Transformation by the Rho-specific Guanine Nucleotide Exchange Factor Dbs Requires ROCK I-mediated Phosphorylation of Myosin Light Chain*

Zhuoming Liu, Elena V. Kostenko, Gwendolyn M. Mahon, Oyenike O. Olabisi, and Ian P. Whitehead

From the Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, Newark, New Jersey 07103

Dbs was identified in a cDNA-based expression screen for sequences that can cause malignant growth when expressed in murine fibroblasts. In previous studies we have shown that Dbs is a Rho-specific guanine nucleotide exchange factor that can activate RhoA and/or Cdc42 in a cell-specific manner. In this current study we have used a combination of genetic and pharmacological approaches to examine the relative contributions of RhoA-PRK and RhoA-ROCK signaling to Dbs transformation. Our analysis indicates that ROCK is activated in Dbs-transformed cells and that Dbs transformation is dependent upon ROCK I activity. In contrast, there appears to be no requirement for PRK activation in Dbs transformation. Dbs transformation is also associated with increased phosphorylation of myosin light chain and stress fiber formation, both of which occur in a ROCK-dependent manner. Suppression of myosin light chain expression by small interfering RNAs impairs Dbs focus formation, thus establishing a direct link between actin-myosin contraction and Rho-specific guanine nucleotide exchange factor transformation.

Members of the Rho subfamily of Ras-related GTPases regulate multiple aspects of cellular behavior including cell movement, cell cycle progression, transcriptional activation, and transformation (1, 2). Like most GTPases, Rho proteins exist in the cell in two states; a biologically active GTP-bound conformation and a biologically inactive GDP-bound conformation. The ratio of active GTP-Rho to inactive GDP-Rho in the cell is regulated by several families of regulatory proteins. The Rho-specific guanine nucleotide exchange factors (RhoGEFs) bind to inactive GDP-RhoA and facilitate the release of GDP, which provides an opportunity for GTP to bind (3). Conversion of Rho to the inactive state is achieved through intrinsic GTP hydrolysis, which can be accelerated by interactions with GTPase-activating proteins (4). A third class of regulators, the guanine nucleotide dissociation inhibitors compete with most GTPases, Rho proteins exist in the cell in two states; a biologically active GTP-bound conformation and a biologically inactive GDP-bound conformation. The ratio of active GTP-Rho to inactive GDP-Rho in the cell is regulated by several families of regulatory proteins. The Rho-specific guanine nucleotide exchange factors (RhoGEFs) bind to inactive GDP-RhoA and facilitate the release of GDP, which provides an opportunity for GTP to bind (3). Conversion of Rho to the inactive state is achieved through intrinsic GTP hydrolysis, which can be accelerated by interactions with GTPase-activating proteins (4). A third class of regulators, the guanine nucleotide dissociation inhibitors compete with the RhoGEFs for binding to GDP-RhoA and sequester the inactive GTPase in the cytoplasm (5).

Although recent studies have begun to reveal the broad assortment of normal biological activities that are regulated by members of the RhoGEF family, historically the family has attracted the most attention for its role in transformation. Chromosomal rearrangements with breakpoints that fall within BCR and LARG occur in distinct subsets of human leukemias, and overexpression of Clg has been implicated in murine leukemias (6–8). Additionally, a large subset of the RhoGEFs has been isolated in screens for proteins whose expression cause deregulated growth in NIH 3T3 mouse fibroblasts (3). Typically NIH 3T3 cells that are transiently transfected with oncogenic RhoGEFs fail to undergo contact inhibition when confluence is reached, forming large dense foci that are morphologically distinct. Cell lines that stably express these oncogenic RhoGEFs also exhibit transformation as measured by a variety of parameters including growth in low serum, anchorage independence, and tumorigenicity in nude mice.

Dbs is a RhoGEF family member that was isolated in a screen for cDNAs whose overexpression cause deregulated growth in NIH 3T3 mouse fibroblasts (9, 10). Like most RhoGEF family members, Dbs contains a tandem DH/PH domain motif that is both necessary and sufficient for Dbs transforming activity (9). DH domains are the catalytic cores of RhoGEFs and contain virtually all of the residues that are required for substrate recognition, binding, and exchange. PH domains have multiple roles in the context of RhoGEF family members (11–14). In Dbs the PH domain contributes several residues to the catalytic interface (15), is a ligand for phosphoinositides (16), and contains a docking site for activated Rac1 (17). Whereas the role of Rac1 in Dbs transformation remains unclear, mutations that block lipid binding are completely impaired in transformation (16).

