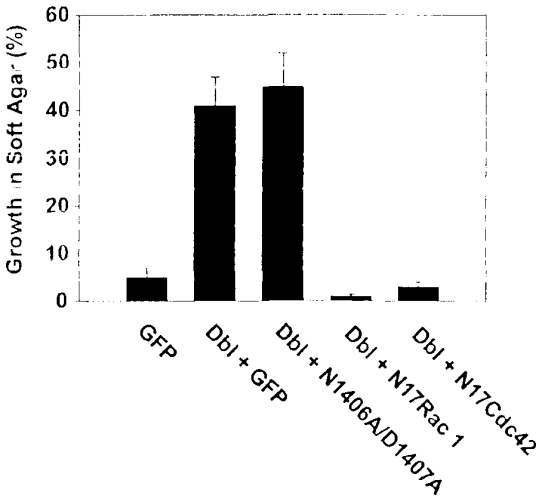

Fig 2.20. Comparison of the effects of dominant negative Trio and Rac1 mutants on the Dbl-induced anchorage-independent growth. NIH 3T3 cells coexpressing oncogenic Dbl and GFP alone, or GFP together with the N1406A/D1407A, N17Rac1 or N17Cdc42, were plated in 0.3 % agar on top of 0.5 % soft agar. Cells were grown for 14 days in DMEM containing 10 % calf serum and the colonies were quantified under a microscope.

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 (Fig. 2.2).

Residues Glu502, Thr506, Arg508, Lys630, Arg633, Leu640, and Lys643-Glu644 of CR1 and CR3 are mostly conserved among DH domains. T506A, corresponding to a loss of function mutation in UNC-73 that is necessary for neuronal pathfinding in Caenorhabditis elegans [Steven et al., 1998], is inactive in the GEF and the Rho GTPase binding assays. Because Thr506 is located on the surface of α1 helix, it is likely that this highly conserved residue is critical in mediating the binding interaction with Rho proteins that is required for GEF catalysis. This conclusion is supported by the NMR data collected with a similar mutation of the DH domain of Trio, showing that such a mutation still maintains the overall integrity of the DH domain [Liu et al., 1998]. The fact that mutations of the charged residues Arg508 and Lys630, which were previously

suggested to be involved in GTPase coupling [Liu et al., 1998; Soisson et al., 1998], do not have detectable effect on the GEF activities indicates that they are not required for Rho GTPase interaction. Arg634, on the other hand, may form an intramolecular salt bridge with Glu507 to contribute to the stabilization of the tertiary folding. The loss of function of R634A, therefore, could be attributed to a possible disruption of the folding pattern of the DH domain. Mutant E502A remained mostly active in binding to the GTPases but was catalytically compromised, suggesting that Glu502, like the C-terminal Asn673-Asp674 residues, is directly involved in GEF catalysis. Previous mutations of residues 640-647, LLLKELL, to IIRDII sequences in DH domains of Cdc24, Dbl, and Vav, inevitably abolished the GEF activities [Ron et al., 1991; Hart et al., 1994; Crespo et al., 1996], and the similar conserved changes in Sos1 led to a significant decrease of transforming activity [Qian et al., 1998]. Our results show that the charged residues Lys643-Glu644 within this stretch of amino acids are not required for the GEF function, whereas the hydrophobic Leu640 is indispensable for both Rho binding and catalysis, providing direct support to the notion that Leu640 and the neighboring leucine residues form a hydrophobic pocket of Rho GTPase interaction.

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 similar point mutant of Ras, which was discovered to be inhibitory for Ras signaling by random mutagenesis screening [Feig and Cooper, 1988]. 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 [Feig, 1999]. 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 [Hart et al., 1994]. Although it seems

logical to expect that the dominant negative Rho proteins may have non-specific effect when applied to sophisticated cellular systems by overexpression because many Dbl-like GEFs shows promiscuity in binding activity towards multiple Rho GTPases [Cerione and Zheng, 1996; Whitehead et al., 1997; van Aelst and D'Souza-Schorey, 1997; Zheng, 2001; Schmidt and Hall, 2002], 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 have 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 non-specific 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 (Figs. 2.8 and 2.9). 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 [Hart et al., 1994]. 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.

