Dbs is a Rho-specific guanine nucleotide exchange factor that was identified in a screen for proteins whose expression causes deregulated growth in NIH 3T3 mouse fibroblasts. Although Rac1 has not been shown to be a substrate for Dbs in either in vitro or in vivo assays, the Rat ortholog of Dbs (Ost) has been shown to bind specifically to GTP-Rac1 in vitro. The dependence of the Rac1/Dbs interaction on GTP suggests that Dbs may in fact be an effector for Rac1. Here we show that the interaction between activated Rac1 and Dbs can be recapitated in mammalian cells and that the Rac1 docking site resides within the pleckstrin homology domain of Dbs. This interaction is specific for Rac1 and is not observed between Rac1 and several other members of the Rho-specific guanine nucleotide exchange factor family. Co-expression of Dbs with activated Rac1 causes enhanced focus forming activity and elevated levels of GTP-RhoA in NIH 3T3 cells, indicating that Dbs is activated by the interaction. Consistent with this, activated Rac1 co-localizes with Dbs in NIH 3T3 cells, and natively expressed Rac1 relocates in response to Dbs expression. To summarize, we have characterized a surprisingly direct pleckstrin homology domain-mediated mechanism through which Rho GTPases can become functionally linked.

The Rho proteins constitute a large branch of the Ras superfamily of small GTPases. Like most small GTPases, Rho proteins function as binary switches cycling between a functionally inert GDP-bound conformation and a biologically active GTP-bound conformation. In their GTP-bound state, Rho proteins are able to participate in signaling pathways by forming productive interactions with effector proteins. The best described cellular functions of Rho proteins are their contributions to the regulation of the actin cytoskeleton, their ability to activate multiple mammalian transcription factors, and their ability to impinge directly on the cell cycle machinery (1, 2). With such a diversity of roles, it is not surprising that Rho proteins have been implicated in a wide variety of cellular activities including invasion and motility, neurite growth and retraction, cytokinesis, cell growth and proliferation, and phagocytosis (2–5).

One family of proteins that regulates the steady state levels of GTP-Rho in cells is the Rho-specific guanine nucleotide exchange factors (RhoGEFs) (6, 7). The mammalian RhoGEF family is thought to consist of over 60 members, a number of which have been identified on the basis of their transforming activity in murine fibroblasts. A well established biochemical model for RhoGEF-mediated GTPase activation has now been established (8). Small GTPases bind to nucleotides with high affinities and are generally unstable in the nucleotide-free state. Upon its own activation, a RhoGEF will form a low affinity ternary complex with the inactive GTPase and its associated GDP, which then rapidly converts to a stable guanine nucleotide exchange factor-GTPase binary complex, displacing both the GDP and Mg$^{2+}$. Although this complex is relatively stable in the absence of exogenous nucleotide, the intracellular levels of GTP are high (generally 30-fold higher than GDP), which favors GTP binding. GTP will, in turn, displace the guanine nucleotide exchange factor, and the GTPase will assume an activated conformation. Once in the activated state, the GTPase is able to establish stable complexes with effector molecules. Thus, whereas RhoGEFs typically have a much greater affinity for the GDP-bound conformation of the GTPase, effector proteins have a greater affinity for the GTP-bound conformation.

Virtually all members of the RhoGEF family share a common 300-amino acid structural motif composed of a Dbl homology (DH) domain arranged in tandem with a pleckstrin homology (PH) domain (6, 7). The DH domain is unique to the RhoGEF family and contains virtually all of the residues required for substrate recognition, binding, and exchange (9–12). Mutations within the DH domain that abrogate exchange activity are invariably associated with loss of signaling and transforming activity (6, 7).

The role of the PH domain in the context of RhoGEFs is uncertain. Like the DH domain, disruption of the PH domain, by truncation or point mutation, is generally associated with loss of transforming and signaling activity (6, 7). Replacement of the PH domains of Dbs and Lfc with a prenylation signal partially restores transforming activity, suggesting that one function of the domain is to promote the translocation of RhoGEFs to plasma membranes (13, 14). Such a function is likely to be facilitated by phosphoinositides, which have been shown to be low affinity (micromolar $K_d$ values) ligands for many PH domains, including the one in Dbs (15, 16). However, since the affinity of PH domains for phosphoinositides is quite low (15), and prenylation signals can only partially restore the transforming activity of Dbs (14), it has been suggested that phospholipid binding may regulate other aspects of RhoGEF

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‡ To whom correspondence should be addressed: Dept. of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, 225 Warren St., Newark, NJ 07103. Tel.: 973-972-4483 (ext. 25215); Fax: 973-972-3644; E-mail: whiteip@umdnj.edu.

