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Requirement For C-terminal Sequences in Regulation of Ect2 Guanine Nucleotide Exchange Specificity and Transformation*

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Ect2 was identified originally as a transforming protein and a member of the Dbl family of Rho guanine nucleotide exchange factors (GEFs). Like all Dbl family proteins, Ect2 contains a tandem Dbl homology (DH) and pleckstrin homology (PH) domain structure. Previous studies demonstrated that N-terminal deletion of sequences upstream of the DH domain created a constitutively activated, transforming variant of Ect2 (designated ΔN-Ect2 DH/PH/C), indicating that the N terminus served as a negative regulator of DH domain function *in vivo*. The role of sequences C-terminal to the DH domain has not been established. Therefore, we assessed the consequences of mutation of C-terminal sequences on Ect2-transforming activity. Surprisingly, in contrast to observations with other Dbl family proteins, we found that mutation of the invariant tryptophan residue in the PH domain did not impair ΔN-Ect2 DH/PH/C transforming activity. Furthermore, although the sequences C-terminal to the PH domain lack any known functional domains or motifs, deletion of these sequences (ΔN-Ect2 DH/PH) resulted in a dramatic reduction in transforming activity. Whereas ΔN-Ect2 caused formation of lamellipodia, ΔN-Ect2 DH/PH enhanced actin stress fiber formation, suggesting that C-terminal sequences influenced Ect2 Rho GTPase specificity. Consistent with this possibility, we determined that ΔN-Ect2 DH/PH activated RhoA, but not Rac1 or Cdc42, whereas ΔN-Ect2 DH/PH/C activated all three Rho GTPases *in vivo*. Taken together, these observations suggest that regions of Ect2 C-terminal to the DH domain alter the profile of Rho GTPases activated *in vivo* and consequently may contribute to the enhanced transforming activity of ΔN-Ect2 DH/PH/C.

Ect2, identified initially in an expression screen to search for novel oncogene proteins expressed in epithelial cells, is a member of the Dbl family of proteins (1). Dbl family proteins act as guanine nucleotide exchange factors (GEFs)[1] for specific members of the Rho family of small GTPases (2, 3). Like Ras, Rho family proteins function as GDP/GTP-regulated molecular switches that cycle between inactive GDP-bound and active GTP-bound states. Dbl family proteins stimulate GDP/GTP exchange to promote formation of Rho-GTP, and the aberrant expression of Dbl family proteins promotes growth transformation by causing persistent activation of Rho GTPases. Rho GTPase activation in turn regulates a wide range of signaling pathways that regulate cell cycle progression, gene expression, actin cytoskeletal organization, and many other cellular processes (4–6).

Ect2 and essentially all Dbl family members possess a Dbl homology (DH) domain followed by a C-terminal pleckstrin homology (PH) domain (2, 3). The DH domain encodes the GEF catalytic activity of these proteins. Some Dbl family members show narrow substrate specificity and activate only specific Rho GTPases, whereas others are more promiscuous and can stimulate exchange on multiple Rho GTPases. The specificity of Ect2 has not been fully resolved. One study demonstrated no activation of RhoA, Rac1, or Cdc42 by Ect2 (1), whereas another found that Ect2 could stimulate exchange on RhoA, Rac1, and Cdc42 *in vitro* (7). In contrast, analyses of Pebble, the *Drosophila* homolog of Ect2 (8), indicated that it is an activator of Rho1, but not of Rac or Cdc42. However, analyses of Ect2-mediated changes in actin organization in mammalian cells suggested that Ect2 did not simply activate RhoA alone (9). The basis for these different observations is unclear, and which Rho GTPases are targets for Ect2 remain to be clarified.

Although PH domains are also found in a variety of non-GEF signaling proteins (10, 11), the invariant, tandem DH/PH domain topography of Dbl family GEFs suggests a critical involvement of the PH domain in DH domain function. Detailed structural, biochemical, and biological analyses have verified the critical role of the PH domain in DH domain function, although the specific role and involvement can vary significantly among different Dbl family proteins. When evaluated, the PH domain has been found to be indispensable for DH domain function (12–18). One exception is Tiam1, where the DH domain-associated PH domain was found to be dispensable for function, and instead, a second PH domain was found to be essential for membrane association and activity (19). Functions ascribed to the PH domain include serving as a membrane-targeting sequence, and the regulation of the intrinsic catalytic activity of the DH domain. Additionally, PH domains can bind phospholipids and facilitate DH domain activation by phospholipid products of phosphatidylinositol 3-kinase (20, 21). The role of the PH domain in Ect2 function has not been determined.

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[1] The abbreviations used are: GEFs, guanine nucleotide exchange factors; DH, Dbl homology; PH, pleckstrin homology; SRF, serum response factor; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione *S*-transferase; HA, hemagglutinin; PBS, phosphate-buffered saline; RBD, Rho binding domain; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate.

Dbl family proteins diverge significantly in the sequences that flank the DH/PH domains. Despite this significant sequence divergence, a common feature of Dbl family proteins is that N-terminal sequences upstream of the DH/PH domains function as negative regulators of DH domain function. Hence, deletion of N-terminal sequences causes the constitutive activation of Ect2 and other Dbl family proteins (*e.g.* Vav, Dbl, Tiam1) (2, 3). Ect2 also contains sequences C-terminal to the DH/PH domain, although no sequence relationship to known functional protein domains or motifs, or to other proteins, have been identified in these sequences. However, our previous analyses suggested that these C-terminal sequences are important for Ect2 transforming activity *in vivo* (9). In this study, we evaluated the contribution of these C-terminal sequences to Ect2 function. We found unexpectedly that the PH domain was dispensable for Ect2 transforming activity. In contrast, sequences C-terminal to the DH/PH domains were critical for full Ect2 transforming activity and altered the Rho GTPase specificity of Ect2 *in vivo*.

