



| | |
|---------------------------|--|
| 学位論文題目 Title | Role of the Sec14-like domain of Db1 family exchange factors in the regulation of Rho family GTPases in different subcellular sites(異なる細胞内領域でのRhoファミリーGTPaseの活性調節に関するDb1ファミリーグアニンヌクレオチド交換因子のSec14様ドメインの役割) |
| 氏名 Author | Ueda, Shuji |
| 専攻分野 Degree | 博士（医学） |
| 学位授与の日付 Date of Degree | 2004-03-31 |
| 資源タイプ Resource Type | Thesis or Dissertation / 学位論文 |
| 報告番号 Report Number | 甲2967 |
| 権利 Rights | |
| JaLDOI | |
| URL | http://www.lib.kobe-u.ac.jp/handle_kernel/D1002967 |

※当コンテンツは神戸大学の学術成果です。無断複製・不正使用等を禁じます。著作権法で認められている範囲内で、適切にご利用ください。

Role of the Sec14-like domain of Dbl family exchange factors in the regulation of Rho family GTPases in different subcellular sites

(異なった細胞内領域での Rho ファミリーGTPase の活性調節に關与する Dbl ファミリーグアニンヌクレオチド交換因子の Sec14 様ドメインの役割)

Authors: Shuji Ueda¹, Tohru Kataoka¹ and Takaya Satoh^{*}

¹Division of Molecular Biology, Department of Molecular and Cellular Biology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan;

*Corresponding author: Dr. Takaya Satoh

Mailing address: Division of Molecular Biology, Department of Molecular and Cellular Biology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Tel.: +81-78-382-5381; Fax: +81-78-382-5399; E-mail: tkysato@med.kobe-u.ac.jp.

Running title: The Sec14-like domain in Dbl family GEFs

Keywords: *Dbl; GEF; Sec14; Cdc42; Rac; Rho*

Abstract

Mechanisms underlying subcellular region-specific regulation of Rho family GTPases through Dbl family guanine nucleotide exchange factors (GEFs) remain totally unknown. Here we show that the Sec14-like domain, which lies in the N-terminus of the Dbl family GEFs Dbl and Ost, directs the subcellular localization of these GEFs and also their substrate Cdc42. When coexpressed with Cdc42 in human adenocarcinoma HeLa cells, Dbl-I and Ost-I, which lack the Sec14-like domain, translocated Cdc42 to the plasma membrane, where Dbl-I or Ost-I was colocalized. In marked contrast, Dbl-II and Ost-II, which contain the Sec14-like domain, were colocalized with Cdc42 in endomembrane compartments. Furthermore, ruffle membrane formation upon epidermal growth factor treatment was mediated by Dbl-I or Ost-I, but neither Dbl-II nor Ost-II, supporting a notion that GEFs with or without the Sec14-like domain are linked to different upstream signals. By employing a novel method to detect the active GTP-bound form of Cdc42 *in situ*, we demonstrate that Dbl-I and Ost-I, but neither Dbl-II nor Ost-II, indeed activate colocalized Cdc42.

1. Introduction

Rho family small GTPases have been implicated in a wide variety of biological responses, such as the determination of cell morphology, the regulation of cell motility, and the establishment of intracellular polarity, in various types of eukaryotic cells [1]. Although the activation of each Rho family GTPase following extracellular stimuli is believed to be tightly regulated, its underlying mechanisms remain incompletely understood. GTPases are activated through conformational change associated with the replacement of bound GDP for GTP, which is stimulated by a family of proteins called guanine nucleotide exchange factors (GEFs). Taking into consideration that another well-characterized GTPase Ras is activated by its specific GEFs in response to upstream signals, GEFs presumably play a pivotal role in the activation of the Rho family as well.

A number of proteins that contain tandem Dbl homology (DH) and pleckstrin homology (PH) domains are putative GEFs for Rho family GTPases, constituting the Dbl family [2, 3]. Whereas DH domains interact directly with Rho family proteins to catalyze GDP/GTP exchange, PH domains exert regulatory functions in the subcellular translocation and the activation of the GEFs. In addition, novel Rho family-targeting GEFs, including Dock180 and Zizimin 1, have recently been identified [4, 5].

Taking into consideration that Rho family proteins serve as molecular switches in a diverse array of signaling pathways, they may localize in various subcellular regions, being regulated by specific GEFs. However, the mechanisms underlying subcellular region-specific regulation remain poorly understood. Furthermore, a gradient of Rac and Cdc42 activities in motile and integrin-stimulated cells was shown by virtue of fluorescent resonance energy transfer technology, implying the spatially orchestrated regulation of the activity of the Rho family [6-8]. Yet, the mechanisms of this regulation are also largely unsolved.

As a step toward understanding how Dbl family GEFs and Rho family GTPases localize and function in a specific subcellular compartment, we particularly focused on

the Sec14-like domain, which resides in the N-terminal portion of a subset of Dbl family GEFs. The Sec14-like domain of Dbl family GEFs, including Dbl, Ost (also named Dbs), Trio, and Kalirin, exhibits sequence homology to the lipid-binding domain of the *Saccharomyces cerevisiae* phosphatidylinositol (PI) transfer protein Sec14p [9-10]. Recently, the Sec14-like domain of the tyrosine phosphatase PTP-MEG2 was shown to interact specifically with phosphatidylserine (PS) [11]. However, ligands for Sec14-like domains of Dbl family GEFs have not been identified, and the role of these domains in the regulation of Rho family GTPases is largely unknown.

Herein, we demonstrate that Sec14-like domains of Dbl and Ost are involved in the regulation of the subcellular localization of these GEFs. In different subcellular compartments, Dbl and Ost splice variants exerted different biological effects. We also developed a novel method to visualize the spatial distribution of the active form of Cdc42 in mammalian cultured cells.

