The Sec14 Homology Domain Regulates the Cellular Distribution and Transforming Activity of the Rho-specific Guanine Nucleotide Exchange Factor Dbs*

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Dbs is a Rho-specific guanine nucleotide exchange factor that was identified in a screen for proteins whose overexpression causes deregulated growth in murine fibroblasts. Dbs contains multiple recognizable motifs including a centrally located Rho-specific guanine nucleotide exchange factor domain, a COOH-terminal Src homology 3 domain, two spectrin-like repeats, and a recently identified NH2-terminal Sec14 homology domain. The transforming potential of Dbs is substantially activated by the removal of inhibitory sequences that lie outside of the core catalytic sequences, and in this current study we mapped this inhibition to the Sec14 domain. Surprisingly removal of the NH2 terminus did not alter the catalytic activity of Dbs in vivo but rather altered its subcellular distribution. Whereas full-length Dbs was distributed primarily in a perinuclear structure that coincides with a marker for the Golgi apparatus, removal of the Sec14 domain was associated with translocation of Dbs to the cell periphery where it accumulated within membrane ruffles and lamellipodia. However, translocation of Dbs and the concomitant changes in the actin cytoskeleton were not sufficient to achieve full transforming activity. Collectively these observations suggest that the Sec14 domain regulates Dbs transformation through at least two distinct mechanisms, neither of which appears to directly influence the in vivo exchange activity of the protein.

The Rho family of small GTPases regulates multiple cellular processes including the remodeling of the actin cytoskeleton, stimulation of transcriptional activity, and progression through the cell cycle (1, 2). Rho proteins function as binary switches cycling between a biologically active GTP-bound conformation (GTP-Rho) and an inactive GDP-bound (GDP-Rho) conformation. When in the active state, Rho proteins are able to propagate cellular signals by forming productive interactions with a wide array of effector molecules (3). Three families of proteins have been identified that regulate the steady state levels of GTP-Rho in cells: the Rho-specific guanine nucleotide exchange factors (RhoGEFs) activate GTPases by stimulating the GTP-GDP exchange rate, the Rho-specific GTPase-activating proteins (RhoGAPs) down-regulate Rho by stimulating the intrinsic rate of hydrolysis, and the Rho-specific guanine nucleotide dissociation inhibitors (RhoGDIs) sequester Rho in inactive complexes.

The RhoGEFs are a large family of proteins that share a common 300-amino acid structural motif comprised of a Dbl homology (DH) domain arranged in tandem with a pleckstrin homology (PH) domain (4, 5). The DH domain is unique to the RhoGEF family and generally contains all of the residues required for substrate recognition, binding, and exchange (4, 6). DH domains interact directly with GTPases to catalyze exchange and, like other exchange factor domains, preferentially bind substrates that are depleted of nucleotide and Mg2+ (7). The role of the non-catalytic PH domain in the context of RhoGEF family members is more uncertain. Several studies have suggested that the PH domains of RhoGEFs can mediate membrane association, although it is unclear whether this is mediated by direct associations with phospholipids or with membrane-bound proteins (8–11).

Many members of the RhoGEF family have been identified as oncogenes due to their ability to transform mouse fibroblasts (5), and several have been shown to be rearranged in the context of human developmental disorders (12) or cancer (13, 14). Numerous studies have now shown that the transforming activity of the RhoGEFs is intimately connected with their ability to activate Rho family GTPases, and that an intact DH/PH domain module is both necessary and sufficient for this activity (4, 6, 15). Although the oncogenic activation of many RhoGEFs occurs as a consequence of the truncation of regulatory motifs that lie outside the DH/PH domain (16–21) there are only a few instances in which the molecular basis for this activation has been identified. For example, both Dbl and Vav1 contain NH2-terminal regulatory domains that inhibit catalytic activity by forming intramolecular contacts with the DH/PH domain module (17, 18). Removal of these domains is required for catalytic activation and oncogenic transformation in vivo. Domains that mediate changes in subcellular distribution have also been implicated in RhoGEF activation. For example, on-

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1 The abbreviations used are: RhoGEF, Rho-specific guanine nucleotide exchange factor; GAP, GTPase-activating protein; DH, Dbl homology; PH, pleckstrin homology; HA, hemagglutinin; PBS, phosphate-buffered saline; WGA, wheat germ agglutinin; BFA, brefeldin A; GST, glutathione S-transferase; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4)P2, phosphatidylinositol 3,4-bisphosphate; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase.
cogenic activation of the Lbc protein requires truncation of its COOH terminus, which is associated with translocation of the protein from the insoluble to the soluble fraction (19). However, the identity of the relevant regulatory sequences and the mechanism through which they mediate cellular distribution have not yet been determined.

Dbs, and its rat ortholog Ost, are RhoGEF family members that were independently identified in screens for cDNAs whose expression causes deregulated growth in NIH 3T3 fibroblasts (20, 22). Like many members of the RhoGEF family, Dbs/Ost has potent transforming activity as measured by loss of contact inhibition, growth in low serum, anchorage-independent growth, and tumorigenicity in nude mice (20, 22, 23). In addition to the canonical DH/PH domain, Dbs encodes a COOH-terminal Src homology 3 domain, two spectrin-like repeats, and a terminal Src homology 3 domain, two spectrin-like repeats, and a domain that must also be relieved for full transformation to occur (24).

As with other RhoGEF family members, the transforming versions of Dbs and Ost contain truncations that are responsible for oncogenic activation (20, 21). In the current study we determined that the transforming activity of the proto-Dbs protein is negatively regulated by the NH₂-terminal Sec14 homology domain. This domain was found to be responsible for phosphoinositide-dependent sequestration of proto-Dbs to a perinuclear region of the cell that corresponds with a marker for the Golgi apparatus. Although a lipid binding mutation in the Sec14 domain caused translocation of proto-Dbs to the cell periphery where it can induce changes in the actin cytoskeleton, this was not sufficient to activate transformation. The Sec14 domain also forms intramolecular contacts with the PH domain that must also be relieved for full transformation to occur. This constitutes a novel mechanism of RhoGEF oncogenic activation that may be relevant to other related members of the RhoGEF family.