Like many members of the RhoGEF family, Dbs has potent transforming activity in NIH 3T3 cells, and this activity has been mapped to the catalytic domain (9, 10). Although Dbs can target RhoA and Cdc42 in vitro (10, 12), substrate usage can vary in a cell type-specific manner. In NIH 3T3 cells Dbs preferentially activates Rac1 (18), in 293T cells it activates Cdc42 (17, 19), and in COS-7 cells it does not appear to activate either GTPase. A recent determination of the crystal structures of Cdc42 in complex with RhoA and Cdc42 has allowed for the design of mutants with more restricted target specificities (18, 19). An analysis of the biological activity of these mutants has determined that Cdc42 activation is dispensable for Dbs transformation (18). Consistent with this, we are able to detect elevated levels of activated RhoA, but not Cdc42, in stable NIH 3T3 cell lines that express activated Dbs, and competitive inhibitors of RhoA specifically block Dbs transformation. Collectively, these observations suggest that RhoA is a relevant target for Dbs transformation in this cell type.

A number of potential target kinases and structural proteins have been described that interact with RhoA and may mediate Dbs transformation. These include ROCK I (20), ROCK II (21), PRK I (22), PRK II

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1 Recipient of Ruth Kirchstein Postdoctoral Fellowship CA113049.
2 Recipient of Ruth Kirchstein Predoctoral Fellowship CA110749.
3 To whom correspondence should be addressed: Dept. of Microbiology and Molecular Genetics, International Center for Public Health, 225 Warren St., Newark, NJ 07103. Tel.: 973-972-4483 (ext. 25215); Fax: 973-972-3644; E-mail: whitehead@umdnj.edu.
4 The abbreviations used are: RhoGEF, Rho-specific guanine nucleotide exchange factor; MLc, myosin light chain; HA, hemagglutinin; GST, glutathione S-transferase; siRNA, small interfering RNA.
5 Z. Liu, E. V. Kostenko, G. M. Mahon, O. O. Olabisi, and I. P. Whitehead, unpublished observations.
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(23), p140mDia (24), rohekmin (25), rhophisin (22), citron kinase (CRK) (26), kinectin (27), p116RIP (28), and the myosin binding subunit of myosin phosphatase (29). However, the evidence linking particular RhoA effector proteins to the transformation phenotype is still somewhat limited. RhoA mutants that are selectively impaired in their ability to interact with specific effectors have implicated ROCK I but not PRK I in RhoA transformation (30). In support of this, a pharmacological inhibitor of ROCK I and a genetic inhibitor of its substrate ezrin, both, block transformation by the RhoGEF family members Net and Dbl (31). However, the RhoA(L-63, V-39) mutant interacts efficiently with ROCK I and is competent in transformation but fails to stimulate actin polymerization (30). This suggests that ROCK I may mediate RhoA transformation independently of its ability to remodel the actin cytoskeleton. Consistent with this possibility, a recent study suggests that ROCK I can stimulate JNK-mediated transcriptional pathways independently of its ability to regulate actin (32). Transformation by RhoA has also been linked to transcriptional activation through ROCK I-independent pathways. For example, dominant inhibitors of MKK3 and MKK6, which are responsible for PRK I-mediated activation of p38-γ, also block RhoA transformation (33). Although these studies suggest that ROCK I- and PRK I-mediated signaling may be necessary for RhoA transformation, it is unclear whether these kinases are directly activated in the context of RhoA transformation or are simply cooperating with additional RhoA-mediated signaling pathways in transformation.

Because both ROCK I and PRK I have been implicated in RhoA transformation, we wished to determine whether RhoA-mediated activation of either kinase contributes to Dbs transformation. Our analysis of the activation status and cellular distribution of these kinases in Dbs-transformed cells suggests a role for ROCK I, but not PRK I, in Dbs transformation. Consistent with this, pharmacological and genetic inhibitors that target ROCK I impair Dbs transformation. ROCK I-mediated hyperphosphorylation of myosin light chain (MLC) was also observed in Dbs-transformed cells, and siRNA targeted against MLC also impaired Dbs transformation. These observations support a model whereby ROCK I-mediated stimulation of actino-myosin contraction contributes directly to Dbs transformation.

MATERIALS AND METHODS

Molecular Constructs—The pAX142 and pCTV3H mammalian expression vectors have been described previously (9). pAX142-dbs-HA6 and pCTV3H-dbs-HA6 contain cDNA fragments that encode a transforming derivative of murine Dbs (onco-Dbs) fused to a hemagglutinin (HA) epitope tag (12), pAX142-ect2-HA3 encode transforming derivatives of the Lfc and Lsc, and pAX142-ect2-HA3 encode transforming derivatives of the Lfc, Lsc, and Ect2 proteins, respectively (34). The plasmids pCAG-ROCK I (11) and pCAG ROCK II (9), which contain full-length Myc-tagged wild-type and kinase-dead ROCK I cDNAs respectively, were kindly provided by S. Narumiya (35). The plasmid pCDNA3-MKK3 AA, containing HA-tagged dominant negative MKK3, and plasmid pCEFL-GST-MKK6 KR, containing kinase-deficient GST-tagged MKK6, were kindly provided by S. Gutkind (33). A plasmid containing wild-type Myc-tagged PRK I was provided by P. Parker (36). The plasmid pCDNA3-Src, which encodes a transforming derivative of murine Src, was provided by C. J. Der.

