THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 38, pp. 33055–33065, September 23, 2005

Transforming Activity of the Rho Family GTPase, Wrch-1, a Wnt-regulated Cdc42 Homolog, Is Dependent on a Novel Carboxyl-terminal Palmitoylation Motif*

Received for publication, October 4, 2004, and in revised form, July 7, 2005 Published, JBC Papers in Press, July 26, 2005, DOI 10.1074/jbc.M507362200

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Wrch-1 is a Rho family GTPase that shares strong sequence and functional similarity with Cdc42. Like Cdc42, Wrch-1 can promote anchorage-independent growth transformation. We determined that activated Wrch-1 also promoted anchorage-dependent growth transformation of NIH 3T3 fibroblasts. Wrch-1 contains a distinct carboxyl-terminal extension not found in Cdc42, suggesting potential differences in subcellular location and function. Consistent with this, we found that Wrch-1 associated extensively with plasma membrane and endosomes, rather than with cytosol and perinuclear membranes like Cdc42. Like Cdc42, Wrch-1 terminates in a C*AAX* tetrapeptide (where C is cysteine, *A* is aliphatic amino acid, and *X* is any amino acid) motif (CCFV), suggesting that Wrch-1 may be prenylated similarly to Cdc42. Most surprisingly, unlike Cdc42, Wrch-1 did not incorporate isoprenoid moieties, and Wrch-1 membrane localization was not altered by inhibitors of protein prenylation. Instead, we showed that Wrch-1 is modified by the fatty acid palmitate, and pharmacologic inhibition of protein palmitoylation caused mislocalization of Wrch-1. Most interestingly, mutation of the second cysteine of the CCFV motif (CCFV > CSFV), but not the first, abrogated both Wrch-1 membrane localization and transformation. These results suggest that Wrch-1 membrane association, subcellular localization, and biological activity are mediated by a novel membrane-targeting mechanism distinct from that of Cdc42 and other isoprenylated Rho family GTPases.

The Rho family of Ras-related small GTPases is a functionally diverse group of proteins that are best known for their roles in regulation of actin cytoskeleton organization, cell polarity, cell adhesion, vesicular trafficking, transcriptional regulation, and cell cycle progression (1, 2). Of the 22 known human Rho GTPases, RhoA, Rac1, and Cdc42 are the most extensively characterized family members (3).

Like Ras, Rho proteins cycle between an inactive GDP-bound state and an active GTP-bound state (2, 4, 5). Guanine nucleotide exchange factors activate Rho proteins by promoting GDP dissociation in exchange for GTP (6, 7), whereas GTPase-activating proteins downregulate Rho protein function by stimulating their intrinsic GTPase activity to hydrolyze GTP to GDP (8). A third regulatory class of proteins includes the Rho guanine nucleotide dissociation inhibitors (RhoGDIs)[4] that bind the carboxyl terminus of Rho GTPases and sequester them in the cytosol (9, 10). Missense mutations within the switch regions of Rho proteins lock them in a GTP-bound conformation and render these proteins GTPase-deficient and constitutively activated. Activated forms of some Rho family GTPases cause growth transformation of NIH 3T3 mouse fibroblasts, and aberrant activity of both regulatory proteins and effectors of the Rho signaling pathways have been linked to human cancers (11–14).

Wrch-1 (Wnt-regulated Cdc42 homolog-1) is a novel member of the Rho subfamily, whose transcription is up-regulated in Wnt-1 transformation of mouse mammary epithelial cells (15). Like many other Rho family members, Wrch-1 activation is regulated by its nucleotide state, and a single missense mutation at residue 107 (analogous to Q61L activating mutation in Cdc42) rendered Wrch-1 more active in signaling (15). Ectopic expression of a constitutively active form of Wrch-1(107L) caused a Wnt1-like change in the cellular morphology of mammary epithelial cells, suggesting a contribution for Wrch-1 in Wnt transformation (15). Additionally, like other Rho family proteins, Wrch-1 activation can promote growth transformation (16).

The correct subcellular localization and function of Ras and Rho family members are dictated by post-translational modification of the carboxyl-terminal hypervariable domain, including the last four amino acids known as the C*AAX* motif (17, 18). The canonical C*AAX* motif consists of a cysteine residue, two aliphatic residues (*AA*), and at the last position any amino acid (*X*). The conserved cysteine residue serves as the site for post-translational modification by either farnesyltransferase (FTase) or geranylgeranyltransferase I (GGTase I), which irreversibly attaches an isoprenoid moiety (19). Although C*AAX*-signaled prenyla-

* This work was supported in part by National Institutes of Health Grants CA63071, CA67771, CA109550 (to A. D. C.), CA51890 (to J. E. B.), GM46372 (to C. A. W.), CA63071, and CA67771 (to C. J. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by National Institutes of Health Predoctoral Fellowship CA103143.
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[4] The abbreviations used are: RhoGDIs, Rho guanine nucleotide dissociation inhibitors; Wrch-1, Wnt-regulated Cdc42 homolog-1; Cdc42, cell division cycle 42; *A*, aliphatic amino acid; *X*, any amino acid; FTase, farnesyltransferase; GGTase I, geranylgeranyltransferase I; GGTase II, geranylgeranyltransferase II; GFP, green fluorescent protein; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; FTI, farnesyltransferase inhibitor; GGTI, geranylgeranyltransferase I inhibitor; 2-BP, 2-bromopalmitate; FPP, farnesylpyrophosphate; GGPP, geranylgeranylpyrophosphate; FITC, fluorescein isothiocyanate; Me_2SO, dimethyl sulfoxide; Btn-BMCC, biotin-1-biotinamido-4-[4′-(maleimidomethyl) cyclohexanecarboxamio] butane; pEGFP, enhanced GFP; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; TBS, Tris-buffered saline; PAK, p21-activated kinase; WT, wild type; GST, glutathione *S*-transferase; PAT, protein *S*-acyltransferase.

tion is necessary (20–25), a second targeting signal immediately upstream of the C*AAX* motif is also required for proper membrane association and subcellular localization. Some small GTPases, such as H-Ras, N-Ras, and the Cdc42-related protein TC10, are covalently modified by addition of a palmitoyl fatty acid on cysteine residues, whereas others, such as K-Ras4B and Cdc42, contain several basic residues that serve as their second targeting signal (18, 23, 26). Finally, additional carboxyl-terminal sequences provide further specificity in targeting Ras and Rho GTPases to distinct plasma membrane microdomains or to endomembrane compartments. Thus, functionally highly related Rho GTPase isoforms (*e.g.* RhoA, RhoB, and RhoC), by virtue of divergent carboxyl-terminal sequences and modifications, can exhibit strikingly different biological functions dependent on distinct subcellular locations (22, 27, 28).

Although Wrch-1 has been shown to localize to plasma membrane and internal membranes (15, 29), the role of the carboxyl terminus in mediating Wrch-1 biological function has not been determined. Like Cdc42, Wrch-1 induces actin reorganization and formation of filopodia and causes activation of the PAK and c-Jun NH_2-terminal kinase serine/threonine kinases (15). However, Wrch-1 contains amino-terminal sequences not found in Cdc42, and we and others recently showed that this amino-terminal extension serves as a negative modulator of Wrch-1 effector interaction (16, 30). In addition, Wrch-1 and Cdc42 differ considerably at their carboxyl termini, sharing only 25% sequence identity. Furthermore, Wrch-1 has an extended carboxyl terminus ending in an unusual C*AAX* sequence (CCFV) not characteristic of known substrates for farnesyl- and geranylgeranyltransferases (31, 32). Therefore, in the present study we assessed the mechanism and role of the unique carboxyl terminus in regulation of Wrch-1 function. As with Cdc42, we found that Wrch-1 activation caused anchorage-dependent growth transformation of NIH 3T3 cells. However, although the carboxyl terminus was critical for Wrch-1 subcellular localization and transforming activity, Wrch-1 function was dependent not on modification by prenylation but on modification by palmitoylation. Thus, although Wrch-1 and Cdc42 share significant functional properties, they exhibit considerable divergence in lipid modifications and subcellular distribution and, consequently, may have divergent roles in cell physiology.

EXPERIMENTAL PROCEDURES

Molecular Constructs—pcDNA3 expression constructs encoding wild type (WT) and GTPase-deficient (Q107L) human Wrch-1 was obtained from Dr. A. Levine (15). PCR-mediated DNA amplification was used to introduce 5′ and 3′ BamHI sites flanking Wrch-1(WT) for subcloning into various epitope-tagged expression vector constructs. To create other constitutively activated mutants of Wrch-1, a glutamate to leucine mutation was generated at residue 107 using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Site-directed mutagenesis was also used to create the carboxyl-terminal CCFV motif mutants by changing cysteine residues at positions 255 and 256 to generate CCFV > SCFV (C255S), CCFV > CSFV (C256S), and CCFV > SSFV (C255S and C256S) point mutants in the activated Wrch-1(Q107L) background. To generate glutathione *S*-transferase (GST), green fluorescence protein (GFP), and hemagglutinin (HA) epitope-tagged Wrch-1 proteins for prenylation, transformation, and localization assays, Wrch-1 coding regions were digested with BamHI and ligated into the BamHI site of pGEX-2T multiple cloning site, the 5′ BglII and 3′ BamHI sites of the pEGFP-C1 (multiple cloning site), and the BamHI site of pCGN-hygro, respectively (33). All sequences were verified by the Genome Analysis Facility at the University of North Carolina, Chapel Hill.