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biological activity (17–19). For example, the binding of phos-
phoinositides to the PH domains of RhoGEFs is also reported to
modulate exchange allosterically (20–22), although this does
not appear to be a general mechanism of RhoGEF regu-
lation (16). Finally, there is also evidence that PH domains can
participate directly in GTPase binding and exchange independ-
ently of phospholipid binding. For example, the crystal struc-
ture of Dbs in complex with Cdc42 reveals a conformation in
which residues from the PH domain contribute directly to the
catalytic interface, primarily through interactions with Switch
2 (23). Substitution of the relevant residues within the PH
domain abrogates exchange activity, both in vitro and in vivo
(17, 23). For the moment, such direct involvement of the PH
domain in regulating catalytic activity appears limited to Dbs,
since the structures of the DH/PH domain modules of Tiam1
and Sos do not share this unique conformation (24, 25).

Dbs is a member of the RhoGEF family that was identified in
a screen for cDNAs whose expression causes deregulated pro-
liferation in NIH 3T3 cells (26). Like many members of the
RhoGEF family, Dbs has potent transforming activity in NIH
3T3 cells as measured by loss of contact inhibition, growth in
low serum, and anchorage-independent growth (14, 26, 27). In
addition to the DH/PH domain, Dbs encodes a putative amin-
terminal Sec14 domain, two spectrin-like repeats, and a car-
boxyl-terminal Src homology 3 domain. However, only the DH
and PH domains are required for cellular transformation.
Throughout its DH/PH domain, Dbs is very similar to Dbl (65%
identity), and like Dbl, it has in vitro catalytic activity for RhoA
and Cdc42 (14, 27). Although Dbs can also target RhoA and
Cdc42 in vivo, substrate usage can vary in a cell type-specific
manner. In NIH 3T3 cells, Dbs preferentially activates RhoA;
293T cells, it activates Cdc42; and in COS-7 cells, it does not
appear to activate either GTPase (28). A analysis of Dbs
mutants that have more restricted target specificities has im-
plicated RhoA as an important mediator of transformation in
NIH 3T3 cells (28).

Despite the fact that we and others have only been able to
detect Dbs-mediated exchange activity on RhoA and Cdc42, it
has been reported that a full-length version of the Rat orthology
doBs (Ost) can specifically interact with Rac1 in an in vitro
binding assay (27). Surprisingly, Ost preferentially interacts
with Rac1, when it is in its GTP-bound state, suggesting that
Ost may be an effector of Rac1 rather than an activator. A
functional relationship between Ost and Rac1 is further sug-
gested by the observation that dominant inhibitory Rac1 can
block Ost transforming activity (29). To further investigate the
possibility that Dbs may be an effector for Rac1 and thus may
have the capacity to coordinate regulation Rac1 and RhoA
activity, we have examined the relationship between Rac1 and
Dbs in vivo. Here we show that an interaction between
GTP-Rac1 and the PH domain of Dbs can be detected in mam-
malian cells and that this interaction has functional conse-
quences for Dbs catalytic and transforming activity. These
studies suggest a simple mechanism through which RhoGEFs
can directly link Rho proteins in regulatory cascades and sug-
gest a novel PH domain-mediated activity that may partially
explain the invariant topography of the DH/PH domain
module.

EXPERIMENTAL PROCEDURES

Molecular Constructs—The pAX142 mammalian expression vector
has been described previously (26). pAX142-23b-H7, pAX142-23b-HA7, pAX142-23b-HA8, pAX142-23b-H1A, pAX142-23b-
DHA6, pAX142-23b-D6HA, pAX142-23b-D6HA, and pAX142-23b-D7HA, and pAX142-23b-H1A contain hemagglutinin (HA)-tagged
derivatives of the respective RhoGEFs that have been described previ-
ously (30). Dbs-HA7 is identical to Dbs-HA8 except that it fuses the
carboxyl terminus to the plasma membrane-targeting sequence (GCM-
SCKCVLS) present at the carboxyl terminus of H-Ras. pAX142-23b-HA, pAX142-23b-HA9, and pAX142-23b-HA10 contain residues 1–1139,
791–967, and 968–1087 of Dbs, respectively, fused at their amino
termini to an HA epitope tag. cDNA sequences of all constructs were
verified by automated sequencing. pAX142-23b (16L), pAX142-
23b (17N), pAX142-23b-HA6L (36L), and pAX142-23b-cdc2 (12V)
have been described previously (14). GST-PBD and GST-
C21 contain the Rho binding domains from the Cdc42/Rac1 effector
protein PAK3 and the Rho effector protein Rhotekin, respectively (28).

