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Association of the proto-oncogene product Dbl with G protein βγ subunits

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Abstract The Rho family of GTP-binding proteins has been implicated in the regulation of various cellular functions including actin cytoskeleton-dependent morphological change. Its activity is directed by intracellular signals mediated by various types of receptors such as G protein-coupled receptors. However, the mechanisms underlying receptor-dependent regulation of Rho family members remain incompletely understood. The guanine nucleotide exchange factor (GEF) Dbl targets Rho family proteins thereby stimulating their GDP/GTP exchange, and thus is believed to be involved in receptor-mediated regulation of the proteins. Here, we show the association of Dbl with G protein βγ subunits (Gβγ) in transient co-expression and cell-free systems. An amino-terminal portion conserved among a subset of Dbl family proteins is sufficient for the binding of Gβγ. In fact, Ost and Kalirin, which contain this Gβγ-binding motif, also associate with Gβγ. c-Jun N-terminal kinase was synergistically activated upon co-expression of Dbl and Gβ in a dominant-negative Rho-sensitive manner. However, GEF activity of Dbl toward Rho as measured by in vitro GDP binding assays remained unaffected following Gβγ binding, suggesting that additional signals may be required for the regulation of Dbl.

Key words: Cdc42; Dbl; G protein βγ subunit; Guanine nucleotide exchange factor; Rac; Rho

1. Introduction

Rho family GTP-binding proteins including Cdc42, Rac, and Rho regulate a variety of physiological responses through actin cytoskeletal rearrangements [1]. In addition to cytoskeletal regulation, Rho family GTP-binding proteins are required for activated Ras-dependent transformation, growth factor-induced cell cycle progression, and generation of reactive oxygen species [1]. Furthermore, Rac, but not Cdc42 and Rho, is implicated in the regulation of cell death triggered by growth factor deprivation [2].

Dbl family proteins act as a guanine nucleotide exchange factor (GEF) for Rho family GTP-binding proteins in vitro, and thus are thought to induce formation of the active GTP-bound form within the cell [3]. The Dbl family is comprised of more than 20 members, which share common motifs in tandem, designated Dbl homology (DH) and pleckstrin homology (PH) domains. The DH domain is essential and sufficient for GEF activity, whereas the PH domain determines proper cellular localization thereby modulating the function of the DH domain. Some Dbl family members are specific for an individual Rho family protein, while others act on all three subgroups. Dbl is a prototype of this family, which acts on Cdc42, Rac1, and RhoA in vitro, and is expressed in brain, adrenal gland, testis, and ovary [4–6]. Truncation of the amino-terminal half enables Dbl to transform NIH3T3 cells, suggesting that the amino-terminal region is involved in the regulation of Dbl functions [4].

As predicted by diverse biological roles, a wide variety of receptors including tyrosine kinase-type, cytokine, and G protein-coupled receptors, trigger the activation of Rho family proteins. However, the mechanisms whereby various upstream stimuli activate Rho family members remain largely unknown.

Recent reports have revealed that Dbl family GEFs play a role in mediating receptor signals to the Rho family. For example, the hematopoietic cell-specific GEF Vav shows Rac-directed GEF activity that becomes evident upon tyrosine phosphorylation [7,8], and thereby acts as a regulator of Rac downstream of receptors such as the high affinity IgE receptor [9]. Signals initiated by G protein-coupled receptors are transduced through both the G protein α subunit (Gα) and βγ subunits (Gβγ) specific to respective receptors. It has been demonstrated that p115 RhoGEF regulates Rho activity downstream of Gα12 and Gα13 through direct interactions [10,11]. In contrast, Gβγ-stimulated pathways linked to the Rho family are less clear although Ras-GRF1, which is expressed exclusively in brain, is able to act as a GEF for Rac following Gβγ stimulation [12].

In this paper, we show the association of the Rho family GEF Dbl with Gβγ. An amino-terminal region conserved among a subset of Dbl family members was sufficient for this interaction. Indeed, other members such as Ost and Kalirin that contain the possible Gβγ interacting motif also bound to Gβγ. These results may implicate Dbl in Gβγ-induced signaling pathways that regulate Rho family GTP-binding proteins.