Cell Culture, Transfection, and Transformation Assays—NIH 3T3 cells were cultured at 37 °C in 10% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum (JRH Biosciences). Primary focus formation assays were performed as described previously (37). Briefly, NIH 3T3 cells were transfected by Lipofectamine reagent (Invitrogen) according to the manufacturer’s recommended protocol. Cells were maintained in growth media for 14 days, and then cells were stained with 0.5% crystal violet to visualize foci. To examine a role for ROCK I in transformation, transfected cells were split into six 6-cm dishes, three of which were treated with 10 μM ROCK inhibitor (2612, Cell Signaling). Cell media was changed, and fresh inhibitor was added every 24 h. To generate NIH 3T3 cells stably transfected by pCTV3H-dbs-HA6 or its cognate vector, the transfected cells were selected in growth media supplemented with hygromycin B (200 μg/ml, Invitrogen) for 10 days. Multiple drug-resistant colonies were pooled together to establish stable cell lines. Three independent cell lines were established and tested for each construct.

GTPase Activation Assay—Affinity purification assays to measure the levels of endogenous GTP-bound RhoA and Cdc42 were performed using the Rho binding domain of Rhotekin (GST-C21) and the Cdc42 binding domain of Pak (GST-PK) as described previously (17, 18). Small Interfering RNA (siRNA) Transfections and Secondary Focus Formation Assay—Validated siRNA oligonucleotides, which were designed to suppress endogenous ROCK I (Santa Cruz, sc-36432), ROCK II (Santa Cruz, sc-36433), PRK I (Santa Cruz, sc-36262), or MLC (Santa Cruz, sc-35940) were transfected by SilEntFect™ lipid reagent (Bio-Rad) according to the manufacturer’s protocol into NIH 3T3 cells, which were stably transfected by pCTV3H-dbs-HA6 or its cognate vector. Scrambled siRNA oligonucleotides (Santa Cruz, sc-37007) were also included in the analysis as negative controls. The medium was replaced 5 h after transfection, and cells were cultured in fresh medium for 2 h before they were used to perform a secondary focus formation assay. Secondary focus formation assays were performed as described previously (12). Briefly, 1 × 105 transfected cells were mixed with 1 × 106 untransfected NIH 3T3 cells and then plated on 60-mm-diameter dishes. Foci were scored after 7 days. Cells from parallel transfections were cultured in fresh medium overnight and then lysed and examined by Western blot analysis. In a parallel assay for growth inhibition, 1 × 105 transfected cells were plated on 60-mm-diameter dishes. Colonies were counted after 7 days.

Western Blot Analysis—Western blot analysis was performed as described (11). All cells were serum-starved for 18 h before lysis. The following antibodies were used for Western blots as indicated: mouse anti-HA (Berkeley Antibody Co., Inc.), rabbit anti-HA (HA-Y11, Santa Cruz), mouse anti-ROCK I (G-6, Santa Cruz), goat anti-ROCK II (C-20, Santa Cruz), mouse anti-PRK I (sc-660B, Transduction Laboratories), rabbit anti-PRK II (2612, Cell Signaling), rabbit anti-phospho-PRK I(Thr-774)/PRK II(Thr-816) (2611, Cell Signaling), mouse anti-ROCK I (C-2, Santa Cruz), mouse anti-HA (26C4, Santa Cruz), mouse anti-c-Myc (9E10, Santa Cruz), mouse anti-GST antibody (B-14, Santa Cruz), rabbit anti-MKK3 antibody (9232, Cell Signaling), rabbit anti-MLC2 antibody (FL-172, Santa Cruz), rabbit anti-phospho-MLC2 (3671, Cell Signaling), rabbit anti-cofilin (ACFL02, Cytoskeleton), and rabbit anti-phospho-cofilin (hSer3, Santa Cruz Biotechnology Inc.). Protein was visualized with enhanced chemiluminescence reagents (Amersham Biosciences).

Subcellular Fractionation Analysis—Fractional distributions were performed as described previously (11). Briefly, NIH 3T3 cells that stably express Dbs-HA6 or its cognate vector were serum-starved in Dulbecco’s modified Eagle’s medium supplemented with 0.5% bovine calf serum for 18 h. Cells were washed by cold phosphate-buffered saline twice and incubated on ice for 30 min with saline containing 10 mM Tris, pH 7.4, 1 mM MgCl2, and a protease inhibitor mixture (Calbiochem). Cells were homogenized and then centrifuged at 500 × g at 4 °C for 10 min. The supernatant was collected, supplemented with 1 mM NaCl to a final concentration of 150 mM, and centrifuged at 100,000 × g at 4 °C for
The protein concentrations of total, particulate, and supernatant fractions were determined by BCA protein assay kit (Pierce). Equal amounts of protein (40 μg) from each fraction were analyzed by SDS-polyacrylamide gel electrophoresis, then transferred to Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences) and analyzed by Western blot.