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 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 [Cerione and Zheng, 1996; Whitehead et al., 1997; van Aelst and D'Souza-Schorey, 1997; Zheng, 2001; Schmidt and Hall, 2002]. We have identified multiple residues of Dbl located in the CR1, CR3 of the DH domain 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 (Figs. 2.2, 2.4 and 2.5). Based on the structural information derived from the complexes of Rac1-Tiam1, Cdc42-Dbs, Cdc42-Intersectin and RhoA-Dbs [Worthylake et al., 2000; Rossman et al., 2002; Snyder et al., 2002], Sondeck and coworkers 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 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 Lys758, Leu759 and Leu766 of Dbs, appear to contribute to Cdc42 or RhoA specificity to a different extent [Cheng et al., 2002; Snyder et al., 2002], 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 ARF GTPase activation [Beraud-Dufour et al., 1998] or an arginine residue of RhoGAPs that is essential for accelerating the GTPase activity of Rho proteins [Nassar et al., 1998], remain to be identified.

In this study 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, in detail. The corresponding mutation of T1244A was first described in C. elegans UNC-73 gene, which resulted in a loss of function [Aghazadeh et al., 1998; Steven et al., 1998], whereas our mutant of Dbl corresponding to N1406A/D1407A of TrioN was found lack of transforming activity [Zhu et al., 2000]. 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 Waal's interaction with the switch I residue Val36 of Rho proteins and the latter involved in hydrogen bonding to the switch II Asp65 of Rho proteins (Fig. 2.10 and [Worthylake et al., 2000; Rossman et al., 2002; Snyder et al., 2002]). Interestingly, T1244A was found 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 completely inactive in the GEF assays but retained the substrate binding activity (Fig. 2.11). N1406A/D1407A was not only inactive in stimulating GDP-dissociation of Rac1 but also inhibited the intrinsic GDP/GTP exchange activity of Rac1 (Fig. 2.11). The reciprocal mutant of D65ARac1 behaved similarly toward TrioN like 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. 2.15). Taken together, these 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 [Nassar et al., 1998] or the glutamic acid residue found in the Sec7 domain in catalyzing the guanine nucleotide exchange of ARF family small G-proteins [Beraud-dufour et al., 1998].

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, we found that the mutant N1406A/D1407A of TrioN could inhibit Rac1 activation by wild type TrioN *in vitro* and effectively blocked the Rac1-mediated membrane ruffling and lamellipodia

formation induced by PDGF in cells (Figs. 2.13 and 2.15). Furthermore, the N1406A/D1407A mutant effectively blocked the TrioN-stimulated SRF transcriptional activity (Fig. 2.16). 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. 2.13, panel B) 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 GDP-bound configuration. On the other hand, N1406A/D1407A was quite effective in inhibiting Rac1 activation in cells, possibly due to the facts that the endogenous Rac GEF level is much lower than the wild type Trio used in the *in vitro* competition assay and N1406A/D1407A may target to the site of Rac activation by endogenous GEF.

To demonstrate the distinction between the dominant negative mutant of TrioN and the conventional dominant negative mutant of Rac1 in inhibiting cellular functions, we further compared the abilities of N1406A/D1407A and T17NRac1 to interfere with PDGF-stimulated DNA synthesis and Dbl-induced anchorage independent growth, both processes that could require Rac1 activation [Hart et al., 1994; Lin et al., 1999]. Despite its inhibitory effect on the PDGF-elicited lamellipodia formation, N1406A/D1407A had no effect on the PDGF-induced DNA synthesis. This is in contrast with the marked inhibition of both of the PDGF-stimulated cellular responses by T17NRac1. Similarly, unlike T17NRac1, N1406A/D1407A did not affect Dbl-induced growth of the cells in soft-agar despite of 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 T17NRac1.

In summary, we presented evidence that non-discriminative administration of dominant negative mutant of Rho proteins such as T17NRac1 may cause nonspecific effect on multiple Rho GTPase-regulated signaling pathways. We showed that the conserved Asp673-Asp674 of Dbl and a conserved pair of amino acid residues of the Trio-Rac complex are 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 N1406ATrioN, as a new generation of Rho GTPasepathway specific inhibitors. We expect that future design of dominant negative mutant similar to N1406A of TrioN made in the full-length backbone of 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.