EXPERIMENTAL PROCEDURES

Molecular Constructs—To generate mammalian expression constructs that encode C-terminal variants of activated ΔN-Ect2 (residues 415–882, amino acid numbers derived from GenBank™ accession no. AAH23881), the pCTV3 HA3-*ect*2 (9) plasmid DNA was used as a template for polymerase chain reaction (PCR)-mediated mutagenesis. The cDNA fragments encoding ΔN-Ect2 DH/PH/C and two C-terminal truncation mutants were generated by PCR to modify the 5′- and 3′-ends to include BamHI restriction sites at each end. These fragments encode the DH domain of Ect2 alone (residues 415–622, designated ΔN-Ect2 DH), the DH and PH domains of Ect2 (residues 415–775; designated ΔN-Ect2 DH/PH), and the DH/PH domains along with the final 107 amino acids of the C terminus of Ect2 (designated ΔN-Ect2) (Fig. 1). The oligonucleotide 5′-AAAGGATCCATGGTTCCTCCAAAGCAGTCA-3′ was used as a forward primer for all three constructs. For the *ect*2 DH construct, the oligonucleotide 5′-TTTGGATCCTTACTCGAGATCAAAAATTTGCTTCTG-3′ was used as the reverse primer. For the *ect*2 DH/PH construct, the oligonucleotide 5′-TTTGGATCCTTAGTCGACCACATACATAAGATTCTC-3′ was used as the reverse primer. For the *ect*2 DH/PH/C construct, the oligonucleotide 5′-TTTGGATCCTTAGTCGACTATCAAGTGAGTTGTAGA-3′ was used as the reverse primer. The QuikChange mutagenesis kit (Stratagene) was used in accordance with the manufacturer's protocol to introduce a missense mutation into the PH domain of ΔN-Ect2, resulting in a change of the invariant Trp present in essentially all Dbl family PH domains to Leu (W752L). The primers used were 5′-CCAAAGGAGAGCTTGCTGAAGATGCTGTGC-3′ and its reverse complement 5′-GCACAGCATCTTCAGCAAGCTCTCCTTTGG-3′. The PCR-amplified fragments were then digested with BamHI and ligated into the BamHI site of the mammalian expression vector pCGN-hygro (gift from Michael Ostrowski, Ohio State University) (22). This vector provides for the attachment of a hemagglutinin (HA) epitope tag to the N terminus of the Ect2 mutants. All constructs were sequence verified.

Guanine Nucleotide Exchange Analyses—To generate expression vectors for expression and preparation of bacterially expressed fragments of the Ect2 DH (residues 415–622) and DH/PH (residues 415–775) domains, the corresponding fragments of Ect2 were subcloned into the NcoI and XhoI sites of pET28a. The resulting protein products contain a C-terminal His_6 tag. Ect2 proteins were expressed in the *Escherichia coli* strain BL21(DE3) for 5 h at 25 °C and purified on a nickel-charged metal chelating column (Amersham Biosciences). Proteins were further purified on an S200 size exclusion column (Amersham Biosciences) equilibrated in a buffer containing 50 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, and 5% glycerol. Expression and purification of Vav2, Dbs, and GST-tagged Rho GTPases have been described previously (23–25).

Guanine nucleotide exchange assays were conducted as described previously (23–25). Briefly, fluorescence spectroscopic analysis of *N*-methylanthraniloyl (mant)-GDP incorporation into GDP-preloaded GST-Rac1, GST-Cdc42 or GST-RhoA was carried out using a PerkinElmer LS 50B spectrometer at 25 °C. Exchange reaction assay mixtures containing 20 mM Tris, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/μl bovine serum albumin, 1% glycerol, 400 nM mant-GDP (Molecular Probes), and 2 μM GTPase was prepared and allowed to equilibrate with continuous stirring. After equilibration, the GEFs were added at the indicated concentrations, and the relative mant fluorescence (λ_{ex} = 360 nm, λ_{em} = 440 nm) was monitored. Experiments were performed in duplicate.

Cell Culture and Transformation Assays—NIH3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (Hyclone). Human embryonic kidney 293T cells were maintained in DMEM supplemented with 10% fetal calf serum (Invitrogen). For focus formation assays, NIH3T3 cells were transfected with 500 ng of the pCGN-*ect*2 mutant plasmid DNA or pCGN-hygro empty vector by calcium phosphate precipitation, followed by a glycerol shock as described previously (26). 14-days post-transfection, the dishes were stained with crystal violet, and the appearance of foci of transformed cells was quantified by visual inspection.

To establish cell lines stably expressing mutant Ect2 protein, NIH3T3 cells were transfected as above. 3-days post-transfection cells were subcultured at a 1:3 split ratio into growth medium supplemented with 400 μg/ml of hygromycin B, and surviving colonies (>50) were pooled for future use. Western blot analyses with anti-HA antibody (Covance) were done to verify protein expression in the established cell lines.

For evaluation of protein expression, NIH3T3 cells were transiently transfected as described. 48-h post-transfection, the cells were lysed in protein sample buffer (27) and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis with anti-HA antibody.

Transient Expression Reporter Gene Assays—For the transcriptional transactivation assay, NIH3T3 cells were transfected by calcium phosphate co-precipitation as described above. 500 ng of the pCGN-hygro empty vector or of pCGN encoding Ect2 sequences were co-transfected together with 2 μg of the luciferase reporter plasmid SRE-Luc (28), where expression is controlled by a minimal promoter from the c-*fos* gene that contains multiple serum response factor-responsive DNA elements. 24 h after transfection, the growth medium was replaced with DMEM supplemented with 0.5% fetal calf serum. After 16–20 h, the cells were lysed and then analyzed for luciferase activity on an enhanced chemiluminescence substrate (BD Biosciences Pharmingen) in a Monolight 2010 luminometer (Analytical Luminescence).

Immunofluorescence and Subcellular Localization Analyses—NIH3T3 cells were used to determine the subcellular location of Ect2 mutants as previously described (29). Briefly, cells that had been plated onto coverslips were transiently transfected (LipofectAMINE-Plus, Invitrogen) with the pCGN-hygro vectors expressing the Ect2 proteins. 5-h post-transfection, the medium was replaced with fresh DMEM containing 0.5% calf serum. 24-h post-transfection, the coverslips were washed twice with phosphate-buffered saline (PBS). The cells were then fixed in 3.7% formaldehyde in PBS for 10 min and incubated for 30 min in PBS supplemented with 0.1% Triton X-100 and 5 mg of bovine serum albumin per ml. The coverslips were incubated with anti-HA antibody, followed by a fluorescein 5′-isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch Laboratories) to visualize HA epitope-tagged Ect2 proteins, or with tetrarhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma) to visualize actin. Images were then collected using an Axioskop 2 fluorescence microscope (Zeiss) and Openlab digital imaging software (Improvision).

Membrane Fractionation Analyses—Subcellular crude fractionations were performed as described previously (27). Briefly, NIH3T3 cells stably expressing each HA epitope-tagged Ect2 mutant protein were washed twice with PBS and resuspended in a hypotonic buffer (10 mM Tris, pH 7.4, 1 mM $MgCl_2$, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) to swell on ice. The cell suspensions were then subjected to Dounce homogenization and separated into cytosolic (S100) and particulate (P100) fractions by ultracentrifugation at 100,000 × *g* in a Beckman TLS-55 rotor. The isolated fractions were incubated with acetone to precipitate the proteins, which were then resolved by SDS-PAGE. Western blot analysis with an anti-HA epitope antibody was used to visualize Ect2 proteins.