**ROCK I Kinase Assay**—The in vitro ROCK I kinase assay was performed as described previously (38). Briefly, NIH 3T3 cells that stably express Dbs-HA6 or cognate vector were transiently transfected with 5 μg pCAG-ROCK I. Lysates were collected at 48 h, clarified by centrifugation, and then immunoprecipitated with an agarose-conjugated anti-Myc antibody (9E10, Santa Cruz). Beads were washed 3 times with buffer containing 50 mM Tris, pH 7.5, 10 mM MgCl2, 50 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 0.03% Brij 35, and a protease inhibitor mixture (Calbiochem). The immunoprecipitate was then split into two parts, one of which was subjected to Western blot using an anti-Myc antibody (9E10, Santa Cruz) to confirm equal expression of Myc-tagged ROCK I. The second fraction was used in an in vitro kinase assay. Immunoprecipitates were washed twice in kinase assay buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 10 mM MgCl2, 50 mM NaCl, 1 mM dithiothreitol, and 0.03% Brij 35. Samples were then resuspended in 30 μl of kinase assay buffer containing 10 μM cold ATP, 50 μg/μl histone H1 (Calbiochem) and 135 μCi/μl [γ-32P]ATP (Amersham Biosciences). After a 30-min incubation at 30 °C, the reaction was terminated by the addition of Laemmli buffer. Samples were resolved by 10% SDS-polyacrylamide gel electrophoresis and subjected to autoradiography. Where indicated, cells were treated with 25 ng/ml recombinant Rat ROCK I. The second fraction was used in an in vitro kinase assay. Immuno-precipitates were washed twice in kinase assay buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 10 mM MgCl2, 50 mM NaCl, 1 mM dithiothreitol, and 0.03% Brij 35. Samples were then resuspended in 30 μl of kinase assay buffer containing 10 μM cold ATP, 50 μg/μl histone H1 (Calbiochem) and 135 μCi/μl [γ-32P]ATP (Amersham Biosciences). After a 30-min incubation at 30 °C, the reaction was terminated by the addition of Laemmli buffer. 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**Immunostaining**—Immunostaining of transiently transfected NIH 3T3 cells was performed as described previously (17). Briefly, NIH 3T3 cells were transiently transfected with 1 μg of pAX142-dbs-HA6 using Lipofectamine reagent (Invitrogen). Cells were replated on coverslips at low cell density 24 h post-transfection. Cells were serum-starved (0.5% FBS) for 24 h post-transfection. Cells were then permeabilized and blocked in 0.1% Triton X-100, 3% bovine serum albumin in phosphate-buffered saline for 30 min. Coverslips were then incubated with appropriate primary antibodies for 1 h in 0.1% Triton X-100 with 3% bovine serum albumin and then in secondary antibodies for 1 h in the dark in 0.1% Triton X-100 with 3% bovine serum albumin. Coverslips were washed in phosphate-buffered saline and mounted on glass slides using SlowFade Antifade kits (Molecular Probes). Cells were viewed with an Zeiss inverted microscope equipped with an ApoTome-based imaging system (Zeiss). Axial stacks were captured for each cell, and a single axial plane is shown. Images were analyzed using Axiosvision software. Immunostaining of F-actin was performed as described above except that cells were imaged using a Zeiss inverted microscope equipped with an ApoTome-based imaging system (Zeiss). Axial stacks were captured for each cell, and a single axial plane is shown. Images were analyzed using Axiosvision software. Immunostaining of F-actin was performed as described above except that cells were imaged using a Zeiss inverted microscope equipped with an ApoTome-based imaging system (Zeiss). Axial stacks were captured for each cell, and a single axial plane is shown. Images were analyzed using Axiosvision software. Immunostaining of F-actin was performed as described above except that cells were imaged using a Zeiss inverted microscope equipped with an ApoTome-based imaging system (Zeiss). Axial stacks were captured for each cell, and a single axial plane is shown. Images were analyzed using Axiosvision software. Immunostaining of F-actin was performed as described above except that cells were imaged using a Zeiss inverted microscope equipped with an ApoTome-based imaging system (Zeiss). Axial stacks were captured for each cell, and a single axial plane is shown. Images were analyzed using Axiosvision software. Immunostaining of F-actin was performed as described above except that cells were imaged using a Zeiss inverted microscope equipped with an ApoTome-based imaging system (Zeiss). Axial stacks were captured for each cell, and a single axial plane is shown. Images were analyzed using Axiosvision software. Immunostaining of F-actin was performed as described above except that cells were imaged using a Zeiss inverted microscope equipped with an ApoTome-based imaging system (Zeiss). Axial stacks were captured for each cell, and a single axial plane is shown. Images were analyzed using Axiosvision software.
NIH 3T3 cells express detectable levels of ROCK I, ROCK II, PRK I, and PRK II, and no changes in overall level of expression of these kinases are observed in response to transient expression of onco-Dbs and activated RhoA or stable expression of onco-Dbs (Fig. 2A). To determine whether Dbs causes activation of PRK I or PRK II, blots were stripped and reprobed with an antibody that detects the activated, phosphorylated form of both kinases. For both PRK I and PRK II, no differences were observed between vector, onco-Dbs, and RhoA(63L)-expressing cells, suggesting that Dbs does not activate either kinase in this cell type (Fig. 2A). In contrast, treatment of parental cells with the phosphatase inhibitor...
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Dbs activates ROCK I in a RhoA-dependent manner in NIH 3T3 cells. For this analysis cells that transiently express onco-Dbs were examined by indirect immunofluorescence to determine the cellular distribution of endogenous PRK I and ROCK II (Fig. 3). Similar to what we observed for ROCK I, we observed that PRK I was redistributed to the particulate fraction in Dbs-expressing cells, whereas PRK II was not.

