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Multiple Sequence Elements Facilitate Chp Rho GTPase Subcellular Location, Membrane Association, and Transforming Activity

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C*dc42 *h*omologous *p*rotein (Chp) is a member of the Rho family of small GTPases and shares significant sequence and functional similarity with Cdc42. However, unlike classical Rho GTPases, we recently found that Chp depends on palmitoylation, rather than prenylation, for association with cellular membranes. Because palmitoylation alone is typically not sufficient to promote membrane association, we evaluated the possibility that other carboxy-terminal residues facilitate Chp subcellular association with membranes. We found that Chp membrane association and transforming activity was dependent on the integrity of a stretch of basic amino acids in the carboxy terminus of Chp and that the basic amino acids were not simply part of a palmitoyl acyltransferase recognition motif. We also determined that the 11 carboxy-terminal residues alone were sufficient to promote Chp plasma and endomembrane association. Interestingly, stimulation with tumor necrosis factor-α activated only endomembrane-associated Chp. Finally, we found that Chp membrane association was not disrupted by Rho guanine nucleotide dissociation inhibitory proteins, which are negative regulators of Cdc42 membrane association and biological activity. In summary, the unique carboxy-terminal sequence elements that promote Chp subcellular location and function expand the complexity of mechanisms by which the cellular functions of Rho GTPases are regulated.

INTRODUCTION

Cdc42 *h*omologous *p*rotein (Chp; also called Wrch-2) is a recently identified member of the Rho family of small GTP binding and hydrolyzing proteins (Aronheim *et al*., 1998). To date, the best-characterized Rho family small GTPases are RhoA, Rac1, and Cdc42 (Wennerberg and Der, 2004). Although Chp exhibits strong sequence, biochemical, and functional similarities with Cdc42, Chp possesses additional ~30-amino acid amino- and carboxy-terminal sequences not found in Cdc42 or other canonical Rho family GTPases. Furthermore, Chp lacks the carboxy-terminal CAAX (C, cysteine; A, aliphatic acid; X, terminal amino acid) tetrapeptide motif that is critical for the function of Cdc42 and other Ras and Rho GTPases and instead terminates in a tripeptide cysteine-phenylalanine-valine sequence.

The presence of a carboxy-terminal CAAX sequence is critical for initiating a series of posttranslational modifications that promote the association of CAAX-containing proteins with the plasma or other cellular membranes (Cox and Der, 1992; Casey and Seabra, 1996; Fu and Casey, 1999). These modifications include 1) covalent modification of the cysteine residue by a geranylgeranyl or farnesyl isoprenoid lipid, 2) Rce1-catalyzed proteolytic removal of the -AAX residues, and 3) carboxylmethylation of the now terminal prenylated cysteine (Ashby *et al*., 1992; Otto *et al*., 1999). CAAX-directed processing increases the overall hydrophobicity of the carboxy terminus of the protein (Der and Cox, 1991; Cox and Der, 1992; Casey and Seabra, 1996; Fu and Casey, 1999), and prevention of these modifications renders Rho GTPases cytosolic and nonfunctional (Murphy *et al*., 1999; Allal *et al*., 2000; Solski *et al*., 2002; Joyce and Cox, 2003). The importance of isoprenoid modification in Rho GTPase function is also indicated by the regulatory mechanism of Rho guanine nucleotide dissociation inhibitors (RhoGDIs; α, β, and γ) (DerMardirossian and Bokoch, 2005). RhoGDIs recognize and mask the isoprenoid group at the carboxy terminus of the Rho GTPase, rendering it cytosolic and inactive. Whether RhoGDIs regulate all members of the Rho GTPase family has not been determined.

Although CAAX-signaled modifications are necessary for Rho GTPase membrane association and function, these modifications alone are not sufficient to promote proper subcellular localization and full biological activity (Hancock *et al*., 1990). Two distinct motifs immediately upstream of the CAAX box have also been implicated in promoting membrane association and are therefore characterized as secondary membrane-targeting signals. The secondary signal may comprise either a cysteine residue that is posttranslationally modified by a palmitate fatty acid or a polybasic amino acid sequence (Hancock *et al*., 1990; Mitchell *et al*., 1994; Dudler and Gelb, 1996; Coats *et al*., 1999). The presence of one of these two motifs, together with prenylation, is necessary and

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Abbreviations used: biotin-BMCC, 1-biotinamido-4-[4′-(maleimidomethyl) cyclohexanecarboxamido] butane; CAAX, cysteine (C) aliphatic amino acid (A) aliphatic amino acid (A) terminal amino acid (X); Chp, Cdc42 homologous protein; HA, hemagglutinin; RhoGDI, Rho guanine nucleotide disassociation inhibitor; GFP, green fluorescent protein; PAK, p21 activated kinase; PBD, p21 binding domain; TNF, tumor necrosis factor; YFP, yellow fluorescent protein.

sufficient for proper localization and biological function of Ras and Rho small GTPases.

The nature of these second signals was first established with CAAX-containing and membrane-localized Ras proteins. Mutation of the cysteine residues upstream of the CAAX motif of H-Ras did not impair CAAX-signaled prenylation but nevertheless caused mislocalization of H-Ras to endomembranes and impairment of transforming activity (Hancock *et al*., 1989). Similarly, pharmacological inhibition of palmitoylation caused H-Ras to mislocalize to endomembranes (Michaelson *et al*., 2002). Furthermore, mutation of lysine basic residues adjacent to the CAAX motif of activated K-Ras4B did not prevent the prenyl modification of K-Ras4B, but nonetheless rendered the protein partially cytosolic and caused a near total elimination of its transforming activity (Hancock *et al*., 1990; Jackson *et al*., 1994). Subsequent studies firmly established the role of palmitoylated cysteines (e.g., RhoB and TC10) or basic residues (e.g., Rac1) as second signals important for the proper membrane targeting and function of Rho family GTPases (Mitchell *et al*., 1994; Dudler and Gelb, 1996; Coats *et al*., 1999).

We recently reported that Chp, which lacks a canonical CAAX motif, is still dependent on an intact carboxy terminus for biological activity and association with the plasma membrane (Chenette *et al*., 2005). We found that mutation of the terminal cysteine resulted in cytoplasmic mislocalization of Chp, analogous to phenotype of a corresponding "SAAX" mutation in Cdc42. Unlike Cdc42, we found that Chp localization and transforming activity depends on palmitoylation rather than prenylation, indicating that Chp membrane association is mediated by a novel posttranslational processing pathway that takes advantage of palmitoylation as the sole carboxyl-terminal lipid modification. Similarly, we also determined that a Chp-related GTPase, Wrch-1, also depends on palmitoylation, and not prenylation, for proper membrane association and function (Berzat *et al*., 2005). Thus, although Chp and Wrch-1 share functional similarities with Cdc42, they diverge significantly in their mode of membrane association.

Recent studies emphasize the critical role of carboxy-terminal variation in supporting functionally distinct roles for otherwise highly related Rho GTPases. For example, although RhoA, RhoB, and RhoC share strong sequence identity, exhibit essentially identical intrinsic biochemical properties, and interact with common regulators and effectors, they serve unique roles in cell physiology (Wheeler and Ridley, 2004). The plasma membrane-associated RhoA is a regulator of actomyosin contractility, whereas endosome-associated RhoB regulates cytokine trafficking and cell survival, and RhoC is important in cell locomotion and has been implicated in cancer cell invasion and metastasis. Divergence in carboxy-terminal sequences account for their distinct subcellular locations, membrane interactions, and biological activities (Wang *et al*., 2003). Similarly, the unique carboxy-terminal sequence and lipid modifications of Chp likely distinguish it functionally from the otherwise highly related Cdc42. Further delineation of the nature of Chp carboxy-terminal sequences and their role in Chp biological function will be needed to address this possibility.

In this study, we found that the carboxy terminus of Chp is necessary for recruitment of Chp effectors to distinct membrane locations. We identified multiple carboxy-terminal sequences important for mediating Chp subcellular location and biological activity, because palmitoylation alone was not sufficient to promote Chp membrane association and function. We found that basic residues as well as an invariant tryptophan residue were not required for Chp modification by palmitoylation, yet were critical for Chp subcellular location and transforming activity. The terminal 11 amino acids of Chp, which contain the palmitoylated cysteine as well as conserved tryptophan and basic amino acids were sufficient to target green fluorescent protein (GFP) to the plasma and endomembranes. Additionally, we found that RhoGDIs failed to prevent Chp membrane association. Thus, although Chp and Cdc42 share significant biochemical similarity, their considerable divergence in carboxy-terminal–mediated membrane targeting and subcellular location help explain their unique roles in cell physiology.