2. Materials and methods

2.1. Plasmids

pCMV5-Myc-Dbl-I is identical to previously described pCMV5-Myc-*proto-Dbl* [12] (GenBank accession no. for Dbl-I is X12556). The cDNA encoding N-terminally Myc-tagged human Ost-I (GenBank accession no. AB116075) was amplified by the polymerase chain reaction (PCR) using KIAA0362 [kindly provided by Takahiro Nagase (Kazusa DNA Research Institute, Japan)] as a template, and subcloned into pCMV5 (GenBank accession no. AF239249), generating pCMV5-Myc-Ost-I. cDNAs encoding N-terminally Myc-tagged human Dbl-II (GenBank accession no. AL117234) and Ost-II (GenBank accession no. AB116074) were obtained by 5'-rapid amplification of cDNA ends from the Marathon-Ready human brain cDNA library (Clontech), and subcloned into pCMV5, generating pCMV5-Myc-Dbl-II and pCMV5-Myc-Ost-II, respectively. cDNAs encoding N-terminally FLAG-tagged Dbl-I and Dbl-II were amplified by PCR, and subcloned into pCMV5, generating pCMV5-FLAG-Dbl-I and pCMV5-FLAG-Dbl-II, respectively. cDNAs encoding Ost-I and Ost-II were amplified by PCR, and subcloned into pFLAG-CMV-2 (Sigma-Aldrich), generating pFLAG-CMV-2-Ost-I and pFLAG-CMV-2-Ost-II, respectively. The cDNA encoding N-terminally FLAG-tagged human Cdc42 was subcloned into pCMV5, generating pCMV5-FLAG-Cdc42(WT). pCMV5-FLAG-Cdc42(G12V) [13], pCMV5-FLAG-Cdc42(G12V/T35A) [14], and pCMV5-FLAG-Cdc42(S17N) [15] were previously described. The cDNA encoding N-terminally hemagglutinin (HA)-tagged human Cdc42 was subcloned into pEF-BOS [16], generating pEF-BOS-HA-Cdc42. For the expression of the N-terminally glutathione *S*-transferase (GST)/Myc-tagged Cdc42/Rac interactive binding (CRIB) domain (amino acids 443-489) of human ACK1, the cDNA encoding Myc-CRIB was subcloned into pGEX-2T (Amersham), generating pGEX-2T-Myc-ACK-CRIB, which is identical to previously described pGEX-ACK-CRIB [17]. For the expression of the N-terminally GST/Myc-

tagged CRIB domain (amino acids 443-489) of human ACK1 with double mutation (H464L/H467L), the cDNA encoding Myc-CRIB(H464L/H467L) was isolated from pCMV5-GST-ACK-CRIB(H464L/H467L) [17] and subcloned into pGEX-6P (Amersham), generating pGEX-6P-Myc-ACK-CRIB(H464L/H467L). The cDNA encoding Myc-Dbl-II(1-210) (an N-terminally Myc-tagged polypeptide consisting of amino acids 1-210 of Dbl-II) was isolated from pCMV5-Myc-Dbl-II, and subcloned into pGEX-6P, generating pGEX-6P-Myc-Dbl-II(1-210). The cDNA encoding Ost-II(1-239)-Myc (a C-terminally Myc-tagged polypeptide consisting of amino acids 1-239 of Ost-II) was amplified by PCR, and subcloned into pGEX-6P, generating pGEX-6P-Ost-II(1-239)-Myc. All PCR products were verified by sequencing.

2.2. Antibodies

Antibodies against the Myc tag (9E10 and A-14) were purchased from Santa Cruz Biotechnology. Anti-FLAG (M2) and anti-HA (3F10) antibodies were obtained from Sigma-Aldrich and Roche Diagnostics, respectively. Antibodies against rabbit or mouse IgG conjugated with horseradish peroxidase were obtained from Amersham. Antibodies against rabbit, mouse, or rat IgG conjugated with AlexaFluor (488, 546, and 647) were obtained from Molecular Probes.

2.3. Cell culture and transfection

HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, and transfected with expression plasmids by using the SuperFect (Qiagen) transfection reagent. After transfection, HeLa cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum for 8 h, followed by further incubation in DMEM for 16 h for serum-starvation. Epidermal growth factor (EGF) was purchased from Takara Bio (Japan).

2.4. Immunofluorescence

HeLa cells were fixed in phosphate-buffered saline (PBS) supplemented with paraformaldehyde [2% (v/v)] on ice for 10 min, followed by treatment with PBS-THS (see below) supplemented with Triton X-100 [0.1% (v/v)] on ice for 10 min. After washing three times with PBS-T [PBS, 0.1% (v/v) Tween 20], cells were incubated with a primary antibody in PBS-T for 1 h. Subsequently, cells were washed three times with PBS-THS [PBS, 0.5 M NaCl, 0.1% (v/v) Tween 20], and then incubated with a secondary antibody conjugated with an appropriate fluorescent dye or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma-Aldrich) in PBS-T for 1 h, followed by washing four times with PBS-THS. Proteins were visualized by a confocal laser scanning microscope (LSM510 META, Carl Zeiss). In some experiments, immunofluorescent analysis was performed in cells subjected to *in situ* Cdc42•GTP detection assay. The Golgi apparatus was stained with BODIPY TR ceramide (Molecular Probes) as described [18].