Rho Activation Assay—This procedure is a modification of a previously described assay for Ras activation (30). Briefly, pGEX constructs (Amersham Biosciences) encoding GST fusion proteins of the Rho binding domain (RBD) of either Rhotekin (amino acids 7–89) (provided by Keith Burridge, University of North Carolina at Chapel Hill) (31) or PAK (amino acids 70–132) (provided by Wang Lu and Bruce Mayer, Harvard University) were expressed in bacteria, and the proteins were then purified from clarified bacterial lysates by coupling to glutathione-Sepharose 4B beads (Amersham Biosciences). The 293T human embryonic kidney epithelial cell line was transiently transfected with the pCGN-*ect*2 constructs using LipofectAMINE Plus according to the manufacturer's protocol. Immediately post-transfection, cells were placed in

medium supplemented with 0.1% serum and incubated for an additional 20 h. The GTP-bound RhoA protein was affinity-precipitated from cell lysates as described previously (31). Briefly, the cells were lysed in lysis buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, 0.1% SDS, 0.5% sodium deoxycholate, 1.0% Triton X-100, and protease inhibitors). Lysates were clarified by centrifugation at 16,000 × *g* for 10 min. 30 micrograms of GST-Rhotekin-RBD or GST-PAK-RBD immobilized on glutathione-Sepharose 4B beads were incubated with 1 mg of clarified 293T cell lysates for 30 min at 4 °C. The beads were washed three times in wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1.0% Triton X-100, and protease inhibitors). Total and precipitated lysate samples were subjected to SDS-PAGE and analyzed by Western blot using anti-RhoA (Santa Cruz Biotechnology), anti-Rac1 (Upstate Biotechnology), and anti-Cdc42 (Transduction Laboratories) antibodies directed against the appropriate GTPase.

In Vitro Rho GTPase Binding Analyses—pGEX 4T-1 constructs for expression of GST-tagged fusion proteins of G15/17A dominant negative mutants of human Cdc42, Rac1, and RhoA were used as described previously (32). Briefly, GST or GST-tagged versions of nucleotide-free Rho proteins (G15A mutation for Rac1 and Cdc42, and G17A mutation for RhoA) were expressed in bacteria, which were lysed in lysis buffer (150 mM NaCl, 20 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1.0% Triton X-100, 1 mM dithiothreitol, and protease inhibitors) and subjected to sonication. Lysates were cleared by centrifugation, incubated with glutathione-Sepharose 4B beads (Amersham Biosciences), and washed three times in lysis buffer. Beads were stored as a 50% slurry in HEPES-buffered saline (HBS) containing 5 mM $MgCl_2$/glycerol (2:1). NIH3T3 cells were transiently transfected with pCGN-ΔN-*ect*2 constructs by using LipofectAMINE Plus as described above. 20-h post-transfection, cells were lysed in lysis buffer, cleared by centrifugation, and incubated with GST-Rho proteins bound to Sepharose beads for 30 min. The beads were washed three times with lysis buffer and the bound material was subjected to SDS-PAGE and Western blot analyses with anti-HA antibody.

RESULTS

The C Terminus, but Not the PH Domain, Is Required for Ect2 Function in Vivo but Not in Vitro—Our previous analyses suggested that sequences C terminus to the DH/PH domains of N-terminally activated Ect2 were important for Ect2 transforming activity (9). However, we found that the isolated fragment of the DH/PH domains of Ect2 alone (Fig. 1) was sufficient for robust GEF activity on RhoA, but not on Rac1 or Cdc42, *in vitro* (Fig. 2). This GEF activity was equivalent to that seen for the isolated DH/PH domain of Dbs, and the Dbs DH/PH fragment is both fully active and highly transforming *in vivo* (33). Additionally, we observed previously that Ect2 caused formation of lamellipodia *in vivo*, indicating activation of Rac rather than of RhoA (9). This apparent discrepancy between our observations of Ect2 *in vitro* GEF activity on RhoA and *in vivo* actin reorganization suggesting activation of Rac prompted our interest in further evaluating the role of the Ect2 C terminus in regulation of DH domain GEF activity and specificity.

Since the boundaries of the original bacterially expressed DH/PH domain fragment were not identical to those of the poorly transforming Ect2 DH/PH domain fragment that we analyzed previously in mammalian cells (9), we generated a mammalian expression vector that encoded the same additional C-terminal sequences (designated ΔN-Ect2 DH/PH). We then compared its transforming activity directly with that of the equivalent construct that includes the remaining 107-residue C-terminal sequence of Ect2 (ΔN-Ect2 DH/PH/C) (Fig. 1). To evaluate a role for the PH domain in Ect2 function, we also generated an Ect2 variant that encodes the isolated DH domain of Ect2 (ΔN-Ect2 DH). All variants of ΔN-Ect2 were expressed as fusion proteins that contain an N-terminal HA epitope tag to monitor expression of the different Ect2 sequences.

We assessed the transforming activity of the different Ect2 variants using the NIH3T3 focus formation transformation assay. Similar to what we described previously, the N-terminally truncated ΔN-Ect2 DH/PH/C protein that includes the C-terminal sequences displayed potent transforming activity