2. Materials and methods

2.1. Antibodies

Mouse monoclonal antibodies against Myc (9E10) and hemagglutinin (HA) (12CA5) epitopes were purchased from BAbCO and Boehringer, respectively. Rabbit antibodies against mouse IgG (55480) and Gβ1 (sc-378) were purchased from Cappel and Santa Cruz Biotechnology, respectively.

2.2. cDNA construction

The cDNA of proto-Dbl [4] was generously provided by Alessandra

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Abbreviations: DH, Dbl homology; Gα, G protein α subunit; Gβγ, G protein βγ subunits; GEF, guanine nucleotide exchange factor; GST, glutathione *S*-transferase; HA, hemagglutinin; HEK, human embryonic kidney; JNK, c-Jun N-terminal kinase; PCR, polymerase chain reaction; PH, pleckstrin homology

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Eva (Institute of Giannina Gaslini, Genoa, Italy). Myc epitope-tagged Dbl mutants were generated using polymerase chain reaction (PCR) and subcloned into the mammalian expression vector pCMV5 and pGEX-2T (Amersham Pharmacia). cDNAs for Myc epitope-tagged Kalirin(1–231) and Ost(1–103) were amplified from a rat brain cDNA library (Clontech) using PCR, and subcloned into pCMV5. pCMV5-Gβ1, pCMV5-Gγ2, and pCMV5-Gαo were kindly provided by Hiroshi Itoh and Junji Yamauchi [13]. All PCR products were sequenced using the DNA sequencer (LI-COR 4000L). pCMV5-FLAG-RhoA(N19) was described elsewhere [12].

2.3. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. For transient expression, plasmid DNAs were introduced into subconfluent HEK293 cells in a 60-mm culture plate by a standard calcium phosphate method. Following 20 h culture, cells were washed and incubated in Dulbecco's modified Eagle's medium supplemented with bovine serum albumin (1 mg/ml) for 24 h for starvation.

2.4. Immunoprecipitation and immunoblotting

Cells were dissolved in buffer A (20 mM Tris-HCl (pH 7.5), 0.5% (v/v) Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 3 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride), and the supernatant of centrifugation (15 000×*g*) for 5 min at 4°C was obtained. Myc-Dbl, Myc-Kalirin, and Myc-Ost were immunoprecipitated with an anti-Myc antibody conjugated with an anti-mouse IgG antibody and protein G-Sepharose (Amersham Pharmacia) for 2 h at 4°C. Immune complexes were washed twice with buffer A and twice with buffer B (20 mM HEPES-NaOH (pH 7.5), 0.05% (v/v) Nonidet P-40, 50 mM NaCl, 0.1 mM EDTA). Immunoprecipitates and lysates were subjected to SDS-PAGE, and proteins were transferred onto a nitrocellulose membrane. Specific proteins were detected with an anti-Myc or anti-Gβ1 antibody and enhanced chemiluminescence detection reagents (DuPont NEN).

2.5. Purification of glutathione S-transferase (GST)-Dbl from Escherichia coli

The *E. coli* strain BL21(DE3) was transformed with pGEX-2T, pGEX-2T-Dbl(53–99), or pGEX-2T-Dbl(1–99). An isolated colony was inoculated and grown in 15 ml of LB medium at 30°C overnight. This culture was diluted in 400 ml of LB and grown at 30°C until A_{600} reached 0.5, when 1 mM isopropyl-1-thio-β-D-galactopyranoside was added. Following incubation for 3 h, cells were harvested and suspended in buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol (DTT). Subsequently, the suspension was briefly sonicated, and cell debris was removed by centrifugation at 15 000×*g* for 20 min at 4°C. The supernatant was applied to a glutathione-Sepharose (Amersham Pharmacia) column, which was subsequently washed with wash buffer (20 mM Tris-HCl (pH 7.5), 2 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT). GST fusion proteins were eluted with elution buffer (50 mM Tris-HCl (pH 8.0), 10 mM glutathione), and dialyzed against dialysis buffer (50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 150 mM KCl). All purification steps were performed under 4°C.