2.5. Preparation of recombinant proteins

The *Escherichia coli* strain BL21DE3pLys carrying each expression plasmid was cultured at 18°C, and the expression of recombinant proteins was induced by isopropyl- β -D-thiogalactopyranoside (0.5 mM) for 24 h. Cells were disrupted by sonication in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 20 mM MgCl₂, 5 mM dithiothreitol, 2% (w/v) sodium cholate, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin], and centrifuged at 100,000 \times g for 60 min. The supernatant was applied to a glutathione-Sepharose affinity column (Amersham), and washed with wash buffer A [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 20 mM MgCl₂, 2 mM dithiothreitol]. GST-Myc-ACK-CRIB and GST-Myc-ACK-CRIB(H464L/H467L) were eluted with elution buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 20 mM MgCl₂, 2 mM dithiothreitol, 20 mM glutathione]. Cdc42, Myc-Dbl-II(1-210), and Ost-II(1-239)-Myc were eluted after

digestion with PreScission protease (Amersham).

2.6. *In situ detection of Cdc42•GTP in HeLa cells*

Cells were fixed in paraformaldehyde buffer [50 mM Hepes-NaOH (pH 7.3), 150 mM NaCl, 20 mM MgCl₂, 0.05% (v/v) Tween 20, 2% (v/v) paraformaldehyde] on ice for 10 min, followed by treatment with Triton buffer [50 mM Hepes-NaOH (pH 7.3), 150 mM NaCl, 20 mM MgCl₂, 0.05% (v/v) Tween 20, 0.1% (v/v) Triton X-100] on ice for 10 min. After washing three times with wash buffer B [50 mM Hepes-NaOH (pH 7.3), 150 mM NaCl, 20 mM MgCl₂, 0.05% (v/v) Tween 20], cells were incubated with GST-Myc-ACK-CRIB or GST-Myc-ACK-CRIB(H464L/H467L) (60 µg/ml) dissolved in wash buffer B supplemented with bovine serum albumin (BSA, 60 µg/ml) on ice for 1 h. Subsequently, cells were washed three times with wash buffer B, and then incubated again in paraformaldehyde buffer on ice for 10 min. After washing three times with wash buffer B, GST-Myc-ACK-CRIB or GST-Myc-ACK-CRIB(H464L/H467L) was probed with an anti-Myc antibody (A-14) and an anti-rabbit IgG antibody conjugated with an appropriate fluorescent dye, and then detected by confocal laser scanning microscopy.

2.7. *Pull down assay for Cdc42•GTP*

Pull down assay for Cdc42•GTP was performed as previously described [17].

2.8. *Lipid-protein overlay assay*

Nitrocellulose membrane strips containing various phospholipids at 100 pmol per spot (PIP-Strips™) were purchased from Echelon Biosciences, and the binding of Sec14-like domains of Dbp-II and Ost-II to these phospholipids was assessed according to the manufacturers' protocol. Myc-Dbp-II(1-210) or Ost-II(1-239)-Myc was probed

with an anti-Myc antibody (A-14) and an anti-rabbit IgG antibody conjugated with horseradish peroxidase. Visualization was carried out with an enhanced chemiluminescence reagent (Amersham).

3. Results

3.1. *Splice variants of Dbl and Ost that contain the Sec14-like domain at the N terminus*

Domain structures of human Dbl and Ost splice variants that we used in this study are shown in Fig. 1. Dbl-II and Ost-II (type II), but not Dbl-I and Ost-I (type I), contain the complete Sec14-like domain consisting of 144 or 149 amino acid residues in the N-terminal portion. Amino acid sequences of Dbl-I (human proto-Dbl [19]) and Dbl-II (version 3 of human Dbl lacking the 16 amino-acid insert encoded by exon D [20]) are identical downstream of the Sec14-like domain. Also, Ost-I and Ost-II (human orthologues of rat Ost- α and Ost- γ that lacks the SH3 domain, respectively [21]) share the same amino acid sequence except for the Sec14-like domain. Dbl-I, Dbl-II, Ost-I, and Ost-II exhibited GEF activity toward all Rho family members tested, including Cdc42, RhoA, Rac1, and RhoG, *in vitro*. No significant difference was observed between GEF activities of type I and type II GEFs (data not shown).

3.2. *Different subcellular localization and biological effects of Dbl and Ost splice variants in HeLa cells coexpressing Cdc42*

To gain insight into the functional difference among Dbl and Ost splice variants, these GEFs were ectopically expressed alone or with their substrate Cdc42 in human adenocarcinoma HeLa cells, and the subcellular localization of these proteins and cell morphology were examined by confocal laser scanning microscopy. When expressed alone, all of the Dbl and Ost splice variants were localized uniformly in endomembrane structures (Fig. 2). Cdc42, when expressed alone, existed mostly in the Golgi apparatus (Fig. 3A). When coexpressed with Dbl-I or Ost-I, Cdc42 was mostly recruited to the plasma membrane, where Dbl-I or Ost-I coexisted (Fig. 3B and D). Microspikes were yielded particularly on the top surface of the cell, where Dbl-I or

Ost-I was colocalized with Cdc42. In marked contrast, Dbl-II and Ost-II were colocalized with Cdc42 in endomembrane compartments, inducing virtually no obvious cell shape change (Fig. 3C and E).

To further clarify a link between a cell surface growth factor receptor and Dbl-I or Ost-I, actin cytoskeletal rearrangements following treatment with EGF was analyzed. In HeLa cells expressing Dbl-I and Cdc42, ruffling membranes were generated following EGF treatment for 5 min, implying that Dbl-I may mediate signals triggered by EGF stimulation (Fig. 4A). EGF did not induce ruffling membrane formation, when Dbl-I was expressed alone (data not shown). Cells expressing Ost-I and Cdc42 exhibited a similar phenotype in response to EGF (data not shown). Time courses of ruffling membrane formation upon EGF stimulation are illustrated in Fig. 4B and C. Membrane ruffling was observed in approximately 50% of Dbl-I and Cdc42-coexpressing cells, but not in Dbl-II and Cdc42-coexpressing cells. Ost-I (but not Ost-II) and Cdc42-coexpressing cells gave similar results. Therefore, only Dbl-I and Ost-I are linked to EGF receptor signaling.