**EXPERIMENTAL PROCEDURES**

**Molecular Constructs**—The mammalian expression vectors pAX142 and pCTV3H have been described previously (28). pAX142-dbs-HA6 and pCTV3H-dbs-HA6 contain cDNAs encoding a fragment of murine Dbs (residues 525–1097) fused to a hemagglutinin (HA) epitope tag (29). pAX142-dbs-HA encodes the full-length Dbs protein (residues 1–1149) fused to a COOH-terminal HA epitope tag (30). Dbs deletion mutant constructs pAX142-dbs-Δ75-HA, pAX142-dbs-Δ235-HA, pAX142-dbs-Δ348-HA, and pAX142-dbs-Δ525-HA were generated based on the pAX142-dbs-HA plasmid using the QuikChange site-directed mutagenesis kit (Stratagene) when possible or PCR-based cloning. To create pCTV3H-based versions of these Dbs proteins, the corresponding cDNAs were subcloned from the pAX142 vector into the pCTV3H vector using an MluI/BsiWI digest. The pAX142-dbs-HA8 construct encodes the isolated DH domain of Dbs (residues 525–813), while pAX142-dbs-HA8 encodes the isolated PH domain (residues 791–967) (30). pAX142-dbs-ΔN1 and pAX142-dbs-ΔN1-FLAG and pAX142-dbs-ΔN1-FLAG contain cDNAs encoding residues 1–525 and 1–235 of Dbs, respectively, fused to the NH₂-terminal FLAG tag. A bacterial expression vector encoding the Sec14 homology domain of Dbs (pTrHiAC-dbs-Sec14) was generated by PCR-based cloning of the Dbs cDNA encoding residues 1–235 into the BamHI site of pTrHiAC vector (Invitrogen). The expressed protein contains a His₆ tag at the NH₂ terminus. When necessary, point mutations were introduced into corresponding plasmids using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. cDNA sequences of all constructs were verified by automated sequencing. pCTV3H-Lac-D7HA encoding residues 1–778 of the Lac operon was described previously (28).

**Cell Culture, Transfection, and Transformation Assays**—NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% bovine calf serum (JRH Biosciences) at 37°C in 10% CO₂. Primary focus formation assays were performed in NIH 3T3 cells as described previously (31). Briefly NIH 3T3 cells were transfected using Lipofectamine reagent (Invitrogen). Focus formation was scored at 14 days by staining with 0.5% crystal violet. NIH 3T3 cells stably transfected with pCTV3H, pCTV3H-dbs-HA6, pCTV3H-dbs-HA, pCTV3H-dbs-Δ75-HA, pCTV3H-dbs-Δ235-HA, pCTV3H-dbs-Δ348-HA, and pCTV3H-dbs-Δ525-HA were generated by calcium phosphate co-precipitation followed by selection for 10 days in growth medium (Dulbecco’s modified Eagle’s medium, 10% bovine calf serum) supplemented with hygromycin B (200 μg/ml). Multiple drug-resistant colonies were pooled to establish multiclonal cell lines.

**Expression and Purification of the Dbs Sec14 Domain**—The pTrHiAC-dbs-Sec14 construct was used to express the Sec14 homology domain of Dbs (residues 1–235) in the Escherichia coli strain DH5α. Cultures were grown at 37°C until A₆₀₀ = 0.8 and then induced with 1 mM isopropyl β-D-thiogalactopyranoside at 18°C for 4 h. The bacterial inclusion was about 50% soluble under these conditions and was purified by immobilized metal affinity chromatography using TALON resin (Clontech) according to the manufacturer’s instructions to an estimated purity of over 90% using Coomassie Blue staining. Western blot analysis with a mouse monoclonal anti-His antibody (H-3, Santa Cruz Biotechnology) showed a single protein band of the expected size.

**Lipid Dot-blot Assays—**Lipid dot-blot strips (PIP Strips) were purchased from Echelon Biosciences Inc., and the recommended protocols were followed. Briefly lipid dot-blots were blocked with 0.1% ovalbumin in TBST (10 mM Tris, 150 mM NaCl, 0.1% Tween 20 at pH 8) for about 1 h and then incubated in TBST containing 0.1% ovalbumin with the protein of interest at 0.5 μg/ml overnight at 4°C. The lipid strips were then washed three times for 10 min each with TBST. To detect lipid binding, the dot-blot was incubated with an anti-His antibody (H-3, Santa Cruz Biotechnology) in TBST containing 0.1% ovalbumin for 1 h at room temperature. Membranes were then washed three times for 10 min each with TBST containing 0.1% ovalbumin and incubated with goat anti-mouse IgG (Calbiochem) for 30 min at room temperature in the same buffer. The bound protein was visualized using Luminol enhanced chemiluminescence reagent (Santa Cruz Bio-technology). To ensure lipid binding specificity, several different concentrations of a protein were tested. The lowest concentration giving specific binding and a detectable signal on a Western blot was chosen for the lipid dot-blot experiments. Control experiments were performed in parallel using no protein or eluants derived from cells that expressed the cognate vector.

**Immunostaining**—Immunostaining of transiently transfected NIH 3T3 cells was performed as described previously (9). Briefly, NIH 3T3 cells were transiently transfected with 2 μg of plasmid using the Lipofectamine reagent (Invitrogen). At 24 h post-transfection cells were trypsinized and plated on poly-L-lysine-coated glass coverslips. At 48 h post-transfection, cells were fixed (see above), and stained with Alexa Fluor 488 conjugate of WGA probes. Where indicated the Golgi complex was visualized by counterstaining with a green fluorescent Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes) in the same buffer.