Next we wished to more precisely characterize the translocation of ROCK I and PRK I in the Dbs-transformed cells. For this analysis cells that transiently express onco-Dbs were examined by indirect immunofluorescence to determine the cellular distribution of endogenous PRK I and ROCK II (Fig. 3). Similar to what we observed for ROCK I, we observed that PRK I was redistributed to the particulate fraction in Dbs-expressing cells, whereas PRK II was not.

Calcineurin A as a positive control for activation resulted in a dramatic increase in the levels of phosphorylated PRK I and PRK II (Fig. 2B).

Next we wished to know whether Dbs can activate ROCK I in the same cells. Unfortunately no antibodies are currently available that can be used to detect the activated form of ROCK I. Thus, we performed an in vitro kinase assay using exogenously expressed, Myc-tagged ROCK I that was immunoprecipitated from cell lines that stably express onco-Dbs (Fig. 2C). As a positive control for ROCK I activation, onco-Dbs-expressing cells were also treated with tumor necrosis factor-α. When compared with vector-expressing cells, ROCK I was immunoprecipitated from Dbs-expressing cells, consistently showed a 3-fold increase in levels of trans- and autophosphorylation, and the level of activation was further increased (5–6-fold) when cells were treated with tumor necrosis factor-α. When we performed an equivalent in vitro kinase assay using exogenously expressed, Myc-tagged PRK I (Fig. 2D), no difference was observed between the vector and Dbs-expressing cells (compare lanes 3 and 4). In contrast, treatment with the PRK I activator, arachidonic acid (40), resulted in a 3-fold increase in activity (compare lanes 1 and 2). These results suggest that ROCK I, but not PRK I, is activated in Dbs-transformed cells.

ROCK I and PRK I Are Translocated to the Plasma Membrane in Dbs-Transformed Cells—It has been shown previously that endogenous ROCK I is translocated from the cytosol to the plasma membrane in response to expression of activated RhoA (43). If oncogenic Dbs activates ROCK I in a RhoA-dependent manner in NIH 3T3 cells, we reasoned that this may be reflected in a similar translocation event. To test this possibility we stably expressed onco-Dbs in NIH 3T3 cells and then performed cell fractionations (Fig. 3). Although we were able to detect ROCK I expression in both the particulate and soluble fractions (Fig. 3A), there was a substantial increase in the amount of ROCK I in the particulate fraction in the Dbs-expressing cells relative to the vector controls (Fig. 3B). The localization of ROCK I coincided with the distribution of onco-Dbs, which was also found predominantly in the particulate fraction (Fig. 3A, lower panel). The overall distribution of ROCK II was not substantially changed in response to Dbs expression (Fig. 3B).

To further examine the possibility that Dbs may modify PRK activity, the equivalent blots were then examined for PRK I and PRK II distributions (Fig. 3). Similar to what we observed for ROCK I, we observed that PRK I was redistributed to the particulate fraction in Dbs-expressing cells, whereas PRK II was not.