3.3. Visualization of the active form of Cdc42 in Dbl-I- or Ost-I-coexpressing cells by means of a novel affinity binding assay

To determine if Dbl-I and Ost-I colocalized with Cdc42 in the cell periphery indeed stimulate guanine nucleotide exchange on Cdc42 to form Cdc42•GTP, we developed a novel affinity binding assay for *in situ* detection of Cdc42•GTP in mammalian cultured cells. Fixation with paraformaldehyde did not interfere with the detection of Cdc42•GTP by pull down assays using the CRIB domain of ACK1 (data not shown). Given these results, we attempted to visualize Cdc42•GTP *in situ* by incubating fixed cells with the GST/Myc-tagged CRIB domain of ACK1 (GST-Myc-ACK-CRIB), which was subsequently detected by immunofluorescent staining. The constitutively active mutant Cdc42(G12V) was stained intensively, whereas wild-type Cdc42 and the dominant-negative mutant Cdc42(S17N) were not stained (Fig. 5A).

Moreover, a mutant that cannot associate with the ACK1 CRIB domain even in its GTP-bound form (Cdc42(G12V, T35A)) [17] was not detected by this assay as expected (Fig. 5A). Furthermore, when a CRIB domain mutant lacking the ability to bind to Cdc42•GTP (GST-Myc-ACK-CRIB(H464L, H467L)) was used, no significant signal was detected (Fig. 5A). These results ensure that GST-Myc-ACK-CRIB specifically interacts with Cdc42•GTP in cells under our assay conditions. Employing this method, we next examined whether Dbl and Ost splice variants activate coexpressed Cdc42 in cells. The GTP-bound form of Cdc42 was in fact detected particularly in the top region of cell periphery, where Dbl-I or Ost-I is colocalized with Cdc42 (Fig. 5B and C). Similarly, Cdc42 colocalized with Dbl-I or Ost-I in the plasma membrane near the bottom of the cell was activated (Fig. 5B and C). In marked contrast, Cdc42 that was colocalized with Dbl-II or Ost-II in endomembrane structures remained inactive (Fig. 5B and C). Consistent with these results, pull down assays to detect Cdc42•GTP revealed that Cdc42•GTP was accumulated in cells harboring Dbl-I or Ost-I, but not Dbl-II- or Ost-II-expressing cells (Fig. 5D).

3.4. Interaction of Sec14-like domains of Dbl and Ost with distinct subsets of phospholipids

We attempted to identify ligands for Sec14-like domains of Dbl and Ost by using nitrocellulose membrane strips containing a variety of lipid compounds. Affinity purified Myc-tagged N-terminal fragments of Dbl and Ost, which contain the whole Sec14-like domain were used for this assay. The Sec14-like domain of Dbl-II interacted with PI3P, PI4P, PI5P, and PI3,5P₂ (Fig. 6A). On the other hand, the Sec14-like domain of Ost-II exhibited affinity to diverse array of inositol phospholipids, such as all PIPs tested, all PIP₂s tested, and PI3,4,5P₃, as shown in Fig. 6B.

4. Discussion

Rho family GTPases act as molecular switches that regulate a wide variety of cellular functions, such as cytoskeletal rearrangements, gene expression, and vesicular transport [1]. To accomplish this, Rho family proteins are distributed to various subcellular compartments, and thereafter activated in response to upstream signals to exert specific functions. With regard to signal-dependent activation of GEF activity, recent studies have revealed a diverse array of mechanisms involving conformational change triggered by covalent modifications, such as phosphorylation [14, 17, 22]. On the other hand, non-covalent protein-protein interactions also modulate GEF activity [23-27]. Several GEFs, such as Vav, mSos1, P-Rex1, and SWAP-70, are also regulated through non-covalent interactions with PI3,4,5P₃ [28-31].

In contrast to the regulation of GEF activity itself, mechanisms underlying subcellular localization-specific signaling remain largely unsolved. Our observation that Dbl family GEFs direct the subcellular localization of Rho family proteins, in addition to stimulating GDP/GTP exchange, may provide novel and important insights into the regulation of small GTPases. Dbl and Ost splice variants that lack the Sec14-like domain (Dbl-I and Ost-I) colocalized with, and then activated Cdc42 in the plasma membrane. Upstream signals triggered by still unidentified extracellular stimulation might activate Dbl-I or Ost-I in the plasma membrane. In contrast, Dbl and Ost containing the Sec14-like domain (Dbl-II and Ost-II) colocalized with Cdc42 in endomembrane structures, where Cdc42 remained inactive. Therefore, it is feasible that Dbl-II and Ost-II are responsible for the regulation of Cdc42 in signaling pathways distinct from those involving Dbl-I and Ost-I. In addition, only Dbl-I and Ost-I mediated signals that induce ruffling membranes downstream of the EGF receptor, further substantiating the notion that Type I and Type II GEFs are implicated in distinct signaling pathways, being localized in distinct subcellular compartments. Signals that activate Dbl-II and Ost-II, thereby allowing their substrate Cdc42 to become GTP-bound remain unknown. Dbl-II and Ost-II may be involved in the regulation of Rho

family proteins that are responsible for membrane traffic or the determination of cell polarity [32, 33].