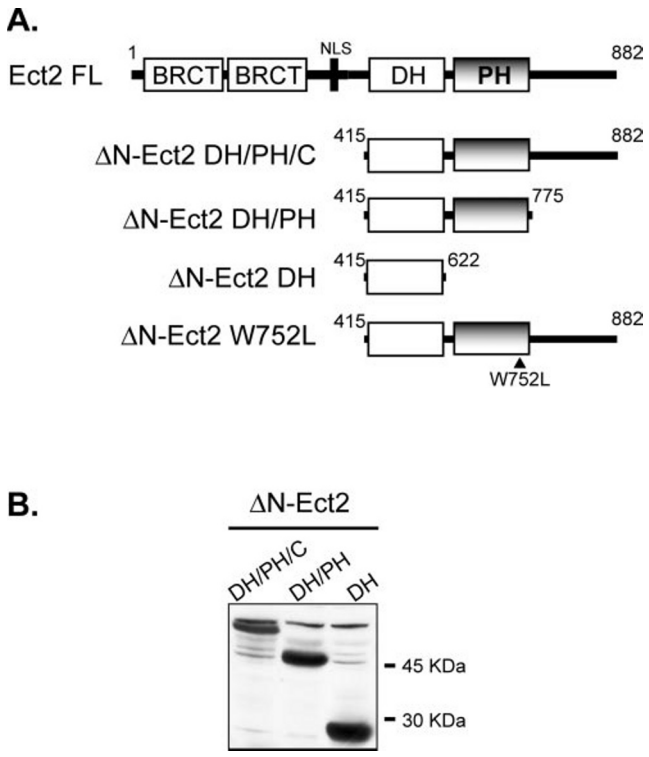

FIG. 1. **Domain structure and protein expression levels of Ect2 mutants.** *A*, the domain structure of full-length and truncation mutants of mouse Ect2. *BRCT* (BRCA1 C terminus domains), *DH* domain, *PH* domain, and *NLS* (nuclear localization signal) are shown. The N-terminal-truncated and constitutively activated Ect2 variant (DH/PH/C) was used to generate three C-terminal mutants that lacked sequences carboxyl to the PH domain (ΔN-Ect2 DH/PH), lacked both the PH domain and flanking C-terminal sequences (ΔN-Ect2 DH) sequences, or contained a missense mutation in the conserved Trp residue of the PH domain (ΔN-Ect2 DH/PH/C W752L). All ΔN-Ect2 variant cDNA sequences were fused in-frame to an N-terminal sequence encoding an HA epitope tag. *B*, NIH3T3 cells were transiently transfected with pCGN-hygro expression plasmids encoding each Ect2 variant. Cell lysates were normalized for total protein (BCA protein assay kit, Pierce). Equivalent amounts of total protein were resolved by SDS-PAGE, and Western blot analyses were done using an anti-HA epitope antibody. Data shown are representative of three independent experiments.

(~45 foci per dish), whereas ΔN-Ect2 DH/PH and ΔN-Ect2DH proteins that lack these sequences showed little to no focus-forming activity (Fig. 3, *A* and *B*). We then determined whether the reduced transforming activity of the DH or DH/PH fragments of Ect2 was due to impaired signaling activity. For these analyses, NIH3T3 cells were transiently co-transfected with Ect2 expression constructs together with a reporter plasmid where expression of the luciferase gene is regulated by a minimal promoter that contains a serum response factor (SRF)-responsive element. We found that ΔN-Ect2 DH/PH/C caused a 7-fold stimulation of SRF activity, whereas the activity of ΔN-Ect2 DH/PH was reduced to 3-fold stimulation (Fig. 3*C*). These results indicate that the reduced transforming activity was due, in part, to reduced signaling activity. Thus, in contrast to some Dbl family proteins (*e.g.* Dbs) where the isolated DH/PH domains alone are sufficient for potent signaling and transforming activity *in vivo* (33), the C terminus is required to facilitate full activity of the Ect2 DH/PH domains *in vivo*.

We next evaluated the role of the PH domain in ΔN-Ect2 DH/PH/C transforming activity. Mutation of the Trp residue (Trp → Leu) conserved in PH domains (Fig. 4*A*) and important for domain structure has been shown to abolish the transforming activity of several Dbl family proteins (12–18). Therefore, we introduced a Trp to Leu substitution into the PH domain of ΔN-Ect2 DH/PH/C (designated W752L) and evaluated the con-

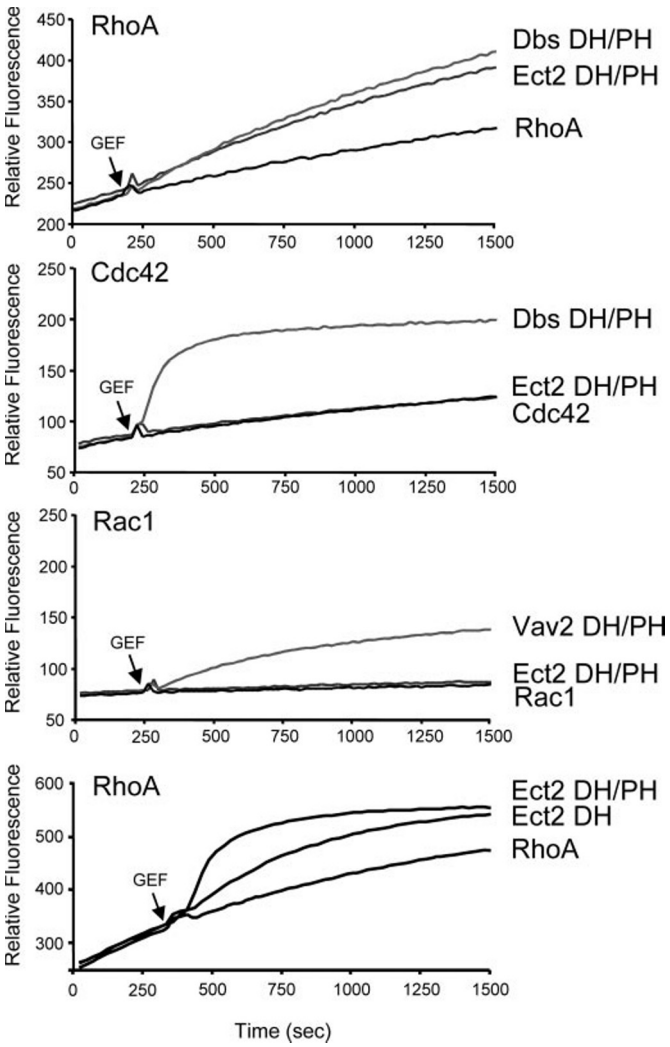

FIG. 2. **Ect2 stimulates incorporation of mant-GTP into RhoA but not Rac1 or Cdc42.** The ability of 100 nM of the isolated Ect2 DH/PH domain to stimulate the incorporation of mant-GTP into 2 μM GST-RhoA, GST-Cdc42, or GST-Rac1 was measured by fluorescence spectroscopy (λ_{ex} = 360 nm, λ_{em} = 440 nm). *Arrows* indicate the time point at which GEF was added to the exchange reaction. Traces labeled RhoA, Cdc42, and Rac1 represent the intrinsic GDP/GTP exchange in each of the assays. Positive controls were 100 nM Dbs DH/PH domain for RhoA and Cdc42, and 200 nM Vav2 DH/PH domain for Rac1. The *bottom panel* is a comparison of the GEF activities of the DH and DH/PH domains from Ect2. The ability of 200 nM Ect2 DH (*middle trace*) or Ect2 DH/PH (*top trace*) to stimulate the incorporation of mant-GTP into 2 μM GST-RhoA was measured by fluorescence spectroscopy as above. Data shown are representative of two independent experiments.