Summary

The Dbl family GEFs are large multidomain proteins that transduce intracellular signals leading to the activation of Rho GTPases. All Dbl family guanine nucleotide exchange factors 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 PH domain is indispensable for their cellular transforming activity. In the past few years a large number of Dbl family members was discovered. However the exact mechaninsm of how they facilitate the GDP/GTP exchange on Rho proteins in not well known. To understand the structure-function relationship of the DH domain, we have investigated the role of specific residues of the DH domain of Dbl in interaction with Rho GTPases. Based on the three-dimensional structures of a few Dbl GEFs recently resolved we have constructed a set of alanine substitution mutants of the Dbl protein and identified a panel of 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.

Catalytic and binding analysis of these mutants revealed that altough five of the mutants displayed decreased catalytic activity in the GEF reaction, the substrate binding activities of two of this set were significantly reduced. Another three mutants, on the other hand, retained the binding capability to the Rho GTPases but lost the GEF catalytic activity.

Trio1 is a member of the Dbl family GEFs containing two DH-PH domains, of which the N-terminal one shows specific catalytic activity toward Rac1, a member of the Rho family small GTPases. Based on the available structural information and our previous investigations we have constructed an N1406A/D1407A alanine substitution mutant of the N-terminal DH domain of Trio1. The N1406A/D1407A mutant of Trio acted dominant negatively *in vitro* by retaining Rac1 binding activity but losing GEF

losing GEF catalytic activity and competitively inhibited Rac1 activation by wild type Trio. Furthermore, the N1406A/D1407A mutant readily blocked the PDGF-induced lamellipodia formation and inhibited the wild type Trio-induced serum response factor activation in 3T3 fibroblasts.

Dominant negative mutants of Rho GTPases, such as T17NRac1, that block the endogenous Rho protein activation by sequestering upstream GEFs have been widely employed to implicate specific members of Rho family in various signaling pathways. 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. In our experiments the N1406A/D1407A mutant was able to selectively inhibit Dbl-induced Rac1 activation without affecting RhoA activity in 3T3 cells, in contrast to the non-discriminative inhibitory effect displayed by T17NRac1. Furthermore the Trio mutant - in contrast to T17NRac1 - was ineffective in inhibiting PDGF-stimulated DNA synthesis and Dbl-induced transformation, revealing the Rac-independent functions of PDGF and Dbl. Our studies also identify the highly conserved amino acid pairing of N1406Trio-D65Rac1 of GEF-Rho GTPase interaction that is likely to be essential to the GEF catalysis of Rho family GTPases.

References

Adams, JM, Houston, H, Allen, J, Lints, T, Harvey, R: The hematopoietically expressed vav proto-oncogene shares homology with the dbl GDP-GTP exchange factor, the bcr gene and a yeast gene (CDC24) involved in cytoskeletal organization. Oncogene 7:611-618, (1992)

Aghazadeh, B, Zhu, K, Kubiseski, TJ, Liu, GA, Pawson, T, Zheng, Y, Rosen, MK: Structure and mutagenesis of the Dbl homology domain. Nat Struct Biol 5:1098-1107, (1998)

Aghazadeh, B, Lowry, WE, Huang, XY, Rosen, MK: Structural basis for relief of autoinhibition of the Dbl homology domain of proto-oncogene Vav by tyrosine phosphorylation. Cell 102:625-633, (2000)

Allen, KM, Gleeson, JG, Bagrodia, S, Partington, MW, MacMillan, JC, Cerione, RA, Mulley, JC, Walsh, CA: PAK3 mutation in nonsyndromic X-linked mental retardation. Nat Genet 20:25-30, (1998)

Anborgh, PH, Qian, X, Papageorge, AG, Vass, WC, DeClue, JE, Lowy, DR: Ras-specific exchange factor GRF: oligomerization through its Dbl homology domain and calcium-dependent activation of Raf. Mol Cell Biol 19:4611-4622, (1999)

Aspenstrom, P: Effectors for the Rho GTPases. Curr Opin Cell Biol 11:95-102, (1999)

Bellanger, JM, Lazaro, JB, Diriong, S, Fernandez, A, Lamb, N, Debant, A: The two guanine nucleotide exchange factor domains of Trio link the Rac1 and the RhoA pathways *in vivo*. Oncogene 16:147-152, (1998)

Beraud-Dufour, S, Robineau, S, Chardin, P, Paris, S, Chabre, M, Cherfils, J, Antonny, B: A glutamic finger in the guanine nucleotide exchange factor ARNO displaces Mg2+ and the beta-phosphate to destabilize GDP on ARF1. EMBO J 17:3651-3659, (1998)