The Sec14-like domain was found in an array of GEFs and GTPase-activating proteins that regulate Ras and Rho family GTPases [10]. Although anticipated to be a lipid-binding domain because of its structural similarity to the *S. cerevisiae* PI transfer protein Sec14p, the role of this domain in the modulation of GEF function remained obscure. We found that Sec14-like domains of two closely related GEFs, Dbl and Ost, indeed bound to subsets of phospholipids, although the specificity of the binding to phospholipids differs from each other (Fig. 6). Indeed, isolated Sec14-like domains of Dbl and Ost are localized in different subcellular compartments (Shuji Ueda, Tohru Kataoka, and Takaya Satoh, unpublished results). Therefore, although intracellular distribution of diverse phospholipids remains unknown in mammalian cells, these GEFs do not seem to simply accumulate in membranes, where phospholipids that bind to the respective Sec14-like domain are enriched. The PH domain adjacent to the DH domain, to which a subset of phospholipids bind, also regulates membrane targeting, the association with cytoskeletal components, and the catalytic activity of Dbl and Ost within the cell [34-36]. Therefore, it is feasible that PH and Sec14-like domains may cooperatively determine the subcellular localization of the GEFs. Detailed mechanisms whereby inositol phospholipids regulate the subcellular localization of GEFs through the binding to the Sec14-like domain will be revealed in future studies.

In an attempt to assess GEF activities of Dbl and Ost in particular subcellular compartments, we were successful in detecting subcellular localization-specific activation of Cdc42 by GEFs in mammalian cells using the CRIB domain of ACK1 as a probe. Similar methodology was applied to *Xenopus* brain sections, providing evidence for crosstalk between Rho family proteins in optic nerve development [37]. Additionally, a green fluorescent protein-fused CRIB domain of the Wiskott-Aldrich syndrome protein was employed to visualize Cdc42 activation *in situ* [38]. However, it was difficult to discriminate signals for active Cdc42 from non-specific ones due to diffuse distribution of this construct (as well as the green fluorescent protein without

the CRIB domain) throughout the cell. In contrast, virtually no binding signal was observed when a control construct (GST-Myc-ACK-CRIB(H464L/H467L)) was used in our assay, which enabled us to illustrate the difference in GEF activities of Dbl and Ost splice variants *in vivo*. Furthermore, the activation of proteins tagged with a short peptide can be detected by this *in situ* affinity binding assays in contrast to fluorescent resonance energy transfer-based assays, where a large fusion construct consisting of fluorescent proteins and the protein of interest is required. Similar approaches to detect the active GTP-bound form of a variety of other Rho family GTPases by the use of specific binding domains will be useful for clarifying the mechanisms for subcellular region-specific activation.

Acknowledgements

We thank Toru Miki and Takahiro Nagase for providing cDNAs, and Masahiro Katsurada for technical assistance. This investigation was supported by Grants-in-aid for Scientific Research on Priority Areas, Scientific Research (B) and (C), and grants for 21st Century COE Research Programs "Signaling Mechanisms by Protein Modification Reactions" and "Center of Excellence for Signal Transduction Disease: Diabetes Mellitus as Model" from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a grant from Hyogo Science and Technology Association.

References

- [1] Etienne-Manneville S, Hall A. *Nature* 2002;420:629-35.
- [2] Zheng Y. *Trends Biochem Sci* 2001;26:724-32.
- [3] Schmidt A, Hall A. *Genes Dev* 2002;16:1587-609.
- [4] Brugnera E, Haney L, Grimsley C, Lu M, Walk SF, Tosello-Tramont AC, Macara IG, Madhani H, Fink GR, Ravichandran KS. *Nat Cell Biol* 2002;4:574-82.
- [5] Meller N, Irani-Tehrani M, Kiosses WB, Del Pozo MA, Schwartz MA. *Nat Cell Biol* 2002;4:639-47.
- [6] Kraynov VS, Chamberlain C, Bokoch GM, Schwartz MA, Slabaugh S, Hahn KM. *Science* 2000;290:333-7.
- [7] Del Pozo MA, Kiosses WB, Alderson NB, Meller N, Hahn KM, Schwartz MA. *Nat Cell Biol* 2002;4:232-9.
- [8] Itoh RE, Kurokawa K, Ohba Y, Yoshizaki H, Mochizuki N, Matsuda M. *Mol Cell Biol* 2002;22:6582-91.
- [9] Sha B, Phillips SE, Bankaitis VA, Luo M. *Nature* 1998;391:506-10.
- [10] Aravind L, Neuwald AF, Ponting CP. *Curr Biol* 1999;9:R195-7.
- [11] Zhao R, Fu X, Li Q, Krantz SB, Zhao ZJ. *J Biol Chem* 2003;278:22609-14.
- [12] Nishida K, Kaziro Y, Satoh T. *FEBS Lett* 1999;459:186-90.
- [13] Nishida K, Kaziro Y, Satoh T. *Oncogene* 1999;18:407-15.
- [14] Kato J, Kaziro Y, Satoh T. *Biochem Biophys Res Commun* 2000;268:141-7.
- [15] Kiyono M, Satoh T, Kaziro Y. *Proc Natl Acad Sci U S A* 1999;96:4826-31.
- [16] Mizushima S, Nagata S. *Nucleic Acids Res* 1990;18:5322.
- [17] Kato-Stankiewicz J, Ueda S, Kataoka T, Kaziro Y, Satoh T. *Biochem Biophys Res Commun* 2001;284:470-7.
- [18] Jin T-G, Satoh T, Liao Y, Song C, Gao X, Kariya K, Hu C-D, Kataoka T. *J Biol Chem* 2001;276:30301-7.
- [19] Ron D, Tronick SR, Aaronson SA, Eva A. *EMBO J* 1988;7:2465-73.