**Co-immunoprecipitations**—Co-immunoprecipitations in 293T cells were performed for 30 min at 37°C in a concentration of 10 μg/ml prior to fixation. After incubation, co-immunoprecipitations were washed with ice-cold PBS, fixed (see above), and stained with Alexa Fluor 488 conjugate of WGA as described above. Cells were viewed with an Olympus IX50 inverted microscope, and images were captured using an Optronics digital CCD camera system.
were performed as described previously (32). Cells were harvested in lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 100 mM NaCl, 10% glycerol, 1% Nonidet P-40) supplemented with a protease inhibitor mixture (Calbiochem). Lysates were precleared with 0.25 g of agarose-conjugated normal mouse IgG (Santa Cruz Biotechnology) by rotation for 30 min at 4 °C and precipitated with anti-FLAG M2 affinity gel (Sigma) by rotation at 4 °C for 2 h. The immunoprecipitates were washed three times with cold lysis buffer and then resuspended in loading dye.

**Protein Expression**—Protein expression in transiently transfected NIH 3T3 cells and stable cell lines was determined by standard Western blot analysis (as described previously (33)) using a rabbit anti-HA antibody (Y-11, Santa Cruz Biotechnology) or an anti-HA mouse monoclonal antibody (BabCo), respectively. Tubulin expression was detected with an anti-α-tubulin monoclonal mouse antibody (clone DM1A, Sigma).

**RhoA Activation Assay**—Affinity purification assays to measure the levels of GTP-bound RhoA were performed using the Rho-binding domain of Rhotekin (GST-C21) for RhoA as described previously (34). Total and GTP-bound RhoA was detected with a mouse monoclonal anti-RhoA antibody (26C4, Santa Cruz Biotechnology). Activation of endogenously expressed RhoA by Dbs proteins was tested in lysates of NIH 3T3 cells stably transfected with pCTV3H-based constructs expressing the indicated Dbs proteins. Foci were stained with crystal violet at 14 days, and the number of foci/pmol of transfected DNA was determined for each construct. C, results of a primary focus formation assay in NIH 3T3 cells transfected with 1 μg of pAX142-based constructs expressing the indicated Dbs proteins. D, the relative transforming activity of the Dbs deletion mutant proteins. Transforming activity of the onco-Dbs was set as 100%.

The data shown are representative of three independent experiments performed on triplicate plates and represent means and S.D. of foci generated. Spec., spectrin-like repeat; SH3, Src homology 3.

**RESULTS**

**Full-length Dbs Has Reduced Transforming Activity Relative to Oncogenic Dbs**—The oncogenic Dbs cDNA encodes a truncated version of the full-length protein (onco-Dbs) that contains tandem DH and PH domains along with short flanking sequences (residues 525–1097) (Fig. 1A). Full-length Dbs (Fig. 1A, proto-Dbs) is an 1149-amino acid-long protein that includes a recently identified NH₂-terminal Sec14 homology domain (residues 75–221), two spectrin-like repeats (residues 235–348 and 354–445), a DH/PH domain module (residues 623–967), and a COOH-terminal Src homology 3 domain (residues 1058–1115). Since many members of the RhoGEF family appear to exist in an inactive, basal state prior to activation (16, 18, 19), we compared the transforming activities of proto-Dbs and oncogenic Dbs to determine whether the missing NH₂- and COOH-terminal sequences can confer inhibitory constraints on the transforming activity of the full-length protein. The transforming activities of proto-Dbs and onco-Dbs were compared in a primary focus formation assay by transfection of a pAX142-dbs-HA6 construct encoding HA-tagged onco-Dbs and a pAX142-dbs-HA construct encoding HA-tagged proto-Dbs into NIH 3T3 mouse fibroblasts. Despite being expressed at equivalent levels (Fig. 1B), proto-Dbs had much weaker transforming activity compared with the Dbs oncogene (Fig. 1, C and D; <3% of transforming activity of onco-Dbs). Although much reduced in number, proto-Dbs foci exhibited characteristic onco-Dbs morphology (not shown). These results are consistent with the previous observation that the rat ortholog of Dbs, Ost, also

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**FIG. 1.** The Sec14 homology domain of Dbs is inhibitory with respect to transformation. A, schematic representation of the panel of Dbs truncation mutants. Numbering corresponds to the proto-Dbs amino acid sequence. B, expression of the Dbs deletion mutant proteins in NIH 3T3 cells. Lysates were collected from cells transiently transfected with pAX142-based constructs expressing HA-tagged versions of Dbs proteins and examined by Western blot using an HA-specific antibody. The membranes were then stripped and reprobed with a tubulin-specific antibody as an internal control for loading. C and D, transforming activity of Dbs deletion mutants in NIH 3T3 cell primary focus formation assay. In three independent experiments performed in triplicate, NIH 3T3 cells were transfected with 0.1, 0.5, or 1 μg of pAX142-based constructs expressing the indicated Dbs proteins. Foci were stained with crystal violet at 14 days, and the number of foci/pmol of transfected DNA was determined for each construct. C, results of a primary focus formation assay in NIH 3T3 cells transfected with 1 μg of pAX142-based constructs expressing the indicated Dbs proteins. D, the relative transforming activity of the Dbs deletion mutant proteins. Transforming activity of the onco-Dbs was set as 100%.
Deletion of the Sec14 Homology Domain Causes Activation of proto-Dbs Transforming Activity—To identify the structural domains that inhibit proto-Dbs transforming activity, we generated a series of proto-Dbs deletion mutants representing successive truncations of NH$_2$-terminal domains (Fig. 1A). Breakpoints for the mutants were chosen with consideration of the protein domain structure to allow correct folding of the truncated protein. For maximum expression efficiency we introduced a Kozak consensus sequence at the NH$_2$ terminus of each mutant. Each cDNA was cloned into the pAX142 vector and then assessed for expression levels in NIH 3T3 cells (Fig. 1B). All truncation mutants showed comparable levels of expression. The transforming potential of the proto-Dbs deletion mutants were then compared in an NIH 3T3 cell primary focus formation assay (Fig. 1C and D). To ensure that primary focus formation assays were not performed under saturated conditions, the transforming activities of the Dbs deletion mutant proteins were determined at several DNA concentrations. NIH 3T3 cells that were transfected with 0.1, 0.5, or 1 μg of DNA demonstrated a clear dose-dependent response in the number of foci generated (not shown). These data were then used to determine the relative transforming activity of the Dbs deletion mutant proteins (Fig. 1D). Completion of the COOH-terminal Src homology 3 domain of onco-Dbs (deletion mutant DbsΔ525) did not result in significant abrogation of the onco-Dbs transforming activity. This suggests that the Src homology 3 domain is not involved in the inhibition of transforming activity in the context of the full-length protein and that the negative regulatory signals reside within the NH$_2$ terminus. Indeed sequential deletion of the NH$_2$-terminal domains resulted in activation of proto-Dbs transforming activity. Removal of the Sec14 homology domain (deletion mutant DbsΔ235) resulted in significant activation of proto-Dbs transforming activity (up to 80% of onco-Dbs), while the full activation of proto-Dbs transforming potential was achieved by deletion of the adjacent spectrin repeat (deletion mutant DbsΔ348). Deletion of the sequences between the NH$_2$ terminus and the Sec14 domain (DbsΔ75) had no effect on transforming activity. Collectively these results suggest that the Sec14 homology domain represents a major regulatory element involved in the negative control of proto-Dbs transforming potential.