Cell Culture and Transfections—NIH 3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Stable NIH cell lines were created by transfection of pCGN-wrch-1 constructs with FuGENE 6 (Roche Applied Science) per the manufacturer's instructions. After 48 h, cells were split into DMEM containing 200 μg/ml hygromycin B (Roche Applied Science) and maintained in antibiotic selection until colonies formed. Colonies (>50) were pooled for use in soft agar assays.

Transformation Assays—For focus forming assays, NIH 3T3 cells were seeded at 2×10^5 cells per 60-mm dish. The following day, cells were transiently cotransfected for 4 h with HA-tagged pCGN constructs encoding empty vector, activated Wrch-1(107L)-CCFV, -SCFV, -CSFV, or -SSFV carboxyl-terminal mutants along with pZIP-NeoSV(x)1 empty vector or pZIP-Raf22W (encoding an amino-terminal truncated and constitutively activated variant of human Raf-1), by calcium phosphate precipitation as described previously (25). After 20–24 days, dishes were washed with 1× phosphate-buffered saline (PBS), fixed with 3:1 (v/v) methanol/acetic acid, and stained with 0.4% crystal violet solution in 20% ethanol. Non-Raf foci of transformed cells (see text) were counted, and the average number of foci found on duplicate sets of dishes was then calculated.

For soft agar assays, NIH 3T3 cells stably expressing HA epitope-tagged pCGN constructs of either empty vector, activated Wrch-1(107L)-CCFV, -SCFV, -CSFV, or -SSFV were suspended in DMEM containing 10% calf serum, 1% penicillin/streptomycin, and 0.4% agar (BD Biosciences) at 5×10^4 cells per 35-mm dish. The single cell suspensions were layered on top of 0.6% agar in DMEM. Colonies that formed after 14–21 days were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and the average number of colonies on triplicate dishes was calculated.

Live Cell Imaging—To analyze subcellular localization and lipid modifications of Wrch-1 proteins, NIH 3T3 cells were seeded onto glass coverslips in 35-mm dishes. The following day, cells were placed in DMEM supplemented with either Me_2SO vehicle, 10 μM FTI-2153, 10 μM GGTI-2166, 10 μM FTI-2153 + GGTI-2166, 20 μM compactin (Sigma), or 150 μM 2-bromopalmitate (2-BP, Sigma) immediately prior to transient transfection with FUGENE, of GFP-tagged pEGFP constructs containing Wrch-1-tail (13 amino acids), Wrch-1(Q107L)-CCFV, -SSFV, Cdc42-tail (20 amino acids), Cdc42(61L), H-Ras(61L), K-Ras4B(12V), or Rab5. FTI-2153 and GGTI-2166 were generous gifts from Andrew D. Hamilton (Yale University) and Saïd M. Sebti (H. Lee Moffitt Cancer Center and Research Institute, University of South Florida). After 24–48 h, live cell images were captured on either an epifluorescent Zeiss Axioskop or Zeiss 510 LSM confocal microscope (Zeiss, Thornwood, NY) using MetaMorph imaging software (Universal Imaging Corp., Downington, PA) or LSM 5 Image browser software (Zeiss, Thornwood, NY).

To visualize localization of GFP-tagged Wrch-1(Q107L) to early endosomes, NIH 3T3 fibroblasts were transiently transfected with pEGFP-Wrch-1(Q107L). 24 h later, cells were serum-starved for 30 min with DMEM, rinsed in 1× PBS, and then treated with DMEM containing 30 μg/ml Texas Red-conjugated transferrin (Molecular Probes). After 10 min of incubation, cells were rinsed in 1× PBS, placed in DMEM, and analyzed for GFP-tagged Wrch-1(Q107L) localization using a fluorescein isothiocyanate (FITC) bandpass filter and Texas Red-conjugated transferrin using a Texas Red (TRITC) bandpass filter. Colocalization of GFP-tagged Wrch-1(Q107L) and Texas Red-conjugated transferrin images was analyzed using MetaMorph imaging software.

Immunofluorescence—NIH 3T3 cells were seeded onto glass coverslips in 35-mm dishes. After 24 h, cells were transfected with HA-tagged pCGN-wrch-1(107L) constructs using FuGENE 6. After 24 h, cells were fixed with 3.7% formaldehyde in PBS and permeabilized with 0.5% Triton X-100 in Tris-buffered saline (TBS). Cells were then incubated with anti-HA antibody (Covance) for 1 h at room temperature. After three washes in 0.1% Triton X-100 in TBS, cells were incubated with Alexa Fluor 488-conjugated secondary antibody for 30 min (Molecular Probes) and washed three times with 0.1% Triton X-100 in TBS. Coverslips were mounted onto glass microslides with Vectashield Hardset mounting medium (Vector Laboratories, Burlingame, CA) and analyzed on the fluorescent microscope as described above.

Metabolic Labeling—NIH 3T3 cells were seeded at 2×10^5 cells per 60-mm dish and transiently transfected with HA epitope-tagged pCGN constructs containing Wrch-1(107L) CCFV, -SCFV, -CSFV, -SSFV, H-Ras(61L), K-Ras(12V), or empty vector with FuGENE 6. After 48 h, cells were labeled for 4 h with 1 mCi/ml [^{3}H]palmitate (American Radiochemical Inc.) in DMEM containing 5 mM sodium pyruvate, 4× nonessential amino acids, 1% glutamine, 20 mM HEPES, pH 7.2, 25 μg/ml cycloheximide, and 10% calf serum. Cells were then rinsed twice with TBS and lysed in Hi-SDS RIPA buffer (1 M Tris, pH 7.0, 5 M NaCl, 10% SDS, 1% sodium deoxycholate, 1% Nonidet P-40, 0.2 M Pefabloc, 0.05–0.10 trypsin inhibitory units/ml aprotinin). For immunoprecipitation, lysates were incubated for 1 h with anti-HA antibody and then incubated for 30 min with protein A/G beads (Santa Cruz Biotechnology). The immunoprecipitates were washed, resuspended in nonreducing protein sample buffer (10% SDS, 1 M Tris-HCl, pH 6.8, 25% sucrose, 0.01% bromphenol blue), resolved on SDS-PAGE, and transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). Membranes were then sprayed with EN^{3}HANCE (PerkinElmer Life Sciences) and exposed to film at −80 °C for 90 days. To detect total amount of immunoprecipitated protein available for labeling, membranes were blocked in 5% nonfat dry milk, and immunoprecipitated proteins were detected with primary mouse anti-HA antibody followed by anti-mouse IgG-horseradish peroxidase (HRP)-conjugated antibody (Amersham Biosciences). Membranes were incubated in SuperSignal West Dura extended duration substrate (Pierce) and developed on film.

Biotin (Btn)-BMCC Fatty Acyl Thioester Bond Labeling—To label Wrch-1 cysteine-palmitate thioester bonds, a modified version of a method described recently for detecting protein palmitoylation was used (34). Briefly, human embryonic kidney 293 cells transiently expressing GFP-tagged Wrch-1 proteins were lysed in BMCC lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.02% NaN_3, and 2% Triton X-100) containing Complete protease inhibitor tablet (Roche Applied Science). Whole cell lysates were cleared, and protein concentrations were normalized using DC Lowry protein assay (Bio-Rad). For immunoprecipitation, protein lysates were pre-cleared with protein A/G-conjugated agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA), incubated with anti-GFP antibody (Clontech) for 2 h, and then incubated for 1 h with protein A/G-conjugated beads (Santa Cruz Biotechnology). Immunoprecipitates were washed, resuspended in 50 mM *N*-ethylmaleimide (Sigma) in BMCC lysis buffer to block free sulfhydryl groups, and incubated for 48 h. Immunoprecipitates were washed, treated for 1 h with 1 M hydroxylamine (Sigma) to hydrolyze any cysteine-palmitate thioester bonds, washed, and then treated with 1 μM Btn-BMCC (Pierce) in 50 mM Tris, pH 7.0, for 2 h to label cleaved thioester bonds. Immunoprecipitates were washed, resuspended in nonreducing protein sample buffer (10% SDS, 1 M Tris-HCl, pH 6.8, 25% sucrose, 0.01% bromphenol blue), resolved on SDS-PAGE, and transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). Membranes were probed with streptavidin-HRP (Pierce) to detect incorporation of Btn-BMCC, incubated in SuperSignal West Dura extended duration substrate (Pierce), and developed on film.

Purified Protein Preparation and in Vitro Prenylation Assay—Recombinant GST-tagged proteins were produced from *Escherichia coli* BL21 strain as described previously (16). Protein induction was confirmed by SDS-PAGE and Coomassie staining.

For isoprenylation of purified proteins, 5 μg of each purified protein was added to the prenylation reaction mixture containing 30 μg of bovine brain high speed supernatant and 1 μM [^{3}H]farnesylpyrophosphate (FPP) or [^{3}H]geranylgeranylpyrophosphate (GGPP) (8–10 Ci/mmol; American Radiochemical, Inc.). Reaction mixture was incubated for 30 min at 30 °C. Reaction was stopped with SDS protein sample buffer. Samples were boiled briefly, run on 4–20% SDS-PAGE, prepared for fluorography, and exposed to preflashed film for 4 days.