2.6. In vitro association assay

GST, GST-Dbl(53–99), or GST-Dbl(1–99) (1.5 μg each) was incubated with 1.5 μg of purified recombinant Gβ1γ2 (kindly provided by Hiroshi Itoh and Takeharu Kawano) and glutathione-Sepharose (Amersham Pharmacia) for 2 h at 4°C in 100 μl of buffer A. Sepharose beads were collected by centrifugation, washed twice with buffer A and twice with buffer B. Precipitated proteins were subjected to immunoblotting using an anti-Gβ1 antibody.

2.7. c-Jun N-terminal kinase (JNK) 1 kinase assay

Plasmids for JNK1 kinase assays were kindly provided by Michael Karin (University of California San Diego, La Jolla, CA, USA). Transfected HEK293 cells were lysed with buffer A, and the supernatant of centrifugation (15 000×*g*) for 15 min at 4°C was obtained. HA-JNK1 was immunoprecipitated with an anti-HA antibody conjugated with protein A-Sepharose (Amersham Pharmacia) by incubation for 1 h at 4°C with gentle mixing. Immunoprecipitates were washed twice with buffer A, twice with buffer containing 20 mM HEPES-NaOH (pH 7.6), 0.05% (v/v) Nonidet P-40, 50 mM NaCl, and 0.1 mM EDTA, and once with buffer C (25 mM HEPES-NaOH (pH 7.6), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 20 mM *p*-nitrophenyl phosphate, 2 mM DTT) followed by incubation within buffer C (30 μl) containing 0.05 mg/ml GST-c-Jun(1–223) and 20 μM [γ-^{32}P]ATP (307 TBq/mol) for 20 min at 30°C. Subsequently, the proteins were separated by SDS-PAGE, and the radioactivity incorporated into the substrate was quantitated.

3. Results

The association of Dbl with Gβγ was investigated by the use of a transient expression system in HEK293 cells. G protein β1 and γ2 subunits were ectopically expressed together with Myc-tagged proto-Dbl followed by immunoprecipitation with the anti-Myc antibody 9E10. As shown in Fig. 1a, Gβ was co-immunoprecipitated with proto-Dbl. The association was detected in the presence or absence of exogenous Gγ, suggesting that Gβ rather than Gγ is primarily responsible for the binding. To substantiate further a possible interaction