- [20] Komai K, Okayama R, Kitagawa M, Yagi H, Chihara K, Shiozawa S. *Biochem Biophys Res Commun* 2002;299:455-8.
- [21] Lorenzi MV, Castagnino P, Chen Q, Hori Y, Miki T. *Oncogene* 1999;18:4742-55.
- [22] Bustelo XR. *Mol Cell Biol* 2000;20:1461-77.
- [23] Kozasa T, Jiang X, Hart MJ, Sternweis PM, Singer WD, Gilman AG, Bollag G, Sternweis PC. *Science* 1998;280:2109-11.
- [24] Hart MJ, Jiang X, Kozasa T, Roscoe W, Singer WD, Gilman AG, Sternweis PC, Bollag G. *Science* 1998;280:2112-4.
- [25] Suzuki N, Nakamura S, Mano H, Kozasa T. *Proc Natl Acad Sci U S A* 2003;100:733-8.
- [26] Kawasaki Y, Senda T, Ishidate T, Koyama R, Morishita T, Iwayama Y, Higuchi O, Akiyama T. *Science* 2000;289:1194-7.
- [27] Shamah SM, Lin MZ, Goldberg JL, Estrach S, Sahin M, Hu L, Bazalakova M, Neve RL, Corfas G, Debant A, Greenberg ME. *Cell* 2001;105:233-44.
- [28] Han J, Luby-Phelps K, Das B, Shu X, Xia Y, Mosteller RD, Krishna UM, Falck JR, White MA, Broek D. *Science* 1998;279:558-60.
- [29] Das B, Shu X, Day GJ, Han J, Krishna UM, Falck JR, Broek D. *J Biol Chem* 2000;275:15074-81.
- [30] Shinohara M, Terada Y, Iwamatsu A, Shinohara A, Mochizuki N, Higuchi M, Gotoh Y, Ihara S, Nagata S, Itoh H, Fukui Y, Jessberger R. *Nature* 2002;416:759-63.
- [31] Welch HC, Coadwell WJ, Ellson CD, Ferguson GJ, Andrews SR, Erdjument-Bromage H, Tempst P, Hawkins PT, Stephens LR. *Cell* 2002;108:809-21.
- [32] Erickson JW, Zhang C, Kahn RA, Evans T, Cerione RA. *J Biol Chem* 1996;271:26850-4.
- [33] Kroschewski R, Hall A, Mellman I. *Nat Cell Biol* 1999;1:8-13.
- [34] Fuentes EJ, Karnoub AE, Booden MA, Der CJ, Campbell SL. *J Biol Chem* 2003;278:21188-96.

- [35] Rossman KL, Cheng L, Mahon GM, Rojas RJ, Snyder JT, Whitehead IP, Sondek J. *J Biol Chem* 2003;278:18393-400.
- [36] Zheng Y, Zangrilli D, Cerione RA, Eva A. *J Biol Chem* 1996;271:19017-20.
- [37] Li Z, Aizenman CD, Cline HT. *Neuron* 2002;33:741-50.
- [38] Kim SH, Li Z, Sacks DB. *J Biol Chem* 2000;275:36999-7005.

FOOTNOTES

*Corresponding author. Tel.: +81-78-382-5381; fax: +81-78-382-5399.

E-mail address: tkysato@med.kobe-u.ac.jp (T. Satoh)

LEGENDS TO FIGURES

Fig. 1. Domain structures of Dbl and Ost splice variants. Sec14-like, spectrin-like, DH, and PH domains and amino acid residue numbers are shown.

Fig. 2. Subcellular localization of Dbl and Ost splice variants expressed in HeLa cells. HeLa cells were transfected with an expression plasmid for a Myc-tagged GEF (Dbl-I, Dbl-II, Ost-I, or Ost-II), and stained with an anti-Myc antibody (A-14). Scale bar, 20 μm .

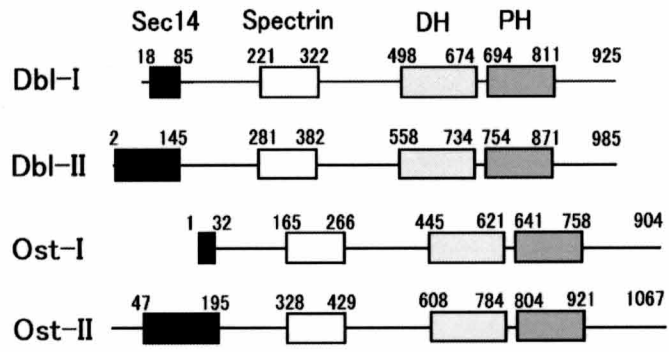
Fig. 3. Different subcellular localization of Dbl and Ost splice variants in HeLa cells coexpressing Cdc42. (A) HeLa cells were transfected with pEF-BOS-HA-Cdc42. Staining of Cdc42 (with an anti-HA antibody) and the Golgi apparatus (with BODIPY TR ceramide) is shown. Scale bar, 20 μm . (B-E) HeLa cells were transfected with an expression plasmid for a Myc-tagged GEF (Dbl-I, Dbl-II, Ost-I, or Ost-II) and pEF-BOS-HA-Cdc42. Staining of Dbl-I (B), Dbl-II (C), Ost-I (D), or Ost-II (E) (with an anti-Myc antibody (A-14)), Cdc42 (with an anti-HA antibody), and F-actin (with TRITC-phalloidin) is shown. The upper figures represent vertical section ($x-z$ or $y-z$) merge images, and lower figures represent horizontal section ($x-y$) images. "Top" and "Bottom" lines indicate positions of horizontal section images. Dotted lines indicate positions of vertical section images. Scale bar, 20 μm .