MATERIALS AND METHODS

Molecular Constructs and Reagents

Mammalian Chp expression vectors were generated by fusing cDNA sequences encoding Chp, Chp[G40V], and ΔN-Chp[G40V] in frame with amino-terminal sequences encoding a hemagglutinin (HA) epitope tag in pBabe-puro-HAII retrovirus expression vector (a generous gift from Teresa Grana, University of North Carolina at Chapel Hill, Chapel Hill, NC) or in frame with amino-terminal sequences encoding green fluorescent protein (GFP) in the pEGFP-c3 expression vector (Clontech, Mountain View, CA). Further missense mutations were created by engineering the appropriate mutation during PCR-mediated DNA amplification to encode the 226Q, 228Q, 229Y, 230Q, and/or 231Q missense mutations in wild-type, activated (G40V), or activated and amino-terminally truncated [ΔN-Chp(40V]). Oligonucleotide DNA sequences designed to engineer the appropriate mutations are as follows: 5′-GGTGTGCGCACGGCCTCTCGCTGTCGC-3′ for a corresponding 224A mutant; 5′-GTGCGCACGCTCGCTCGCTGTCGCTGG-3′ for a corresponding 225A mutant; 5′-CGCACGCTCTCTCAGTGTCGCTGGAAG-3′ for a corresponding 226Q mutant; 5′-CTCTCTCGCTGTCAGTGGAAGAAGTTC-3′ for a corresponding 228Q mutant; CGCACGCTCTCTCAGTGTCAGTGGAAGAAGTTC-3′ for a corresponding 226Q/228Q mutant; 5′-CTCTCGCTGTCGCTATAAGAAGTTCTTCTG-C-3′ for a corresponding 229Y mutant; 5′-CGCTGGCAGAAGTTCTTCTGC-3′ for a corresponding 230Q mutant; 5′-CGCTGGAAGCAGTTCTTCTGC-3′ for a corresponding 231Q mutant; 5′-CGCTGGCAGCAGTTCTTCTGC-3′ for a corresponding 230Q/231Q mutant; and 5′-ACGCTCTCTCAATGTCAATGGCAGCAGTTCTTC-TGC-3′ for the corresponding 4Q mutant. Truncation mutants were created by designing oligonucleotide primers for PCR-mediated DNA amplification of the appropriate length of Chp carboxy-terminal sequence, and fusing the PCR product in a pEGFP-c3 expression vector. pcDNA3.1-RhoGDI expression vectors were obtained from the Guthrie cDNA Resource (Sayre, PA).

The yellow fluorescent protein (YFP)-p21 activated kinase (PAK)-p21 binding domain (PBD) probe was generated by fusing a cDNA sequence encoding the Cdc42/Rac PBD of human PAK1 in frame with the amino-terminal YFP sequence in a pcDNA3-YFP expression vector (Mitin *et al*., 2004). The PBD corresponds to amino acids 74-133 of PAK1 and was cloned into pcDNA3-YFP in 5′EcoRI and 3′XhoI restriction sites. Oligonucleotide primers used to amplify PAK-PBD are as follows: 5′-GCGGAATTCGAGATTTGTCTCCC-3′ and 5′-GCGTATTTACCTCGAGGATTACGAGTTGTA-3′.

Tissue Culture and Transformation Assays

NIH 3T3 cells were maintained in DMEM supplemented with 10% calf serum (Sigma-Aldrich, St. Louis, MO), and 100 U/ml penicillin and 100 μg/ml streptomycin (designated growth medium). Unless otherwise indicated, cells were fed every 2 d with 4 ml of growth medium.

To evaluate anchorage-independent growth in soft agar, we first established mass populations of NIH 3T3 cells that were stably transfected with the empty pBabe-HAII expression vector or vectors encoding wild-type (WT) Chp, ΔN-Chp(40V), ΔN-Chp(40V/229Y), ΔN-Chp(40V/230Q), or ΔN-Chp (40V/231Q). NIH 3T3 cells stably expressing wild type or mutant Chp proteins were used in soft agar assays according to procedures described previously (Cox and Der, 1994; Clark *et al*., 1995; Solski *et al*., 2000). Colony formation was quantitated and photographed after 35 d. Expression of HA-tagged wild-type or mutant Chp in stably transfected mass populations of NIH 3T3 cells was assessed by Western blot with the rat anti-HA 3F10 antibody (Roche Diagnostics, Indianapolis, IN). Comparable protein loading was verified using an anti-β-actin antibody (clone AC-15; Sigma-Aldrich).

Fluorescence Microscopy

For GFP-Chp localization analyses, NIH 3T3 cells grown in growth medium were seeded in 35-mm glass-bottomed plates (MatTek, Ashland, MA). Cells were transiently transfected with 0.5 μg of the pEGFP-c3 constructs using Lipofectamine Plus lipid transfection reagents (Invitrogen, Carlsbad, CA). Three hours after transfection, cells were washed and grown in phenol red-free DMEM/F-12 supplemented with 10% calf serum. Live cells were viewed 20 h after transfection using a Zeiss 510 LSM confocal microscope. Images were captured using the Zeiss LSM 510 imaging software, and bright-

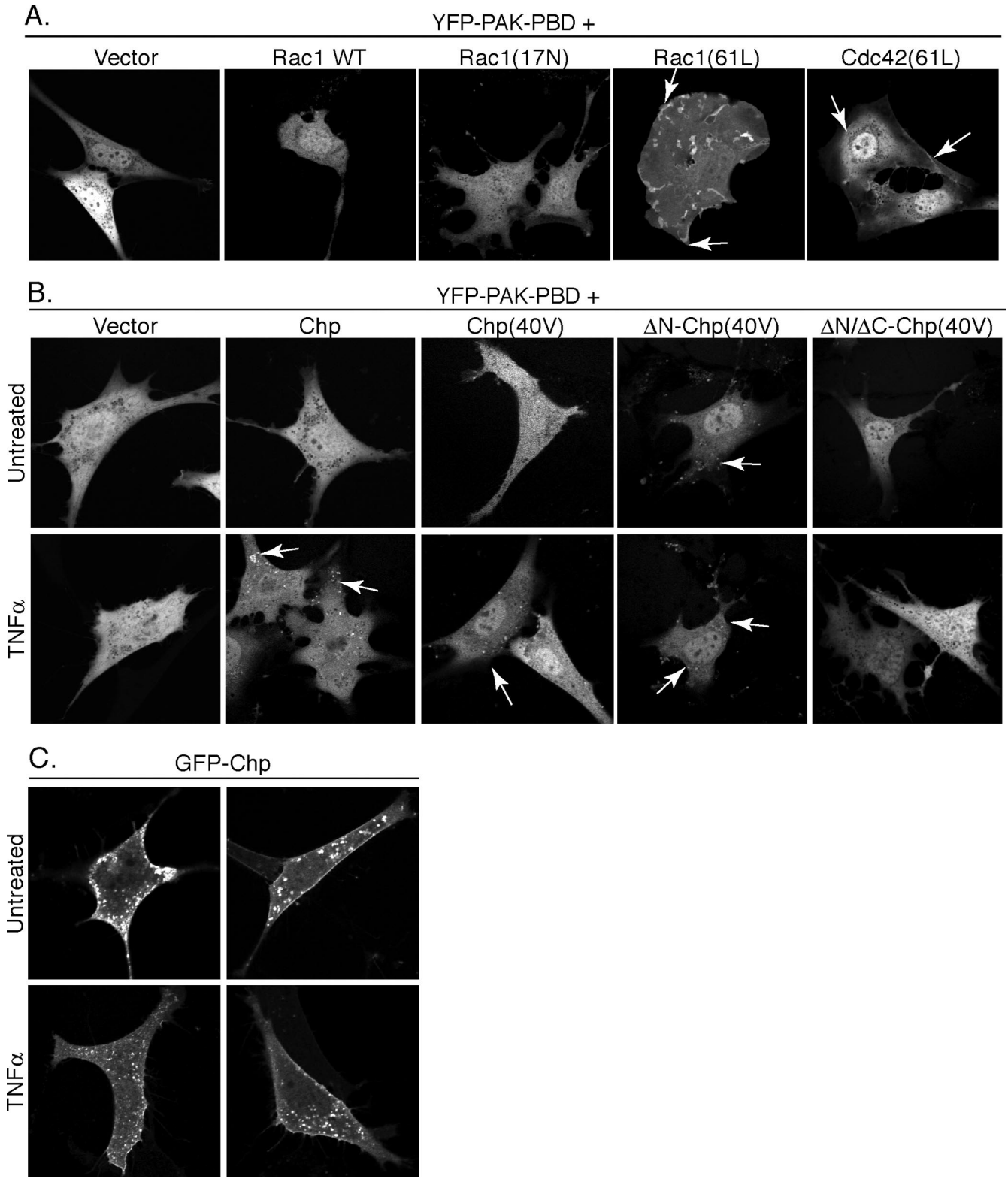


Figure 1. TNF-α–stimulated activation of endomembrane-associated Chp. (A) YFP-PAK-PBD colocalizes with activated Rac and Cdc42 small GTPases. NIH 3T3 cells were transiently transfected with 100 ng of YFP-PAK-PBD and 1 μg of the indicated pcDNA 3.1 construct [vector, Rac1, Rac1(17N), Rac1(61L), or Cdc42(61L)]. YFP fluorescence in live cells was visualized 20 h after transfection using a Zeiss 510 LSM confocal microscope, and 0.4-μm Z-slices were captured. Images shown are representative of at least two independent experiments, with >30 cells examined in each assay. YFP-PAK-PBD localized to the cytoplasm in cells that express vector, Rac1, or dominant negative Rac1(17N). Expression of constitutively active Rac1(61L) recruited YFP-PAK-PBD to membrane ruffles (arrows), whereas Cdc42(61L) recruited PAK-PBD to Golgi and plasma membranes (arrows). (B) Constitutively activated Chp recruits YFP-PAK-PBD to distinct cellular domains. NIH 3T3 cells were transiently transfected with 100 ng of YFP-PAK PBD and 1 μg of the indicated pcDNA3 construct [vector, Chp, Chp(40V), ΔN-Chp(40V),

ness/contrast adjustments were made in Adobe Photoshop (Adobe Systems, Mountain View, CA).

For PAK-PBD recruitment studies, live cells were plated as described above and transfected with 100 ng of pcDNA3-YFP-PAK-PBD and 1 μg of the indicated pcDNA-Chp expression vector using Lipofectamine Plus. Three hours after transfection, cells were washed and grown in phenol red-free DMEM/F-12 supplemented with 0.5% calf serum overnight. Cells were treated with 10 ng/ml tumor necrosis factor (TNF)-α (Roche Diagnostics) for 15 min at 37°C where indicated. Cells were viewed live as described above, 0.4-μm Z-slices were captured using the Zeiss LSM 510 imaging software, and brightness/contrast adjustments were made in Adobe Photoshop.