Bi, F, Debreceni, B, Zhu, K, Salani, B, Eva, A, Zheng, Y: Autoinhibition mechanism of proto-Dbl. Mol Cell Biol 21:1463-1474, (2001)

Billuart, P, Bienvenu, T, Ronce, N, des, P, V, Vinet, MC, Zemni, R, Roest, CH, Carrie, A, Fauchereau, F, Cherry, M, Briault, S, Hamel, B, Fryns, JP, Beldjord, C, Kahn, A, Moraine, C, Chelly, J: Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation. Nature 392:923-926, (1998)

Bishop, AL and Hall, A: Rho GTPases and their effector proteins. Biochem J 348 Pt 2:241-255, (2000)

Blomquist, A, Schworer, G, Schablowski, H, Psoma, A, Lehnen, M, Jakobs, KH, Rumenapp, U: Identification and characterization of a novel Rho-specific guanine nucleotide exchange factor. Biochem J 352 Pt 2:319-325, (2000)

Bustelo, XR: Vav proteins, adaptors and cell signaling. Oncogene 20:6372-6381, (2001)

Cerione, RA and Zheng, Y: The Dbl family of oncogenes. Curr Opin Cell Biol 8:216-222, (1996)

Cheng, L, Rossman, KL, Mahon, GM, Worthylake, DK, Korus, M, Sondek, J, Whitehead, IP: RhoGEF specificity mutants implicate RhoA as a target for Dbs transforming activity. Mol Cell Biol 22:6895-6905, (2002)

Cherfils, J and Chardin, P: GEFs: structural basis for their activation of small GTP-binding proteins. Trends Biochem Sci 24:306-311, (1999)

Crespo, P, Bustelo, XR, Aaronson, DS, Coso, OA, Lopez-Barahona, M, Barbacid, M, Gutkind, JS: Rac-1 dependent stimulation of the JNK/SAPK signaling pathway by Vav. Oncogene 13:455-460, (1996)

Daniels, RH, Zenke, FT, Bokoch, GM: alphaPix stimulates p21-activated kinase activity through exchange factor-dependent and -independent mechanisms. J Biol Chem 274:6047-6050, (1999)

Das, B, Shu, X, Day, GJ, Han, J, Krishna, UM, Falck, JR, Broek, D: Control of intramolecular interactions between the pleckstrin homology and Dbl homology domains of Vav and Sos1 regulates Rac binding. J Biol Chem 275:15074-15081, (2000)

Debant, A, Serra-Pages, C, Seipel, K, O'Brien, S, Tang, M, Park, SH, Streuli, M: The multidomain protein Trio binds the LAR transmembrane tyrosine phosphatase, contains a protein kinase domain, and has separate rac- specific and rho-specific guanine nucleotide exchange factor domains. Proc Natl Acad Sci U S A 93:5466-5471, (1996)

Debreceni, B, Gao, Y, Guo, F, Zhu, K, Jia, B, Zheng, Y: Mechanisms of guanine nucleotide exchange and Rac-mediated signaling revealed by a dominant negative trio mutant. J Biol Chem 279:3777-3786, (2004)

Engers, R, Zwaka, TP, Gohr, L, Weber, A, Gerharz, CD, Gabbert, HE: Tiam1 mutations in human renal-cell carcinomas. Int J Cancer 88:369-376, (2000)

Etienne-Manneville, S and Hall, A: Rho GTPases in cell biology. Nature 420:629-635, (2002)

Eva, A and Aaronson, SA: Isolation of a new human oncogene from a diffuse B-cell lymphoma. Nature 316:273-275, (1985)

Feig, LA and Cooper, GM: Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. Mol Cell Biol 8:3235-3243, (1988)

Feig, LA: Tools of the trade: use of dominant-inhibitory mutants of Ras-family GTPases. Nat Cell Biol 1:E25-E27, (1999)

Feltham, JL, Dotsch, V, Raza, S, Manor, D, Cerione, RA, Sutcliffe, MJ, Wagner, G, Oswald, RE: Definition of the switch surface in the solution structure of Cdc42Hs. Biochemistry 36:8755-8766, (1997)

Fritz, G, Just, I, Kaina, B: Rho GTPases are over-expressed in human tumors. Int J Cancer 81:682-687, (1999)

Fukuhara, S, Murga, C, Zohar, M, Igishi, T, Gutkind, JS: A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho. J Biol Chem 274:5868-5879, (1999)