Fig. 4. Induction of ruffling membranes upon EGF treatment in HeLa cells expressing either Dbl-I or Ost-I with Cdc42. (A) HeLa cells expressing HA-Cdc42 and Myc-Dbl-I were stimulated with EGF (50 ng/ml) for 5 min ("5 min") or left unstimulated ("0 min"). Staining of Dbl-I (with an anti-Myc antibody (A-14)), Cdc42 (with an anti-HA antibody), and F-actin (with TRITC-phalloidin) is shown. Horizontal section ($x-y$) images at the top position are shown. Arrows indicate ruffling membranes. Scale bar, 20 μm . (B and C) HeLa cells expressing HA-Cdc42

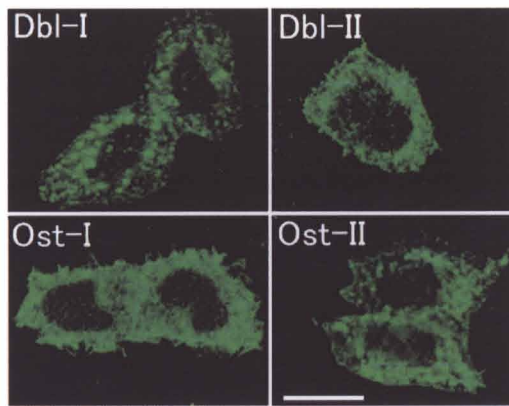
alone (open squares), HA-Cdc42 plus Myc-Dbl-I (B) or Myc-Ost-I (C) (closed circles), or HA-Cdc42 plus Myc-Dbl-II (B) or Myc-Ost-II (C) (open circles) were stimulated with EGF (50 ng/ml) for the indicated times. The percentage of cells containing ruffling membranes is shown. The values are expressed as the means \pm S.D. ($n = 3$).

Fig. 5. *In situ* detection of Cdc42•GTP in HeLa cells. (A) HeLa cells expressing indicated FLAG-Cdc42 mutants were subjected to *in situ* detection of Cdc42•GTP. GST-Myc-ACK-CRIB or GST-Myc-ACK-CRIB(H464L/H467L) as a probe for Cdc42•GTP was stained with an anti-Myc antibody (A-14). Staining of Cdc42 (with an anti-FLAG antibody) is also shown. Scale bar, 20 μ m. (B and C) HeLa cells expressing HA-Cdc42 plus FLAG-tagged Dbl-I, Dbl-II, Ost-I, or Ost-II were subjected to *in situ* detection of Cdc42•GTP. GST-Myc-ACK-CRIB as a probe for Cdc42•GTP was stained with an anti-Myc antibody (A-14). Staining of Cdc42 (with an anti-HA antibody) and Dbl-I (B), Dbl-II (B), Ost-I (C), or Ost-II (C) (with an anti-FLAG antibody) is also shown. Scale bar, 20 μ m. (D) Cdc42•GTP levels in HeLa cells expressing FLAG-Cdc42 plus Myc-tagged Dbl-I, Dbl-II, Ost-I, or Ost-II were assessed by pull-down assays. Aliquots of cell extracts were also subjected to immunoblotting ("*input*"). Cdc42 were stained with an anti-FLAG antibody.

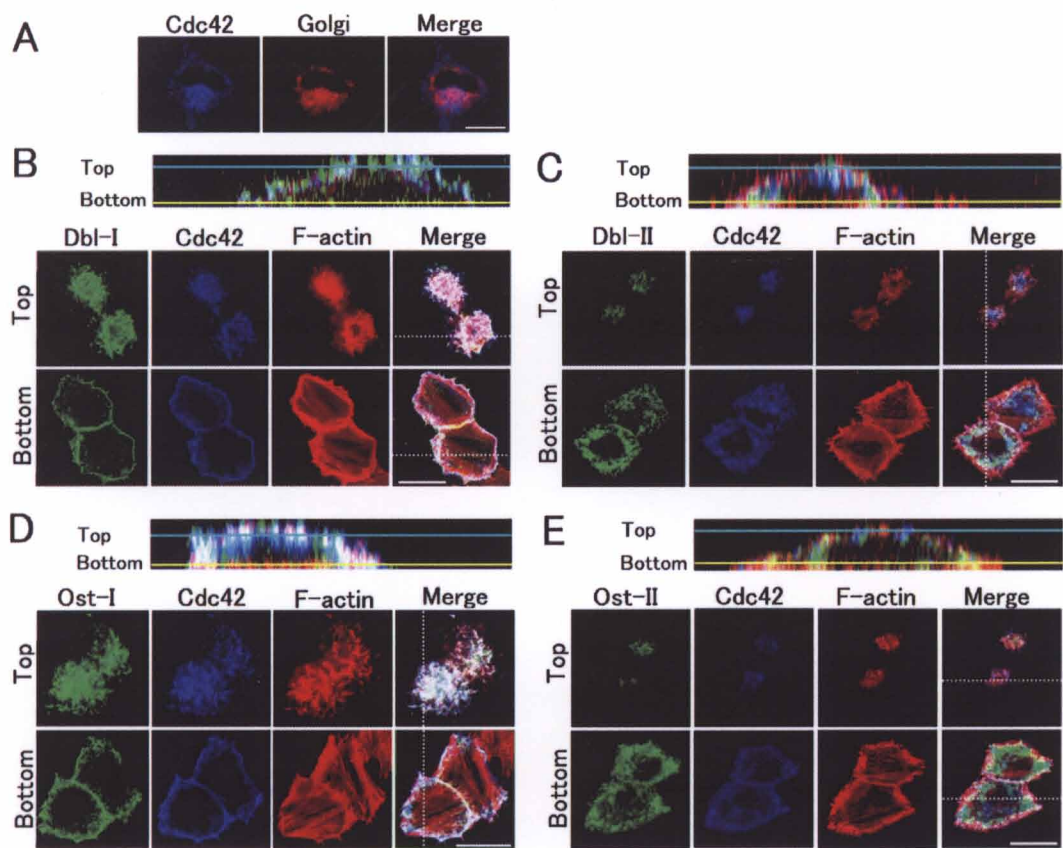
Fig. 6. Interaction of Sec14-like domains of Dbl and Ost with phospholipids. A nitrocellulose membrane strip containing various phospholipids was probed with Myc-Dbl-II(1-210) (A) or Ost-II(1-239)-Myc (B), which was subsequently detected by an anti-Myc antibody (A-14). A representative result of three independent experiments is shown.