For RhoGDI localization studies, live cells were plated and transfected as described above and visualized using a Zeiss 510 LSM confocal microscope. Images were captured using the Zeiss LSM 510 imaging software and brightness/contrast adjustments were made in Adobe Photoshop.

1-Biotinamido-4-[4′-(maleimidomethyl) Cyclohexanecarboxamido] Butane (Biotin-BMCC) Labeling

Direct analyses of protein palmitoylation were done as described in Drisdel and Green (2004). Briefly, 293T cells were transfected with 7 μg of the indicated pEGFP construct using a calcium phosphate transfection technique. Forty-eight hours after transfection, cells were lysed in lysis buffer and incubated with 5 μg of anti-GFP monoclonal antibody (JL-8; Clontech) at 4°C for 1 h, at which point 20 μg of protein G (Invitrogen) was added to the lysates and incubated at 4°C for 1 h. Bound protein was washed and incubated with lysis buffer containing 50 mM *N*-ethylmaleimide (Sigma-Aldrich) for 48 h at 4°C. Bound protein was then washed and treated with 1 M hydroxylamine, pH 7.4, to cleave thioester bonds for 1 h at 25°C, washed again, and treated with biotin-BMCC (Pierce Chemical, Rockford, IL), which recognizes free sulfhydryl groups, for 2 h at 25°C. Bound protein was washed again, resuspended in 50 μl of 2× sample loading buffer, and resolved by 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Labeled protein was detected by incubating with streptavidin-horseradish peroxidase (HRP; Pierce Chemical), and membrane was washed and exposed to x-ray film. Twenty microliters of lysate was resolved by SDS-PAGE, transferred, incubated with anti-GFP primary antibody and anti-mouse IgG-HRP secondary antibody, and exposed to x-ray film to verify the presence of all GFP-tagged proteins.

RESULTS

TNF-α Causes Activation of Endomembrane-associated Chp

Our previous studies indicated the critical requirement of an intact carboxy terminus in promoting Chp membrane association and transforming activity (Chenette *et al*., 2005). To evaluate the importance of the carboxy terminus in promoting effector interactions, we created a novel fluorescent probe to detect the subcellular location of activated, GTP-bound Chp. This technique involved designing a probe similar to the fluorescent probe developed by Philips and colleagues to demonstrate Ras activation on internal membranes (Bivona *et al*., 2003). An advantage of this approach for evaluating Chp activation, when compared with the standard pull-down assay, is the unbiased spatial determination of Chp activity (Philips, 2005). A potential limitation of this approach is that it is not sensitive enough to detect activation of endogenous GTPase activation. However, this limitation can be exploited to selectively evaluate activation of a specific GTPase that is expressed ectopically. The p21-binding domain of the PAK1 serine/threonine kinase (PAK-PBD) binds preferentially to the activated, GTP-bound forms of Chp as well as Cdc42 and Rac1 and has been used for the development of fluorescence-based sensors for Rac1 and Cdc42 activation (Kraynov *et al*., 2000; Itoh *et al*., 2002). We created a fusion protein where the yellow fluorescent protein (YFP) was added to the amino terminus of PAK-PBD (YFP-PAK-PBD) and then evaluated the ability of this probe to determine the subcellular localization of activated Rho GTPases.

We initially performed analyses to verify the specificity of the probe for detection of the activated forms of Rac1 and Cdc42. When coexpressed with empty vector, YFP-PAK-PBD was localized to the cytoplasm and nucleus of cells (Figure 1A). No plasma membrane or endomembrane association was seen. As expected, coexpression of wild-type Rac1 or dominant negative Rac1(17N), both GDP-bound, did not cause any reorganization of YFP-PAK-PBD. However, expression of constitutively active GTP-bound Rac1(61L) recruited YFP-PAK-PBD to lamellipodia, which is a canonical site of activated Rac1 localization. This result is similar to that seen with other fluorescence-based PAK-PBD probes for Rac activation (Itoh *et al*., 2002). Likewise, expression of constitutively active Cdc42(61L) recruited YFP-PAK-PBD to the plasma and Golgi membranes, which are known sites of activated Cdc42 localization (Michaelson *et al*., 2001; Aspenstrom *et al*., 2004). These results verified that YFP-PAK-PBD can detect the activated GTP-bound forms of Rac1 and Cdc42 in their specific subcellular locations.

We next evaluated the ability of Chp to recruit YFP-PAK-PBD to distinct cellular domains. We previously determined that wild-type and activated Chp are both localized to plasma and endomembranes (Chenette *et al*., 2005). As expected, expression of wild-type, presumably GDP-bound Chp did not alter the cytoplasmic location of YFP-PAK-PBD (Figure 1B). Expression of Chp(40V) likewise did not appreciably alter localization of YFP-PAK-PBD, which is in keeping with our previous results that found that Chp(40V) had only modest transforming activity above that of wild-type Chp (Chenette *et al*., 2005). However, expression of amino-terminally truncated, GTPase-deficient ΔN-Chp(40V) that we previously determined to have potent transforming activity was able to recruit a portion of YFP-PAK-PBD to punctate endomembrane structures, reminiscent of the endosomal localization of Chp. An intact carboxy terminus was required for YFP-PAK-PBD recruitment, because the putative constitutively GTP-bound ΔN/ΔC-Chp(40V) mutant was not able to cause redistribution of YFP-PAK-PBD to endomembranes. Deletion of the carboxy terminus was shown previously to impair PAK1 binding (Aronheim *et al*., 1998) as well as Chp localization and cellular transformation (Chenette *et al*., 2005). Thus, the ability of the different Chp variants to cause YFP-PAK-PBD redistribution correlated directly with their transforming activity.

Previous analyses found that TNF-α stimulation of c-Jun NH_2-terminal kinase (JNK) activation was impaired by ectopic expression of a putative Chp dominant negative protein (Aronheim *et al*., 1998). This observation suggests that TNF-α may stimulate Chp activation. To address this directly, we next determined whether TNF-α treatment would cause Chp-dependent relocalization of YFP-PAK-PBD. The localization of GFP-Chp was examined in cells that were

Figure 1 (cont). or ΔN/ΔC-Chp(40V)]. YFP fluorescence in live cells was visualized 20 h after transfection using a Zeiss 510 LSM confocal microscope, and 0.4-μm Z-slices were captured. Images shown are representative of at least two independent experiments, with >30 cells examined in each assay. YFP-PAK-PBD is localized to the cytoplasm in cells that express vector, wild-type Chp, Chp(40V), and ΔN/ΔC-Chp(40V). Expression of ΔN-Chp(40V) recruited PAK-PBD to discrete and punctate intercellular domains (arrow), reminiscent of Chp localization. Treatment of cells that expressed vector or ΔN/ΔC-Chp(40V) with 10 ng/ml TNF-α for 15 min did not alter cytoplasmic localization of PAK-PBD. Wild-type Chp and Chp(40V) recruited YFP-PAK-PBD to endomembrane structures following TNF-α treatment. YFP-PAK-PBD retained its endomembrane localization in cells that expressed ΔN-Chp(40V) after TNF-α treatment. (C) TNF-α signaling does not cause redistribution of wild-type GFP-Chp. NIH 3T3 cells were transiently transfected with 1 μg of pEGFP-Chp and left untreated or stimulated with 10 ng/ml TNF-α. Images were captured as described above 15 min after treatment.