Habets, GG, Scholtes, EH, Zuydgeest, D, van der Kammen, RA, Stam, JC, Berns, A, Collard, JG: Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. Cell 77:537-549, (1994)

Hall, A: Rho GTPases and the actin cytoskeleton. Science 279:509-514, (1998)

Han, J, Luby-Phelps, K, Das, B, Shu, X, Xia, Y, Mosteller, RD, Krishna, UM, Falck, JR, White, MA, Broek, D: Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. Science 279:558-560, (1998)

Hart, MJ, Eva, A, Evans, T, Aaronson, SA, Cerione, RA: Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the dbl oncogene product. Nature 354:311-314, (1991)

Hart, MJ, Eva, A, Zangrilli, D, Aaronson, SA, Evans, T, Cerione, RA, Zheng, Y: Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product. J Biol Chem 269:62-65, (1994)

Hart, MJ, Jiang, X, Kozasa, T, Roscoe, W, Singer, WD, Gilman, AG, Sternweis, PC, Bollag, G: Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13. Science 280:2112-2114, (1998)

Hirshberg, M, Stockley, RW, Dodson, G, Webb, MR: The crystal structure of human rac1, a member of the rho-family complexed with a GTP analogue. Nat Struct Biol 4:147-152, (1997)

Hoffman, GR and Cerione, RA: Signaling to the Rho GTPases: networking with the DH domain. FEBS Lett 513:85-91, (2002)

Ihara, K, Muraguchi, S, Kato, M, Shimizu, T, Shirakawa, M, Kuroda, S, Kaibuchi, K, Hakoshima, T: Crystal structure of human RhoA in a dominantly active form complexed with a GTP analogue. J Biol Chem 273:9656-9666, (1998)

Jin, S and Exton, JH: Activation of RhoA by association of Galpha(13) with Dbl. Biochem Biophys Res Commun 277:718-721, (2000)

Johnson, DI and Pringle, JR: Molecular characterization of CDC42, a Saccharomyces cerevisiae gene involved in the development of cell polarity. J Cell Biol 111:143-152, (1990)

Khosravi-Far, R, Chrzanowska-Wodnicka, M, Solski, PA, Eva, A, Burridge, K, Der, CJ: Dbl and Vav mediate transformation via mitogen-activated protein kinase pathways that are distinct from those activated by oncogenic Ras. Mol Cell Biol 14:6848-6857, (1994)

Kourlas, PJ, Strout, MP, Becknell, B, Veronese, ML, Croce, CM, Theil, KS, Krahe, R, Ruutu, T, Knuutila, S, Bloomfield, CD, Caligiuri, MA: Identification of a gene at 11q23 encoding a guanine nucleotide exchange factor: evidence for its fusion with MLL in acute myeloid leukemia. Proc Natl Acad Sci U S A 97:2145-2150, (2000)

Kozma, R, Ahmed, S, Best, A, Lim, L: The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol Cell Biol 15:1942-1952, (1995)

Kutsche, K, Yntema, H, Brandt, A, Jantke, I, Nothwang, HG, Orth, U, Boavida, MG, David, D, Chelly, J, Fryns, JP, Moraine, C, Ropers, HH, Hamel, BC, van Bokhoven, H, Gal, A: Mutations in ARHGEF6, encoding a guanine nucleotide exchange factor for Rho GTPases, in patients with X-linked mental retardation. Nat Genet 26:247-250, (2000)

Laurent, E, Talpaz, M, Kantarjian, H, Kurzrock, R: The BCR gene and philadelphia chromosome-positive leukemogenesis. Cancer Res 61:2343-2355, (2001)

Li, R and Zheng, Y: Residues of the Rho family GTPases Rho and Cdc42 that specify sensitivity to Dbl-like guanine nucleotide exchange factors. J Biol Chem 272:4671-4679, (1997)

Li, R, Debreceni, B, Jia, B, Gao, Y, Tigyi, G, Zheng, Y: Localization of the PAK1-, WASP-, and IQGAP1-specifying regions of Cdc42. J Biol Chem 274:29648-29654, (1999)

Lifshitz, B, Fainstein, E, Marcelle, C, Shtivelman, E, Amson, R, Gale, RP, Canaani, E: bcr genes and transcripts. Oncogene 2:113-117, (1988)