Cell Culture, Transfection, and Transformation Assays—NIH 3T3
and 293T cells were maintained in Dulbecco’s modified Eagle’s medium
(high glucose) supplemented with either 10% bovine calf serum (NIH
3T3; JRH) or 10% fetal bovine serum (293T; Sigma). Primary focus
formation assays were performed in NIH 3T3 cells exactly as described
previously (31). Briefly, NIH 3T3 cells were transfected by Lipofect-
AMINE reagent (Invitrogen), and focus formation was scored visually
at 14 days.

Protein Expression—Protein expression in transiently transfected
293T and NIH 3T3 cells, was determined by Western blot analysis as
described previously (13). Protein was visualized with ECL reagents
(Amersham Biosciences).

Co-immunoprecipitations—Co-immunoprecipitations in 293T and
NIH 3T3 cells were performed as described previously (32). Briefly, 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
protease inhibitor mixtures (Calbiochem). Lysates were preclarified with
0.25 μg of agarse-conjugated normal mouse IgG (Santa Cruz Biotech-
nology, Inc., Santa Cruz, CA) by rotation for 30 min at 4 °C and pre-
clarified with agarose-conjugated mouse monoclonal HA antibody (anti-
Dbs, Santa Cruz Biotechnology) by rotation at 4 °C for 2 h. The immunopre-
cipitate was washed four times with cold lysis buffer and then resus-
pended in loading dye.

Cdc42, Rac1, and RhoA Activation Assays—Affinity purification assays
to measure cellular levels of GTP-Rac1, GTP-Cdc42, and GTP-RhoA were
performed as described previously (28). Total amount of RhoA, Cdc42,
and Rac1 were detected by monoclonal antibodies (Cdc42 and RhoA
(Santa Cruz Biotechnology) and Rac1 (BD Transduction Laboratories)).

Immunostaining—NIH 3T3 cells were transiently co-transfected with
1 μg of either pAX142 or pAX142-23b-H6A and 1 μg of either
pAX142 rac1(61L) or pAX142 rhoa(60L) using LipofectAMINE reagent
according to the manufacturer’s instructions (Invitrogen). At 24 h post-
transfection, cells were trypsinized and plated on coverslips at low
density. At 48 h post-transfection, cells were fixed with 3.7% formalde-
yde (in phosphate-buffered saline) for 10 min and then permeabilized
and blocked in 0.1% Triton X-100, 3% bovine serum albumin in phos-
phate-buffered saline for 30 min. Coverslips were then incubated in a
humidity chamber with a rabbit anti-HA polyclonal antibody (Y-11;
Santa Cruz Biotechnology) in conjunction with either a mouse anti-
Rac1 monoclonal antibody (clone 102; BD Transduction Laboratories)
or a mouse anti-RhoA monoclonal antibody (clone 119; Santa Cruz
Biotechnology), for 1 h in 0.1% Triton X-100 with 0.1% bovine serum
albumin. Coverslips were then washed in phosphate-buffered saline
and incubated with green-fluorescent Alexa Fluor 488-conjugated goat
anti-rabbit to detect HA-tagged Dbs and red fluorescent Alexa Fluor
568-conjugated goat anti-mouse IgG to detect either RhoA or Rac1
(0.1% Triton X-100, 0.1% bovine serum albumin; Molecular Probes, Inc.,
Eugene, OR), for 30 min in the dark. Coverslips were washed in phos-
phate-buffered saline on glass slides, and coverslips were mounted using
FluorSave reagent (Calbiochem). Cells were viewed with an Olympus IX50
inverted microscope, and images were captured using the optronics
digital CCD camera system. Images were analyzed in Adobe Photoshop.

RESULTS

Dbs Interacts with Rac1 in a GTP-dependent Manner in Vivo—Dbs-HA6 is an HA-tagged, oncogenic version of Dbs that contains
intact DH and PH domains along with flanking sequences (30). Rac1(61L) and Cdc42(12V) are GTPase-defective, GAP-insensitive
mutants that accumulate in the GTP-bound form, whereas Cdc42(17N) and Rac1(17N) are dominant inhibitory
mutants that accumulate in the GDP-bound form. We have shown previously in in vitro assays that Dbs-HA6 can
catalyze the exchange of GDP for GTP on Cdc42 and RhoA but not Rac1 (14). Consistent with the in vitro data, when we