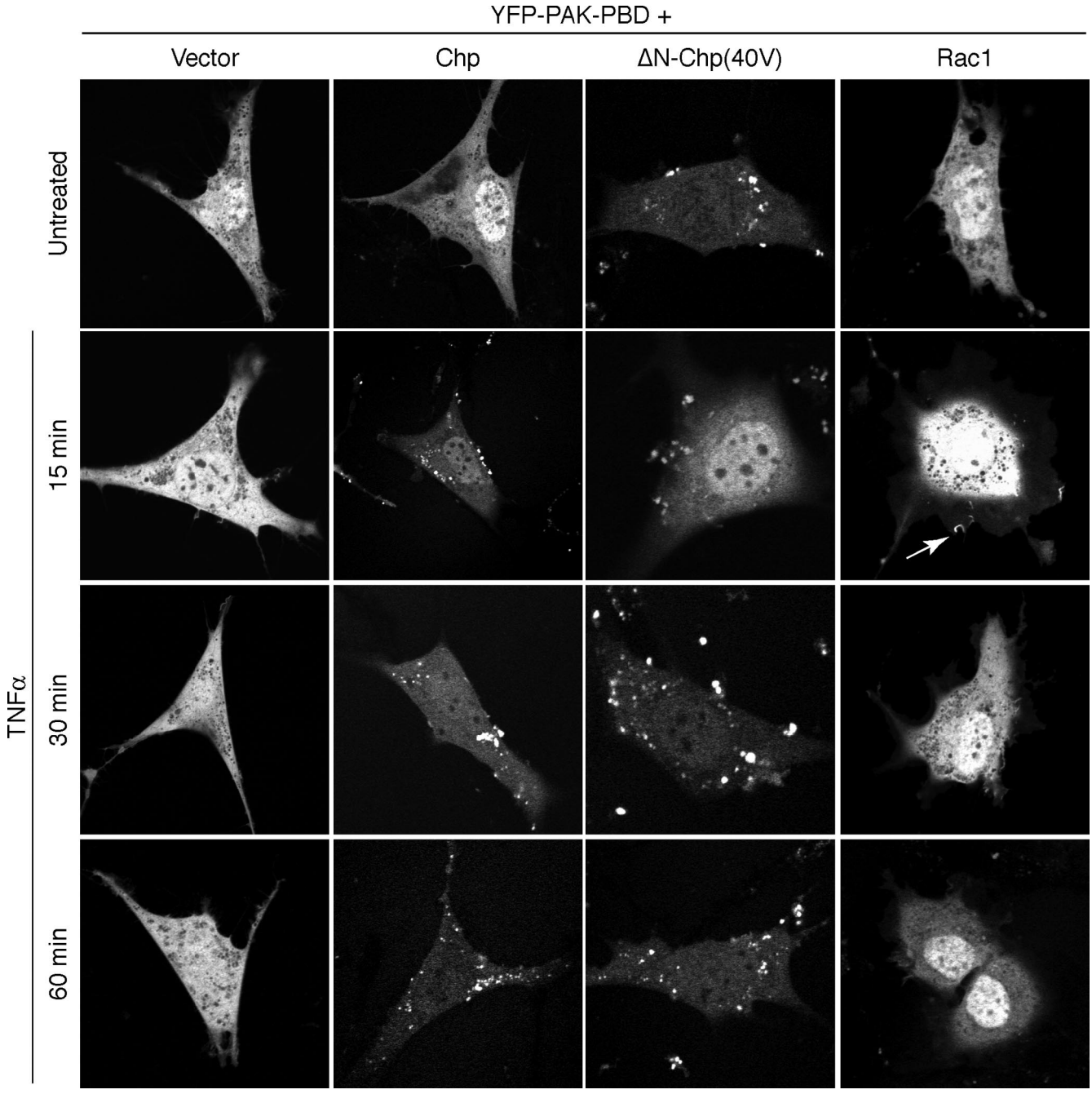


Figure 2. TNF-α causes prolonged Chp activation at the endomembrane. NIH 3T3 cells were transiently transfected with 1 μg of the indicated pcDNA construct [vector, Chp, ΔN-Chp(40V), or Rac1] together with 100 ng of YFP-PAK-PBD. Twenty hours after transfection, cells were then either incubated with growth medium (untreated) or growth medium supplemented with 10 ng/ml TNF-α. YFP fluorescence in live cells was visualized at the indicated times by using a Zeiss 510 LSM confocal microscope, and 0.4-μm Z-slices were captured. Images shown are representative of at least two independent experiments, with >30 cells examined in each assay. Arrows indicate Rac1-mediated recruitment of YFP-PAK-PBD to membrane structures (arrows) with concomitant clearing of cytoplasm.

stimulated with TNF-α to ensure that extracellular stimulation did not affect Chp localization (Figure 1C). We next treated cells that coexpressed YFP-PAK-PBD and wild-type or mutant Chp with TNF-α to assess the requirement for an intact carboxy or amino terminus in TNF-α–mediated activation of Chp. The addition of TNF-α did not alter the localization of YFP-PAK-PBD in cells that also expressed control empty vector or vector encoding ΔN/ΔC-Chp(40V) compared with untreated cells (Figure 1B). TNF-α treatment did not alter the punctate endomembrane localization of cells that expressed active ΔN-Chp(40V). Interestingly, we observed a redistribution of YFP-PAK-PBD to punctate endomembrane, but not plasma membrane, structures upon TNF-α treatment in cells that expressed wild-type Chp or Chp(40V), suggesting that TNF-α treatment activated endomembrane-associated Chp. These results indicate that endomembrane-associated Chp possesses effector binding and signaling activity.

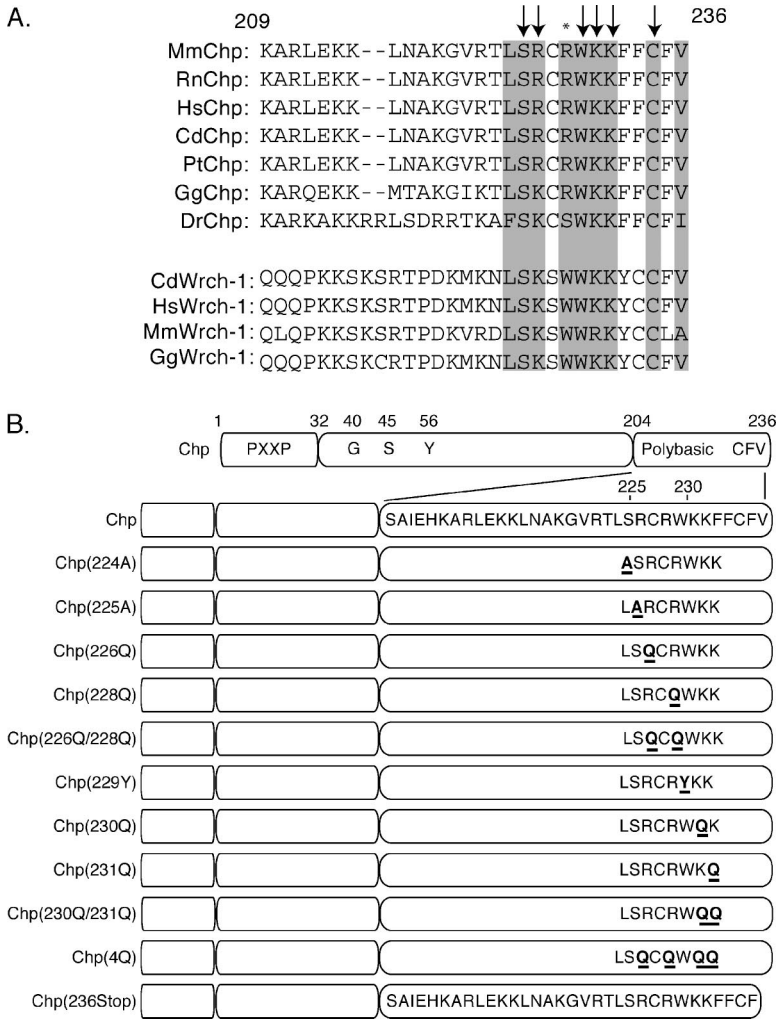


Figure 3. Conserved residues in the carboxy termini of Chp and Wrch-1. (A) Sequence comparison of orthologues of Chp and Wrch-1 reveals the presence of conserved residues that may also play an important role in the membrane-targeting function of the Chp carboxy terminus. Gray boxes indicate amino acids that were mutated to aid in understanding of Chp membrane targeting. Arrows delineate basic amino acids that are conserved between Chp orthologues and homologues. Asterisk indicates Arg residue that is incompletely conserved between Chp homologues. Mm, *Mus musculus* (mouse); Rn, *Rattus norvegicus* (rat); Hs, *Homo sapiens* (human); Cd, *Canis domesticus* (dog); Pt, *Pan troglodyte* (chimp); Gg, *Gallus gallus* (chicken); Dr, *Danio rerio* (zebrafish). (B) Missense mutations were engineered in the carboxy terminus of Chp to evaluate the importance of basic amino acids as well as a conserved Leu, Ser, and Trp residues at positions 224, 225, and 229, respectively, in Chp localization. The terminal Val [Chp(236Stop)] was also removed to determine whether, as with the CAAX motif, the distance of the Cys residue from the carboxy terminus is critical for proper membrane targeting and localization.

To determine the kinetics of TNF-α–mediated recruitment of YFP-PAK-PBD, we analyzed PAK-PBD location in live cells that coexpressed Chp, constitutively active Chp, and Rac1 for 60 min after stimulation with TNF-α (Figure 2). As expected, the localization of YFP-PAK-PBD did not change appreciably over the course of 60 min in cells that coexpressed vector or constitutively active ΔN-Chp(40V). TNF-α–mediated stimulation of Rac1 promoted transient redistribution of YFP-PAK-PBD, with PAK-PBD occurring at cell membranes after 15 min. After 30 min, the majority of YFP-PAK-PBD had returned to the cytoplasm in cells that coexpressed Rac1. After 60 min, the Rac1-expressing cells were indistinguishable from untreated cells. TNF-α–mediated stimulation of Chp promoted recruitment to endomembrane structures after 15 min. Unlike Rac1, coexpression of Chp caused YFP-PAK-PBD to persist at endomembrane structures for over 60 min in stimulated cells. TNF-α stimulation did not affect localization of GFP-Chp during the course of the experiment (Figure 1C; our unpublished data).