Western Blot Analysis—NIH 3T3 cells transiently expressing GFP-tagged or HA-tagged Wrch-1 proteins were lysed in 1% Triton X-100 lysis buffer containing protease inhibitors (5 μg/ml aprotinin, 10 μM leupeptin, 20 nM β-glycerophosphate, 12 mM *p*-nitrophenyl phosphate, 0.5 mM Pefabloc, and 0.1 mM sodium vanadate) or magnesium lysis buffer (25 mM HEPES, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10% glycerol, 10 mM $MgCl_2$, and 1 mM EDTA, pH 8.0, containing Complete protease inhibitor tablet). Whole cell lysates were cleared, and protein concentration was determined using DC Lowry protein assay. Fifteen μg of protein lysates were prepared in sample buffer, resolved on SDS-PAGE, and transferred to Immobilon-P membrane. Membranes were then blocked in 5% nonfat dry milk and probed for HA-tagged Wrch-1 proteins using mouse anti-HA antibody, for β-actin as a loading control using mouse anti-β-actin (Sigma), for endogenous phosphorylated PAK using rabbit anti-phospho-PAK1 (Ser-144)/PAK2 (Ser-141), for total endogenous PAK using rabbit anti-PAK1/2/3, or for GFP-tagged proteins using mouse anti-GFP antibody (Clontech), followed by anti-mouse HRP-conjugated antibody or anti-rabbit HRP-conjugated antibody and SuperSignal West Dura extended duration substrate as above.

RESULTS

The Carboxyl-terminal Nine Residues of Wrch-1 Promote a Subcellular Distribution Distinct from That Seen for Cdc42—As shown in Fig. 1*A*, the carboxyl termini of Wrch-1 and Cdc42 exhibit sequence differences that may result in different functional roles for Wrch-1. First, Wrch-1 terminates in an atypical C*AAX* motif. Second, Wrch-1 contains an additional 21 residues that have no counterpart in the Cdc42 carboxyl terminus. To determine the role of Wrch-1 carboxyl-terminal sequences in dictating Wrch-1 membrane association and subcellular location, and to compare these properties with those of Cdc42, we expressed GFP-tagged Wrch-1 and Cdc42 in NIH 3T3 mouse fibroblasts and performed live cell imaging analyses (Fig. 1*B*). Whereas GFP alone localized to the cytosol and prominently in the nucleus, both GFP-Cdc42 and GFP-Wrch-1 were excluded from the nucleus. Consistent with previous observations (18), we detected Cdc42 localization strongly in the cytosol, with its predominant membrane staining in the perinuclear region. Wrch-1, however, distributed mainly to the plasma membrane and to internal membranes reminiscent of endosomes and polarized, perinuclear Golgi, demonstrating an only partially overlapping localization pattern with that of Cdc42 (Fig. 1*B*) (15, 29).

We next wanted to determine which of the unique carboxyl-terminal sequences of Wrch-1 were sufficient to dictate its unique localization. Philips and colleagues (18) demonstrated previously that the carboxyl-terminal C*AAX*- and hypervariable domain-containing 20 amino acids of Cdc42 and other Rho family GTPases were sufficient to determine their membrane targeting. As described previously, a GFP fusion pro-

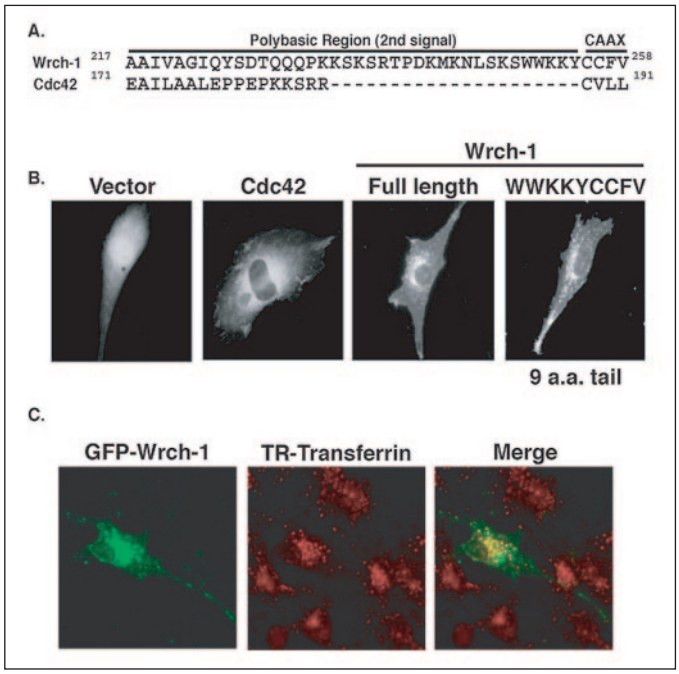

FIGURE 1. **The carboxyl-terminal hypervariable domain contributes to differences in subcellular localization of Cdc42 and Wrch-1.** *A,* sequence alignment of the hypervariable domains of Cdc42 and Wrch-1 demonstrates differences in length and composition. *B,* Wrch-1 and Cdc42 exhibit distinct subcellular locations. NIH 3T3 fibroblasts were transiently transfected with pEGFP empty vector or pEGFP constructs encoding GFP-tagged full-length Cdc42 or Wrch-1, or GFP fused to the carboxyl-terminal nine residues of Wrch-1, which are sufficient to promote Wrch-1 membrane association. *a.a.,* amino acids. *C,* endosomal localization of Wrch-1. Colocalization of GFP-Wrch-1 (*green, left panel*) and the early endosomal marker Texas Red-conjugated transferrin (*red, middle panel*) is indicated by the *yellow areas* in the merged image (*right panel*). Live cells were imaged after 24 h. Images are representative of at least three independent experiments.

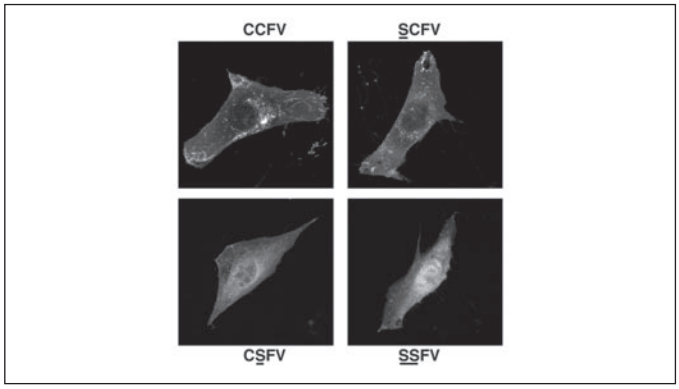

FIGURE 2. **The second cysteine of the Wrch-1 (C)CFV motif is required for Wrch-1 membrane localization.** NIH 3T3 cells were transiently transfected with the following expression constructs: HA-tagged Wrch-1(Q107L)(CCFV) or Wrch-1 bearing carboxyl-terminal mutations at cysteine residues 255 (CCFV > SCFV), 256 (CCFV > CSFV), or both (CCFV > SSFV). Cells were fixed and stained with anti-HA primary and FITC-conjugated anti-mouse secondary antibody. Wrch-1 proteins lacking cysteine 256 (CSFV, SSFV) accumulated in the cytosol. Images are representative of three independent experiments.

tein terminating in the 20 carboxyl-terminal residues of Cdc42 exhibited the same subcellular distribution as authentic Cdc42 (data not shown) (18, 22). Despite the extended length of the Wrch-1 carboxyl terminus, we found that even fewer residues were sufficient for authentic Wrch-1 localization, as a GFP fusion protein terminating in the nine most carboxyl-terminal residues of Wrch-1 displayed the same plasma membrane and endosomal localization as authentic Wrch-1 (Fig. 1*B*). To confirm that the internal membrane structures were indeed endosomes, we compared directly the localization of GFP-Wrch-1 and Texas Red-transferrin, which marks early endosomes. As shown in Fig. 1*C*, merging these images demonstrated that Wrch-1 on internal membranes colocalizes with transferrin. Thus, although Wrch-1 and Cdc42 regulate similar downstream effector functions (15, 29), they exhibit significant differences in their subcellular distribution.

Wrch-1 Membrane Localization Is Not Dependent Upon a CAAX Motif—By analogy to Cdc42 and other C*AAX*-terminating small GTPases, the existence of a putative C*AAX* motif (CCFV) in Wrch-1 suggested that the cysteine at residue 255 (CCFV) is likely to be a target for prenylation and critical for membrane localization. To evaluate this possibility, we generated HA-tagged Wrch-1 proteins with missense mutations in the C*AAX* motif. Cysteine to serine substitutions of the cysteine residue of the C*AAX* motif abolishes the prenylation of Ras and Rho GTPases, resulting in cytosolic and inactive proteins (20–23, 25). Therefore, we generated the analogous mutant of Wrch-1 (C255S, SCFV). Most surprisingly, this mutant did not localize exclusively to the cytosol as seen in similarly mutated Ras and Rho GTPases (20–24). Instead, it exhibited similar subcellular localization as wild type Wrch-1 (Fig. 2). However, it did show reduced asymmetrical "Golgi-like" localization near the nucleus, suggesting that the first cysteine may function as a Golgi targeting or retention signal (Fig. 2). Thus, in contrast to Cdc42 and other Rho GTPases, an intact C*AAX* motif is not required for membrane localization.

The Wrch-1 C*AAX* motif contains a second cysteine residue at position 255 and is similar to the carboxyl-terminal C*CXX* motif present on proteins modified by geranylgeranyltransferase II (GGTase II). Presently, all known substrates of GGTase II are members of the Rab small GTPase family. Thus, we speculated that Wrch-1 may possess an atypical prenylation signal sequence that is dependent instead on this second cysteine residue, alone or in combination with the first cysteine. Therefore, we generated a mutant of this cysteine residue, either alone or together with mutation of Cys-255. Most surprisingly, both Wrch-1 (C256S, CSFV) and Wrch-1 (C255S/C256S, SSFV) were mislocalized to the cytosol in a pattern similar to that of unprocessed small GTPases (Fig. 2). However, the CSFV mutant did retain minimal plasma membrane localization. Of particular note, mutation of the second or both cysteines resulted in nucleoplasm, but not nucleosome, accumulation of the HA-tagged Wrch-1 protein, implicating a potential nuclear localization signal within the carboxyl terminus of Wrch-1. These results suggested that Wrch-1 may be prenylated at Cys-256 rather than at Cys-255 and that, like other Ras and Rho family proteins, additional sequences within the carboxyl terminus may function as a secondary membrane targeting signal and/or nuclear localization signal in conjugation with modification of these cysteine residues.