2 L. Cheng, G. M. Mahon, E. V. Kostenko, and I. P. Whitehead, unpublished observations.

PH Domain-mediated Activation of Dbs by Rac1
expressed Dbs-HA6 in 293T cells, we observed elevated levels of GTP-Cdc42 but not GTP-Rac1 (Fig. 1A). To determine whether the in vitro association of Dbs with GTP-Rac1 could be recapitulated in this cell type, we co-expressed Dbs-HA6 with either Rac1(61L), Rac1(17N), Cdc42(12V), or Cdc42(17N) and then performed co-immunoprecipitations using an anti-HA (F7; Santa Cruz Biotechnology) monoclonal antibody. Although we were readily able to detect an interaction between Dbs and Rac1(61L), we were only able to detect a weak interaction between Dbs and Rac1(17N) (Fig. 1B). In contrast, we were not able to detect an association between Dbs-HA6 and Cdc42(12V) but were able to detect an interaction between Cdc42(17N) and Dbs (Fig. 1C). Our observation that Dbs binds to GDP-Cdc42 but not GTP-Cdc42 is typical of an interaction between a RhoGEF and its substrate. However, our observation that Dbs has a higher affinity for GTP-Rac1 than GDP-Rac1 confirms the in vitro data (27) and suggests that Dbs may function as an effector for Rac1 in vivo.

Since an interaction between full-length Ost and Rac1 has been demonstrated in vitro (27), we also wondered whether Rac1(61L) could interact with full-length Dbs in vivo. To confirm such an interaction, we constructed a full-length HA-tagged version of Dbs (designated Dbs-HA). When we co-expressed Dbs-HA with Rac1(61L) in 293T cells, we were readily able to detect an interaction by co-immunoprecipitation (Fig. 1D).

**Specificity of the Rac1/Dbs Interaction**—To determine whether the ability to form stable complexes with activated Rac1 in 293T cells is a characteristic of all RhoGEFs, we examined four additional RhoGEF family members for an interaction with Rac1(61L). Dbl-HA1, Lfc-D6HA, Lsc-D7HA, and Ect2-HA1 are constitutively activated, HA-tagged versions of RhoGEFs that contain intact DH/PH domain modules (30). Each RhoGEF family member was co-expressed with Rac1(61L) in 293T cells, and co-immunoprecipitations were performed. Dbs-HA6 was also included in the panel as a positive control for binding. Despite the fact that all panel members were expressed at roughly equivalent levels, we were only able to detect an interaction between Rac1(61L) and Dbs-HA6 (Fig. 2). This suggests that the interaction with GTP-Rac1 does not extend to all RhoGEF family members and may reflect a novel relationship between Rac1 and Dbs.

**GTP-Rac1 Interacts with the PH Domain of Dbs**—Since Dbs does not interact with Rac1 in a manner that is consistent with a substrate interaction, we wondered whether the Rac1 binding site in Dbs lies outside of the catalytic DH domain. To address this possibility, we subdivided Dbs-HA6 into several smaller fragments (Fig. 3A) and examined them for their ability to interact with Rac1(61L) (Fig. 3B). Dbs-HA8 is a transformation-defective version of Dbs-HA6 that lacks all sequences carboxyl-terminal to the DH domain. Dbs-HA7 is equivalent to Dbs-HA8 except that it is fused to an isoprenylation signal derived from H-Ras. We have shown previously that this membrane localization signal restores transforming activity to the transformation-defective Dbs-HA8 derivative (14). Dbs-HA9 contains the isolated PH domain, and Dbs-HA10 contains the residues that are located carboxyl-terminal to the PH domain. Each member of the panel as well as Dbs-HA6 was co-expressed with Rac1(61L) in 293T cells, and co-immunoprecipitations were performed (Fig. 3B). Although all derivatives were expressed at roughly equivalent levels, an interaction was only observed with Dbs-HA6 and Dbs-HA9. We conclude that the Rac1 docking site within Dbs resides within the PH domain.

It has been shown previously that the PH domain of Dbs contributes several residues (Tyr889 and Lys885) to the catalytic interface with its substrate, Cdc42 (23), and that substitution of these residues blocks exchange activity, both in vitro and in vivo (17, 23). To determine whether the PH domain-mediated interaction with Rac1 requires a contribution from these residues, we introduced the relevant substitutions into Dbs-HA6