Non-transforming Derivatives of Dbs Are Catalytically Active in Vivo—Previously we characterized the transforming activity of onco-Dbs in NIH 3T3 mouse fibroblasts and demonstrated that RhoA is a physiologically relevant substrate for the Dbs-mediated transformation (34). NIH 3T3 cell lines stably expressing onco-Dbs demonstrate many parameters of oncogenic transformation and reliably show elevated levels of activated RhoA. Thus we were interested in comparing the guanine nucleotide exchange activities of our panel members toward RhoA in vivo. For this analysis we established polyclonal NIH 3T3 cell lines that stably express each member of the panel at roughly equivalent levels (Fig. 2). Empty cDNA pCTV3H vector was included in this analysis as a negative control. To measure endogenous levels of activated RhoA, the Rho-binding domain of Rhoetkin was used to affinity precipitate GTP-RhoA from cell lysates. Consistent with our previous observations, cells expressing onco-Dbs had elevated levels of GTP-RhoA (Fig. 2). Surprisingly, however, all members of the panel (both transforming and non-transforming) showed a similar increase in levels of GTP-RhoA relative to vector controls. These observations suggest that the reduced transforming activity associated with proto-Dbs expression cannot be simply attributed to intramolecular constraints on its RhoGEF activity.

The Dbs Sec14 Homology Domain Regulates the Subcellular Localization of the Dbs Protein—Compartmentalization and/or changes in subcellular localization provide alternative mechanisms for regulating intracellular signaling events. Thus, we compared the subcellular localization of our panel of Dbs truncation mutants using indirect immunofluorescence (Fig. 3, upper panel). The highly transforming onco-Dbs protein showed primarily a plasma membrane localization where its expression coincided with membrane ruffles. Additionally a weak diffuse staining was consistently observed in the cytoplasm. In contrast, proto-Dbs localized predominantly in the perinuclear region. We also observed punctate cytoplasmic staining that was never observed in onco-Dbs-expressing cells and was especially noticeable at higher levels of the proto-Dbs protein expression. This staining may represent vesicular structures, the nature of which we did not identify in this study.

The morphology of the actin cytoskeleton in cells expressing the onco- and proto-Dbs proteins was also quite different (Fig. 3, lower panel). Actin structures and the general cell morphology of cells transfected with the full-length protein resembled those of control vector-transfected cells, while the actin cytoskeleton of cells expressing onco-Dbs underwent a major reorganization with increased level of actin polymerization, formation of dense ringlike structures on the cell periphery, and a more rounded cell morphology. Next we investigated the intracellular localization of the proto-Dbs truncation mutants. All the derivatives that lacked the Sec14 homology domain (DbsΔ235, DbsΔ48, and DbsΔ525) displayed subcellular localization and actin cytoskeleton structures similar to onco-Dbs (Fig. 3). In contrast, DbsΔ75, which is missing the first 75 residues from the NH$_2$ terminus but contains the intact Sec14 homology domain, had a subcellular localization and actin cytoskeleton structures, which were very similar to the proto-Dbs protein. The DbsΔ75 protein showed a generally more diffuse perinuclear staining when compared with proto-Dbs, which might be explained by partial distortion of the Sec14 domain structure due to loss of the NH$_2$-terminal amino acids. To summarize, only Dbs derivatives containing the Sec14 domain exhibited perinuclear localization suggesting that this domain may regulate the spatial distribution of the protein.

Proto-Dbs Associates with Golgi Structures in NIH 3T3 Cells—The S. cerevisiae Sec14 protein is a major phosphatidyl-
choline/phosphatidylinositol transfer protein, which is involved in the secretion of proteins from a late Golgi compartment (35). Yeast Sec14 is a cytoplasmic protein partially associated with Golgi complex (36). Since the Sec14 homology domain of proto-Dbs is responsible for localization of the protein to a perinuclear region, we tested whether proto-Dbs is localized to the Golgi complex. For this analysis we performed dual label indirect immunofluorescence (Fig. 4A) using a fluorescent conjugate of WGA as a marker for the Golgi complex. WGA is a lectin that selectively binds to N-acetyl-β-D-glucosaminyl residues, which are predominately found on the proteins in the Golgi apparatus as well as on the plasma membrane and nuclear envelope (37). WGA has been successfully used in several studies to visualize Golgi apparatus in fixed cell preparations and is considered to be a reliable marker for this organelle (38). We transiently expressed our panel of Dbs deletion mutants in NIH 3T3 cells and determined the intracellular distribution of Dbs proteins using an anti-HA antibody. The Golgi complex was then counterstained with green fluorescent WGA. The non-transforming proto-Dbs and DbsΔ75 proteins demonstrated perinuclear staining that coincided with the Golgi marker, whereas the oncogenic DbsΔ235, DbsΔ348, DbsΔ525, and onco-Dbs proteins did not.