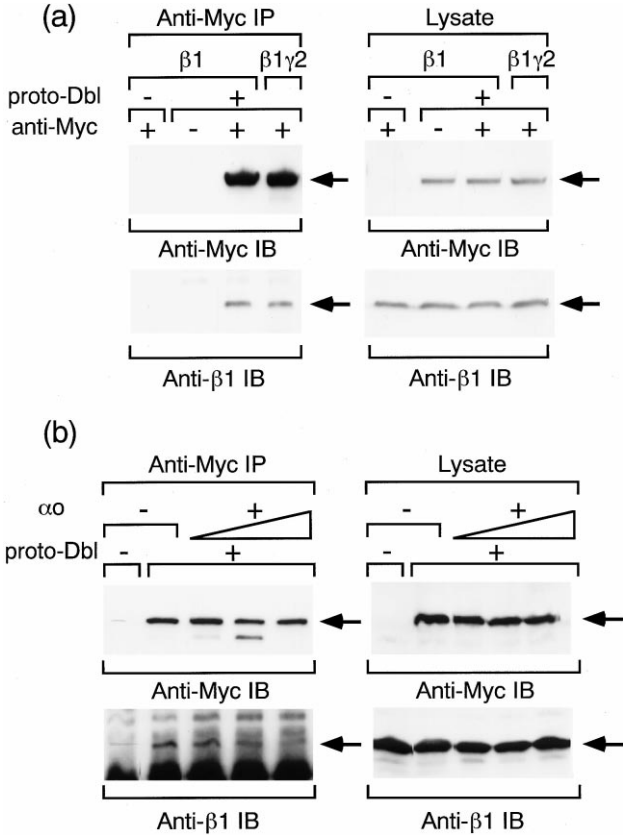

Fig. 1. Association of proto-Dbl with Gβ1. a: Co-immunoprecipitation of proto-Dbl with Gβ1. HEK293 cells were transfected with cDNAs for Gβ1, Gγ2, and Myc-proto-Dbl as indicated. Cell lysates were subjected to immunoprecipitation with (+) or without (−) the anti-Myc antibody 9E10, followed by SDS-PAGE and immunoblotting (left panels). Aliquots of cell lysates were also subjected to SDS-PAGE and immunoblotting (right panels). b: Effect of co-expression of Gαo on the interaction between proto-Dbl and Gβ1. HEK293 cells were transfected with cDNAs for Gβ1, FLAG-Gγ2, Myc-proto-Dbl, and increasing amounts of Gαo (2–6-fold excess against Gβ) as indicated. Cell lysates were prepared, and anti-Myc immunoprecipitates (left panels) and lysates (right panels) were subjected to SDS-PAGE, followed by immunoblotting.