Lin, R, Bagrodia, S, Cerione, R, Manor, D: A novel Cdc42Hs mutant induces cellular transformation. Curr Biol 7:794-797, (1997)

Lin, R, Cerione, RA, Manor, D: Specific contributions of the small GTPases Rho, Rac, and Cdc42 to Dbl transformation. J Biol Chem 274:23633-23641, (1999)

Liu, X, Wang, H, Eberstadt, M, Schnuchel, A, Olejniczak, ET, Meadows, RP, Schkeryantz, JM, Janowick, DA, Harlan, JE, Harris, EA, Staunton, DE, Fesik, SW: NMR structure and mutagenesis of the N-terminal Dbl homology domain of the nucleotide exchange factor Trio. Cell 95:269-277, (1998)

Liu, X, Constantinescu, SN, Sun, Y, Bogan, JS, Hirsch, D, Weinberg, RA, Lodish, HF: Generation of mammalian cells stably expressing multiple genes at predetermined levels. Anal Biochem 280:20-28, (2000)

Mattingly, RR, Sorisky, A, Brann, MR, Macara, IG: Muscarinic receptors transform NIH 3T3 cells through a Ras-dependent signalling pathway inhibited by the Ras-GTPase-activating protein SH3 domain. Mol Cell Biol 14:7943-7952, (1994)

Nassar, N, Hoffman, GR, Manor, D, Clardy, JC, Cerione, RA: Structures of Cdc42 bound to the active and catalytically compromised forms of Cdc42GAP. Nat Struct Biol 5:1047-1052, (1998)

Nimnual, AS, Yatsula, BA, Bar-Sagi, D: Coupling of Ras and Rac guanosine triphosphatases through the Ras exchanger Sos. Science 279:560-563, (1998)

Nishida, K, Kaziro, Y, Satoh, T: Association of the proto-oncogene product dbl with G protein betagamma subunits. FEBS Lett 459:186-190, (1999)

Nobes, CD and Hall, A: Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81:53-62, (1995)

Olson, MF, Ashworth, A, Hall, A: An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. Science 269:1270-1272, (1995)

Olson, MF, Pasteris, NG, Gorski, JL, Hall, A: Faciogenital dysplasia protein (FGD1) and Vav, two related proteins required for normal embryonic development, are upstream regulators of Rho GTPases. Curr Biol 6:1628-1633, (1996)

Otsuki, Y, Tanaka, M, Yoshii, S, Kawazoe, N, Nakaya, K, Sugimura, H: Tumor metastasis suppressor nm23H1 regulates Rac1 GTPase by interaction with Tiam1. Proc Natl Acad Sci U S A 98:4385-4390, (2001)

Pasteris, NG, Cadle, A, Logie, LJ, Porteous, ME, Schwartz, CE, Stevenson, RE, Glover, TW, Wilroy, RS, Gorski, JL: Isolation and characterization of the faciogenital dysplasia (Aarskog-Scott syndrome) gene: a putative Rho/Rac guanine nucleotide exchange factor. Cell 79:669-678, (1994)

Qian, X, Vass, WC, Papageorge, AG, Anborgh, PH, Lowy, DR: N terminus of Sos1 Ras exchange factor: critical roles for the Dbl and pleckstrin homology domains. Mol Cell Biol 18:771-778, (1998)

Qiu, RG, Chen, J, McCormick, F, Symons, M: A role for Rho in Ras transformation. Proc Natl Acad Sci U S A 92:11781-11785, (1995)

Qiu, RG, Chen, J, Kirn, D, McCormick, F, Symons, M: An essential role for Rac in Ras transformation. Nature 374:457-459, (1995)

Qiu, RG, Abo, A, McCormick, F, Symons, M: Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation. Mol Cell Biol 17:3449-3458, (1997)

Ren, XD, Kiosses, WB, Schwartz, MA: Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J 18:578-585, (1999)

Ren, Y, Li, R, Zheng, Y, Busch, H: Cloning and characterization of GEF-H1, a microtubule-associated guanine nucleotide exchange factor for Rac and Rho GTPases. J Biol Chem 273:34954-34960, (1998)

Ridley, AJ, Paterson, HF, Johnston, CL, Diekmann, D, Hall, A: The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70:401-410, (1992)

Ridley, AJ and Hall, A: The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70:389-399, (1992)