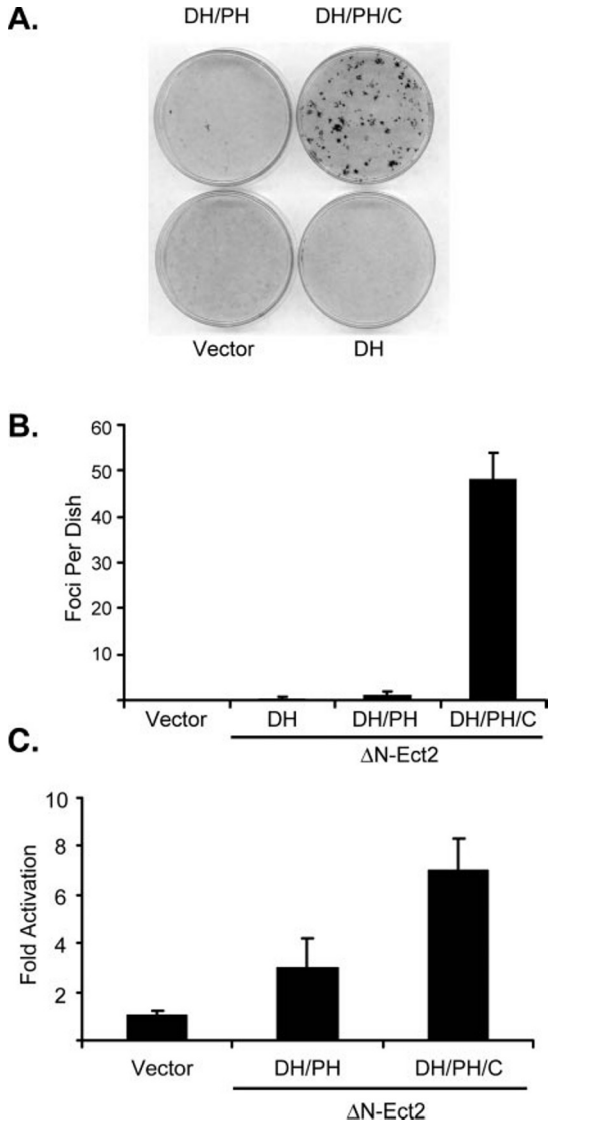

FIG. 3. **Deletion of Ect2 C-terminal sequences causes impairment in transformation and signaling.** NIH3T3 cells were transfected by calcium phosphate precipitation to express pCGN-hygro expression vectors encoding the indicated proteins. Cells were maintained in growth medium for 16 days. The cells were fixed and stained with 0.4% crystal violet (*A*), and the number of foci of transformed cells was quantified by visual inspection using phase contrast microscopy (*B*). Data shown are the mean of triplicate dishes with standard error indicated by the *bars* and are representative of three independent experiments. *C*, NIH3T3 cells were co-transfected with pCGN-hygro expression vectors encoding the indicated proteins and with the SRF-Luc reporter plasmid in which luciferase gene expression is controlled by a minimal *fos* promoter containing tandem SRF-responsive DNA elements. Transcriptional activation was determined by measuring luciferase activity in cell lysates. Fold activation was determined by the number of luciferase units in the presence of Ect2 relative to the number of units seen with empty vector control. Values represent the mean of three independent measurements ± S.E. Data shown are representative of three independent experiments.

sequence of this missense mutation on signaling, transformation, and subcellular location. The PH domain mutant of ΔN-Ect2 was not significantly altered in the level of protein expressed when evaluated in transiently (Fig. 4*B*) or stably transfected (data not shown) NIH3T3 cells. Surprisingly, ΔN-Ect2 DH/PH/C W752L showed comparable signaling and transforming activity (Fig. 4, *C* and *D*) and showed the same subcellular location (data not shown) as the nonmutated counterpart. Thus, in contrast to other Dbl family proteins, this PH domain mutation did not impair Ect2 function.

The C Terminus Does Not Alter Protein Stability or Degree of Membrane Association—One possible mechanism by which the C terminus may enhance ΔN-Ect2 DH/PH transforming activity may involve increased protein stability, resulting in elevated steady state levels of protein expression. To address this possibility, we transiently transfected NIH3T3 cells with each of the pCGN-*ect*2 constructs (Fig. 1*B*) and analyzed the cell lysates by SDS-PAGE and Western blot analysis with an anti-HA epitope antibody. The ΔN-Ect2 DH/PH and DH variants lacking the C-terminal sequences showed expression levels that were comparable to or higher than that seen with ΔN-Ect2 DH/PH/C. The same results were seen when protein expression was evaluated in NIH3T3 cells stably transfected with the different Ect2 expression vectors (data not shown). Thus, we conclude that the reduced transforming activity associated with loss of the C terminus is not a consequence of decreased protein stability or expression.