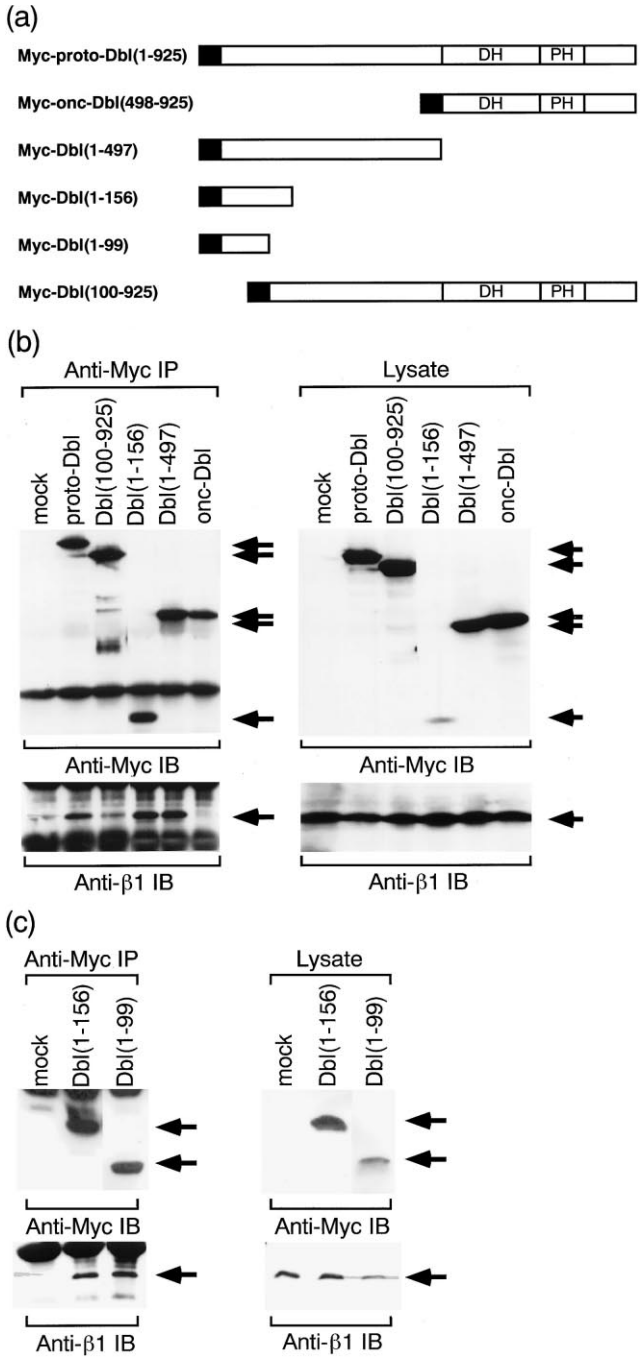

Fig. 2. Association of Dbl mutants with Gβ1. a: Schematic representation of Myc epitope-tagged Dbl mutants. Filled squares indicate the Myc epitope tag. b: Co-immunoprecipitation of Dbl mutants with Gβ1. HEK293 cells were transfected with the cDNA for Gβ1 plus vector DNA (mock) or the cDNA for a Myc-tagged mutant of proto-Dbl as indicated. Cell lysates were prepared, and anti-Myc immunoprecipitates (left panels) and lysates (right panels) were subjected to SDS-PAGE, followed by immunoblotting. c: Co-immunoprecipitation of Dbl(1–99) with Gβ1. HEK293 cells were transfected with the cDNA for Gβ1 plus vector DNA (mock), the cDNA for Myc-Dbl(1–156) or Myc-Dbl(1–99). Cell lysates were prepared, and anti-Myc immunoprecipitates (left panels) and lysates (right panels) were subjected to SDS-PAGE, followed by immunoblotting.

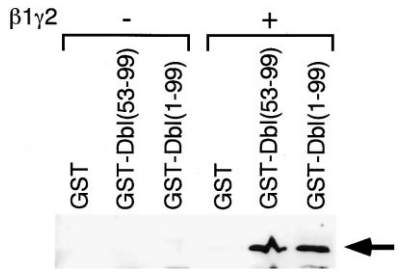

Fig. 3. In vitro association of Dbl with Gβ1γ2. GST, GST-Dbl(53–99), or GST-Dbl(1–99) was incubated with (+) or without (−) recombinant Gβ1γ2, and precipitated with glutathione-Sepharose beads. Samples were subjected to SDS-PAGE, followed by immunoblotting with an anti-Gβ1 antibody.

between proto-Dbl and Gβγ, the effect of Gα overexpression was examined (Fig. 1b). Increasing amounts of Goα co-expressed with proto-Dbl and Gβγ effectively diminished the association between proto-Dbl and Gβγ. Thus, it is likely that, in receptor-mediated signaling, proto-Dbl interacts with Gβγ released upon dissociation of heterotrimeric G proteins by receptor stimulation.

To identify the sequence of proto-Dbl that is involved in the association with Gβγ, an array of Myc-tagged deletion mutants were generated, and examined for their ability to bind Gβγ (Fig. 2). The amino-terminal half of Dbl (Dbl(1–497)) was associated with Gβγ, whereas the carboxy-terminal half (onc-Dbl) failed to bind to Gβγ (Fig. 2b). Further, both Dbl(1–156) and Dbl(1–99) were efficiently associated with Gβγ (Fig. 2b,c). In contrast, deletion of the amino-terminal 99 amino acids rendered the protein incapable of binding to Gβγ (Fig. 2b). Hence, we conclude that the amino-terminal 99 amino acids are sufficient for the binding to Gβγ. The formation of the Dbl/Gβγ ternary complex was further confirmed by co-immunoprecipitation of Myc-tagged Dbl(1–156) with FLAG-tagged Gγ in the presence of ectopically expressed Gβ (data not shown).

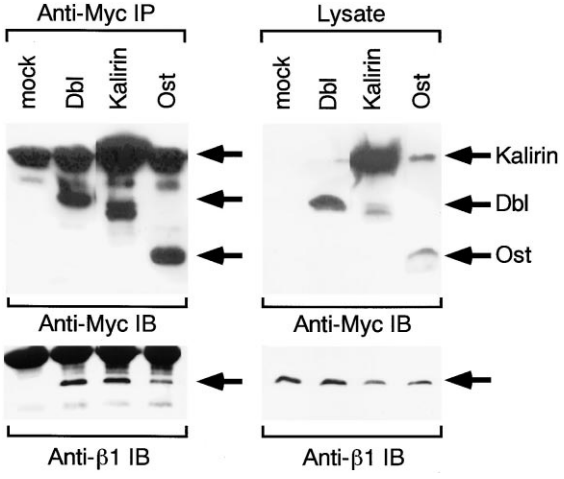

Fig. 4. Association of Ost and Kalirin with Gβ1. HEK293 cells were transfected with the cDNA for Gβ1 plus vector DNA (mock), the cDNA for Myc-Dbl(1–156), Myc-Kalirin(1–231), or Myc-Ost(1–103). Cell lysates were prepared, and anti-Myc immunoprecipitates (left panels) and lysates (right panels) were subjected to SDS-PAGE, followed by immunoblotting. The upper band of the Ost lane in the right upper panel is not reproducible. The lower bands of Kalirin lanes in the upper panels represent degradation products.