Ron, D, Zannini, M, Lewis, M, Wickner, RB, Hunt, LT, Graziani, G, Tronick, SR, Aaronson, SA, Eva, A: A region of proto-dbl essential for its transforming activity shows sequence similarity to a yeast cell cycle gene, CDC24, and the human breakpoint cluster gene, bcr. New Biol 3:372-379, (1991)

Rossman, KL, Worthylake, DK, Snyder, JT, Siderovski, DP, Campbell, SL, Sondek, J: A crystallographic view of interactions between Dbs and Cdc42: PH domain-assisted guanine nucleotide exchange. EMBO J 21:1315-1326, (2002)

Sahai, E and Marshall, CJ: RHO-GTPases and cancer. Nat Rev Cancer 2:133-142, (2002)

Schmidt, A and Hall, A: Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev 16:1587-1609, (2002)

Snyder, JT, Worthylake, DK, Rossman, KL, Betts, L, Pruitt, WM, Siderovski, DP, Der, CJ, Sondek, J: Structural basis for the selective activation of Rho GTPases by Dbl exchange factors. Nat Struct Biol 9:468-475, (2002)

Soisson, SM, Nimnual, AS, Uy, M, Bar Sagi, D, Kuriyan, J: Crystal structure of the Dbl and pleckstrin homology domains from the human Son of sevenless protein. Cell 95:259-268, (1998)

Sprang, SR and Coleman, DE: Invasion of the nucleotide snatchers: structural insights into the mechanism of G protein GEFs. Cell 95:155-158, (1998)

Steven, R, Kubiseski, TJ, Zheng, H, Kulkarni, S, Mancillas, J, Ruiz, MA, Hogue, CW, Pawson, T, Culotti, J: UNC-73 activates the Rac GTPase and is required for cell and growth cone migrations in C. elegans. Cell 92:785-795, (1998)

Suwa, H, Ohshio, G, Imamura, T, Watanabe, G, Arii, S, Imamura, M, Narumiya, S, Hiai, H, Fukumoto, M: Overexpression of the rhoC gene correlates with progression of ductal adenocarcinoma of the pancreas. Br J Cancer 77:147-152, (1998)

Symons, M and Settleman, J: Rho family GTPases: more than simple switches. Trends Cell Biol 10:415-419, (2000)

Toksoz, D and Williams, DA: Novel human oncogene lbc detected by transfection with distinct homology regions to signal transduction products. Oncogene 9:621-628, (1994)

Van Aelst, L and D'Souza-Schorey, C: Rho GTPases and signaling networks. Genes Dev 11:2295-2322, (1997)

Wei, Y, Zhang, Y, Derewenda, U, Liu, X, Minor, W, Nakamoto, RK, Somlyo, AV, Somlyo, AP, Derewenda, ZS: Crystal structure of RhoA-GDP and its functional implications. Nat Struct Biol 4:699-703, (1997)

Whitehead, I, Kirk, H, Tognon, C, Trigo-Gonzalez, G, Kay, R: Expression cloning of lfc, a novel oncogene with structural similarities to guanine nucleotide exchange factors and to the regulatory region of protein kinase C. J Biol Chem 270:18388-18395, (1995)

Whitehead, IP, Campbell, S, Rossman, KL, Der, CJ: Dbl family proteins. Biochim Biophys Acta 1332:F1-23, (1997)

Worthylake, DK, Rossman, KL, Sondek, J: Crystal structure of Rac1 in complex with the guanine nucleotide exchange region of Tiam1. Nature 408:682-688, (2000)

Zhang, B, Chernoff, J, Zheng, Y: Interaction of Rac1 with GTPase-activating proteins and putative effectors. A comparison with Cdc42 and RhoA. J Biol Chem 273:8776-8782, (1998)

Zhang, B, Zhang, Y, Wang, Z, Zheng, Y: The role of Mg2+ cofactor in the guanine nucleotide exchange and GTP hydrolysis reactions of Rho family GTP-binding proteins. J Biol Chem 275:25299-25307, (2000)

Zheng, Y, Fischer, DJ, Santos, MF, Tigyi, G, Pasteris, NG, Gorski, JL, Xu, Y: The faciogenital dysplasia gene product FGD1 functions as a Cdc42Hs-specific guanine-nucleotide exchange factor. J Biol Chem 271:33169-33172, (1996)

Zheng, Y: Dbl family guanine nucleotide exchange factors. Trends Biochem Sci 26:724-732, (2001)