Wrch-1 Localization Is Not Dependent on Isoprenoid Modification—The importance of Cys-256 suggested that perhaps Wrch-1 terminates in an atypical prenylation signal sequence. To address this possibility, we first determined whether Wrch-1 is a substrate for GGTase I or for FTase. We treated cells expressing GFP-tagged Wrch-1 with pharmacological inhibitors of GGTase I (GGTI) and of FTase (FTI). We have shown previously that inhibition of prenylation of GFP-tagged small GTPases results in nuclear accumulation because of the loss of the membrane targeting lipid and to the nuclear localization signal present within the GFP protein (20, 25). Therefore, sensitivity of the GFP-tagged GTPase to drug treatment was measured by redistribution of nuclear excluded, membrane-bound proteins to the cytosol and nucleus. Efficacy and specificity of drug treatment were confirmed by their ability to cause relocalization of GFP-tagged H-Ras (farnesylated), Cdc42 (geranylgeranylated), and K-Ras (alternatively prenylated) proteins to the nucleus and cytosol upon treatment with FTI, GGTI, and FTI plus GGTI, respectively (Fig. 3). As expected, FTI treatment caused mislocalization of H-Ras but not K-Ras4B or Cdc42, whereas GGTI treatment caused mislocalization of Cdc42 but not H-Ras or K-Ras4B. Most surprisingly, Wrch-1 localization was completely unaffected by treatment with either GGTI or FTI (Fig. 3). To eliminate the possibility that

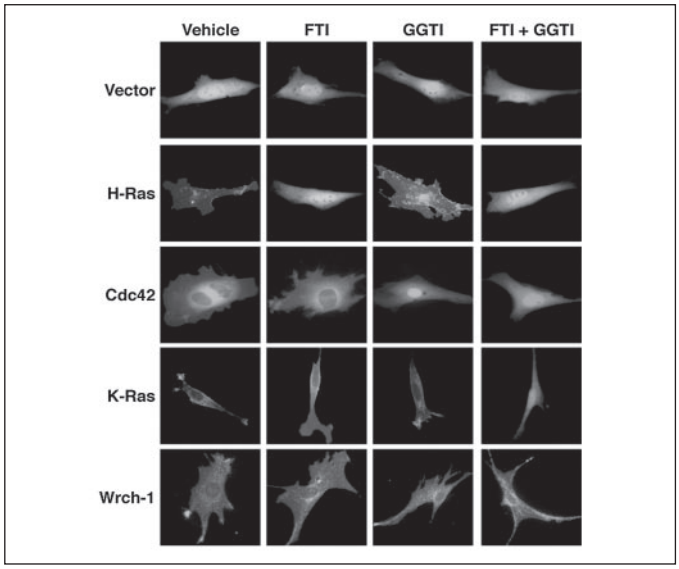

FIGURE 3. **Wrch-1 is not prenylated by either FTase or GGTase I.** NIH 3T3 cells were transiently transfected with either empty pEGFP vector or pEGFP constructs encoding GFP-tagged H-Ras (farnesylated, FTI-sensitive), Cdc42 (geranylgeranylated, GGTI-sensitive), or Wrch-1 in the presence of 10 μM vehicle, FTI-2153, GGTI-2166, or a combination of both, and live cells were imaged 24 h post-transfection. Fully processed GTPases are excluded from the nucleus, whereas accumulation of GFP-tagged proteins in the nucleus following treatment with prenyltransferase inhibitors is indicative of unprocessed protein. Images are representative of at least three independent experiments.

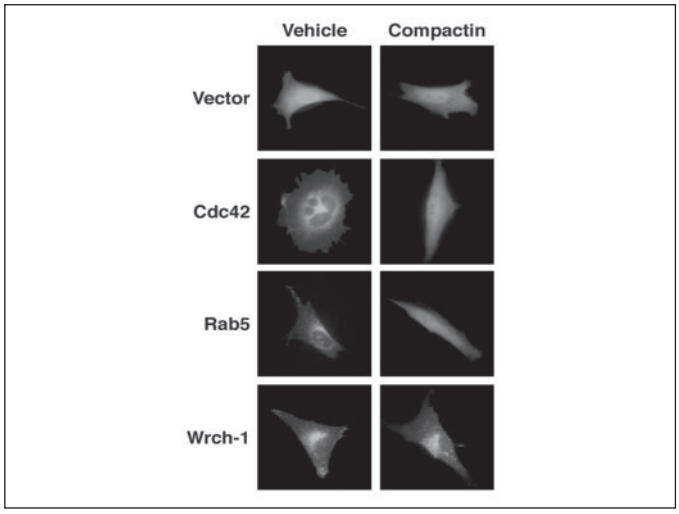

FIGURE 4. **Wrch-1 localization is not dependent on any isoprenoid modification.** NIH 3T3 cells transiently expressing either empty pEGFP vector or EGFP constructs encoding GFP-tagged Cdc42 (geranylgeranylated by GGTase I; compactin-sensitive), Rab5 (geranylgeranylated by GGTase II; compactin-sensitive), or Wrch-1 fusion proteins were treated overnight with either Me_2SO vehicle or 20 μM compactin (an inhibitor of all isoprenoid precursors). As above, mislocalization of GFP-tagged proteins to cytosol and nucleus after compactin treatment is indicative of disruption of isoprenoid-dependent localization. Images are representative of at least three independent experiments.

Wrch-1, like K-Ras, is alternatively prenylated by geranylgeranylation when farnesylation is blocked, cells were also treated with a combination of both FTI and GGTI. Although H-Ras, Cdc42, and K-Ras were all sensitive to the combination treatment, Wrch-1 still did not mislocalize (Fig. 3). These results suggested that, unlike most Rho proteins, Wrch-1 is not post-translationally modified by either FTase or GGTase I and may, therefore, be a novel substrate for prenylation by the Rab GGTase, GGTase II.

We next evaluated whether Wrch-1 is a substrate for GGTase II or for an unknown prenyltransferase. Because there are currently no pharmacological inhibitors available that specifically target GGTase II, we used compactin to treat NIH 3T3 cells transiently transfected with pEGFP-Wrch-1, -Rab5, -Cdc42, or empty vector. Compactin, an inhibitor of hydroxymethylglutaryl-CoA reductase, prevents the formation of all isoprenoid precursors, thereby preventing the formation of the farnesyl and geranylgeranyl isoprenoid moieties used by all prenyltransferases, including GGTase II. Vehicle-treated cells showed nuclear exclusion and membrane and cytosolic localization of each GFP-tagged GTPase, as expected (Fig. 4). Compactin treatment caused both Cdc42 and the GGTase II substrate Rab5 (*CAAX* = CCSN) to mislocalize to the nucleus and cytosol (Fig. 4). In contrast, Wrch-1 was entirely resistant to the treatment and remained excluded from the nucleus and targeted to the membranes and cytosol (Fig. 4). Given that both Rab5 and Cdc42 were susceptible to compactin treatment, these results clearly demonstrate that Wrch-1 membrane association is not dependent on modification with any isoprenoid moiety.

To confirm that Wrch-1 does not utilize isoprenoid lipid groups for its membrane targeting, we performed *in vitro* prenylation assays on purified, un-lipidated Wrch-1 protein to directly label Wrch-1 protein with either [^{3}H]farnesylpyrophosphate (FPP) or GGPP. Purified bacterially expressed GST-tagged Ras and Rho family proteins are unprenylated because of absence of FTase, GGTase I, or GGTase II enzymes in *E. coli*. Purified H-Ras, H-Ras (FTase substrate) CVLL mutant (contains serine to leucine mutation of the *CAAX* motif, GGTase I substrate), and Rab5 (GGTase II substrate) proteins served as standard controls for correct incorporation of [^{3}H]FPP and [^{3}H]GGPP by prenyltransferases. Incubation of purified, bacterially expressed Wrch-1 protein with bovine brain lysate, containing endogenous FTase, GGTase I, and GGTase II enzymes, and [^{3}H]prenylpyrophosphates followed by autoradiography demonstrated that, unlike H-Ras and Rab5, Wrch-1 was unable to incorporate [^{3}H]FPP or [^{3}H]GGPP (Fig. 5). To confirm further the absence of isoprenyl modification of purified Wrch-1 protein, radioactivity of each [^{3}H]FPP- or [^{3}H]GGPP-bound protein reaction was determined by counting in a scintillation counter. As expected, no radioactivity was detected with Wrch-1, whereas [^{3}H]FPP radioactivity counts were present for H-Ras WT and [^{3}H]GGPP counts for H-Ras CVLL and Rab5 WT (data not shown). Taken together with the above prenyltransferase inhibitor data, these data indicate that, unlike most Ras and Rho family proteins, Wrch-1 is not a substrate of FTase, GGTase I, or GGTase II, and its membrane localization is independent of isoprenylation.