To further confirm an association of proto-Dbs and DbsΔ75 with the Golgi, cells were treated with BFA (Fig. 4B). Treatment of cells with BFA has been shown to lead to disruption of the Golgi complex due to inhibition of the ADP-ribosylation factor GTPase, which then triggers fusion of the Golgi membranes with the endoplasmic reticulum (39). As shown in Fig. 4B, there was a strong convergence of the proto-Dbs- and DbsΔ75-specific signals with WGA-positive perinuclear structures that was lost in response to treatment with BFA. These results suggest that a large portion of the overexpressed non-transforming proto-Dbs in NIH 3T3 cells localizes to Golgi membranes.

The Dbs Sec14 Homology Domain Binds Lipid—The Sec14 homology domain is shared by several lipid-binding proteins such as cellular retinaldehyde-binding protein (40, 41), α-tocopherol transfer protein (42), yeast Sec14 protein (25), and several RhoGEFs and RasGAPs (24). Because of this significant sequence homology to lipid-binding proteins, we wondered whether the Sec14 domain of proto-Dbs may also bind lipids that may regulate its intracellular localization. Since protein domains that specifically bind phosphoinositides have been shown to be involved in both subcellular recruitment and allosteric modulation of enzymatic activities (43, 44), we determined whether the Dbs Sec14 domain could bind phosphoinositides. For this analysis, we performed lipid dot-blot assays using PIP Array membranes (Echelon Biosciences Inc.) that contain eight major phosphoinositides spotted on a nitrocellulose membrane in different concentrations (range, 1.6–100 pmol) and the purified, bacterially expressed Sec14 homology domain of Dbs. As shown in Fig. 5A, we detected specific binding of the Sec14 homology domain of Dbs to phosphatidylinositol 3,5-bisphosphate, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2), and phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2) with highest affinity for the phosphatidylinositol 4,5-bisphosphate form. No binding was observed in control experiments in which membranes were incubated with no Sec14 protein or with eluants derived from bacterial cells that expressed the cognate vector (not shown).

A Mutation in the Dbs Sec14 Homology Domain That Diminishes Phosphoinositide Binding Results in Loss of Perinuclear Localization—Next we wished to determine whether lipid binding to the Sec14 domain is a major factor determining the Golgi localization of proto-Dbs. For this analysis we wanted to construct point mutants of proto-Dbs that disrupt lipid binding and then determine the effect of these mutations on proto-Dbs intracellular localization and transforming activity. Two substitutions (Dbs G218D and Dbs K191A) were introduced into the context of the full-length Dbs protein. Substitution of Dbs G218D corresponds to a well characterized temperature-sensitive mutant of the yeast Sec14 protein (G266D) (45), while K191A corresponds to the K239A mutant of the yeast Sec14 protein known to disrupt phosphoinositide binding (46). Intracellular localization of the proto-Dbs mutant proteins were tested by indirect immunofluorescence. Only one of the mutant proteins (G218D) demonstrated loss of the perinuclear localization (Fig. 5B). Interestingly this mutant showed the same cellular distribution and changes in actin cytoskeleton architecture as the oncogenic versions of Dbs. To determine whether the loss of perinuclear localization of G218D mutant occurred due to loss of the ability of its Sec14 domain to bind a specific lipid, we performed PIP Array analysis on this mutant. We introduced the equivalent point mutation into the context of the Sec14 domain of Dbs encoded in a bacterial expression vector and then performed a lipid dot-blot analysis using an equivalent concentration of the mutated protein. As shown in Fig. 5C, the mutant protein had reduced affinity to PtdIns(3,4)P2 and PtdIns(4,5)P2, although its affinity for phosphatidylinositol 3,5-bisphosphate was not significantly affected. This suggests that the binding of the Dbs Sec14 homology domain to PtdIns(4,5)P2 and PtdIns(3,4)P2 may be required for sequestration of proto-Dbs on Golgi membranes. In con-
contrast, the affinity of the K191A mutant of the Dbs Sec14 domain to phospholipids was not affected (not shown).

Next we wished to determine whether loss of association with the Golgi is sufficient to activate Dbs transformation. For this analysis we tested the transforming activity of the proto-Dbs G218D mutant in a primary focus formation assay in NIH 3T3 cells. Onco-Dbs and proto-Dbs were included as positive controls, and vector transfected cells were included as a negative control. Surprisingly we observed only a slight increase (2-fold) in the transforming activity of the proto-Dbs G218D mutant in comparison to wild-type proto-Dbs (Fig. 5D) despite the fact that the mutant was able to induce the formation of actin structures similar to what we observed for oncogenic Dbs. This would suggest that the ability to modify the actin cytoskeleton is not sufficient to account for Dbs transformation. In addition, simply sequestration of the proto-Dbs on the perinuclear Golgi membranes cannot fully explain the reduced transforming activity of the proto-Dbs protein.

The Sec14 Homology Domain Interacts with the PH Domain of Dbs—Autoinhibition of a RhoGEF exchange activity by an intramolecular interaction between the regulatory NH2-terminal sequences and the DH/PH domain module has been shown to be a mechanism of regulation of the transforming activity of Dbl and Vav. Although our finding that proto-Dbs can function as an active exchange factor in vivo argues against such a mechanism of proto-Dbs regulation, the data obtained with the proto-Dbs G218D mutant demonstrated that differential intracellular targeting of proto-Dbs could not fully explain the dramatic differences in the transforming activity of the full-length and oncogenic Dbs proteins. Thus, we wondered whether the Sec14 domain might have an additional function in the regulation of Dbs transforming activity. Initially we felt it would be