Zhu, K, Debreceni, B, Li, R, Zheng, Y: Identification of Rho GTPase-dependent sites in the Dbl homology domain of oncogenic Dbl that are required for transformation. J Biol Chem 275:25993-26001, (2000)

Zhu, K, Debreceni, B, Bi, F, Zheng, Y: Oligomerization of DH domain is essential for Dbl-induced transformation. Mol Cell Biol 21:425-437, (2001)

Acknowledgements

I would like to thank Dr. Yi Zheng, my mentor at the University of Tennessee, for his solid support and guidance. I also would like to thank my friends and collegues at UT, especially Kejin Zhu, Yuan Gao, Baoqing Gia and Baolin Zhang, with whom I shared a really great time. I am also in debt to professors Gyula Mozsik, Balazs Sumegi and Attila Sandor for they constitutive support in my research career. I am grateful to Dr. Gabor Tigyi for his encouragement for completing my work.

List of Publications

Publications supporting the dissertation:

Debreceni, B, Gao, Y, Guo, F, Zhu, K, Jia, B, Zheng, Y: Mechanisms of guanine nucleotide exchange and Rac-mediated signaling revealed by a dominant negative trio mutant. J Biol Chem 279:3777-3786, (2004)

Zhu, K, Debreceni, B, Li, R, Zheng, Y: Identification of Rho GTPase-dependent sites in the Dbl homology domain of oncogenic Dbl that are required for transformation. J Biol Chem 275:25993-26001, (2000)

Other papers:

Bi, F, Debreceni, B, Zhu, K, Salani, B, Eva, A, Zheng, Y: Autoinhibition mechanism of proto-Dbl. Mol Cell Biol 21:1463-1474, (2001)

Zhu, K, Debreceni, B, Bi, F, Zheng, Y: Oligomerization of DH domain is essential for Dbl-induced transformation. Mol Cell Biol 21:425-437, (2001)

Fischer, GM, Nemeti, B, Farkas, V, Debreceni, B, Laszlo, A, Schaffer, Z, Somogyi, C, Sandor, A: Metabolism of carnitine in phenylacetic acid-treated rats and in patients with phenylketonuria. Biochim Biophys Acta 1501:200-210, (2000)

Li, R, Debreceni, B, Jia, B, Gao, Y, Tigyi, G, Zheng, Y: Localization of the PAK1-, WASP-, and IQGAP1-specifying regions of Cdc42. J Biol Chem 274:29648-29654, (1999)

Sebok, A, Nusser, N, Debreceni, B, Guo, Z, Santos, MF, Szeberenyi, J, Tigyi, G: Different roles for RhoA during neurite initiation, elongation, and regeneration in PC12 cells. J Neurochem 73:949-960, (1999)

Debreceni, A, Debreceni, B, Mozsik, G: A study of the actions of naloxone and morphine on gastric acid secretion and gastric mucosal damage in the rat. J Physiol Paris 91:189-197, (1997)

Debreceni, L, Hartmann, G, Debreceni, B: Effect of morphine on hypothalamic catecholamine and serotonin level in relation to the stress-induced pituitary-adrenocortical activation in the rat. Exp Clin Endocrinol

Abstracts and posters:

G Varbiro, B Debreceni, P Csere, B Sumegi, Gy Mozsik: Analysis of heat shock proteins with monoclonal antibodies in rat lingual and buccal mucosa following 2',3'-dideoxycytidine (ddC) treatment.
Balatonaliga, Gasztroenterológus Kongresszus, 1995
Z Gastroenterologie (1995) 5:314

P Csere, B Debreceni, G Varbiro, Gy Mozsik, B Sumegi, Gy Kispal: Function of a mitochondrial multi-drug resistance transporter homolog, Atm1p.
Balatonaliga, Gasztroenterológus Vándorgyűlés, 1996
Z Gastroenterologie (1996) 5:306

G Varbiro, B Debreceni, P Csere, B Sumegi, Gy Mozsik: The Role of haet shock proteins in gastric cytoproection.
Balatonaliga, Gasztroenterológus Vándorgyűlés, 1996
Z Gastroenterologie (1996) 5:338

P Csere, B Debreceni, Gy Mozsik, R Lill, B Sumegi, Gy Kispal: The function of CPV motif.
Balatonaliga, Gasztroenterológus Vándorgyűlés, 1997
Z Gastroenterologie (1997) 35:372