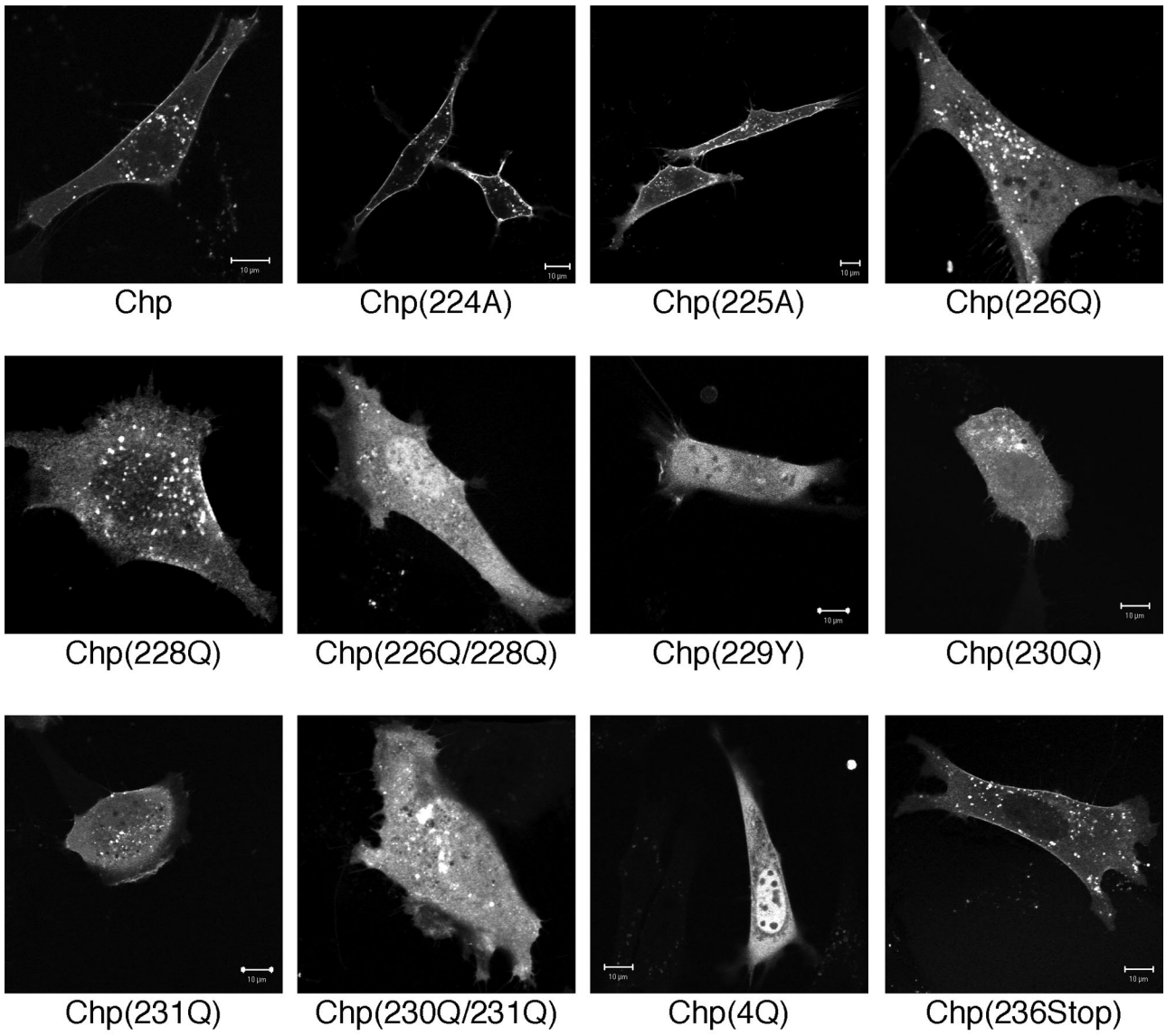
Basic Residues are Critical for Chp Subcellular Location

Our previous studies determined that palmitate modification of cysteine 234 in the carboxy terminus of Chp was critical for membrane association and transforming activity. Because palmitate modification alone is not expected to be sufficient for full Chp membrane targeting (Mitchell *et al*., 1994; Booden *et al*., 1999; Dong *et al*., 2003), we evaluated a possible role for basic residues adjacent to cysteine 234 in Chp processing and localization.

As seen in Figure 3A, the positions of three basic amino acid residues are completely conserved between the various Chp orthologues and between the closely related Wrch-1 proteins: Arg-226, Lys-230, and Lys-231 (arrows). A fourth basic residue, Arg-228, is present in only a subset of Chp/Wrch-1 proteins (asterisk). We hypothesize that these conserved residues, in particular the tandem Lys residues at positions 230 and 231, are important for Chp localization to the plasma membrane. Previous studies substituted Gln residues for Lys or Arg residues to demonstrate the importance of carboxy terminal basic residues in Ras membrane targeting (Hancock *et al*., 1990; Jackson *et al*., 1994). To assess the contribution of the basic amino acids in Chp localization, we introduced single Gln substitutions for Lys and/or Arg residues at positions 226, 228, 230, and 231 in the rat Chp protein and designated Chp(226Q), Chp(228Q), Chp(230Q), and Chp(231Q), respectively. We also created mutant proteins with tandem substitutions [Chp(226Q/228Q) and Chp(230Q/231Q)] as well as a mutant with Gln substitutions at all four basic residues, designated Chp(4Q) (Figure 3B).

To determine the contribution of these basic amino acids to Chp plasma membrane association and subcellular localization, we generated expression vectors encoding GFP-

A.



B.

	Terminal Residues	Localization
GFP-Chp:	LSRCRWKKFFCFV	PM, endomembrane
GFP-Chp(224A):	**A**	PM, endomembrane
GFP-Chp(225A):	**A**	PM, endomembrane
GFP-Chp(226Q):	**Q**	cyto, endomembrane
GFP-Chp(228Q):	**Q**	cyto, endomembrane, PM
GFP-Chp(226Q/228Q):	**Q Q**	cyto, endomembrane
GFP-Chp(229Y):	**Y**	cytoplasm
GFP-Chp(230Q):	**Q**	cyto, endomembrane
GFP-Chp(231Q):	**Q**	cyto, endomembrane
GFP-Chp(230Q/231Q):	**QQ**	cyto, endomembrane
GFP-Chp(4Q):	**Q Q QQ**	cytoplasm
GFP-Chp(236Stop):	LSRCRWKKFFCF	PM, endomembrane

Figure 4.

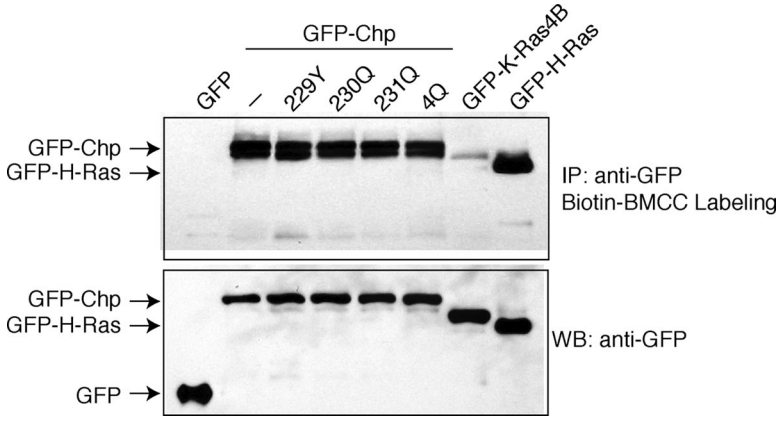


Figure 5. Basic residues are not part of the protein acyltransferase motif. 293T cells were transiently transfected with the indicated pEGFP constructs, lysed 48 h after transfection, and subjected to a biotin-BMCC labeling assay (Drisdel and Green, 2004). Nonpalmitoylated K-Ras was included as a negative control for this assay (top). An anti-GFP Western blot shows that equivalent concentration of GFP and GFP-tagged versions of Chp, Chp(229Y), Chp(230Q), Chp(231Q), Chp(4Q), K-Ras, and H-Ras protein was used in the assays (bottom). Data shown are representative of three independent experiments.

tagged versions of each Chp mutant protein. NIH 3T3 cells transiently transfected with the GFP empty vector or encoding wild-type or mutant Chp proteins were then used for live cell fluorescence analyses of GFP expression (Figure 4). As we described previously, GFP alone showed nuclear and cytoplasmic localization, whereas GFP-Chp showed localization to both the plasma membrane and to endomembrane structures (Chenette *et al*., 2005). In contrast to GFP-Chp, we found that GFP-Chp(226Q) and GFP-Chp(228Q) showed an intermediate phenotype, with significantly diminished plasma membrane association, increased cytoplasmic localization, but retention of endomembrane localization. Partial plasma membrane association was seen in ~30% of cells that expressed GFP-Chp(226Q), and a slight majority (60%) of cells that expressed GFP-Chp(228Q). However, the extent of plasma membrane association (e.g., proportion of membrane that contained Chp) was significantly decreased compared with wild-type Chp. The double mutant [Chp(226Q/228Q)] exhibited no plasma membrane binding, diminished association with endomembranes, and significantly increased cytoplasmic localization. This localization pattern is representative of 100% of the cells examined. Mutation of residues 230 or 231 [GFP-Chp(230Q) and GFP-Chp(231Q)] exhibited a phenotype similar to the Chp(226Q/228Q) mutant, with no association with the plasma membrane, increased cytoplasmic localization, and modest retention of association with endomembranes or vesicles. The double Chp(230Q/231Q) mutant exhibited a similar pattern, although with increased cytoplasmic localization. Less than half of cells (~30%) examined exhibited a total lack of endomembrane localization. Not surprisingly, GFP-Chp 4Q was mislocalized to the nucleo- and cytoplasm in 100% of cells visualized, demonstrating the importance of basic residues in Chp localization.

The Invariant Carboxy-Terminal Tryptophan Residue Is Essential for Chp Subcellular Location

There are several other nonbasic amino acids that are conserved between Chp orthologues (arrows) (Figure 3A). We were especially interested in the contribution of a Trp residue at position 229, conserved in all Chp/Wrch proteins and not found in the carboxy termini of other Rho GTPases (except RhoD). To test the importance of this conserved residue in Chp localization, we created a GFP-Chp(229Y) mutant and visualized its localization in live NIH 3T3 cells. Interestingly, GFP-Chp(229Y) was mislocalized to the cyto- and nucleoplasm in 100% of cells visualized (Figure 4). This altered localization is essentially identical to that seen with ΔC-Chp, where the entire carboxy-terminal sequence is deleted (Chenette *et al*., 2005). There are no known modification events that occur on Trp residues, so it is possible that the tryptophan is required for recognition by a protein acyltransferase (PAT) enzyme.