**Fig. 4. Localization of proto-Dbs and DbsΔ75 to the Golgi apparatus.** A, NIH 3T3 cells were transiently transfected with pAX142-based constructs encoding the indicated versions of the Dbs protein. After 48 h cells were examined by indirect immunofluorescence for expression of Dbs proteins (anti-HA, red signal). Perinuclear Golgi structures were visualized using a green fluorescent conjugate of WGA. Shown is a representative example of over 50 cells examined in each condition. B, BFA treatment disrupts perinuclear localization of the proto-Dbs and DbsΔ75 proteins. NIH 3T3 cells were transiently transfected with pAX142-based constructs encoding the proto-Dbs or DbsΔ75 proteins. After 48 h cells were treated with 10 μg/ml BFA for 30 min at 37 °C (+BFA) or were left untreated. Cell were fixed and examined by indirect immunofluorescence for expression of Dbs proteins (anti-HA, red signal). The Golgi complex was visualized as described above. In all images the blue signal indicates nuclear staining with 4,6-diamidino-2-phenylindole. Images were merged to compare the pattern of the Dbs proteins expression and the location of Golgi structures (merge).
of interest to determine whether the NH2-terminal sequences of Dbs that contain the Sec14 domain can interact with the DH/PH module. For this analysis we co-expressed onco-Dbs (residues 523–1097, containing the DH/PH module with short flanking sequences) with the N1-FLAG peptide that contains residues 1–525 of Dbs in 293T cells and performed co-immunoprecipitations using an anti-FLAG antibody. As shown in Fig. 6A, we were able to detect an association of the NH2-terminal fragment with onco-Dbs. Next we wished to determine the docking site for the NH2-terminal domain within the DH/PH module. We co-expressed the N1-FLAG peptide with the isolated DH and PH domains of Dbs encoded in the pAX142-dbs-HA8 and pAX142-dbs-HA9 constructs, respectively, in 293T cells and performed co-immunoprecipitations. Although the DH and PH domains were expressed at similar levels, an interaction was only observed with the PH domain of Dbs (Fig. 6B). Finally we co-expressed a similar fragment that contained the isolated Sec14 homology domain of Dbs (residues 1–235) encoded in the pAX142-dbs-HA9 plasmid in 293T cells and performed co-immunoprecipitations using anti-FLAG antibody. As shown in Fig. 6C, we were readily able to detect binding of this fragment with the PH domain of Dbs. These observations suggest that the Sec14 domain of Dbs is not only able to form lipid-mediated interactions with the Golgi but may also direct intramolecular interactions with the PH domain.

The G218D Mutation Does Not Affect the Binding of the Sec14 Homology Domain to the PH Domain of Dbs—Although the interaction between the Sec14 domain and the PH domain
The NH2-terminal Sec14 homology domain interacts with the PH domain of the onco-Dbs protein. 293T cells were transiently transfected in the indicated combinations with the constructs encoding onco-Dbs, the DH domain of Dbs (DH-Dbs), the PH domain of Dbs (PH-Dbs), the NH2-terminal peptide (residues 1–525) of proto-Dbs (proto-Dbs, DH-Dbs, PH-Dbs), the PH domain of Dbs, the NH2-terminal peptide (residues 1–525) of proto-Dbs (Sec14), and the G218D mutant of the Sec14 homology domain of proto-Dbs (Sec14-G218D). Lysates were collected 48 h after transfection and examined by Western blot for expression of the DH domain using an anti-FLAG antibody (total HA). Co-immunoprecipitations were then performed using an anti-FLAG M2 antibody, and precipitates were examined by Western blot with either an anti-HA or an anti-FLAG M2 antibody to detect the interactions. IP indicates antibody used for immunoprecipitations, while IB indicates antibody used for Western blot. A, onco-Dbs interacts with the PH domain and does not appear to regulate Dbs catalytic activity in vivo (see Fig. 2), this interaction may still regulate Dbs transformation by interfering with an alternative function of the PH domain. Several of such functions have been assigned to the PH domain of Dbs including lipid-mediated regulation of cellular localization and direct interactions with regulatory partners. Thus the failure of the G218D mutant to be fully activated in transformation may reflect its ability to maintain an interaction with the PH domain and mask these additional functions that are required for transformation. To test this possibility we co-expressed the G218D mutant of the Sec14 homology domain encoded in pAX142-Sec14-G218D plasmid with the PH domain of Dbs in 293T cells and performed co-immunoprecipitations using anti-FLAG antibody. As shown in Fig. 6D, the G218D mutant retained its ability to interact with the PH domain.

The Sec14 Domain of Proto-Dbs Inhibits Onco-Dbs Transformation When Expressed in trans—To directly test whether the binding of the Sec14 homology domain to the PH domain of Dbs can interfere with the PH domain function and thus inhibit transforming activity, we cotransfected either pAX142-N1-FLAG or pAX142-Sec14-FLAG with onco-Dbs into NIH 3T3 cells and performed primary focus formation assays. When compared with the vector controls, N1-FLAG reduced onco-Dbs transforming activity by about 60%, while the isolated Sec14 domain consistently inhibited onco-Dbs transformation by about 30% (Fig. 7A). The inhibition of onco-Dbs transforming activity by co-expression of regulatory NH2-terminal sequences was specific since transformation of onco-Lsc was not inhibited by co-expression with the Dbs-N1-FLAG or Dbs-Sec14 fragments. Taken together our results suggest that the Sec14 domain inhibits proto-Dbs transforming activity through two distinct mechanisms: through intramolecular interaction with the PH domain and through lipid-mediated sequestration of proto-Dbs on the Golgi membrane. Surprisingly neither mechanism regulates Dbs catalytic activity, and both need to be relieved to achieve full transformation (Fig. 7B).