**Fig. 1. Dbs interacts with, but does not activate Rac1 in vitro.** A. Dbs activates Cdc42 but not Rac1 in 293T cells. Lysates were collected from 293T cells that express Dbs-HA6 or cognate vector. Lysates were then examined by Western blot for expression of Rac1 (Total Rac1), Cdc42 (Total Cdc42), or Dbs (HA). Each lysate was then split into two parts, each of which was normalized for expression of Rac1 or Cdc42. Each lysate was then subjected to affinity purification with immobilized GST-PBD, and then GTP-bound Rac1 (GTP-Rac1) or Cdc42 (GTP-Cdc42) was visualized by Western blot. Rac1(61L) and Dbl-HA1 were included as positive controls for Rac1 and Cdc42, respectively. B–D, 293T cells were co-transfected with the indicated combination of plasmids. Lysates were collected at 48 h and then examined by Western blot for expression of Rac1 (Total Rac1) or Cdc42 (Total Cdc42). Immunoprecipitations were then performed with an anti-HA monoclonal antibody (F7; Santa Cruz Biotechnology), and precipitates were examined by Western blot with either an anti-Rac1 or anti-HA monoclonal antibody to detect an interaction. IP, antibody used for immunoprecipitation; IB, antibody used for Western blot. B, Dbs-HA6 interacts preferentially with GTP-Rac1. C, Dbs-HA6 interacts preferentially with GDP-Cdc42. D, full-length Dbs interacts with GTP-Rac1. Shown are representative assays that were performed in triplicate.

**Fig. 2. Specificity of the Rac1/Dbs interaction.** 293T cells were transiently transfected with the indicated combination of plasmids. Lysates were collected at 48 h and then examined by Western blot for expression of Rac1 (Total Rac1). Co-immunoprecipitations were then performed as described in the legend to Fig. 1. Shown is a representative assay that was performed in triplicate. IP, antibody used for immunoprecipitation; IB, antibody used for Western blot.
Activated Rac1 Potentiates Dbs Transforming Activity—Next we wished to determine whether the interaction with GTP-Rac1 has functional consequences for Dbs. Although the native function of Dbs has not yet been determined, its transforming activity in NIH 3T3 cell mouse fibroblasts has been well characterized. We have shown previously that Dbs is strongly transforming when expressed in NIH 3T3 cells (as measured by focus formation) and that this transforming activity is mediated by its in vivo substrate, RhoA (28). To confirm that Rac1(61L) can interact with Dbs in this cell type, we transiently co-expressed Dbs-HA6 with either Rac1(61L) or Rac1(17N) and performed co-immunoprecipitations. Consistent with what we observed in 293T cells, we could readily detect an interaction with Rac1(61L) but not Rac1(17N) (Fig. 4A). Next, we wondered whether the Rac1(61L) interaction could influence Dbs focus formation activity in this cell type. Although Rac1(61L) also exhibits several parameters of transformation when stably expressed in NIH 3T3 cells, in our hands it has no activity in a focus formation assay (Fig. 4B). However, when we co-expressed Rac1(61L) with Dbs-HA6, we observed markedly enhanced focus-forming activity (>100%; Fig. 4B). The potentiation in transformation assays was not observed when we co-expressed activated RhoA(63L) with Dbs, despite the fact that RhoA appears to be a bona fide substrate for Dbs in this cell type. Potentiation was also not observed between an oncogenic derivative of Dbl (Dbl-HA1) and Rac1(61L), which is consistent with our observation that Dbl-HA1 cannot interact with Rac1 (see Fig. 2). These results suggest that GTP-Rac1 enhances the transforming potential of Dbs but not all RhoGEFs.

Although the potentiation of Dbs transformation by Rac1 is consistent with a functional interaction between the two proteins, it is possible that Rac1 contributes to Dbs transformation in a manner that does not require direct binding. Dbs-HA7 is a transforming derivative of Dbs in which the PH domain has been replaced by an isoprenylation signal derived from H-Ras (14). Although Dbs-HA7 has transforming activity when expressed alone in NIH 3T3 cells, it cannot interact with Rac1(61L) (see Fig. 3). If Rac1(61L) influences Dbs transformation through binding to its PH domain, then Dbs-HA7 should not be responsive to Rac1(61L) expression. Consistent with this, we found that Rac1(61L) was unable to influence the transforming activity of Dbs-HA7 in an NIH 3T3 focus formation assay (Fig. 4C).

As is the case for many RhoGEFs, the biological activity of full-length Dbs (including transformation) is controlled by negative regulatory sequences located in its amino terminus. Thus, full-length Dbs has only a very weak transforming activity in NIH 3T3 cells when compared with the amino-terminal truncated Dbs-HA6 derivative (27). Since full-length Dbs can interact with Rac1(61L) in vivo (see Fig. 1D), we wondered whether Rac1 could also influence the transforming activity of full-length Dbs. Interestingly, co-expression with Rac1(61L) consistently doubled the transforming activity of Dbs-HA, suggesting that Rac1 binding may relieve the amino-terminal inhibition (Fig. 4C). However, since transformation was still much lower than the fully activated Dbs-HA6 mutant, it seems that expression of Rac1(61L) is not sufficient to fully activate Dbs.