We also evaluated the consequence of mutation of conserved leucine and serine residues at positions 224 and 225, respectively (Figure 4). An alanine point mutation at either of these sites had no effect on localization in any of the cells examined, suggesting that these residues are not part of the carboxy-terminal targeting motif of Chp.

Other Rho GTPases require an intact CAAX motif for recognition by protein prenyltransferases. Removal of the -X residue results in a lack of prenylation and cytoplasmic mislocalization of the GTPase (Jackson *et al*., 1994). Although Chp lacks a CAAX motif, we determined the importance of the terminal residues in Chp localization. As seen in Figure 4, removal of the terminal valine had no effect on Chp localization in any of the cells examined, further emphasizing the unique properties of the carboxy terminus of Chp.

Basic and Conserved Amino Acids Are Not Part of the Protein Acyltransferase Recognition Motif

One simple explanation for the mislocalization seen with the Chp basic residue mutants is that the basic and conserved residues comprise part of the protein acyltransferase recognition motif. Aside from the cysteine residue, however, no clear consensus sequence for the carboxy-terminal PAT motifs of small GTPases has been established (Smotrys and Linder, 2004). To determine the importance of carboxy-terminal residues in protein palmitoylation, we used an assay to detect posttranslational palmitoylation of Chp and Chp mutant proteins that involved hydrolyzing the thioester bond that links palmitate groups to cysteines and treating with a biotinylated compound that recognizes and binds to the free sulfhydryl group generated upon cleavage of the thioester bond (Drisdel and Green, 2004). We applied this assay previously to demonstrate the palmitate modification of Chp and Wrch-1 (Berzat *et al*., 2005; Chenette *et al*., 2005). As expected, we were able to detect labeling of H-Ras, which is palmitoylated, but not K-Ras or GFP, which are not palmitoylated (Figure 5). As we found

Figure 4 (facing page). Chp membrane localization is dependent on basic amino acids. (A) NIH 3T3 cells were transiently transfected with 0.5 μg of the pEGFP expression vector encoding GFP fusion proteins of the indicated Chp proteins. Live cells were visualized 20 h after transfection using a Zeiss 510 LSM confocal microscope. Images shown are representative of at least two independent experiments, with >50 cells examined in each assay. Bars, 10 μm. (B) Summary of mutations engineered in the carboxy terminus of Chp and the effect of each mutation on subcellular localization of GFP-Chp. PM, plasma membrane; cyto, cytoplasm.

previously, we were able to detect palmitate modification of Chp. Interestingly, we were able to detect palmitoylation of four Chp mutants with altered localization phenotypes (Chp 229Y, 230Q, 231Q, and 4Q), suggesting that the mislocalization of the Chp basic residue mutants is not due to a lack of palmitoylation.

Basic Amino Acids Are Required for Chp Transforming Activity

We previously demonstrated that amino-terminally truncated and GTPase-deficient Chp [ΔN-Chp(40V)] caused growth transformation of NIH 3T3 cells and that the transforming activity of Chp was dependent on an intact carboxy-terminal extension. To determine whether mutation of the basic or conserved amino acids in the carboxy terminus of Chp affected Chp transforming activity, we subjected mass populations of NIH 3T3 cells that stably expressed Chp, ΔN-Chp(40V), ΔN-Chp(40V/229Y), ΔN-Chp(40V/230Q), and ΔN-Chp(40V/231Q) (Figure 6A) to a soft agar assay to monitor anchorage-independent growth potential. As we described previously, cells that express wild-type Chp were unable to facilitate colony formation in soft agar, whereas cells that express ΔN-Chp(40V) formed many multicellular colonies (Figure 6B). Mutation of the conserved tryptophan residue (229Y) or the basic residues at positions 230 or 231 inhibited the ability of activated Chp to promote anchorage-independent growth. Although our transient expression analyses indicated that these mutations did not alter protein stability (Figure 5), we did find that their steady-state levels were lower than that of ΔN-Chp(40V) (Figure 6A). Thus, although reduced level of expression may contribute to their diminished transforming activity, we also suggest that these results support the importance of proper localization for Chp transforming activity. Additionally, we suggest that partial Chp localization to endosomal structures [as seen in Chp(230Q) and Chp(231Q)] is still not sufficient to promote activation of effector pathways important for transformation.

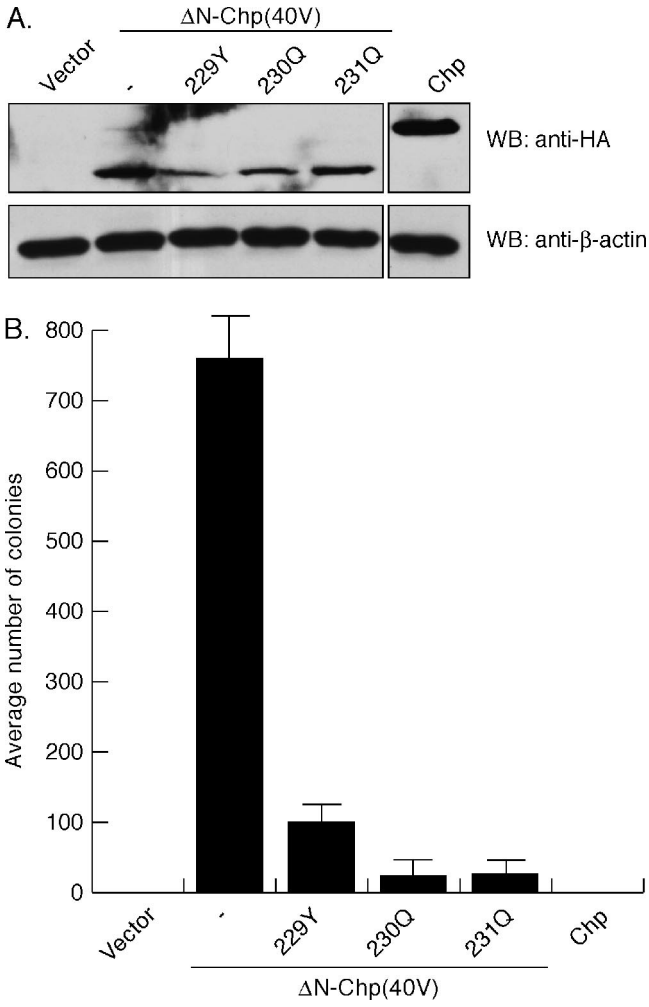


Figure 6. Basic residues are required for Chp transforming activity. Mass populations of NIH 3T3 cells that stably express the indicated Chp proteins were established and evaluated for expression of HA-tagged Chp by Western blot analyses with anti-HA antibody (A; top). Western blot analyses with anti-β-actin were done to verify equivalent total protein for each cell lysate (bottom). An intervening lane was removed between the mutant and wild-type Chp proteins in both blots, with crop boundaries as shown in the figure. (B) Mutant Chp proteins were analyzed for their ability to promote growth of NIH 3T3 cells in soft agar. The number of colonies was quantitated after 35 d. Data shown are representative of three independent experiments, with SE indicated by the bars.

The Carboxyl Terminal 11 Residues of Chp Are Sufficient to Promote Chp Subcellular Membrane Association

Work done by Michaelson *et al*. (2001) determined that the carboxy-terminal 21 amino acids of Cdc42 were sufficient to target a heterologous protein (GFP) to the same cellular membranes as authentic Cdc42. Unlike Cdc42, Chp possesses additional proline-rich amino-terminal sequences that may be involved in interaction with Src homology domain 3-containing proteins (Shutes *et al*., 2004). Therefore, it is possible that the carboxy terminal sequences of Chp alone may be necessary, but not sufficient, for proper Chp subcellular localization.

To determine whether the carboxy terminus alone is sufficient for localization and to identify the minimum membrane targeting sequence of Chp, we generated a series of GFP fusion proteins that terminated with different lengths of the Chp carboxy terminus (Figure 7A), including 7-amino acid, 11-amino acid, 15-amino acid, and 37-amino acid tails. The 7-amino acid tail contains the Cys 234, Lys 230, and Lys 231 residues but excludes the Trp that we showed above to be critical for localization, whereas the 11- and 15-amino acid tails include the conserved Trp residue and various portions of the basic motif. The 37-amino acid tail of Chp corresponds to the 21-amino acid tail of Cdc42. NIH 3T3 cells transiently expressing the indicated GFP-Chp fusion proteins were used for live cell fluorescence analyses (Figure 7B). The GFP fusion construct containing the seven carboxyl-terminal amino acids of Chp, including the palmitoylated Cys 234 residue [GFP-Chp(230-236)], exhibited a diffuse cytosolic localization with no plasma membrane component. This localization pattern was seen in 100% of cells examined and is identical to that seen with the Trp mutant of Chp [GFP-Chp(229Y)]. This finding again demonstrates that palmitoylation alone is not sufficient to promote membrane association. In contrast, the last 11 amino acids of Chp [Chp(226-236)] were sufficient to target GFP to both plasma and endomembranes, in a localization pattern reminiscent of wild-type Chp. The addition of other upstream residues [Chp(222-236) and Chp(200-236)] did not further alter the plasma- and endomembrane localization pattern seen with Chp(226-236). Thus, the carboxy-terminal 11 amino acids of Chp are sufficient to promote membrane association and subcellular localization.