Next we wished to more precisely characterize the translocation of ROCK I and PRK I in the Dbs-transformed cells. For this analysis cells that transiently express onco-Dbs were examined by indirect immunofluorescence to determine the cellular distribution of endogenous PRK I and ROCK II (Fig. 4). ROCK II was included in this analysis as an additional control. siRNAs targeted against each of the kinases were used to demonstrate the specificity of the antibodies (lower panels). Whereas endogenous ROCK I and PRK I exhibited dispersed punctate staining throughout the cytosol in control cells, in Dbs-expressing cells a discrete fraction of the ROCK I and PRK I immunoreactivity was observed at the cell periphery. An equivalent accumulation at the plasma membrane was not observed for ROCK II. These observations are consistent with the fractionation studies and suggest that the accumulation of PRK I and ROCK I in the particulate fraction can be partially explained by their translocation onto the plasma membrane.
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Dbs Transformation in NIH 3T3 Cells Requires ROCK-mediated Signaling—Because the results of our kinase and cellular localization studies suggest that natively expressed ROCK I may be activated in Dbs-transformed cells, we wondered whether ROCK activation is necessary for Dbs transformation. For this analysis we utilized the Y27632 inhibitor, which has been shown previously to be a selective inhibitor of ROCK I and ROCK II. Cells were transiently transfected with either cognate vector or onco-Dbs, and then primary focus formation assays were performed in the presence or absence of the inhibitor. As an additional control, the inhibitor was also tested on an unrelated oncogene (Src) that has potent focus-forming activity in this cell type. Previous studies have shown that Src transformation in NIH 3T3 cells is independent of ROCK (42). In the presence of the inhibitor, onco-Dbs-transforming activity was reduced to background levels, whereas the inhibitor had no effect on transformation by Src (Fig. 5A). We then confirmed that the inhibition could not be attributed to an effect on overall expression levels of ROCK I, ROCK II, PRK I, PRK II, or onco-Dbs (Fig. 5B). The inhibition could also not be attributed to a nonspecific inhibition of PRK I and PRK II activity since the levels of phospho-PRK I and phospho-PRK II also did not change.

To confirm the dependence of Dbs transformation on ROCK I signaling, we utilized a kinase-dead dominant-inhibitory version of ROCK I. When we co-expressed this mutant with onco-Dbs in NIH 3T3 cells, we observed a dose-dependent inhibition of Dbs transformation (Fig. 5C). In contrast, the mutant had no effect on Src transformation, again confirming that Src and Dbs transform cells through distinct mechanisms.

Additional Members of the RhoGEF Family Transform NIH 3T3 Cells through a ROCK-dependent Manner—Because many members of the RhoGEF family were isolated based on their transforming activity in NIH 3T3 cells, we wondered whether they transform this cell type through an equivalent mechanism. We have previously described a panel of transforming derivatives of RhoGEF family members that includes Ect2, Lfc, and Lsc (34). NIH 3T3 cells were transiently transfected with all members of the panel, and then focus formation assays were performed in the presence or absence of the inhibitor (Fig. 5A). Similar to what we observed for Dbs, all members of the panel exhibited greatly reduced transforming activity when tested in the presence of the inhibitor. When viewed in the context of previous studies (31, 44), these results strongly suggest that there is common ROCK-dependent mechanism through which RhoGEF family members transform NIH 3T3 cells.

Dbs Transformation Does Not Require PRK-mediated Signaling—To further examine the possible contribution of PRK-mediated signaling to Dbs transformation, we also determined whether expression of dominant-inhibitory versions of MKK3 and MKK6 could block Dbs transformation (Fig. 5D). Both of these kinases are downstream of PRK, and it has been shown previously that both of these mutants can block transformation by constitutively activated RhoA. Although a dominant inhibitory version of RhoA effectively blocked Dbs transformation, no effect was observed with the dominant-inhibitory MKK3 and MKK6 despite the fact that they were expressed at significant levels (Fig. 5D, lower panels). We conclude that the PRK I-mediated signaling pathway that has been previously implicated in RhoA transformation does not contribute to Dbs transformation in NIH 3T3 cells.

siRNA Targeted against ROCK I, but Not ROCK II, Blocks Dbs Transformation—To confirm a specific role for ROCK I in Dbs transformation, siRNAs were used to target ROCK I, ROCK II, and PRK I in Dbs-transformed cells (Fig. 6, A and B). For this analysis cells were transiently transfected with siRNAs and then examined in secondary focus formation (Fig. 6C) and clonogenicity (Fig. 6D) assays. Although siRNAs targeted against ROCK I consistently inhibited Dbs transformation by ~45% (Fig. 6C), we observed that ROCK II expression was also suppressed in these cells (Fig. 6B). However, siRNAs targeted against PRK I and ROCK II were much more specific in their action and had no effect on Dbs transformation. The reduced transforming activity associated with the ROCK I siRNAs could not be attributed to nonspecific growth inhibition as demonstrated by the clonogenicity assays (Fig. 6D). These results are consistent with genetic and pharmacological inhibitors and suggest that endogenous ROCK I, but not ROCK II or PRK I, is required for Dbs transformation.