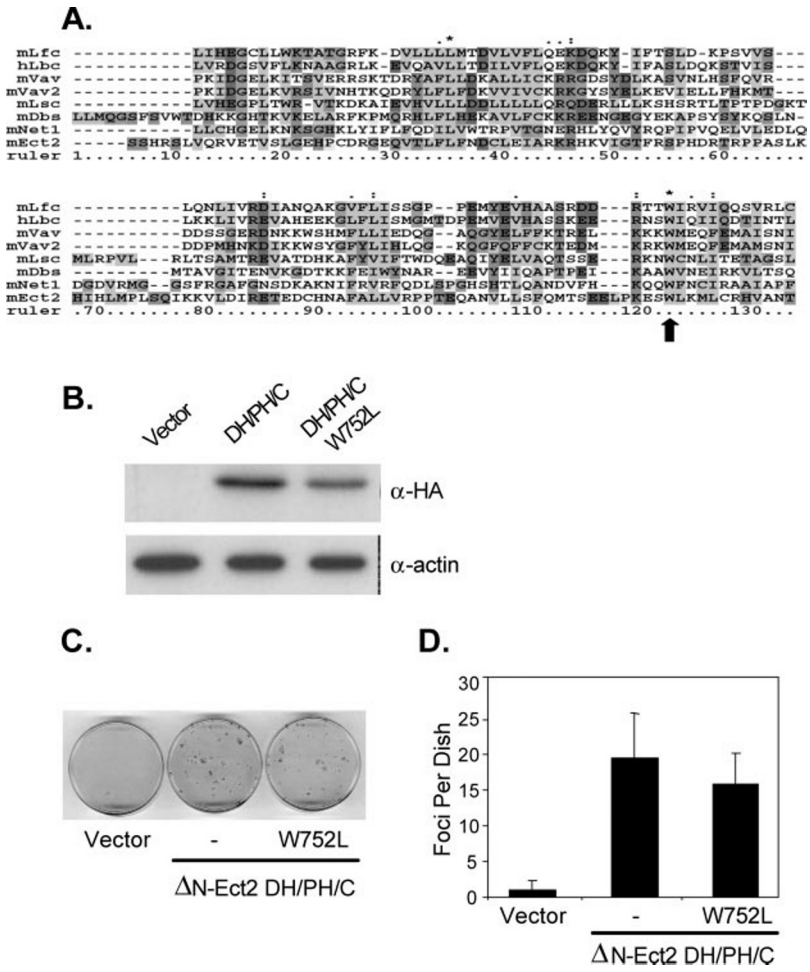

FIG. 4. **Mutation of the invariant Trp residue of the PH domain does not impair ΔN-Ect2 DH/PH/C transformation.** *A*, alignment of sequences flanking the invariant tryptophan residue (indicated by *arrow*) found in the PH domains of all (except Tiam1) Dbl family proteins. Previous studies found that mutation of the Trp residue to Leu (Trp → Leu) abolished transforming activity of Dbs (12), Lsc (13), Lfc (14) Lbc (15), Vav (16), Vav2 (17), and Net1 (18). *B*, mutation of the PH domain of ΔN-Ect2 does not alter protein expression. pCGN-hygro expression vectors encoding the indicated proteins were transiently transfected into NIH3T3 cells. 24-h post-transfection, cells were lysed, equivalent amounts of total protein were resolved by SDS-PAGE, and HA epitope-tagged Ect2 proteins were visualized by immunoblotting with anti-HA antibody. Blot analysis for actin was done to verify equivalent loading of total protein. *C*, mutation of the PH domain does not impair ΔN-Ect2 transforming activity. pCGN-hygro expression vectors encoding the indicated proteins were transfected into NIH3T3 cells, and focus formation was assessed after 14 days. *D*, quantification of focus forming activity. Data shown are representative of three independent experiments performed in duplicate.

It is possible that the C terminus of Ect2 plays a role in facilitating Ect2 membrane association, which could influence Ect2 protein interaction with its membrane-bound RhoA substrate. We therefore utilized fractionation analysis to address this possibility. We subjected cell lysates from NIH3T3 cells stably expressing the Ect2 variant proteins to high speed centrifugation (100,000 × *g*) to separate proteins into crude membrane and cytoskeletal P100 and cytosolic S100 fractions (Fig. 5). Western blot analysis with anti-HA antibody showed that ΔN-Ect2 DH/PH/C and ΔN-Ect2 DH/PH demonstrated similar degrees of association with the membrane-containing P100 fraction (~50%, Fig. 5). This result indicates that the C terminus of Ect2 does not significantly influence the degree of membrane association of Ect2.

The C Terminus Is Required for Ect2-mediated Changes in NIH3T3 Cellular Morphology—In addition to biochemical fractionation, we also utilized indirect immunofluorescence to determine whether the C terminus modulated the subcellular localization of ΔN-Ect2. We visualized the different ΔN-Ect2 proteins when transiently expressed in NIH3T3 cells by using an anti-HA epitope antibody and a fluorescein-conjugated secondary antibody (Fig. 6). Whereas ΔN-Ect2 DH showed a diffuse, cytoplasmic staining, ΔN-Ect2 DH/PH showed a similar, but more punctate pattern, of cytoplasmic staining, with limited association with the plasma membrane (Fig. 6*A*). ΔN-Ect2 DH/PH/C showed increased staining at the periphery and along membrane ruffles (Fig. 6*B*). Thus, the C terminus of ΔN-Ect2 may influence subcellular location and association with specific membrane compartments.

We observed that the morphology of cells expressing ΔN-Ect2 DH/PH/C, but not ΔN-Ect2 DH or DH/PH, was altered

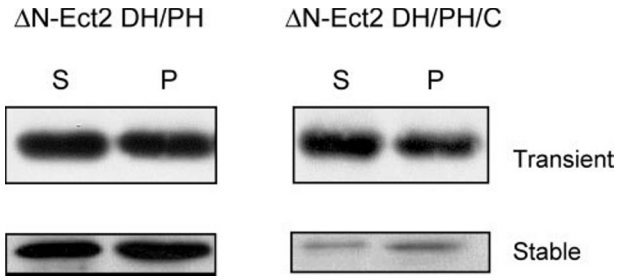

FIG. 5. **Ect2 DH/PH and DH/PH/C show a similar degree of membrane association.** NIH3T3 cells stably or transiently expressing each HA epitope-tagged Ect2 mutant protein were lysed by Dounce homogenization and then separated into cytosolic (S100) and particulate (P100) fractions by ultracentrifugation at 100,000 × *g*. Proteins were acetone-precipitated, resolved by SDS-PAGE, and visualized by Western blot analysis using anti-HA epitope antibody. Data shown are representative of three independent experiments.

when compared with control, empty vector-transfected NIH3T3 cells. The ΔN-Ect2 DH- and DH/PH-expressing cells looked similar to control NIH3T3 cells (Fig. 6), whereas ΔN-Ect2 DH/PH/C-expressing cells displayed membrane ruffling and a less fibroblast-like, rounded cell morphology, suggesting different consequences on actin organization. Therefore, we compared the actin cytoskeletal organization of these cells by immunofluorescence analyses using rhodamine-conjugated phalloidin.

ΔN-Ect2 DH-expressing cells and the control empty vector-transfected cells displayed similar staining for actin stress fibers, whereas the ΔN-Ect2 DH/PH-expressing cells displayed a significantly increased degree of staining and showed many thick actin stress fibers traversing the cell (Fig. 6*A*). These

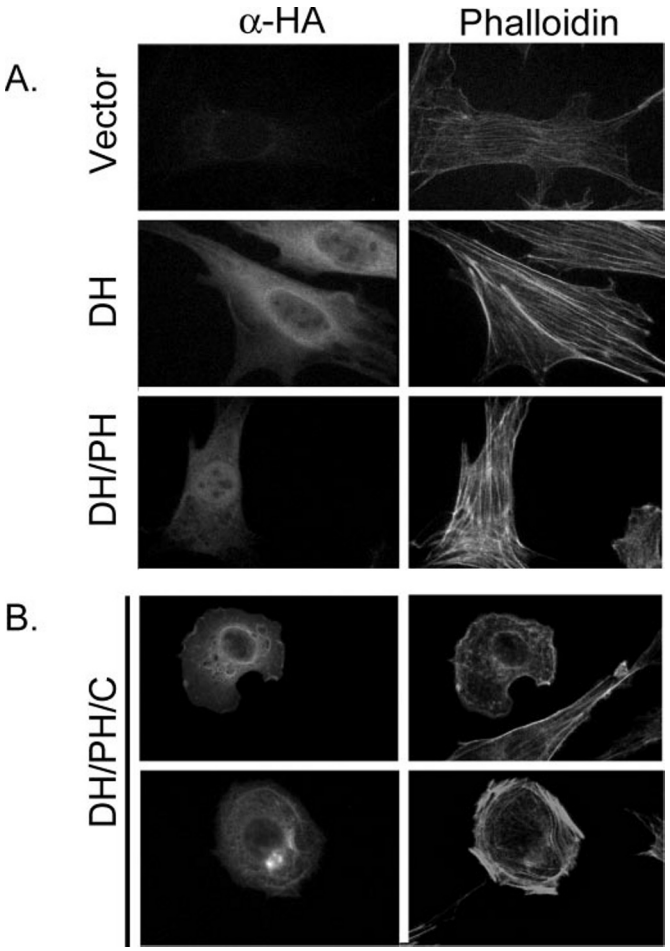

FIG. 6. **Ect2 DH/PH/C and DH/PH expressing cells exhibit different cellular morphology and actin reorganization.** NIH3T3 cells were transiently transfected with pCGN-hygro expression vectors encoding the DH or DH/PH (*A*), or DH/PH/C (*B*) fragments of Ect2, and immunofluorescence was performed as described under "Experimental Procedures." Protein expression was detected with anti-HA antibody followed by FITC-conjugated secondary antibody. Actin-containing structures were visualized with TRITC-conjugated phalloidin. Data shown are representative of three independent experiments.