Wrch-1 Subcellular Localization Is Dependent on Palmitoylation—The data above indicate that, unlike those of Cdc42, the last four amino acids of Wrch-1 do not function as a canonical "*CAAX*" motif to specify prenylation. However, because a carboxyl-terminal cysteine is clearly important for Wrch-1 localization, we then investigated other potential post-translational modifications that might occur at cysteine residues. Palmitoylation is the reversible attachment of a palmitoyl fatty acid to cysteines via a thioester bond (35, 36). Although no consensus signal sequence exists to aid in the prediction of which cysteine residues are likely to be palmitoylated (35, 36), palmitoylation of cysteines in the hypervariable domains of prenylated small GTPases is common, although *CAAX*-signaled prenylation is a critical prerequisite for this fatty acid modification (18, 26, 37, 38). To determine whether the carboxyl-terminal cysteine residues of Wrch-1 are susceptible to thioester linkage to acyl groups like palmitates, we performed a recently described nonradioactive method for determining protein acylation (34). This method utilizes the ability of hydroxylamine to cleave thioester bonds resulting in free sulfhydryl groups that can then interact with biotin-conjugated 1-biotinamido-4-[4′-(maleimidomethyl)cyclohexanecarboxamido] butane (Btn-BMCC) sulfhydryl-specific reagent, effectively labeling acylated cysteine residues. Transiently expressed GFP-tagged Wrch-1 protein immunoprecipitated from human embryonic kidney 293 cells, was sus-

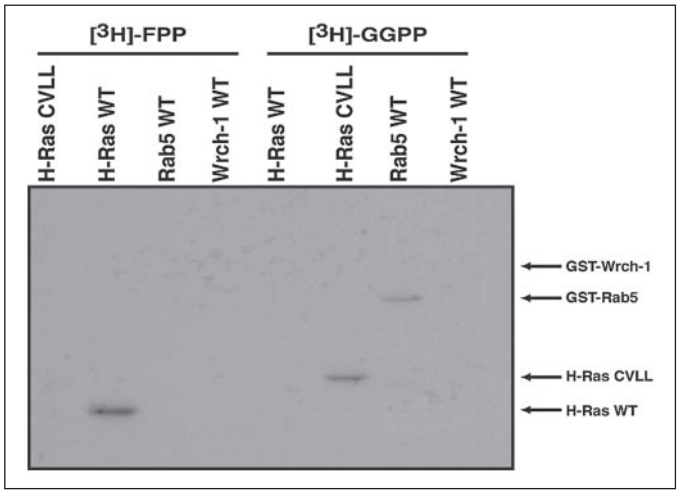

FIGURE 5. **Wrch-1 does not incorporate isoprenoid moieties.** Recombinant, purified H-Ras (FTase substrate), H-Ras CVLL mutant (GGTase I substrate), Rab5 WT (GGTase II substrate), and Wrch-1 proteins were incubated in reaction mixture with bovine brain lysate containing endogenous prenyltransferase activity and [^{3}H]farnesylpyrophosphate (*FPP*) or [^{3}H]geranylgeranylpyrophosphate (*GGPP*). To detect incorporation of radioactive prenylpyrophosphates, reactions were then analyzed by SDS-PAGE and autoradiography. The presence of a band demonstrates incorporation of indicated isoprenyl groups.

pended in *N*-ethylmaleimide to block any free, nonacylated cysteine residues, treated with hydroxylamine to cleave thioester bonds, and incubated with Btn-BMCC reagent to label newly exposed sulfhydryl groups, followed by Western blot analysis with streptavidin to detect any fatty acid modifications. Like H-Ras (palmitoylated on Cys-181 and Cys-184), Wrch-1 was labeled with the Btn-BMCC reagent, suggesting that Wrch-1 contains one or more cysteine residues that are acylated (Fig. 6*A*, *Wrch-1 CCFV lane, top band*). The nonspecific lower band seen in Fig. 6*A*, *Wrch-1 lanes*, is also observed at the same molecular weight in the *H-Ras lane*. As expected, the Btn-BMCC signal for K-Ras4B (no palmitoylated cysteines) was notably absent (Fig. 6*A*).

Because Wrch-1 contains two cysteine residues at its carboxyl terminus that may be palmitoylated, we wanted to determine whether one or both cysteine residues were susceptible to acylation. We anticipated that the second cysteine, Cys-256, would be the preferred site for acylation because of the cytosolic and nuclear subcellular distribution when mutated to a serine residue (CSFV). However, the CSFV mutant did retain some membrane localization, and it is possible that the first cysteine (Cys-255) may also be lipid-modified. As seen with parental Wrch-1 (CCFV), Wrch-1 (C255S, SCFV) labeled with Btn-BMCC, albeit at a lower efficiency, suggesting that the first cysteine residue (Cys-255) may regulate acylation of the second cysteine (Cys-256) (Fig. 6*A*, *Wrch-1 SCFV lane, top band*). As expected, neither the Wrch-1 (C256S, CSFV) nor (C255S/C256S, SSFV) mutants were labeled with Btn-BMCC (Fig. 6*A*, *Wrch-1 CSFV, -SSFV lanes, top band*). Additionally, treatment of Wrch-1 with the palmitate analog 2-BP, a potent inhibitor of small GTPase palmitoylation (37), caused a dramatic decrease in Wrch-1 fatty acid modification, suggesting that palmitate lipids are the fatty acid moieties utilized by Wrch-1 (Fig. 6*B*, *top panel*). Expression of each protein was confirmed by Western blot analysis (Fig. 6*A*, *bottom panel*). These results indicate that the second cysteine residue of Wrch-1 (Cys-256) is the primary cysteine residue for palmitate fatty acid modification.

To investigate directly whether the fatty acid modification of Wrch-1 detected by the above Btn-BMCC method is due to incorporation of palmitoyl groups at its carboxyl-terminal cysteines, similar to H-Ras, we used [^{3}H]palmitate to metabolically label NIH 3T3 cells transiently expressing Wrch-1 (38). Correct incorporation of [^{3}H]palmitate was confirmed using cells expressing H-Ras (palmitoylated on Cys-181 and Cys-184) and K-Ras (no palmitoylatable cysteines) as positive and negative controls, respectively. Immunoprecipitation of the exogenous HA-tagged Wrch-1 protein from labeled cells, followed by autoradiography, showed that Wrch-1, like H-Ras, incorporated [^{3}H]palmitate (Fig. 6*C*, *top panel*). The reduced level of incorporation seen in Wrch-1 compared with H-Ras may be due in part to potential differences in palmitoyl turnover and, therefore, steady-state levels of the palmitoylated GTPases and in part to the single palmitoylated cysteine residue in Wrch-1 compared with the two palmitoylatable cysteines in H-Ras. Western blot analysis of the total lysate input and immunoprecipitated K-Ras4B protein confirm that the absence of a [^{3}H]palmitate signal in the K-Ras-expressing cells was not due simply to a lack of protein expression or immunoprecipitation (Fig. 6*C*, *middle* and *bottom panel*). These results directly demonstrate that Wrch-1 is modified by palmitoylation.

Next, we investigated whether one or both Wrch-1 carboxyl-terminal cysteine residues are substrates for palmitoylation. Given that the Wrch-1 (C255S, SCFV) mutant, but not the Wrch-1 (C256S, CSFV) or -(C255S/C256S, SSFV) mutants, retained an acylated cysteine residue, we anticipated the second cysteine would also prove to be the important cysteine residue for palmitoylation. Like the Wrch-1 (CCFV) parent, Wrch-1 (C255S, SCFV) mutant incorporated the [^{3}H]palmitate signal, whereas Wrch-1 (C255S, CSFV)- and -(C255S/C256S, SSFV)-expressing cells lacked a [^{3}H]palmitate and, therefore, failed to incorporate [^{3}H]palmitate (Fig. 6*D*, *top panel*). Expression of each protein is confirmed by Western blot analysis of total lysate input and immunoprecipitated CSFV and SSFV protein (Fig. 6*D*, *middle* and *bottom panels*). These data show that the second cysteine residue is the required cysteine for palmitoylation. Thus, as with other palmitoylated small GTPases, this post-translational modification may be important for Wrch-1 subcellular localization.

To determine whether palmitoylation influences Wrch-1 localization, we treated NIH 3T3 cells transiently expressing GFP-tagged Wrch-1, H-Ras, K-Ras, or empty vector with 2-BP. 2-BP has been shown previously to cause the redistribution of palmitoylated GTPases, most recently the Cdc42 homologous protein Chp/Wrch-2, and other proteins within the cell (18, 37, 39). As expected, the lack of palmitoylatable sites within the K-Ras carboxyl terminus rendered its localization insensitive to the effects of 2-BP, whereas H-Ras was mislocalized from plasma membrane to endomembranes, thereby demonstrating substrate specificity of 2-BP for carboxyl-terminal cysteine-containing proteins (Fig. 7). Upon treatment with 2-BP, GFP-Wrch-1 relocalized dramatically to the cytosol and accumulated in the nucleus (Fig. 7). That Wrch-1 localization is sensitive to 2-BP treatment is consistent with our Btn-BMCC and metabolic labeling studies showing that Wrch-1 is palmitoylated. These results suggest that palmitate(s) could be the sole lipid modification normally causing retention of Wrch-1 in the cytosol and membranes.

