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 CAAX 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 (CCFFV > 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). Guanine nucleotide exchange factors activate Rho proteins by promoting GDP dissociation in exchange for GTP (6, 7), whereas GTPase-activating proteins down-regulate 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 Wnt1 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 CAAX motif (17, 18). The canonical CAAX 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 CAAX-signaled prenylation

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; FFt, farnesyltransferase inhibitor; GGtI, geranylgeranyltransferase I inhibitor; 2-BP, 2-bromopalmitate; FPP, farnesylpyrophosphate; GGPP, geranylgeranylpyrophosphate; FTIC, fluorescence isothiocyanate; MeSO, dimethyl sulfoxide; Bnn-BMCC, biotin-1-biotinamido-4-(maleimidomethyl) cyclohexanecarboxamidoxbutane; pEGFP, enhanced GFP; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; HRP, horse radish peroxidase; TBS, Tris-buffered saline; P21, activated kinase; WT, wild type; GST, glutathione S-transferase; PAT, protein S-acyltransferase.
Palmitoylation Not Prenylation of Wrch-1 GTPase

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 × 10^3 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, -SSFV, -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, -SSFV, -CSFV, or -SSFV were suspended in DMEM containing 10% calf serum, 1% penicillin/streptomycin, and 0.4% agar (BD Biosciences) at 5 × 10^5 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 Me2SO vehicle, 10 μM FTI-2153, 10 μM GGT1-2156, 10 μM FTI-2153 + GGT1-2166, 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 Saı ¨d 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 and Zeiss 510 LSM confocal microscope (Zeiss, Thornwood, NY) using MetaMorph imaging software (Universal Imaging Corp., Downingtown, 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(107L). 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(107L) 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(107L) and Texas Red-conjugated transferrin images was analyzed using MetaMorph imaging software.

EXPERIMENTAL PROCEDURES

Molecular Constructs—pcDNA3 expression constructs encoding wild type (WT) and GFPase-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 Quick-Change 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.
Palmitoylation Not Prenylation of Wrch-1 GTPase

**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. Cov- erslips were mounted onto glass microslides with Vectashield Hardset mounting medium (Vector Laboratories, Burlingame, CA) and ana- lyzed 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 [3H]palmitate (American Radiochemical Inc.) in DMEM containing 5 mM sodium pyruvate, 4X 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 inhibitor 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 nonreduc- ing protein sample buffer, and incubated for 30 min at 30 °C. Reaction was stopped with SDS protein sam- ple buffer. Samples were boiled briefly, run on 4–20% SDS-PAGE, pre- pared for fluorography, and exposed to prefloshed 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 mM β-glycero phosphatase, 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 MgCl₂, 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 mem- brane. 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 Subcellu- lar Distribution Distinct from That Seen for Cdc42—As shown in Fig. 1A, 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 CAAX motif. Second, Wrch-1 con- tains 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 fibro- blasts and performed live cell imaging analyses (Fig. 1B). 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. 1B) (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 CAAX- 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 localizations. 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 (CCFV) 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 (CCFV, SCFV) accumulated in the cytosol. Images are representative of 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 GTase, 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 [3H]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 [3H]FPP and [3H]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 [3H]prenylpyrophosphates followed by autoradiography demonstrated that, unlike H-Ras and Rab5, Wrch-1 was unable to incorporate [3H]FPP or [3H]GGPP (Fig. 5). To confirm further the absence of isoprenyl modification of purified Wrch-1 protein, radioactivity of each [3H]FPP- or [3H]GGPP-bound protein reaction was determined by counting in a scintillation counter. As expected, no radioactivity was detected with Wrch-1, whereas [3H]FPP radioactivity counts were present for H-Ras WT and [3H]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 GTases is common, although CAAX-signaled palmitoylation 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-[maleimidomethyl] cyclohexanecarboxamidol butane (Btm-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-
The carboxyl-terminal cysteine residues of Wrch-1 are necessary for its subcellular localization. Upon treatment with 2-BP, GFP-Wrch-1 relocalized dramatically to the cytosol and accumulated in the nucleus (Fig. 7). Upon treatment with 2-BP, GFP-Wrch-1 localized to the cytosol and redistributed to the nucleus. This redistribution of GFP-Wrch-1 is consistent with our previous studies that showed that the carboxyl-terminal cysteine residues of Wrch-1 are necessary for its subcellular localization. The carboxyl-terminal cysteine residues of Wrch-1 are necessary for its subcellular localization. Upon treatment with 2-BP, GFP-Wrch-1 relocalized dramatically to the cytosol and accumulated in the nucleus (Fig. 7). Upon treatment with 2-BP, GFP-Wrch-1 relocalized dramatically to the cytosol and accumulated in the nucleus (Fig. 7). Upon treatment with 2-BP, GFP-Wrch-1 relocalized dramatically to the cytosol and accumulated in the nucleus (Fig. 7). Upon treatment with 2-BP, GFP-Wrch-1 relocalized dramatically to the cytosol and accumulated in the nucleus (Fig. 7).
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 phospho-rylated 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 mod-
ification 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 palmitoylation would also affect Wrch-1 biological activity. The transforming activity of prenylated Rho family proteins is impaired when palmitoylation 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 for-
mation, 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 GTGases 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. 9A). In cooperation with Raf, however, activated Wrch-1 formed foci that were similar to those of Cdc42 and Rac1 (Fig. 9A).

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. 9B). These results are consistent with a critical role for the second cysteine (CCFV), rather than the canonical CAAX.
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 CAAX 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 CAAX 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 CAAX 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 CAAX 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 CAAX motif and a “second
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 CAAX-like motif, CCFV, is not a canonical, prenylated CAAX.

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 CAAX-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 CAAX motif and, instead, terminate in CCXX, CXC, and XXCC sequences that, in combination with upstream residues, serve as targeting motifs for GGTase II modification (54, 55). We have demonstrated here that the CCXX 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 CAAX motif and should, therefore, not be modified by prenyltransferases (56). However, it shares with Wrch-1 a CFV (CXX) 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 (CXX) 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 CAAX nor in CXX 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 non tandem 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, 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 (t½ ~ 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 terminal 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.

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