DISCUSSION

The transforming potential of several RhoGEFs including Dbl, Ost, Vav, Asef, Tiam, Ect2, and Net1 can be activated by deletion of their NH2-terminal sequences (17, 18, 21, 47, 48), while COOH-terminal truncations activate p115-RhoGEF and Lbc (19, 49). Although these observations may suggest a simple autoinhibitory model of RhoGEF exchange activity, it is becoming increasingly clear that the activation of RhoGEF transformation is a multifactorial and possibly multistage process involving the regulation of RhoGEF catalytic activity itself, recruitment of a RhoGEF to the correct intracellular location, and, in some cases, oligomerization or binding to appropriate regulatory protein partners. For example, activation of Vav requires phosphorylation of Tyr-174 by the Src family kinases, which causes the NH2-terminal peptide to become unstructured and relieve inhibitory constraints on GEF activity (17). However, there is a second regulatory stage in the activation of Vav: when bound to the PtdIns(4,5)P2 the PH domain of Vav strongly interacts with the DH domain, and this inhibitory interaction can be weakened by binding of PtdIns(3,4,5)P3. In addition, binding of this phosphoinositide to the PH domain of Vav facilitates the phosphorylation of Vav by Lck (50). This suggests that initial weakening of the DH/PH domain interaction is a multifactorial and possibly multistage process involving the regulation of proto-Dbl transforming potential. The transforming activity of Dbl can be activated by removal of the NH2-terminal sequences (18). Further it was demonstrated that the catalytic activity of the full-length Dbl protein is inhibited due to binding of an NH2-terminal region to the PH domain that interferes with access of the substrates to the catalytic center of the DH domain (18). However, other levels of complexity of proto-Dbl regulation include differences in the intracellular localization of the onco- and proto-Dbl proteins as well as the necessity of the onco-Dbl DH homology domain to form oligomers to display transforming activity (18, 51).

Many cellular functions of RhoGTPases require their com-
partmentalized activation. Correspondingly, the catalytic activity of RhoGEFs, upstream regulators of Rho proteins, should also be regulated in a spatiotopic manner. For example, the biological activity of Tiam1 and Lbc appears to be determined by sequences that regulate their intracellular localization (19, 52). Another interesting example is Ect2 whose intracellular localization is strictly regulated during cell cycle progression by its nuclear sequestration away from its GTPase substrate.

**Fig. 7.** The NH$_2$-terminal domain (residues 1–525) and the Sec14 homology domain of Dbs (residues 1–235) specifically inhibit onco-Dbs transforming activity. A, NIH 3T3 cells were transfected with pAX142-based constructs encoding onco-Dbs together with constructs encoding the NH$_2$-terminal peptide of Dbs (N1-FLAG, residues 1–525), the Sec14 homology domain of Dbs (Sec14-FLAG, residues 1–235), or the cognate vector. Foci were scored 14 days after transfection. The focus forming activity of onco-Dbs cotransfected with NH$_2$-terminal peptides was determined in four independent experiments performed on duplicate plates using different concentrations of onco-Dbs (0.05, 0.1, and 0.5 μg/6-cm dish) and an excess of NH$_2$-terminal domains (2 μg/6-cm dish). To control the specificity of inhibition, in parallel experiments, the NH$_2$-terminal fragments of Dbs were cotransfected with the pAX142-based construct encoding the Lsc oncogene. The focus forming activity of onco-Dbs and Lsc cotransfected with the empty vector was set at 100%. The data shown represent means and S.D. of foci generated. B, a model of the Sec14 domain-mediated regulation of proto-Dbs transforming activity. The Sec14 domain blocks Dbs transformation by sequestering proto-Dbs onto Golgi membranes and by blocking functions associated with the PH domain. A point mutant that diminishes lipid binding (G218D) is sufficient to translocate Dbs to the cell periphery but not activate transformation. Removal of the Sec14 domain (i.e., DbsΔ235) relieves the intramolecular constraints on the PH domain and thus fully activates transformation. This would allow the PH domain to perform non-catalytic functions required for transformation such as mediate interactions with the plasma membrane (as shown). SH3, Src homology 3.
Truncation of the Ect2 NH2 terminus containing nuclear localization signals causes oncogenic activation of the full-length molecule. Interestingly sequences COOH-terminal to the Ect2 PH domain were also shown to be important for the Ect2 transforming activity and involved in the control of RhoGTPase specificity (53). The PH domains of Vav, Sos1, Tiam, Trio, Dbs, and Dbl (9, 54–57) are ligands for the regulatory phosphoinositides (9, 54–57). Additionally the PH domains of Trio and Dbs are directly involved in the regulation of the catalytic activity of these Dbl family members (9, 57). However, additional functions of the PH domain of RhoGEFs include the regulation of intracellular localization and, in some cases, binding to regulatory protein partners (30, 58, 59). In the current study, we investigated the mechanism of oncogenic activation of Dbs, the mouse ortholog of the rat Ost-γ RhoGEF. We found that both non-transforming and oncogenic Dbs variants displayed equivalent catalytic activity toward RhoA in vivo, yet removal of the NH2-terminal Sec14 homology domain was necessary for the activation of proto-Dbs transforming activity. This observation is similar to what has been shown previously for Lbc where full-length and oncogenic versions of Lbc displayed similar catalytic activity in vivo. This data suggests that intramolecular autoinhibition of the DH domain function cannot explain the reduced transforming activity of proto-Dbs.

In contrast, our examination of a panel of proto-Dbs deletion mutants demonstrated a strong correlation between intracellular localization and the transforming activity of Dbs. All highly transforming Dbs derivatives (onco-Dbs, DbsΔ235, DbsΔ348, and DbsΔ525) displayed cytoplasmic and plasma membrane localization, while the poorly transforming proto-Dbs and DbsΔ75 proteins localized primarily to the perinuclear region. Indirect immunofluorescence using a Golgi-specific marker suggested that proto-Dbs is localized on Golgi membranes when expressed in NIH 3T3 cells and that this association is inhibitory with respect to transformation. Our data suggest that the intracellular localization of proto-Dbs to the Golgi is regulated by the Sec14 homology domain and that this association occurs in a phosphoinositide-specific manner. We showed that the Sec14 domain of Dbs is a ligand for lipids and that a point mutation within the Dbs Sec14 homology domain that harbors reduced affinity for PtdIns(4,5)P2 and PtdIns(3,4)P2 caused loss of perinuclear localization of proto-Dbs. During preparation of this manuscript, a study demonstrating the regulation of Dbl and Ost intracellular localization by their Sec14 homology domains was published (60). Similar to our data, the authors demonstrated Golgi localization of the proto-Dbl and Ost GEFs and tested lipid binding specificities of the Sec14 domain of these proteins. In contrast to our data that proto-Dbs can function as a fully catalytically active exchange factor in vivo, only truncated versions of Dbl and Ost missing the Sec14 homology domains were capable of activating over-expressed Cdc42 in HeLa cells. It is possible that this discrepancy is due to cell type-dependent specificity of proto-Dbs catalytic activity. Interestingly, however, the in vitro catalytic activity of the full-length Dbl and Ost was indistinguishable from their truncated variants (60), which is consistent with our observations in NIH 3T3 cells.