Dbs Transformation Requires ROCK-mediated Phosphorylation of MLC—Because onco-Dbs causes profound changes in the actin cytoskeleton including the formation of stress fibers, we wondered whether targets of ROCK that regulate actino-myosin contraction contribute to transformation. Initially we measured the levels of phosphorylated cofilin and MLC2 in Dbs-transformed cells (Fig. 7A). Whereas we observed a 3-fold increase in phosphorylated MLC2 in Dbs-trans-
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FIGURE 6. Suppression of ROCK I blocks Dbs transformation. NIH 3T3 cells that stably express onco-Dbs (Dbs) or cognate vector (vector) were examined by Western blot for expression of the indicated proteins. Cells treated with the ROCK inhibitor were also included in the analysis. p-MLC and p-cofilin indicate activated phosphorylated forms of the proteins. Antibodies are as described under "Materials and Methods." A, NIH 3T3 cells that stably express onco-Dbs were transiently transfected with control siRNAs or with siRNAs that target MLC2. Relative levels of protein expression were determined by Western blot (upper blot) and then quantified using an Odyssey IR imager (lower bar graph). Integrated intensity units were determined and then expressed as a percentage of siRNA control levels. Data shown are an average of three independent experiments and show S.D. Cells transfected in parallel experiments were tested in assays for secondary focus formation (C) or for clonogenicity (D). Data shown are an average of three independent experiments and show S.D.

FIGURE 7. Dbs transformation requires ROCK-mediated phosphorylation of MLC. A, NIH 3T3 cells that stably express onco-Dbs (Dbs) or cognate vector (vector) were examined by Western blot for expression of the indicated proteins. Cells treated with the ROCK inhibitor were also included in the analysis. B, NIH 3T3 cells that stably express onco-Dbs were transiently transfected with control siRNAs or with siRNAs that target MLC2. Relative levels of protein expression were determined by Western blot (upper blot) and then quantified using an Odyssey IR imager (lower bar graph). Integrated intensity units were determined and then expressed as a percentage of siRNA control levels. Data shown are an average of three independent experiments and show S.D. SiCtrl control siRNA. Cells transfected in parallel experiments were tested in assays for secondary focus formation (C) or for clonogenicity (D). Data shown are an average of three independent experiments and show S.D.

formed cells relative to vector controls, no differences in activated cofilin were noted. Treatment of Dbs-transformed cells with the ROCK inhibitor reduced the level of MLC2 phosphorylation to basal levels (Fig. 7A) and disrupted the formation of actin stress fibers in Dbs-transformed cells (Fig. 7E). Interestingly, the accumulation or cortical actin that is associated with Dbs expression was not affected by the inhibitor, suggesting that Dbs can also modify the actin cytoskeleton through ROCK-independent events. To determine whether phosphorylation of MLC2 is required for Dbs transformation, Dbs-transformed cells were transfected with siRNAs targeted at MLC2 (Fig. 7B) and then assessed in a secondary focus formation assay (Fig. 7C). A reduction in transforming activity by about 40% was observed in the MLC suppressed cells, which is consistent with what we observed for siRNAs targeted against ROCK I in the equivalent assay (see Fig. 6). Successful silencing of MLC was demonstrated by Western blot using lysates derived from parallel transfections (Fig. 7B), and the reduced transforming activity associated with the MLC2 siRNAs could not be attributed to nonspecific growth inhibition (Fig. 7D). These results suggest that Dbs causes phosphorylation of MLC in a ROCK-dependent manner and suggests that actin-myosin contraction is required to support Dbs transformation.

DISCUSSION

Although members of the RhoGEF family are well known for their transforming activity in NIH 3T3 cells, the identification of the molecular mechanisms that underlie this activity has remained elusive. In recent studies (including the current one), we have identified RhoA as a relevant physiological target for Dbs transformation in this cell type (12, 18). We have shown previously that onco-Dbs mutants that cannot activate Cdc42 retain full transforming activity (18), whereas in this current study we showed that a mutant that is selectively impaired in RhoA activity shows reduced transformation. Because Dbs transformation is blocked by RhoA inhibitors and activated mutants of RhoA produce foci that are phenotypically indistinguishable from Dbs foci, a role for RhoA in Dbs transformation seems apparent. Based on these observations we have attempted to identify RhoA effector proteins that may contribute to Dbs transformation. Although many of such proteins have been described, two recent studies suggest that pathways that are regulated by ROCK I and PRK I are essential for RhoA transforming activity (33, 42). Thus, we chose to focus our attention on these two kinases.