The Carboxyl-terminal Cysteines Are Necessary for Wrch-1 Signaling to PAK—Because inhibition of palmitoylation and mutation of the carboxyl-terminal cysteines to serines rendered Wrch-1 improperly distributed, we predicted that loss of the palmitate modification would also affect Wrch-1 downstream signaling pathways. Previous studies have shown that activated, constitutively GTP-bound Wrch-1 results in increased auto-phosphorylation of the serine/threonine kinase PAK (15, 16). PAK1 is a well known effector of several Rho family proteins, including Cdc42 and Rac (3). To determine whether mutation of the carboxyl-terminal cysteines of Wrch-1 would affect its ability to promote auto-phosphorylation of PAK in NIH 3T3 fibroblasts, we transiently expressed the cysteine to serine mutants in an activated

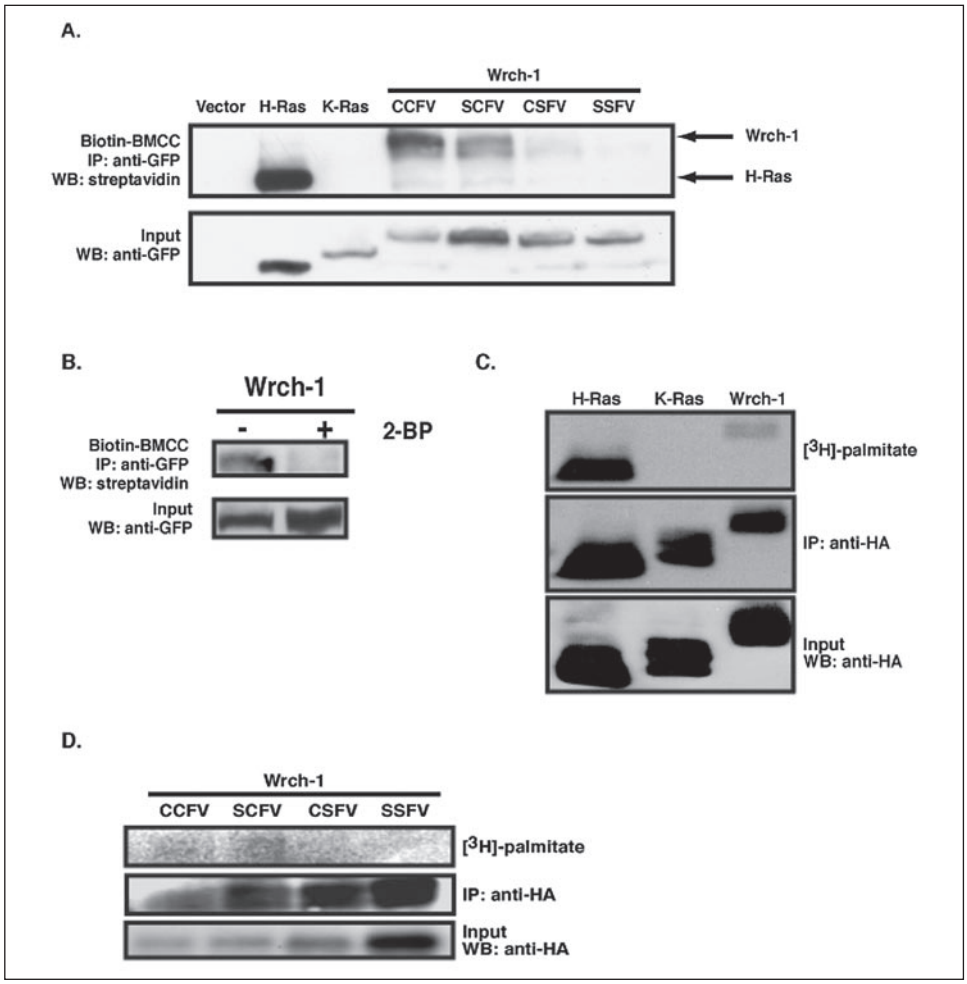

FIGURE 6. **Wrch-1 is palmitoylated.** NIH 3T3 cells were transiently transfected with either pEGFP constructs encoding GFP-tagged (*A* and *B*) or pCGN constructs encoding HA-tagged (*C* and *D*) H-Ras (palmitoylated positive control), K-Ras (nonpalmitoylated negative control), or Wrch-1 (CCFV, SCFV, CSFV, and SSFV). Two days post-transfection, proteins were immunoprecipitated with anti-GFP antibody, treated with hydroxylamine, and labeled with biotin-BMCC to detect acylated cysteine residues (*A*). A band indicates fatty acid modification (*top panel*). *Bottom panel* detects amount of protein available for immunoprecipitation (*IP*). *WB*, Western blot. *B*, Wrch-1-expressing cells were treated with either Me_2SO vehicle or 150 μM 2-BP 12 h prior to immunoprecipitation for Btn-BMCC labeling. *C* and *D*, cells were labeled metabolically with a 4-h pulse of [^{3}H]palmitate, and the GTPases were immunoprecipitated with anti-HA antibody. Immunoprecipitates were subjected to SDS-PAGE and exposed to film for 4–6 weeks. *Top panel*, [^{3}H]palmitate autoradiogram shows that Wrch-1- and H-Ras-positive control, but not K-Ras4B-negative control, incorporated [^{3}H]palmitate (*C*). Mutation of carboxyl-terminal cysteines affects Wrch-1 incorporation of [^{3}H]palmitate (*D*). *Middle panel*, to detect total amount of GTPases retrieved for autoradiography, immunoprecipitates were probed for GTPases by Western blot using anti-HA antibody (*IP*). *Bottom panel*, to detect amount of starting protein available for immunoprecipitation (*input*), total cell lysates were probed by Western blot using anti-HA antibody.

Wrch-1(Q107L) background and looked for differences in the PAK phosphorylation status. As shown previously by us and others (15, 16), the Wrch-1(Q107L) CCFV parent resulted in an increase in phosphorylated PAK levels when compared with vector-expressing cells (Fig. 8). Consistent with the loss of membrane association, the ability of the Wrch-1 SCFV, -CSFV, and SSFV mutants to induce phosphorylation of PAK was considerably reduced when compared with the CCFV parent. This suggests that correct localization of Wrch-1 via its palmitate modification is necessary for downstream signaling molecules.

The Carboxyl-terminal Cysteine 256 (CCFV), but Not 255 (CCFV), Is Required for Wrch-1 Transformation—Given that loss of palmitate modification affected Wrch-1 downstream signaling, we predicted that loss of the palmitate modification would also affect Wrch-1 biological activity. The transforming activity of prenylated Rho family proteins is impaired when palmitate modification is blocked (25). We sought to determine whether loss of palmitoylation would interfere with Wrch-1 transformation.

Previous studies showed that activated Raf can cooperate with constitutively activated Rho family members such as Cdc42 to cause synergistic transformation of NIH 3T3 cells (40–43). To determine whether Wrch-1, like Cdc42, could also cooperate with Raf to cause focus formation, we transiently cotransfected into NIH 3T3 cells pCGN-hygro constructs expressing activated Wrch-1(Q107L), Cdc42(Q61L), or Rac1(Q61L), together with either pZIP-Raf22W or the empty pZIP-NeoSV(x)1 vector. We then evaluated the dishes for the appearance of foci of transformed cells. Raf-induced foci of transformed cells are indistinguishable in appearance from those caused by activated Ras and are characterized by large, spreading foci of highly refractile, spindle-shaped, morphologically transformed cells. In contrast, activated Rho GTPases cause foci of transformed cells that are very distinct from those of Raf, and are characterized by tight clusters of rounded, refractile cells that are frequently multinucleated. We anticipated that the appearance of Wrch-1 foci might resemble those of Cdc42 more than those of Raf-1. As expected, activated Wrch-1 alone was unable to induce focus formation (Fig. 9*A*). In cooperation with Raf, however, activated Wrch-1 formed foci that were similar to those of Cdc42 and Rac1 (Fig. 9*A*).

Next, we evaluated whether loss of the carboxyl-terminal cysteines, individually or together, impaired Wrch-1 focus formation in cooperation with Raf. The ability of activated Wrch-1-CSFV and -SSFV to form foci of transformed cells was greatly reduced compared with that of the parental Wrch-1 (Fig. 9*B*). These results are consistent with a critical role for the second cysteine (CCFV), rather than the canonical C*AAX*

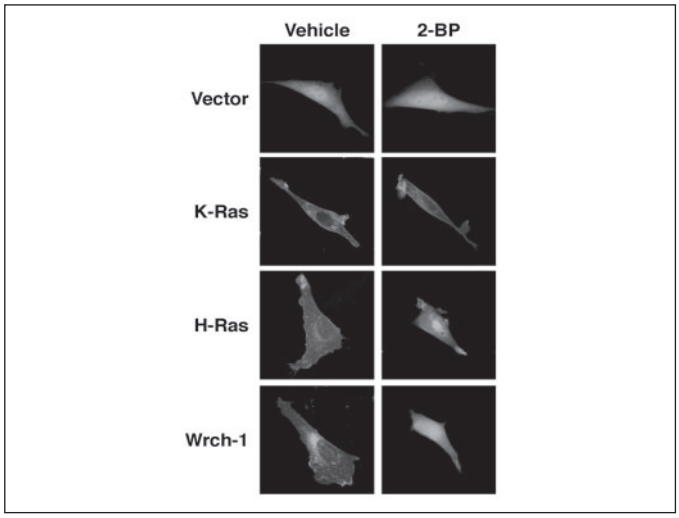

FIGURE 7. **Palmitate analog 2-BP causes mislocalization and cytosolic accumulation of Wrch-1.** NIH 3T3 cells were transiently transfected with pEGFP empty vector or pEGFP constructs encoding GFP-tagged K-Ras4B (nonpalmitoylated negative control), H-Ras (palmitoylated positive control), or Wrch-1. Cells were treated overnight with either Me_2SO vehicle or 150 μM 2-BP. The following day, 2-BP-treated cells were assessed for cytosolic and/or nuclear accumulation by live cell imaging. Images are representative of at least three independent experiments.