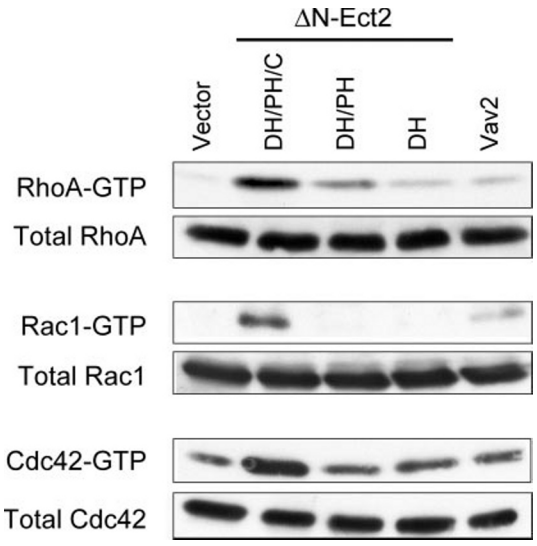

FIG. 7. **The C terminus of Ect2 promotes activation of RhoA, Rac1, and Cdc42 *in vivo*.** Human embryonic kidney cells (293T) cells were transiently transfected with pCGN-hygro expression vectors encoding each ΔN-Ect2 C-terminal variant protein. GTPase activation was assessed by the ability to bind GST-Rhotekin (RhoA) or GST-PAK (Rac1 and Cdc42) that was precoupled to glutathione-Sepharose beads. Total and precipitated lysates were analyzed by Western blot using antibodies against the appropriate GTPase. Data shown are representative of three independent experiments.

results are consistent with the RhoA-specific activity that we observed for ΔN-Ect2 DH/PH *in vitro*, and with our observation that ΔN-Ect2 DH showed significantly reduced RhoA GEF activity *in vitro* (Fig. 2). In contrast, we found two predominant patterns of actin staining in ΔN-Ect2 DH/PH/C-expressing cells. Generally, these cells displayed fewer actin stress fibers when compared with control NIH3T3 cells, and instead, lamellipodia formation was observed in approximately half of the cells (Fig. 6*B*, *upper panel*). Approximately 25% of the ΔN-Ect2 DH/PH/C-expressing cells displayed the same rounded morphology, but instead possessed actin stress fibers that were aligned and concentrated along the cell periphery (Fig. 6*B*, *lower panel*). Thus, whereas ΔN-Ect2 DH/PH caused changes in actin organization consistent with activation of RhoA, ΔN-Ect2 DH/PH/C caused changes that are more consistent with Rac1 activation. These results suggested that the C terminus of Ect2 may influence the ability of Ect2 to mediate different types of actin reorganization by altering its GTPase specificity.

The C-terminal Sequence of Ect2 Plays a Role in GTPase Specificity in Vivo—The analyses of actin organization suggested different Rho GEF specificities for ΔN-Ect2 DH/PH/C and ΔN-Ect2 DH/PH. To address this possibility, we determined the ability of the two Ect2 variants to activate Rho GTPases *in vivo* by using pull-down analyses to measure the formation of GTP-bound Rho GTPases. These analyses utilized GST fusion proteins containing the isolated, GTP-dependent binding domains of a Rac/Cdc42 effector (GST-PAK RBD) or of a RhoA-specific effector (GST-Rhotekin RBD) (34–37). As expected, compared with control cells, cells expressing ΔN-Ect2 DH/PH, a variant of Ect2 that lacks the C-terminal sequences, displayed increased formation of GTP-bound RhoA, but not of Rac1 or Cdc42 (Fig. 7). Surprisingly, we found that ΔN-Ect2 DH/PH/C-expressing cells showed increased formation not only of GTP-bound RhoA, but also of GTP-bound Rac1 and GTP-bound Cdc42. These data suggest that the C-terminal sequences of Ect2 may influence the GTPase specificity of the DH domain *in vivo*.

To determine if the C terminus alters the intrinsic GTPase specificity of the DH domain, we undertook two approaches. First, we generated a bacterial expression vector to express ΔN-Ect2 DH/PH/C as recombinant protein to determine whether it could stimulate exchange on RhoA, Rac1, and Cdc42 *in vitro*. However, this expression construct did not yield protein that was useful for *in vitro* GEF analyses. Therefore, we instead utilized dominant negative mutants of RhoA, Rac1, and Cdc42 to assess alterations in GTPase recognition. We showed recently that the ability of dominant negative mutants of Rho GTPases to bind to a particular Dbl family protein corresponds with the Rho GTPase specificity of its exchange activity (32). As expected, dominant negative RhoA, but not Rac1 or Cdc42, was able to form a stable complex with ΔN-Ect2 DH/PH expressed in cell lysates (Fig. 8). However, the same specificity of complex formation was also seen with ΔN-Ect2 DH/PH/C. Thus, it does not appear that the C terminus significantly altered the ability of the Ect2 DH domain to associate with Rac1 or Cdc42. Therefore, the basis for the ability of ΔN-Ect2 expression to cause activation of Rac1 and Cdc42 *in vivo* does not appear to be due simply to altered substrate binding. It remains possible that the C terminus does directly influence Ect2 DH domain activity *in vivo*, or alternatively, indirectly by a mechanism involved in interaction with other proteins that then facilitate Rac and Cdc42 activation.