PRK I is a lipid activated protein serine/threonine kinase that was originally identified as a binding partner for activated RhoA. Although the native function of PRK I is unknown, a recent study has implicated PRK I in RhoA transformation in NIH 3T3 cells (33). PRK I was shown to activate p38 and members of the MKK3- and MKK6-dependent mechanisms, and dominant-inhibitory mutants of MKK3 and MKK6 were effective in blocking RhoA transformation. However, despite the fact that we are able to detect PRK I and PRK II expression in Dbs-transformed cells, we found no evidence of elevated kinase activity and no evidence that the integrity of PRK-mediated signaling pathways is required to support Dbs transformation. Thus, although Dbs transforms NIH 3T3 cells in a RhoA-dependent manner, the mechanism of transformation by onco-Dbs and the constitutively activated RhoA (63L) mutant may be mechanistically distinct. Because constitutively activated RhoA mutants have a much weaker transforming activity than onco-Dbs, these mutants may not mimic activation of the endogenous GTPase by
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Dbs and, thus, may utilize a distinct roster of signaling pathways to mediate transformation. Unlike PRK I, the evidence linking Dbs transformation to ROCK I activation seems clearer. An elevated level of ROCK I kinase activity was observed in Dbs-transformed cells, and both genetic and pharmacological inhibitors of ROCK block Dbs focus-forming activity. Previous studies that utilized the same ROCK inhibitor have implicated ROCK signaling as necessary for transformation by both RhoA (42) and several members of the RhoGEF family (Tim, Dbl, and Net1) (31, 44). In these studies however, it was unclear whether RhoGEFs directly activate ROCK to support transformation or whether endogenous ROCK I simply cooperates with RhoGEFs in transformation. In this current study we have found evidence to suggest that ROCK I is activated by onco-Dbs expression. Endogenous ROCK I is redistributed to the plasma membrane in response to Dbs expression, and Dbs can strongly activate ectopically expressed ROCK I.

Interestingly, Dbs had no discernible effect on the cellular distribution of ROCK II despite the fact that it is expressed at detectable levels in these cells. In addition, siRNAs targeted at ROCK I blocked Dbs transformation, whereas those targeted at ROCK II did not. Thus, Dbs-mediated activation of RhoA appears to be associated with the recruitment of only a subset of the available RhoA effectors in this cell type. This would be consistent with previous studies which suggest that Rho effectors can also serve as scaffolding proteins that mediate the association of GTPases with specific RhoGEFs (45, 46).

This study expands the roster of members of the RhoGEF family that transform NIH 3T3 cells through a ROCK-dependent mechanism and suggests that RhoA may be a common target of transformation in this cell type. Interestingly, despite the fact that the foci that are induced by RhoGEFs and Src are virtually indistinguishable, both we and others see no effect of the ROCK inhibitor on Src transformation (42). Thus, if Src and RhoA transform NIH 3T3 cells through equivalent mechanisms, this would suggest that Src acts downstream of ROCK I activity in this pathway. Because activated ROCK I has no focus-forming activity when expressed alone in these cells, it is likely that additional pathways that are commonly activated by both RhoGEFs and Src are also necessary for the full transformed phenotype. In support of this we have observed that the ability of Dbs to stimulate cortical actin or to activate the SRE family, whereas those targeted at ROCK II did not. Thus, Dbs-mediated ROCK II targeted at ROCK II did not. Thus, Dbs-mediated activation of RhoA appears to be associated with the recruitment of only a subset of the available RhoA effectors in this cell type. This would be consistent with previous studies which suggest that Rho effectors can also serve as scaffolding proteins that mediate the association of GTPases with specific RhoGEFs (45, 46).

The research of ROCK substrates in the context of Dbs transformation suggests an important role for MLC. We observed an elevated level of phosphorylated MLC in Dbs-transformed cells that was effectively inhibited focus formation by Dbs, which implies a direct role for actinomyosin contraction in the transformation phenotype. The phosphorylation of MLC causes the bundling of filamentous actin into stress fibers (47, 48), and ROCK has been shown to stimulate actinomyosin contractility by directly phosphorylating MLC (49). We have shown previously that cells stably express onco-Dbs are more rounded and exhibit elevated actin stress fibers and a dense ring of cortical actin (41). Interestingly, we observed in this current study that the ROCK inhibitor blocks the formation of stress fibers, which is consistent with the down-regulation of MLC but has little effect on the accumulation of cortical actin. This suggests that Dbs can activate changes in the actin cytoskeleton that are independent of ROCK but that such changes are dispensable for transformation.

Although it has been reported that transformation by the RhoGEF family members Dbl and Net1 is blocked by genetic inhibitors of ezrin (31), we were unable to see any evidence of increased ezrin phosphorylation in Dbs-transformed cells5 nor were we able to see changes in phosphorylation status of cofilin. Thus, although it is possible that Dbs transformation is dependent on the activation status or integrity of these actin regulatory proteins, they do not appear to be targets for ROCK I in the context of Dbs transformation.

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