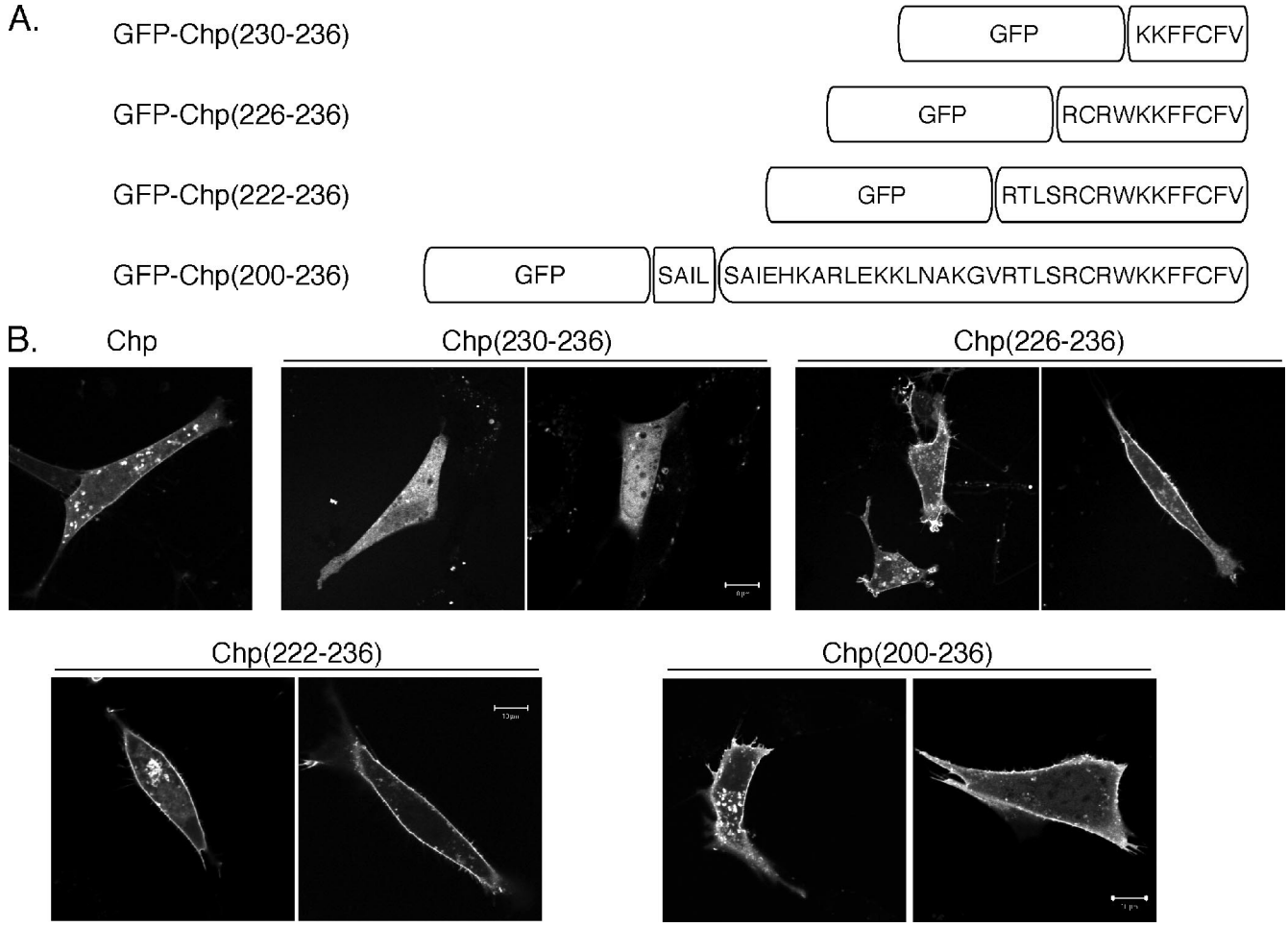


Figure 7. The last 11 amino acids of Chp are necessary and sufficient for membrane targeting. (A) Truncation mutants were engineered to determine the minimal region of the carboxy terminus of Chp that was sufficient to target a heterologous protein (GFP) to cellular membranes. (B) NIH 3T3 cells were transiently transfected with 0.5 μg of the pEGFP expression vector encoding GFP fusion proteins that terminate with the indicated Chp carboxy-terminal sequences. GFP fluorescence in live cells was visualized 20 h after transfection using a Zeiss 510 LSM confocal microscope. Images are shown in duplicate and are representative of at least three independent experiments, with >50 cells examined in each assay.

Chp Subcellular Location Is Not Regulated by RhoGDIs

RhoGDIs form stable complexes with Rho GTPases, in part by recognizing and masking the isoprenoid moiety, and render the GTPase cytosolic and inactive. Although Chp membrane association is not dependent on prenylation, it is still possible that RhoGDIs may serve as regulators of Chp subcellullar localization. Michaelson *et al*. (2001) showed that the expression levels of RhoGDIs were limiting in the cell and that ectopic overexpression of RhoGDIs was sufficient to cause mislocalization of Rho GTPases to the cytosol. We used this same approach to determine whether coexpression of the three known RhoGDIs would prevent Chp association with the plasma membrane. As shown previously, Rac1 was mislocalized to the cytoplasm following ectopic RhoGDI expression (Figure 8). In contrast, Chp localization was not disrupted by coexpression of RhoGDI-α, -β, or -γ, although coexpression of RhoGDI-α severely affects cell morphology. These results suggest that, unlike Cdc42, Chp is not a target for regulation by RhoGDIs, as was expected based on its unusual carboxy terminus.

DISCUSSION

Variations in carboxy-terminal sequences and posttranslational modifications promote the divergent biological properties of otherwise highly related Rho GTPases (Figure 9). In the present study, we found that stimulation with TNF-α caused carboxy-terminus–dependent activation of endosome- but not plasma membrane-associated Chp. We determined that basic residues as well as a conserved tryptophan residue are required for proper Chp subcellular localization and transformation, but they are not essential for palmitate modification. Additionally, unlike the classical Rho GTPases, Chp membrane association is not regulated by RhoGDIs. Together, these observations demonstrate that the membrane-targeting function of Chp is complex and distinct from that of other human Rho family GTPases.

The activation of the classical Rho GTPases involves extracellular stimulus-mediated activation of various cell surface receptors. Previously, Abo and colleagues found that TNF-α caused Chp-dependent JNK activation (Aronheim *et al*., 1998). Because we found Chp localized to both the plasma membrane and to endomembranes, we wanted to

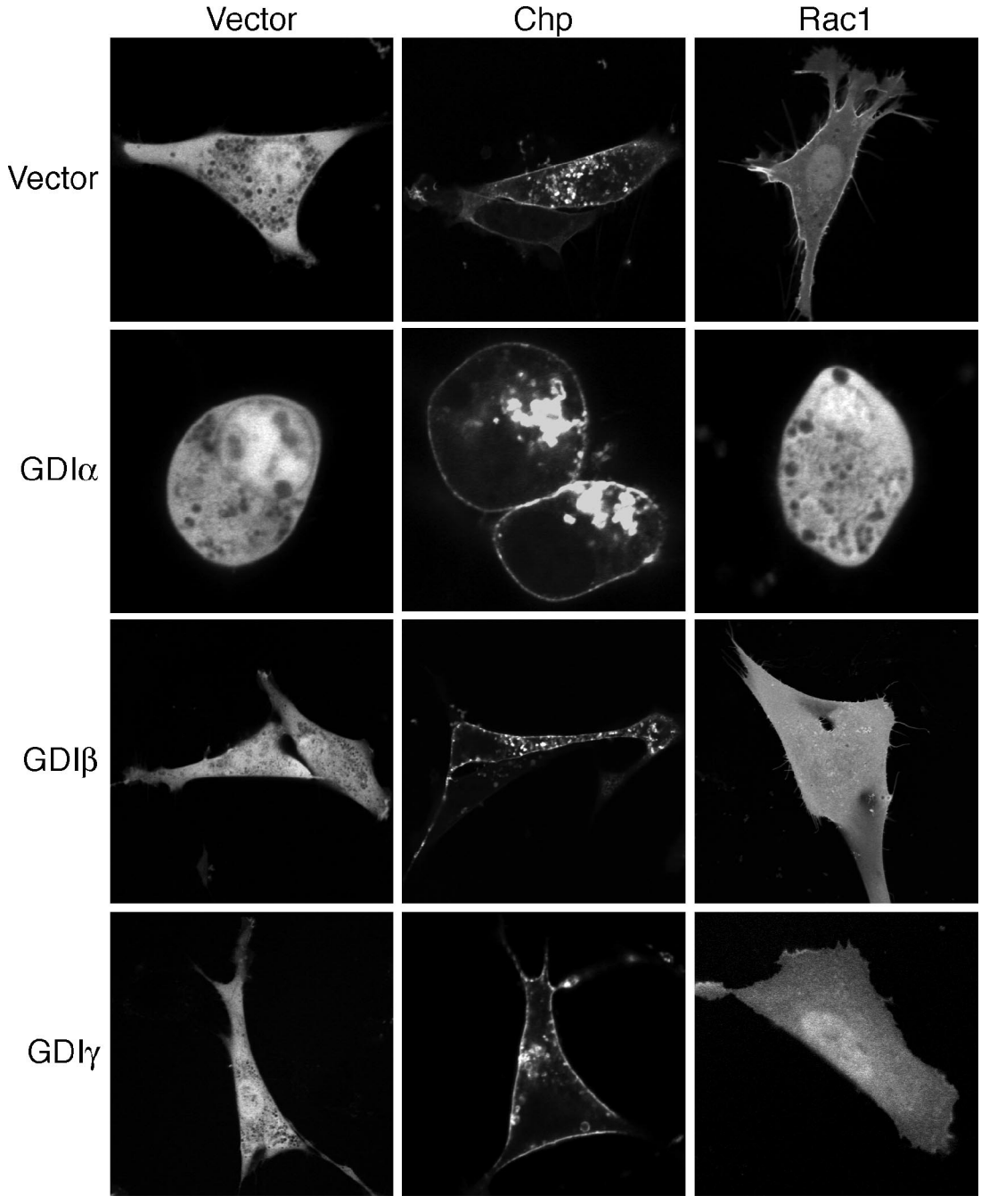


Figure 8. Chp membrane association is not regulated by RhoGDIs. NIH 3T3 cells were transiently transfected with 0.5 μg of the indicated GFP-tagged construct and 1 μg of pcDNA3.1 expression plasmid DNA encoding the indicated RhoGDI. Images shown are representative of at least three independent experiments, with >50 cells examined per assay. Live cells were visualized and photographed 20 h after transfection using a Zeiss 510 LSM confocal microscope.

determine which subcellular pool might be activated by TNF-α. We applied an approach developed by Philips and colleagues to evaluate the spatial distribution of activated Ras (Philips, 2005) to perform a similar analysis with Chp. Surprisingly, we found that TNF-α stimulation caused wild-type Chp activation only at endosomes. These results are similar to those made with Ras, where different extracellular stimuli resulted in activation of either plasma membrane or Golgi localized Ras (Bivona *et al*., 2003). Because Src and Cdc42 activation also caused Chp-dependent JNK activation (Aronheim *et al*., 1998), it will be interesting to determine whether these, or other signals, can activate plasma membrane-associated Chp.