Next we wondered whether the elevated transforming activity associated with Dbs and Rac1 co-expression is dependent upon Dbs catalytic activity. Since Rac1(61L) is known to be transforming in NIH 3T3 cells, as measured by other parameters of transformation (34), it is possible that the potentiation of transformation that we observe can be attributed to enhanced Rac1 transforming capacity in this cell type. However, when we co-express Rac1(61L) with a DH domain minus ver-

Fig. 3. Rac1 interacts with the PH domain of Dbs. A, the domain structure of Dbs-HA6 is illustrated on the upper line, whereas the lines below indicate the regions of the protein included in predicted translation products of the various cDNA derivatives. B and C, 293T cells were co-transfected with Rac1(61L) along with the indicated plasmids. Lysates were collected at 48 h and examined by Western blot for expression of Rac1 (Total Rac1). Co-immunoprecipitations were then performed as described in the legend to Fig. 1. All assays were performed in triplicate. B, Rac1 interacts with the PH domain of Dbs. C, Dbs PH domain mutations that disrupt the catalytic interface do not impair Rac1 binding. Both mutations are in the background of Dbs-HA6. IP, antibody used for immunoprecipitation; IB, antibody used for Western blot.

(Y889F and K885A) and then examined these mutants for Rac1 binding (Fig. 3C). Neither of these two mutants was impaired in its ability to bind with Rac1(61L), again suggesting that the interaction between Rac1 and Dbs differs from the conventional interaction between this RhoGEF and its substrates.

The GTP-Rac1/Dbs Interaction Can Occur Independently of Phosphoinositide Binding—Many RhoGEFs, including Dbs, have been shown to interact with phosphoinositides in a PH domain-dependent manner (16). In a previous study, we identified the lipid binding pocket within the PH domain of Dbs and characterized point mutations that are completely defective in phosphoinositide binding (17). Since activated Rac1 is thought to be localized to the plasma membrane in NIH 3T3 cells (33), we wondered whether Rac1(61L) binding in vivo is dependent upon an association between the PH domain and lipids. To address this possibility, several of the Dbs phosphoinositide-binding mutations (K892E, K849E/K851E, and R855E/K857E) were introduced into the background of Dbs-HA6 and co-expressed with Rac1(61L) in 293T cells, and then co-immunoprecipitations were performed (data not shown). We observed that all mutants were able to interact with Rac1(61L), suggesting that the association does not occur in a phosphoinositide-dependent manner.
L759I/L766M mutations were tested in the background of Dbs-HA6. C, not RhoA, potentiates Dbs transforming activity. The data presented are representative of three independent experiments performed on triplicate plates. The error bars indicate S.D. B, Rac1, but not RhoA, potentiates Dbs transforming activity. C, potentiation of Dbs transforming activity by Rac1 requires intact PH and DH domains. The L759I/L766M mutations were tested in the background of Dbs-HA6.

In NIH 3T3 cells, co-expression of Dbs (Dbs-HA9) or a catalytically dead mutant (Dbs-HA6(L759I/L766M)), no enhancement of focus-forming activity is observed (Fig. 4C), despite the fact that both mutants are able to bind to activated Rac1 (see Fig. 2 and data not shown). Thus, potentiation appears to require a structurally intact and catalytically active DH domain.

**Co-expression of Dbs with Rac1(61L) Is Associated with Elevated Levels of RhoA-GTP**—We have shown previously that Dbs transforming activity in NIH 3T3 cells correlates with its ability to utilize RhoA as a substrate and that NIH 3T3 cells that stably express oncogenic Dbs exhibit elevated levels of GTP-RhoA (28). Thus, we wondered whether Rac1(61L) potentiates Dbs transforming activity by stimulating its ability to catalyze exchange on RhoA. However, despite repeated attempts, we were unable to establish stable cell lines that express detectable levels of both Rac1(61L) and Dbs-HA6, suggesting that high levels of co-expression may be growth-inhibitory. As an alternative approach, we asked whether transient co-expression of Dbs-HA6 with Rac1(61L) in this cell type would produce increased levels of GTPase activation. We have shown previously that we cannot detect elevated levels of activated RhoA, Cdc42, or Rac1 when Dbs-HA6 is transiently expressed in NIH 3T3 cells, which we attributed to poor levels of Dbs expression (28). However, when we transiently co-express Rac1(61L) with Dbs-HA6, we are able to observe low (~3-fold) but reproducible increases in levels of GTP-RhoA (Fig. 5) but not GTP-Cdc42 (not shown). We observed no change in GTP-RhoA levels when we expressed Rac1(61L) with an oncogenic Dbs derivative that lacks the PH domain (Dbs-HA7) or with vector alone.