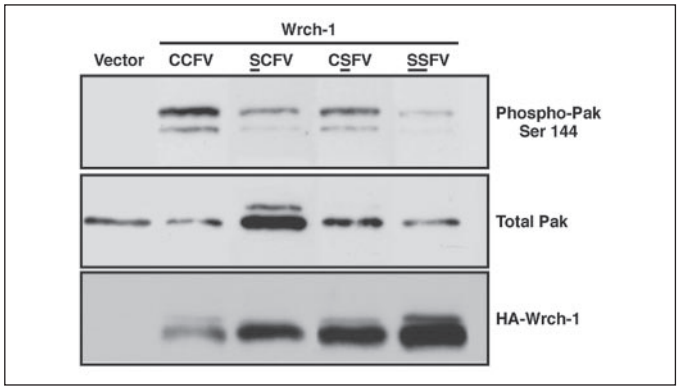

FIGURE 8. **Carboxyl-terminal cysteines regulate Wrch-1 downstream signaling to PAK.** NIH 3T3 cells were transiently transfected with pCGN constructs encoding activated Wrch-1(Q107L) and Wrch-1(Q107L) carboxyl-terminal mutants (SCFV, CSFV, and SSFV). Whole cell lysates were collected 24 h post-transfection and analyzed by Western blot for changes in endogenous phosphorylated PAK status (*top panel*). For controls, total PAK and HA-Wrch-1 protein levels were analyzed (*middle, bottom panels*). Blots are representative of three separate experiments.

cysteine (CCFV), in mediating Wrch-1 membrane association. Most unexpectedly, mutation of the first cysteine residue, SCFV, did not impair Wrch-1 focus forming ability but rather enhanced it. This result suggests that the roles of the two cysteines are distinct and that the upstream cysteine plays a negative regulatory role in Wrch-1 biological activity.

To determine whether these results also applied to other aspects of the transformed phenotype, we evaluated the ability of activated Wrch-1 carboxyl-terminal mutants to promote anchorage-independent growth in soft agar. In contrast to their activity in focus formation assays, Rho proteins alone, including Wrch-1 (16), are sufficient to confer anchorage-independent growth and do not require cooperation with Raf in soft agar assays. Therefore, NIH 3T3 cells stably expressing activated, HA-tagged Wrch-1(Q107L) were seeded into agar and analyzed for colony forming activity. Comparable expression of each Wrch-1 protein was seen (data not shown). Activated Wrch-1 potently induced colony formation in soft agar (Fig. 9, *C* and *D*). Consistent with the focus formation data, mutation of either the second cysteine (C256S, CSFV) or both cysteines (C255S/C256S, SSFV) resulted in a strong reduction of colony formation, whereas the cysteine to serine mutation at residue 255 (SCFV) led to a significant increase in Wrch-1-induced colony formation (Fig. 9, *C* and *D*). Taken together, our focus formation and soft agar analyses suggest that the second cysteine residue of the CCFV motif is required for Wrch-1 transforming activity, whereas the first cysteine may function instead as a negative regulator.

DISCUSSION

Wrch-1 gene expression is up-regulated in Wnt-1-transformed cells, and Wrch-1 activation can phenocopy the changes in cellular morphology caused by Wnt-1 (15). Wrch-1 shares significant amino acid sequence and functional identity with Cdc42 but exhibits significant divergence in carboxyl-terminal sequences. Wrch-1 terminates in an atypical C*AAX* tetrapeptide motif, and its hypervariable domain possesses an additional 21 amino acid residues not found in Cdc42. In the present study, we evaluated the role of these unique carboxyl-terminal features in Wrch-1 membrane association and biological activity. Although we found that the carboxyl-terminal nine residues of Wrch-1 alone were sufficient to promote Wrch-1 membrane association, Wrch-1 and Cdc42 exhibited very distinct patterns of subcellular localization, with significant amounts of Wrch-1 found at the plasma membrane and early endosomes. Most surprisingly, we found that an intact C*AAX* motif was not required for Wrch-1 membrane association, but instead, mutation of a second carboxyl-terminal cysteine significantly reduced Wrch-1 membrane association. Furthermore, Wrch-1 membrane association was not dependent on isoprenoid modification but was instead dependent on palmitoylation of the second cysteine residue. Finally, we found that Wrch-1, like Cdc42, can also promote growth transformation of NIH 3T3 cells and that the palmitoylated cysteine was critical for this activity.

Highly related Rho and Ras GTPases exhibit distinct cellular functions that can be attributed in part to subcellular localizations dictated by their distinct hypervariable domains (22, 27, 28). For example, RhoA shares 90% identity with RhoB and RhoC, and these three proteins share common regulators and effectors (3). However, despite these strong similarities, whereas RhoA can promote growth transformation, there is evidence that RhoB may function in an opposite fashion and exhibit tumor suppressor function (20, 44). RhoC but not RhoA has been associated with tumor cell invasion (45, 46). These three related Rho GTPases show the greatest sequence divergence in their carboxyl-terminal sequences, and this divergence results in differences in subcellular localization that in turn promote different cellular functions (22, 27, 28). Philips and co-workers (18) eloquently demonstrated that the last 20 amino acids of several Rho and Ras proteins, including Cdc42, mimic the subcellular localization of the full-length proteins. We have shown here that at least the last 9 amino acids of Wrch-1 are sufficient to confer proper subcellular distribution. This finding is consistent with other studies that illustrate that all of the membrane targeting information is located in the carboxyl terminus.

Thus, although Wrch-1 and Cdc42 share significant sequence identity and functional overlap, their divergent carboxyl-terminal sequences may also impart different biological roles to these biochemically related proteins. Mutation of the cysteine residue of the C*AAX* motifs of Cdc42 and other Rho GTPases to prevent prenylation results in loss of membrane association and biological activity (20–24). Therefore, we were surprised that the analogous mutation of the Wrch-1 C*AAX* motif (C255S, SCFV) did not cause complete mislocalization or loss of transforming activity. Analogously to Ras family GTPases, the membrane localization of conventional Rho family proteins generally requires either a geranylgeranyl (*e.g.* Cdc42) or a farnesyl lipid group (*e.g.* Rnd3/RhoE) attached to the cysteine residue of the C*AAX* motif and a "second

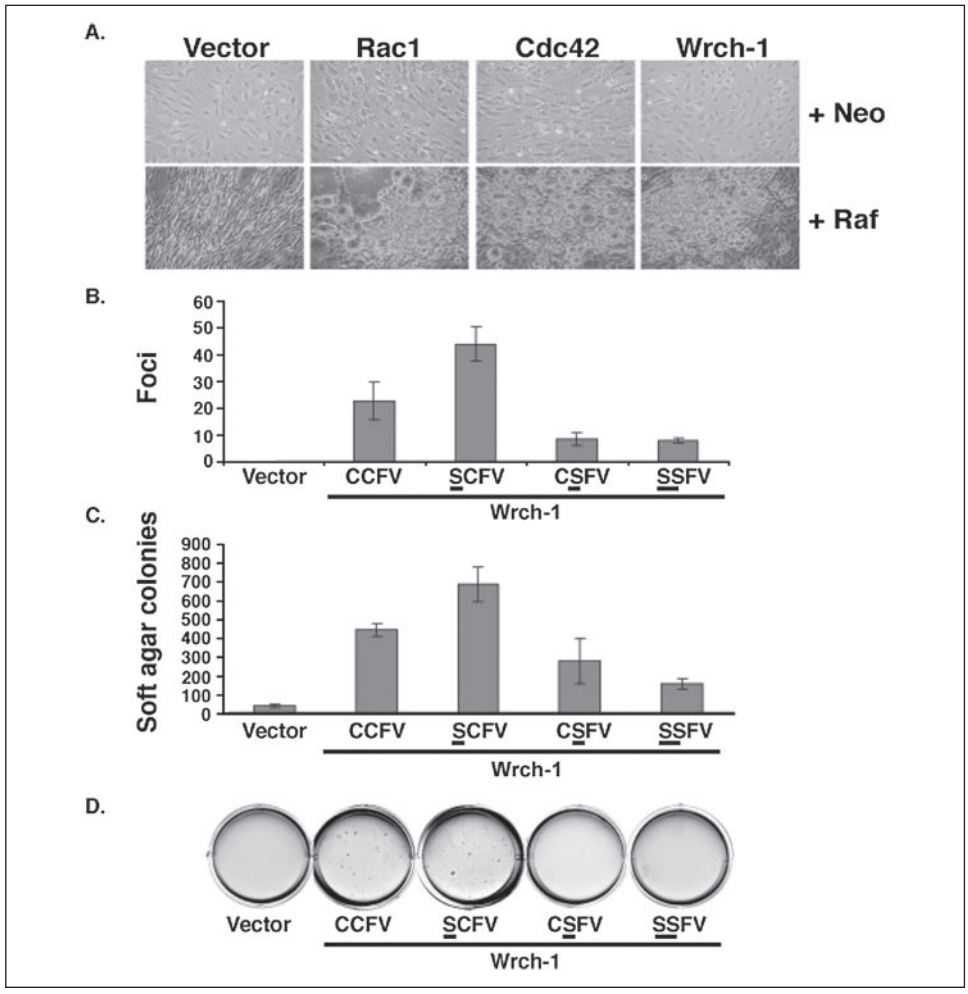

FIGURE 9. **Carboxyl-terminal cysteines of the hypervariable domain differentially modulate Wrch-1 transforming activity.** NIH 3T3 cells were transiently cotransfected with either empty vector pZIPneo or activated pZIP-Raf22W, along with pCGN constructs encoding activated Rac1(Q61L), Cdc42(Q61L), or Wrch-1(Q107L). After 14 days, dishes were fixed and stained with crystal violet, and foci of transformed cells were counted. Images are representative of at least three separate experiments carried out in duplicate. *A*, activated Wrch-1 cooperates with activated Raf to form Cdc42-like foci of transformed cells. *B*, cooperation with Raf by Wrch-1 containing carboxyl-terminal mutations at cysteine residues 255 (CCFV > SCFV), 256 (CCFV > CSFV), or both (CCFV > SSFV). *C*, NIH 3T3 cells stably expressing the above-mentioned pCGN Wrch-1 constructs were seeded into soft agar and analyzed for their ability to induce anchorage-independent growth. Colonies formed after 14–21 days were stained and scanned (*D*) and quantified (*C*). *Images* and *bar graphs* are representative of at least two separate experiments carried out in triplicate.

signal" consisting of either several basic residues or palmitoylated cysteine residues in the upstream hypervariable domain (3). In direct contrast, we show here that the Cdc42 homolog Wrch-1 does not utilize either a geranylgeranyl or farnesyl isoprenoid moiety for membrane targeting. Instead, its localization is regulated by a palmitoyl fatty acid, demonstrating that the Wrch-1 C*AAX*-like motif, CCFV, is not a canonical, prenylated C*AAX*.