While the involvement of the Dbs Sec14 homology domain in the regulation of proto-Dbs intracellular localization is clear, its role in the regulation of proto-Dbs transforming activity is more complex. Removal of the Sec14 homology domain of proto-Dbs resulted in significant activation of its transforming potential. However, a point mutation in the domain that destroys proto-Dbs perinuclear localization caused only a modest activation of its transforming activity. This suggests that in addition to sequestering proto-Dbs on Golgi membranes, the Sec14 homology domain plays an additional role in the control of Dbs activity and that simply losing perinuclear localization is not sufficient for proto-Dbs activation.

This additional function of the Sec14 homology domain became apparent in our studies of the intramolecular interactions of the Dbs NH2-terminal sequences with the DH/PH module, which demonstrated that the Sec14 homology domain interacts with the PH domain of Dbs. Our previous studies demonstrated a critical role of the PH domain of Dbs in the control of onco-Dbs catalytic activity (9) and intracellular localization (23). Additionally phosphoinositides binding to the PH domain of Dbs were shown to be important for the catalytic and transforming activity of onco-Dbs in vivo. Thus, interaction of the Sec14 homology domain with the PH domain of Dbs might affect several functions of the PH domain that are critical for Dbs transforming activity.

The G218D substitution, which diminishes phosphoinositide binding to the Sec14 homology domain of Dbs, did not affect the ability of this domain to interact with the PH domain. Thus, the inhibitory effect of the Sec14 domain binding to the PH domain might explain the low transforming activity of the proto-Dbs G218D mutant. Since this point mutant is not completely impaired in its ability to bind phosphoinositides, it is also possible that its effect may be to simply translocate Dbs to a membrane subdomain that does not fully support transformation. Elucidation of the crystal structure of the Dbs Sec14 domain may facilitate the design of mutants that are much more restricted in their lipid binding properties and help to determine physiologically relevant lipid binding partners.

Interestingly the proto-Dbs G218D mutant showed low transforming activity in focus formation assays but demonstrated actin cytoskeleton remodeling similar to oncogenic Dbs. This points to the possibility of the uncoupling of Dbs signaling to actin cytoskeleton rearrangements from its ability to activate pathways triggering oncogenic transformation. It is reasonable to hypothesize that the Sec14 domain of Dbs is involved in determining specificity of downstream signaling events possibly through control of Dbs recruitment into different signaling complexes.

In addition to Dbs, Sec14 homology domains are found in a number of other mammalian proteins including several additional members of the RhoGEF family (Dbl, Trio, Kalirin, and UNC-73) (24). Although the function and lipid binding specificity of the domain in the context of other RhoGEFs has yet to be determined, recent studies of the Sec14 homology domain of the protein phosphatase MEG2 demonstrated that this domain is responsible for regulation of the MEG2 catalytic activity (61) and that it modulates intracellular localization of the protein through specific binding to phosphatidylinositol (62). Our demonstration that the Sec14 domain of Dbs has similar properties suggests a conserved function for these sequences in the context of mammalian proteins. Interestingly deletion of 100 amino acids from the NH2-terminal of Dbl, which spans the Sec14 domain, has been reported to cause only slight activation of proto-Dbl transforming activity (18). The failure of this deletion to more fully activate Dbl transformation can likely be attributed to the observation that Dbl RhoGEF activity is also regulated through intramolecular autoinhibition of its RhoGEF activity. Removal of the Sec14 domain may not be sufficient to relieve this second level of regulation.

Since we have shown previously that RhoA is a target of Dbs transforming activity in NIH 3T3 cells (34), our data demonstrating the ability of the proto-Dbs protein to activate RhoA in vivo seems to be in contradiction with its low transforming activity. One explanation for this apparent discrepancy may be that proto-Dbs and onco-Dbs differ in their spatio-specific acti-
vation of RhoA. The importance of the spatial regulation of signaling events can be illustrated using the Ras/mitogen-activated protein kinase pathway. Thus, Sef, a MEK/ERK scaffold at the Golgi apparatus, binds to active MEK, inhibits dissociation of MEK/ERK complexes, and blocks nuclear translocation of ERK (63). Consequently this blocks activation of ERK nuclear targets but does not affect ERK activity toward cytoplasmic substrates. It is possible that the activation of RhoA by proto-Dbs, when localized on the Golgi membrane, engages a different signaling cascade than RhoA activated by onco-Dbs present on the plasma membrane and that only activation of plasma membrane-localized RhoA can lead to the appearance of the transformed phenotype. Development of molecular probes allowing visualization of localized activation of native Rho proteins within living cells and investigation of the factors determining spatiotemporal activation of RhoGTPases will be necessary to examine this possibility.

Data presented in this study suggest that the Sec14 homology domain regulates the transforming activity of proto-Dbs through a dual mechanism (see Fig. 7B), which includes control of proto-Dbs intracellular localization through lipid-mediated sequestration of proto-Dbs on Golgi membranes and inhibitory interaction with the PH domain. The exact mechanism by which the Sec14 homology domain inhibits a function of the PH domain of Dbs as well as the molecular events that relieve this inhibitory interaction remains to be elucidated. Similar to other RhoGEFs such as Vav, Dbl, and Sos, full activation of Dbs transforming activity requires several intracellular events. It is possible that, depending on cell requirements under certain growth conditions and in response to cellular events, it is possible that, depending on cell requirements, the Sec14 homology domain regulates the transforming activity of proto-Dbs present on the plasma membrane and that only activation of the PH domain of Dbs as well as the molecular events that relieve this inhibitory interaction remains to be elucidated.

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The Sec14 Homology Domain Regulates the Cellular Distribution and Transforming Activity of the Rho-specific Guanine Nucleotide Exchange Factor Dbs
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