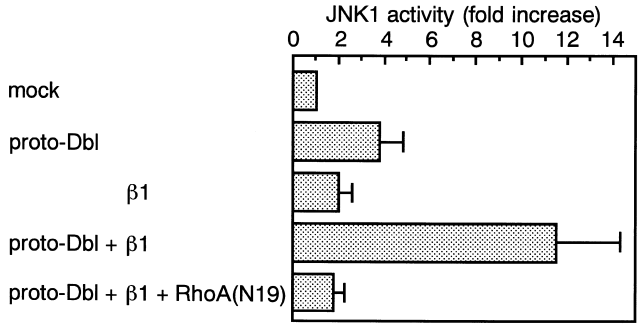

Fig. 5. JNK1 activation following co-expression of proto-Dbl and Gβ1. HEK293 cells were transfected with the HA-JNK1 expression plasmid plus cDNAs for Myc-proto-Dbl, Gβ1, and FLAG-RhoA(N19) as indicated. Relative kinase activities of immunoprecipitated HA-JNK1 are shown (mean ± S.E.M., $n = 3$).

Fig. 3 shows in vitro association of the amino-terminal portion of Dbl with Gβγ. Dbl mutants fused to GST were mixed with purified recombinant Gβ1γ2, and subjected to pull down assays using glutathione-Sepharose. In agreement with the results obtained from transient expression studies, Gβ was co-precipitated with GST-Dbl(1–99). Furthermore, GST-Dbl(53–99) suffices to form a complex with Gβγ. Taken together, these results suggest that the interaction between Dbl and Gβγ is direct, and amino acids 53–99 of Dbl are sufficient for this interaction.

Although highly conserved, no function has been attributed to the amino-terminal region of any of the Dbl family proteins, including Dbl, Ost, and Kalirin [14]. As anticipated from the results described above, this region may represent a general Gβγ-binding motif. Thus, Myc-tagged constructs of this region from other Rho family GEFs, Ost and Kalirin, were expressed, and the interaction with Gβ was assessed (Fig. 4). As predicted, both Ost and Kalirin were co-immunoprecipitated with Gβ as efficiently as Dbl, suggesting that the conserved amino-terminal sequence is responsible for the binding to Gβ.

To further study the physiological significance of this interaction, the activity of JNK, which acts downstream of Rho family proteins, was measured following co-expression of proto-Dbl and Gβ. Whereas either proto-Dbl or Gβ alone stimulated JNK activity only slightly, co-expression of both molecules caused synergistic activation of JNK1 (Fig. 5). Dominant-negative RhoA(N19) diminished the synergistic activation almost completely, indicative of the involvement of Rho family members (Fig. 5). These results suggest that the binding of Gβ triggers the activation of the proto-Dbl-dependent pathway in the cell. However, we were unable to detect any significant increase in GEF activity of proto-Dbl towards RhoA and Cdc42 following Gβγ binding (data not shown).

4. Discussion

Stimulation of G protein-coupled receptors leads to the activation of Rho family GTP-binding proteins through Gβγ ([15] and Nishida et al., unpublished results). However, little is known regarding the target for Gβγ that is responsible for signal transduction to Rho family members.