It is unclear at this juncture whether Wrch-1 also requires a second signal for proper localization to plasma membranes. However, the carboxyl terminus of Wrch-1 contains several basic residues that could form a polybasic second signal to complement the palmitate modification (47, 48), and are included in the short stretch of nine amino acids that constitutes a minimal targeting sequence. Alternatively, by analogy to C*AAX*-containing palmitoylatable small GTPases, Wrch-1 may require other poorly defined but essential motifs surrounding palmitoylatable cysteines (38, 49). The Wrch-1 carboxyl terminus also contains uncommon but conserved residues such as tandem tryptophans and a tyrosine. The tandem tryptophan residues may represent a di-aromatic motif of the kind frequently associated with endosomal sorting (50) and may help to direct Wrch-1 to endosomes. The contribution of these residues to Wrch-1 membrane targeting and function is currently under investigation.

More distantly related Rho and Ras proteins also target to membranes but do not depend on carboxyl-terminal lipid modification. For example, the Rho-related proteins, RhoBTB-1/2 and Miro-1/2, as well as the Ras-related proteins, Rit, Rin, Gem, and Rem2, are not known to undergo lipid modification, yet display distinct membrane associations (29, 51–53). Conversely, although Rab proteins are prenylated, they lack a conventional C*AAX* motif and, instead, terminate in CC*XX*, C*XC*, and *XX*CC sequences that, in combination with upstream residues, serve as targeting motifs for GGTase II modification (54, 55). We have demonstrated here that the CC*XX* motif of Wrch-1 is not a target for GGTase II but rather for palmitoylation. Most interestingly, Chp/Wrch-2, the closest relative of Wrch-1, lacks a C*AAX* motif and should, therefore, not be modified by prenyltransferases (56). However, it shares with Wrch-1 a CFV (C*XX*) motif, incorporates a fatty acid modification at its carboxyl-terminal cysteine residue, and Chp membrane association is also disrupted by 2-BP treatment (39). It is interesting to speculate that the CFV (C*XX*) motif may be a novel recognition site for post-translational modification by palmitoyltransferases. Although other mammalian Rho GTPases are also palmitoylated, their palmitate modification is dependent on prior modification by prenylation. Therefore, Wrch-1 and Chp undergo unique lipid modification-dependent membrane targeting not seen with other known mammalian Ras family GTPases. Most interestingly, atypical Rho-like proteins have been described in the plant *Arabidopsis* that also undergo a prenyl-independent, palmitoyl modification; however, these small GTPases terminate neither in conventional C*AAX* nor in C*XX* motifs (57, 58). In addition, their carboxyl-terminal sequences lack the basic residues found in Wrch-1 and Chp, and multiple palmitoylated cysteines appear to be required for full membrane association.

Our finding that the Wrch-1 carboxyl-terminal Cys to Ser mutants have differential effects on Wrch-1 localization and function is unusual for small GTPases and suggests that each cysteine has a distinct contri-

bution to Wrch-1 function. Consistent with this possibility, we have shown that mutation of Cys-255 (SCFV) resulted in increased transforming activity of Wrch-1, suggesting that this residue has a negative regulatory effect on Wrch-1 localization and function, whereas mutation of Cys-256 (CSFV) abrogated membrane localization and transformation. Most interestingly, we have made a similar observation with Chp, where mutation of the cysteine residue of the CFV motif caused mislocalization and loss of transforming activity, whereas mutation of an upstream cysteine did not alter membrane association, yet caused a significant enhancement of transforming activity (39). It is formally possible that one cysteine regulates acylation of the second cysteine; more sensitive methods for detection of such modifications will be necessary to determine whether this is the case.

Palmitoylation of cysteines in the hypervariable domain of Wrch-1 suggests that Wrch-1 may traffic and signal similarly to other palmitate-containing small GTPases such as H-Ras and TC10 rather than Cdc42. For example, palmitoylated H-Ras, but not polybasic domain-containing K-Ras, transports to the plasma membrane via a Golgi-mediated process leading to the association of H-Ras with cholesterol-rich lipid rafts (59–61). Lipid rafts are specialized microdomains that contain distinct composition of lipids and signaling proteins that may organize signals impinging on the cell surface into distinct cascades (62). For some palmitoylated small Rho GTPases, such as TC10, lipid raft localization is critical to their downstream activity. For example, one study showed that TC10 could control Glut 4 activity only if specifically targeted to lipid rafts (63). TC10 has two nontandem upstream cysteines that are substrates for palmitoylation. Mutation of the cysteine immediately upstream of the *CAAX* motif prevented endomembrane localization of TC10, whereas the other cysteine had no effect on TC10 subcellular distribution (64). Because palmitoylation favors association of proteins to lipid rafts, it is possible that Wrch-1 may also traffic through the exocytic pathway to interact with these lipid-rich microdomains, thereby introducing different Wrch-1 protein-protein interactions that Cdc42, lacking a palmitoylation site, may not encounter. These differences in subcellular localization suggest a potential mechanism for functional diversity.

Palmitoylation of Wrch-1 may also provide another level of regulation for Wrch-1 protein interactions and biological function. Rho and Ras protein activities are regulated by both nucleotide binding and subcellular location. For example, RhoGDIs negatively regulate Cdc42 and other Rho family proteins by binding their prenoid moieties and sequestering the proteins to the cytosol (9, 10). Because Wrch-1 lacks a prenyl group and does not bind RhoGDIs,[5] the dynamic, reversible nature of palmitoylation could serve instead as a "RhoGDI-like" regulatory entity for Wrch-1 localization. The turnover rate for H-Ras palmitoylation is a rapid ($t_{1/2}$ ~20 min) (65, 66), and H-Ras lacking palmitoylatable cysteines fails to target the plasma membrane and is functionally deficient (26, 38, 66). Most recently, a de/reacylation cycle on H-Ras has been shown to regulate its localization and activation subcellularly (67). Palmitoylation targets H-Ras not only to the plasma membrane but specifically to lipid rafts where dynamic GTP-dependent shifts of H-Ras in and out of rafts occur (68). Similar palmitoylation and de-palmitoylation kinetics for Wrch-1 palmitoylation could similarly cause Wrch-1 to quickly enter and exit lipid rafts and regulate Wrch-1 downstream activity in a dynamic manner. Our recent data demonstrate that Wrch-1 exchanges GDP unusually rapidly (69). Thus, rapid movement of Wrch-1 in and out of lipid rafts may combine with the fast-cycling nature of Wrch-1, leading to regulatory control of Wrch-1 based in part on its localization.

To date, there are no published human protein *S*-acyltransferases (PATs) shown to modify Wrch-1 or other palmitoylated small GTPases. However, recent yeast genetic screens for PAT components have identified PAT genes that are necessary for palmitoylation of yeast Ras (70). These yeast PAT genes contain a cysteine-rich domain and a DHHC motif required for PAT activity, and this DHHC-cysteine-rich domain motif has been found in several human proteins that are involved in the *S*-acylation of specific neuronal proteins (70–73). Given that there are several DHHC-cysteine-rich domain-containing genes, characterization of these genes as PATs could reveal potential regulatory proteins for Wrch-1 localization and, ultimately, its downstream activity and serve as potential targets for pharmacological inhibitors.

Because Cdc42 and other Rho GTPases have been implicated in human oncogenesis (11–14), inhibitors of GGTase I have been considered for cancer therapy (31, 74). However, because Cdc42 function is important for normal cell proliferation, one concern is that GGTIs may exhibit significant normal cell toxicity. Because Wrch-1 and Chp exhibit functional overlap with Cdc42, and their functions are not dependent on GGTase I activity, perhaps these atypical Rho GTPases will provide some protection against GGTase I suppression of Cdc42 function in normal cells.

In summary, our recent delineation of a unique regulatory function of the amino terminus of Wrch-1 (69) together with the unusual nature of the Wrch-1 carboxyl terminus in mediating subcellular localization identified in this study make Wrch-1 highly distinct from the classical Rho family GTPases. Wrch-1 together with Chp represent a new class of mammalian Rho GTPases whose membrane targeting and biological activity are dependent on lipidation by palmitoyl fatty acids but not by isoprenoids. Our studies with structural mutants suggest distinct functional contributions of palmitoylation at different carboxyl-terminal cysteines. Further studies are needed to determine how the other residues of the hypervariable domain affect Wrch-1 localization and function. Additional studies of Wrch-1 and other palmitoylation-only lipidated small GTPases like Chp/Wrch-2 and *Arabidopsis* Rac proteins will also be necessary to clarify how this modification affects their ability to localize and to target downstream signaling pathways. The novel mechanism by which Wrch-1 and Chp function is regulated by carboxyl-terminal sequences and lipid modifications adds further to the complexity by which carboxyl-terminal variation may diversify the biological roles of proteins that otherwise exhibit strong biochemical similarity.

[5] Berzat, A. C., Brady, D. C., Fiordalisi, J. J., and Cox, A. D. (2005) *Methods Enzymol*, in press.

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