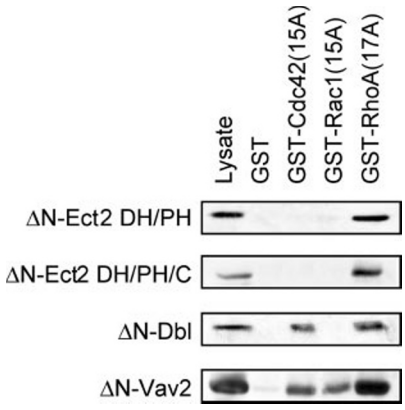

FIG. 8. **The C terminus does not alter Ect2 interaction with RhoA, Rac1, or Cdc42 *in vitro*.** HA epitope-tagged versions of ΔN-Ect2 DH/PH/C or ΔN-Ect2 DH/PH proteins, or of control Vav2 and Dbs proteins, were expressed transiently in NIH3T3 cells. Dominant negative Rho GTPases were expressed in bacteria as GST fusion proteins and precoupled to glutathione-Sepharose beads. Cell lysates were passed over the beads, and stable complexes formed between the GEFs and Rho proteins that remained bound following centrifugation and washing were resolved on SDS-PAGE and detected by immunoblotting with anti-HA antibody. Data shown are representative of three independent experiments.

DISCUSSION

In addition to the strictly conserved tandem DH/PH domain structure found in essentially all Dbl family Rho GEFs, Dbl family proteins typically show significant divergence in sequences that flank these two domains (2, 3). Like many Dbl family members, Ect2 becomes constitutively active when N-terminal sequences upstream of the DH/PH domains are deleted from the protein (1). Thus, these sequences serve a negative regulatory role for DH domain function *in vivo*. Little is known regarding the PH domain and sequences C-terminal to the DH/PH domains of Ect2. In contrast to many other Dbl family GEFs, the Ect2 C terminus lacks any known functional domains or motifs. Our previous studies suggested that the C terminus was important for Ect2 transforming potential (9), yet in the present study we found that the isolated DH/PH domains of Ect2 alone displayed potent GEF activity *in vitro*. Surprisingly, we determined that these C-terminal sequences altered the Rho GTPase specificity of the Ect2 DH domain *in vivo*. Whereas the Ect2 DH/PH domains alone activated RhoA *in vitro* and *in vivo*, the DH/PH/C fragment activated not only RhoA, but also Rac1 and Cdc42 *in vivo*. Thus, sequences beyond the DH and PH domains may influence the GTPase specificity of Ect2. Finally, in contrast to other Dbl family proteins, mutation of the conserved Trp residue in the PH domain did not impair Ect2 subcellular location or transforming activity. Therefore, the presence of the C-terminal sequences may compensate for the loss of Ect2 PH domain function.

The invariant DH/PH domain topography seen in the majority of Dbl family GEFs suggests the critical role of the PH domain in DH domain function (2, 3). Consistent with this possibility, previous studies found that mutation of the conserved PH domain Trp residue to Leu (Trp → Leu) abolished the transforming activity of all Dbl family proteins evaluated, including Dbs (12), Lsc (13), Lfc (14), Lbc (15), Vav (16), Vav2 (17), and Net1 (18). In contrast, the adjacent PH domain was found to be dispensable for Tiam1 function, and instead, a second PH domain N-terminal to the DH/PH domains was found to be essential for GEF activity *in vivo* (19). Interestingly, this Trp residue is not conserved in the DH domain-associated PH domain of Tiam1; instead, Tiam1 contains a Phe at this position. Thus, we were surprised to find that mutation of the Trp residue in the Ect2 PH domain did not impair ΔN-Ect2 DH/PH/C transforming activity. We would not have predicted this result based on our observation that the DH/PH domain fragment showed greater activity than the DH domain alone *in vitro*. One possible explanation may be that, while mutation of this residue is generally considered to abolish PH domain function, perhaps this is not the case with the Ect2 PH domain. Alternatively, perhaps the C terminus provides a function that renders the PH domain dispensable.

Studies examining the Rho GTPase substrate specificity of Ect2 have reached conflicting conclusions. Miki and co-workers (7) showed that full length Ect2, immunoprecipitated from mammalian cells, catalyzed exchange on RhoA, Rac1, and Cdc42 *in vitro*. In contrast, the function of the *Drosophila* homolog of Ect2, Pebble, suggests that it acts as a RhoA-specific Rho GEF (8). Furthermore, our analyses in the present study found that the isolated DH/PH domains of Ect2 acted as a RhoA-specific exchange factor *in vitro* and *in vivo*. The DH domain of Ect2 exhibits strongest sequence identity with the DH domain of GEF720/KIAA0720 (2), a RhoA-specific GEF (38). However, when we characterized Rho GEF activity *in vivo* for the DH/PH/C fragment, we showed that this mutant caused increased levels of RhoA-GTP, Rac1-GTP and Cdc42-GTP *in vivo*. One possible explanation for the conflicting results may be that different fragments of Ect2 protein (*i.e.* full-length, DH/PH/C, or DH/PH) have been used in different studies. Our results are consistent with those of Miki and co-workers (7), who isolated full length Ect2 expressed in COS cells to perform their exchange assays. Although this protein is not N-terminally truncated, it does contain the full C-terminal sequence. Taken together, these results all suggest that when the C-terminal end is not deleted, ΔN-Ect2 can activate RhoA, Rac1, and Cdc42. The manner in which the Ect2 protein is expressed (mammalian *versus* bacterial) may also influence the specificity. Ect2 contains two putative Cdc2 phosphorylation sites, one of which is located in the C terminus. Miki and co-workers (7) stated that phosphorylation of Ect2 is required for the exchange activity, though they showed only the exchange activity on Rac1 protein. It is possible that the GEF activity on Rac1 requires the C terminus of Ect2 to be phosphorylated, which would occur when expressed in mammalian cells, but not when expressed in bacteria. This may explain why Miki and colleagues first found that Ect2 did not display exchange activity for Rac1 (1). Unfortunately, we have not been able to isolate a bacterially expressed version of the Ect2 DH/PH/C to evaluate the possibility that the C terminus can influence DH domain specificity *in vitro*.

The lamellipodia-like structures that we observed with ΔN-Ect2 DH/PH/C also support our Rho GTPase activation results and indicate that Ect2 is an activator of Rac1 *in vivo*. Our previous study found that ΔN-Ect2 caused an unusual cellular morphology with lamellipodia-like structures similar to that induced by Rac1 (9). It is also possible that Rho GTPases other than the well studied Rac1, Cdc42, and RhoA could be targets for Ect2. For example, TCL and RhoG can also promote lamellipodia formation (39). Therefore, to fully understand the consequences of Ect2 function, a complete understanding of its Rho GTPase targets will be needed.

In summary, the studies presented here indicate that the C terminus of Ect2 is important for its transforming activity and signaling properties, and can influence the GTPase specificity of the DH domain. With the possible exception of Vav, where the cysteine-rich domain adjacent to the PH domain may influence substrate specificity (40), the Rho GTPase specificity of Dbl family GEFs typically resides in the DH domain. How the C terminus influences the Rho GTPase specificity of the Ect2

DH domain, and whether other GTPases are also activated, remain to be determined.

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