**Dbs Co-localizes with Rac1 in NIH 3T3 Cells**—If Rac1 influences Dbs exchange and transforming activity in NIH 3T3 cells through a direct interaction with its PH domain, we would anticipate that we could co-localize Rac1(61L) and Dbs-HA6 in this cell type. To examine this possibility, we co-expressed Dbs-HA6 with either Rac1(61L) or RhoA(63L) and performed indirect immunofluorescence to determine the cellular distribution of each protein (Fig. 6). Cells that co-express Dbs-HA6 with either Rac1(61L) or RhoA(63L) had a more rounded morphology than untransfected cells, and membrane ruffling and large lamellipodia were readily apparent. Immunostaining of the Dbs- and Rac1(61L)-expressing cells revealed an accumulation of both proteins within membrane ruffles as well as a discrete and strong accumulation of both in a subcortical ring. Merging of images consistently revealed a precise convergence in the cellular distribution of both proteins. In contrast, in cells that co-express Dbs-HA6 and RhoA(63L), RhoA exhibited both a plasma membrane and cytoplasmic staining and did not localize to the subcortical ring or membrane ruffles associated with Dbs staining. Merging of images revealed discrete yet overlapping staining patterns. We conclude that Rac1 precisely colocalizes with Dbs in NIH 3T3 cells, but RhoA does not.

**Dbs Can Influence the Cellular Localization of Endogenous Rac1 in NIH 3T3 Cells**—Although Dbs transformation in NIH 3T3 cells requires PH domain-mediated membrane association, this association does not appear to be regulated by phospholipid binding (17). Thus, we wondered whether an interaction...
with endogenous Rac1 could influence the cellular distribution of oncogenic Dbs. To explore this possibility, we examined the cellular localization of native Rac1 in NIH 3T3 cells that express vector, compared with cells that express Dbs-HA6 (Fig. 7). In vector-expressing cells, we did not observe any of the lamellipodia or membrane ruffles associated with co-expression of Dbs and Rac1(61L). In these cells, Rac1 is primarily located in the cytoplasm and in discrete pockets, adjacent to the plasma membrane. In Dbs-expressing cells, lamellipodia and membrane ruffling was consistently observed, albeit with less frequency than Dbs/Rac1-expressing cells. In the Dbs expressing cells, both Dbs and native Rac1 are found predominantly in the dense staining subcortical ring. The precise co-localization of the proteins is readily apparent when the images are merged. These observations suggest that Dbs can interact with endogenous Rac1 in NIH 3T3 cells and that these proteins may influence one another’s cellular distribution. This raises the possibility that this interaction may contribute to some of the signaling and transforming activities associated with Dbs expression in this cell type.

**DISCUSSION**

It has been shown previously that Ost/Dbs interacts specifically with GTP-Rac1 in an in vitro binding assay (27). In the current study, we have demonstrated that this association can be recapitulated in live cells and have provided further evidence that Dbs may function as an effector molecule for Rac1. Unlike a typical association of a RhoGEF with its substrates, the Rac1 docking site resides within the PH domain of Dbs, and the association is only observed when Rac1 is in a GTP-bound state. Although the PH domain of Dbs is unusual in that it contributes several residues to the catalytic interface, mutation of these residues has no effect on Rac1 binding, suggesting that the Rac1 and substrate docking sites are distinct. These observations are in accordance with several previous studies, all of which concluded that Dbs cannot function as an exchange factor for Rac1 (27, 28). These observations also contrast sharply with what has been observed for the association between Dbs and its bona fide substrate, Cdc42. A stable association between Cdc42 and Dbs can only be observed when Cdc42 is in the GDP-bound form, and Dbs can utilize Cdc42 as a substrate both in vitro and in vivo.

Dbs was originally identified as an oncogene based on its focus formation activity in NIH 3T3 cells (26). Here we show that co-expression of activated Rac1 and Dbs causes enhanced focus formation activity and that this influence of Rac1 is specific. In a previous study, we have established that RhoA is a physiologically relevant substrate for Dbs transformation in NIH 3T3 cells (28), and in the current study we have shown that transient co-expression of Dbs-HA6 and activated Rac1 causes elevated levels of GTP-RhoA in the same cell type. However, since we are unable to detect elevated levels of RhoA when we transiently express Dbs alone in these cells, we cannot exclude the possibility that an additional GTPase is responsible for the enhanced transforming activity.

Although it is possible that Dbs and Rac1 activate independent pathways that cooperate in transformation, we have made several observations that suggest that this is not the case. First, activated Rac1 does not cooperate with Dbl in transformation, although Dbl is closely related to Dbs and activates many of the same signaling pathways in NIH 3T3 cells (14, 30). Second, although Rac1 can potentiate the transforming activity of versions of Dbs that contain the Rac1 binding site (Dbs-HA and Dbs-HA6), it cannot influence an oncogenic Dbs derivative that does not contain the Rac1 binding site (Dbs-HA7).