Here, we show that Gβγ bind to a subset of Dbl family GEFs, including Dbl, Ost, and Kalirin, suggesting the involvement of these Dbl family members in Gβγ-mediated regulation of Rho family proteins. Furthermore, co-expression of Gβ and proto-Dbl caused synergistic enhancement of JNK activity (Fig. 5), implying that the interaction of Gβ and proto-Dbl is indeed important for Dbl activation within the cell. In vitro GEF activity of proto-Dbl, however, remained unaffected upon the interaction with Gβγ (data not shown). Therefore, it is possible that transient modifications or labile interactions with other molecules, which are not preserved in our in vitro system, may be required for the enhancement of GEF activity. For instance, phosphorylation-dependent interaction with still unknown regulatory proteins may be implicated in the regulation of GEF activity. Also, second messengers such as Ca^{2+} or lipid metabolites may modulate the activity. Alternatively, without affecting GEF activity, the binding of Gβγ may elicit conformational alterations of Dbl, which allow Dbl to access to a molecule that directs the interaction between Dbl and its downstream targets. These issues will be clarified further in future studies.

In the yeast *Saccharomyces cerevisiae*, Gβγ (Ste4·Ste18) function downstream of mating pheromone receptors. Upon dissociation of heterotrimeric G proteins, Gβγ stimulate signaling pathways that regulate events associated with mating. Yeast Cdc42, which belongs to the Rho family, controls asymmetric cellular organization through actin cytoskeletal rearrangements. Cdc24, which is also involved in the regulation of cell polarity, possesses the DH/PH domains, and thereby functions as a GEF for Cdc42. Interestingly, direct interaction between Ste4 and Cdc24 has been shown through the use of the yeast two-hybrid system and cell-free pull down assays [16,17]. Thus, considered as a whole, the signaling cascade consisting of Gβγ, a Dbl family GEF, and Rho family GTP-binding proteins is conserved among yeast and mammalian cells. Yet, the precise mechanism whereby Gβγ associates with proto-Dbl or Cdc24 seems different: the interaction of Cdc24 with Ste4 requires the γ subunit Ste18 because deletion of STE18 abolished the interaction [17]. Additionally, the Ste4-binding site locates between amino acids 181 and 199 of Cdc24 (corresponding to 385–403 of proto-Dbl), whereas an amino-terminal region (amino acids 53–99) of proto-Dbl is sufficient for the binding of Gβ (Fig. 2).

The Gβγ complex binds to, and thereby regulates numerous effector molecules including adenylyl cyclases, phospholipases, ion channels, G protein-coupled receptor kinases, phosphatidylinositol 3-kinase, PH domain-containing tyrosine kinases, and the serine/threonine kinase c-Raf-1. Amino acid residues essential for Gβγ binding were characterized for several effectors including β-adrenergic receptor kinase 1, adenylyl cyclase 2, and phospholipase C β2 [18,19]. However, primary structures highly conserved among the effectors have not been identified although a very limited sequence similarity was found among Gβγ-binding regions in β-adrenergic receptor kinases and adenylyl cyclases [18]. On the other hand, the regions of Gβγ that interact with different effectors may be very close to each other if not overlapping because a short amino acid peptide effectively blocks Gβγ interaction with several effectors [18]. In agreement with this, mutational analysis of the Gα-binding surface of Gβ revealed that different, but partially overlapping subsets of Gβ residues are required for the interaction with individual effectors [20]. In fact, overexpression of Gαo also diminished the interaction of proto-Dbl with Gβγ (Fig. 1b). In addition to residues that make

contact with Gα, other regions of Gβ such as the carboxy-terminal region are implicated in effector interactions [13].

The yeast β subunit Ste4 also binds to multiple molecules including the scaffold proteins Ste5 [21] and Far1 [22]. For these cases, RING finger domains of Ste5 and Far1 mediate the interaction with Ste4. Additionally, Ste4 binds to the serine/threonine kinase Ste20, a yeast homologue of mammalian p21-activated protein kinase [23]. The Ste4-binding site of Ste20 is conserved among p21-activated protein kinases and c-Raf-1 [23,24], yet shows no similarity to the RING finger domain. Consistently, a different set of Ste4 residues seems to be involved in this interaction compared to the case of Ste5 [23]. To date, we were unable to find a significant sequence similarity between the amino-terminal Gβγ-interacting site of proto-Dbl and known Gβγ-interacting domains described above. Thus, the structural basis of the interaction between proto-Dbl and Gβγ remains to be further investigated.

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