We determined that basic residues likely comprise a second signal, which, together with palmitoylation, can facilitate effective and functional membrane targeting. Although this combination of signals has not been described for any mammalian Ras or Rho family GTPase, similar genetically engineered sequences have been shown to target Ras proteins to cellular membranes (Mitchell *et al*., 1994; Booden *et*

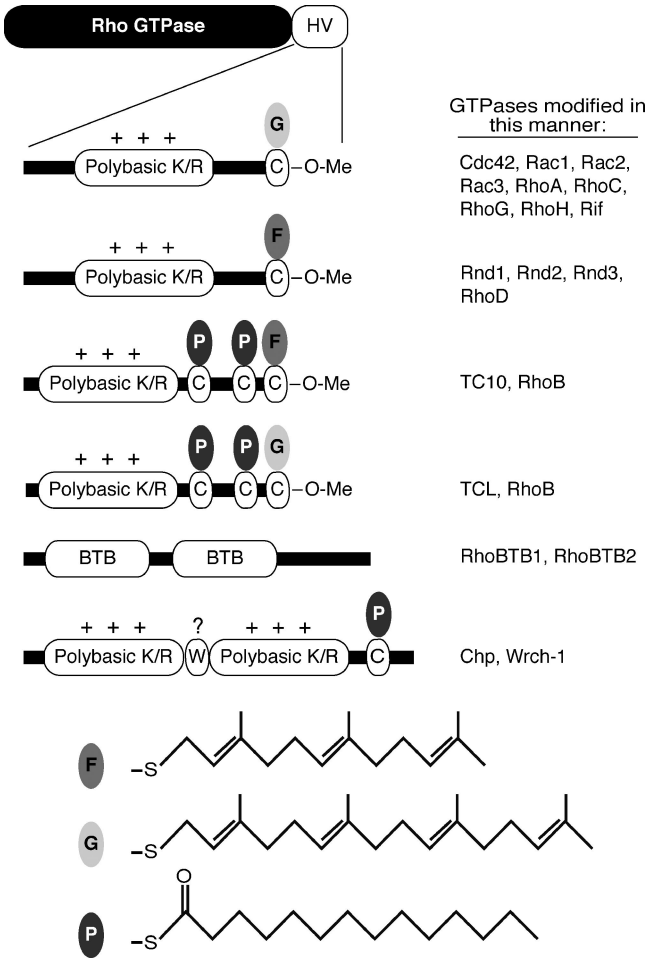


Figure 9. Rho GTPase carboxy-terminal membrane-targeting sequences. The greatest sequence divergence between otherwise closely-related Rho GTPases lies in the hypervariable (HV) motif at the carboxy terminus of the protein. Specific sequences in the HV motif signal for posttranslational modifications and direct localization of Rho GTPases to distinct membrane compartments. The majority (16 of 20) of human Rho family GTPases terminates with CAAX motifs that signal for modification by either farnesyl (F) or geranylgeranyl (G) isoprenoids, AAX proteolysis, and carboxymethylation (O-Me). These GTPases also possess a second membrane-targeting signal: dual palmitoylated cysteines (P), positively charged amino acid stretches (polybasic K/R), or a combination of both signals. Chp lacks a CAAX motif and is modified by palmitoylation of a single cysteine. At least two additional carboxy-terminal sequence elements (polybasic amino acids and an invariant tryptophan residue) are required for Chp subcellular membrane localization. RhoBTB proteins lack any known carboxy-terminal lipid modifications and instead terminate with tandem BR-C, ttk, and bab (BTB) domains.

al., 1999). For example, Deschenes and colleagues determined that yeast RAS2 terminating with a CCIIKLIKRK sequence no longer underwent prenylation, but it retained wild-type membrane association and function (Mitchell *et al*., 1994).

Our analyses of the role of individual basic residues in Chp function revealed two unexpected findings. First, we found that mutation of a single basic residue (K230Q or K231Q) abolished Chp association with the plasma membrane. These results contrast with observations made with K-Ras4B, where mutation of multiple basic residues was required to disrupt its targeting function: mutation of two of the six basic residues in the carboxy terminus of K-Ras4B did not significantly alter the subcellular localization of K-Ras, and ~50% of mutant K-Ras4B was able to reach cellular membranes upon replacement of the entire polybasic domain with glutamine residues (Jackson *et al*., 1994). One possible explanation for this difference may be that the bond between palmitate and cysteine is dynamic and reversible, whereas the prenyl-cysteine bond is permanent and irreversible (Mumby, 1997; Bijlmakers and Marsh, 2003; Linder and Deschenes, 2003). Therefore, it is possible that Chp is less tightly associated with the plasma membrane and has an increased dependency on basic residues to maintain its membrane association. Consistent with this possibility, Booden *et al*. (1999) found that a variant Ras protein that was targeted to membranes by palmitoylation, and basic residues showed only partial membrane association compared with the authentic prenylated Ras protein. We also found that the basic residues are more critical for Chp association with the plasma membrane than with endomembranes.

In addition to the palmitoylated cysteine and basic residues, our sequence alignment of the carboxy termini of Chp homologues and orthologues identified additional conserved residues that we speculated might be important for Chp subcellular location and function. We found that a conserved tryptophan residue was critical for Chp localization and transforming activity. Tryptophan residues have not been described to be important in the subcellular localization of other small GTPases; perhaps this Trp residue comprises a novel membrane-targeting motif. Thus, we speculate that this residue may constitute a third carboxy terminal element that, together with basic residues and palmitoylation, is essential to promote Chp function. Our finding that Chp membrane association is mediated by three separate signals expands the known mechanisms of carboxy-terminal directed membrane targeting to at least six distinct motifs (Figure 9).

One possible role of the tryptophan and basic residues is that they comprise the sequence that signals for Chp palmitoylation. However, we found that none of the Chp mutants tested was defective in palmitate modification. Therefore, we conclude that the mislocalization of Chp mutants is not due to lack of palmitoylation but rather to loss of net positive charge in the carboxy terminus (as is the case with mutant K-Ras [Jackson *et al*., 1994] and the basic Chp mutants) or some other unknown mechanism (in the case of the Trp mutant).

We determined that the carboxy-terminal 11 amino acids of Chp were sufficient to direct a heterologous protein, GFP, to the same gross membrane and subcellular location as wild-type Chp. This result implies that these amino acids are involved in critical associations with the plasma membrane. Comparing the sequences of GFP-Chp(230-236) (no membrane association) to GFP-Chp(226-236) (plasma and endomembrane association) reveals that a few amino acids may tip the balance toward membrane association. Predictably, these amino acids include the two basic residues that we showed were important for membrane targeting (R226 and R228) as well as the tryptophan residue (W229) that was essential for proper localization. Future studies will be directed toward determining the importance of these individual residues in dictating endosome versus plasma membrane localization of Chp.

Our finding that Chp is not a target for mislocalization by RhoGDIs is not entirely surprising. RhoGDIs recognize Cdc42 and other Rho family GTPases and extract them from membranes. RhoGDI association with GTPases requires an

isoprenyl moiety at the carboxy terminus of the protein (DerMardirossian and Bokoch, 2005). Because Chp does not undergo prenylation, RhoGDIs may not be able to recognize Chp. Furthermore, RhoGDI recognition is also dependent on specific sequences in the switch II domain of Rho GTPases (Hoffman *et al*., 2000). Because Chp shows divergence in these sequences compared with Cdc42, the failure of RhoGDIs to recognize Chp may be due to both primary sequence divergence and the lack of isoprenoid modification. Clearly, the regulation of Chp is a complex issue, most likely managed by multiple interactions at the carboxy terminus of the protein.

In conclusion, the data presented here exemplify the complex and multifaceted mechanisms of regulation of Rho GTPase membrane association. Canonical Rho GTPases rely on prenylation in combination with palmitoylated cysteines and/or positively charged residues for proper localization. In contrast, Chp function requires palmitoylation, basic residues, and an invariant tryptophan residue for proper subcellular localization. The unprecedented way by which Chp reaches cellular membranes as well as its insensitivity to RhoGDI regulation further emphasizes the idiosyncratic means of localization of the Rho family of small GTPases. It also illuminates how otherwise highly conserved proteins achieve distinct subcellular localization profiles and biological functions.

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