We have shown previously that the PH domain can be replaced by a plasma membrane targeting sequence to maintain the transforming activity of Dbs, suggesting a role for the PH domain in membrane localization (14). Consistent with this, we have shown that the PH domain of Dbs interacts with phosphoinositides and that mutants that lack phospholipid binding in vivo are impaired in their transforming activity (17). However, we also observed that these PH domain mutants were able to maintain the proper subcellular localization and that the PH domain binds phosphoinositides with an affinity that is typically considered insufficient to recruit proteins to plasma membranes \( K_d \approx 10 \mu M \). Thus, we and others have proposed that although phospholipids may be required to reorient DH and PH domains to engage effectively to Rho GTPases, they may not be responsible for membrane recruitment (17, 19). Our observation that Rac1 can bind to the PH domain of Dbs and potentiates its transforming activity provides an alternate function for the PH domain with respect to Dbs transforming activity. In such a model, activated Rac1 would serve to recruit Dbs to a specific membrane compartment (Fig. 8). The interaction would then be further stabilized through an interaction with phospholipids, which would position Dbs in a manner that it could efficiently engage substrates such as RhoA. Consistent with this model, we observed that either endogenous or overexpressed Rac1 co-localizes with Dbs in NIH 3T3 cells, suggesting that these proteins may influence one another’s cellular localization. Additionally, our observation that we can immunoprecipitate Rac1 and Dbs in the absence of lipid binding supports the notion that Dbs engages Rac1 prior to interacting with phosphoinositides.

Although microinjection studies have revealed that members of the Rho family such as RhoA, Rac1, and Cdc42 can have
discrete effects on the cytoskeleton, there is also evidence to suggest that these GTPases may be linked in activating or inhibitory cascades (35–37). For example, in Swiss 3T3 fibroblasts, activation of Cdc42 leads to activation of Rac1, which in turn leads to activation of RhoA (38, 39). However, this sequence of activation is not observed in all cell types, and in several instances Rho GTPases have been shown to inhibit rather than activate other members of the family. For example, the activities of Rac1 and RhoA appear to oppose each other in neuronal cells (40, 41), and both Rac1 and Cdc42 appear to inhibit RhoA function when expressed in NIH 3T3 mouse fibroblasts (42). Although the mechanism through which Rac1 can inhibit RhoA activation in NIH 3T3 cells has not yet been elucidated, in HeLa cells it has been proposed that Rac1 can down-regulate RhoA through an indirect mechanism involving generation of reactive oxygen species and subsequent activation of p190 Rho-specific GTPase-activating protein (43). Our observation that co-expression of Rac1 with Dbs leads to increased GTP-RhoA levels in NIH 3T3 cells further suggests a functional link between the two GTPases in this cell type. However, since Dbs is not normally expressed in this cell type and appears to facilitate Rac1-mediated activation of RhoA, it is likely that the regulatory mechanism that we have identified is distinct from, and perhaps more direct than, the mechanisms that have been described previously.

Several lines of evidence provide the precedent that GTPases can be linked directly in RhoGEF-mediated cascades. In Schizosaccharomyces pombe, Sgcd/Ral1 directly links Ras and Cdc42 activation by functioning as an effector for GTP-Ras and a RhoGEF for Cdc42 (44). These three elements act cooperatively in the regulation of cell morphology and mating. Similarly, in mammalian cells it has been shown that Tiam-1, a Rac1-specific RhoGEF, is activated by forming complexes with GTP-bound Ras (45). The observation that RhoGEF family members such as Trio and Ber contain multiple domains through which they may interact with distinct Rho family members has also fuelled speculation that RhoGEFs may also serve to functionally couple different members of the Rho family (7). Our observation that Dbs can bind with and is activated by GTP-Rac1 provides strong evidence for such a regulatory paradigm.

Although to our knowledge there have been no previous reports of a GTPase interacting with the PH domain of a RhoGEF, PH domain/GTPase interactions have been reported for other types of signaling molecules. For example, it has been shown that GTP-Rac1 can activate phospholipase C through an interaction with its PH domain (46). Additionally, a recent report has documented the direct binding of RhoA to the amino-terminal PH domain of the Btk family tyrosine kinase Etk (47). Although it is an intriguing possibility that the PH domains of RhoGEFs have a common function as docking sites for regulatory proteins such as Rac1, we have been unable to observe the Rac1 interaction with several other members of the RhoGEF family member. Thus, at this time, we cannot discount the possibility that the Dbs/Rac1 interaction is unique. Studies are currently under way to determine whether this regulatory interaction can be observed between additional members of the Rho and RhoGEF families.

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