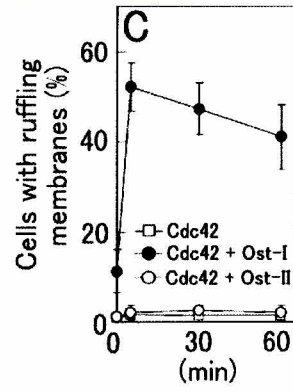
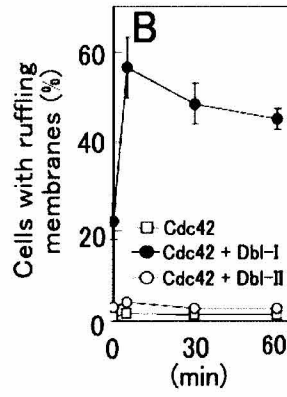
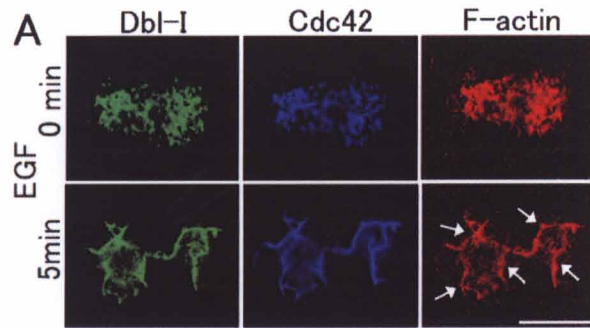
Ueda et al. Fig. 1



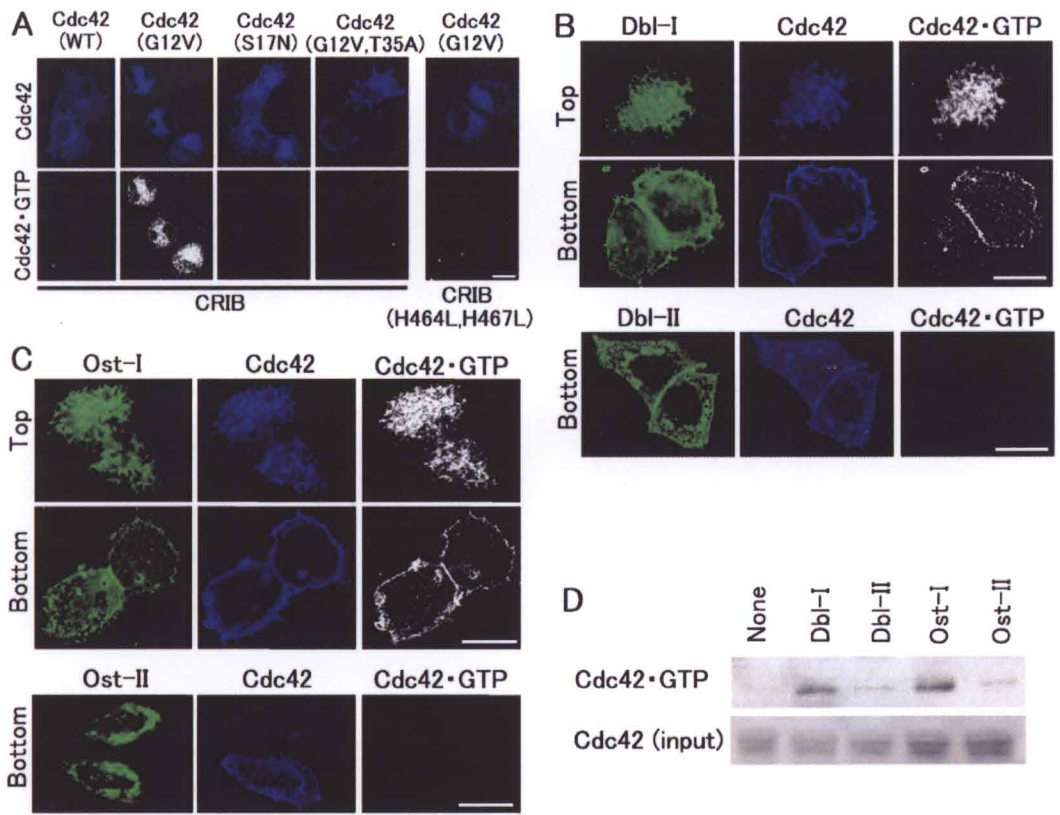
Ueda et al. Fig. 2



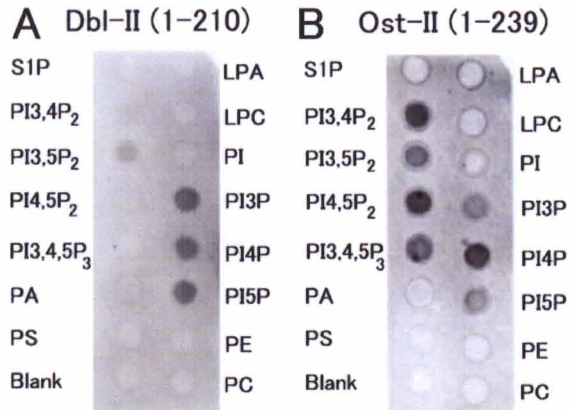
Ueda et al. Fig. 3



Ueda et al. Fig. 4



Ueda et al. Fig. 5



Ueda et al. Fig. 6