Regulation of Membrane Trafficking by a Novel Cdc42-related Protein in *Caenorhabditis elegans* Epithelial Cells

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Submitted August 31, 2004; Accepted January 11, 2005
Monitoring Editor: Martin A. Schwartz

Rho GTPases are mainly known for their implication in cytoskeleton remodeling. They have also been recently shown to regulate various aspects of membrane trafficking. Here, we report the identification and the characterization of a novel *Caenorhabditis elegans* Cdc42-related protein, CRP-1, that shows atypical enzymatic characteristics in vitro. Expression in mouse fibroblasts revealed that, in contrast with CDC-42, CRP-1 was unable to reorganize the actin cytoskeleton and mainly localized to *trans*-Golgi network and recycling endosomes. This subcellular localization, as well as its expression profile restricted to a subset of epithelial-like cells in *C. elegans*, suggested a potential function for this protein in polarized membrane trafficking. Consistent with this hypothesis, alteration of CRP-1 expression affected the apical trafficking of CHE-14 in vulval and rectal epithelial cells and sphingolipids (C₆-NBD-ceramide) uptake and/or trafficking in intestinal cells. However, it did not affect basolateral trafficking of myotactin in the pharynx and the targeting of IFB-2 and AJM-1, two cytosolic apical markers of intestine epithelial cells. Hence, our data demonstrate a function for CRP-1 in the regulation of membrane trafficking in a subset of cells with epithelial characteristics.

INTRODUCTION

Rho GTPases are molecular switches cycling between “off,” GDP-bound, and “on,” GTP-bound conformations. On their on state, they propagate downstream signaling through interaction with effectors. These GTPases exhibit two intrinsic enzymatic activities: i) a nucleotide-exchange activity prevented by guanine nucleotide-dissociation inhibitors (GDIs) and enhanced by guanine nucleotide-exchange factors (GEFs) and ii) a GTP-hydrolysis activity catalyzed by GTPase-activating proteins (GAPs). To date, 23 genes have been identified as coding for functional Rho GTPases in *Homo sapiens*, 7 in *Drosophila melanogaster*, and only 5 in *Caenorhabditis elegans*. In human, the Rho family of GTPases is divided in six groups including RhoA-related proteins (RhoA, RhoB, RhoC), Rac1-related proteins (Rac1, -2, -3, and RhoG), Cdc42-related proteins (Cdc42, TC10, TCL, Wrch-1, and Chp), Rnd proteins (Rnd1, -2, and -3), RhoBTB proteins (RhoBTB1, -2, and -3), and Miro proteins (Miro1, -2). Three other members of the Rho family of GTPase, RhoD, Rif, and RhoH have not been classified in any group yet. Members of

each group have similar but not identical functional characteristics (Wennerberg and Der, 2004), thus generating a functional redundancy whose biological interest remains unclear.

The prototype members of the Rho GTPase family, namely RhoA, Rac1, and Cdc42, have been extensively studied for their role in actin cytoskeleton remodeling (Ridley, 2001). Indeed, in mouse fibroblasts, the activation of RhoA, Rac1, and Cdc42 promote formation of stress fibers, lamellipodia, and filopodia, respectively (Hall, 1998), resulting in the alteration of cell shape and movement. Consequently, Rho GTPases have been implicated in a wide range of biological functions requiring remodeling of actin structures such as cytokinesis, cell polarization, adhesion and migration, axon guidance, host-pathogen interactions, and intracellular membrane trafficking. Independently of their role on cell morphogenesis, Rho GTPases have also been shown to regulate cell cycle progression and gene expression (Etienne-Manneville and Hall, 2002).

Rho GTPases are highly conserved throughout evolution at both structural and functional levels (Wherlock and Mellor, 2002). In *C. elegans*, the Rho family of proteins can be subdivided in three sequence-related groups, namely Rac1-related proteins (CED-10, RAC-2, MIG-2), Cdc42-related proteins (CDC-42), and RhoA-related proteins (RHO-1). Similar to their respective mammalian orthologues, CDC-42 plays a major role in cell polarization (Gotta *et al.*, 2001; Kay and Hunter, 2001), whereas CED-10 together with RAC-2 and MIG-2 control axon guidance and cell corpse phagocytosis (Zipkin *et al.*, 1997; Reddien and Horvitz, 2000; Lundquist *et al.*,

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-08-0760>) on January 19, 2005.

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2001; Wu *et al.*, 2002) and CED-10, MIG-2, and RHO-1 participate in cell migration events (Zipkin *et al.*, 1997; Reddien and Horvitz, 2000; Lundquist *et al.*, 2001; Spencer *et al.*, 2001; Wu *et al.*, 2002).

The sequence of complete genomes allowed the identification of novel Rho GTPase domain encoding genes such as RhoBTBs and Miros (Rivero *et al.*, 2001; Fransson *et al.*, 2003). Similarly, we identified, in the *C. elegans* genome, a gene potentially encoding a novel Rho GTPase, and related to CDC-42. We report here the functional characterization of this protein, named CDC-42-related protein-1 (CRP-1). We show that CRP-1 is an atypical small GTP-binding protein unable to hydrolyze GTP and to promote actin cytoskeleton remodeling when expressed in mouse fibroblasts. Consistent with the exclusive epithelial expression pattern of CRP-1 and its localization at the *trans*-Golgi network and recycling endosomes, phenotypic characterization of *crp-1* mutant and *crp-1(RNAi)* animals revealed a role for CRP-1 in membrane trafficking in epithelial cells.

MATERIALS AND METHODS

Antibodies and Reagents

Mouse monoclonal anti-Myc and rabbit anti-clathrin heavy chain antibodies were purchased from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Rabbit BiP antiserum was kindly provided by Dr. Linda Hendershot (St. Jude Children's Research Hospital, Memphis TN) and anti-alpha mannosidase II antibodies were a kind gift from Dr. Marilyn Farquhar (UCSD, San Diego, CA). MH27, MH33, and MH46 ascite supernatants were obtained from the Developmental Studies Hybridoma Bank at The University of Iowa. Secondary FITC- and TRITC-conjugated anti-mouse and anti-rabbit antibodies as well as TRITC-conjugated phalloidin were purchased from Sigma (St. Louis, MO). C₆-NBD-ceramide was purchased from Molecular Probes (Eugene, OR). pEGFP-ERGICp58 and pEGFP-transferrin receptor were kindly provided by Dr. John Presley (McGill University, Montreal, PQ). LR and BP recombinases were purchased from Invitrogen (Carlsbad, CA).

DNA Constructs

Gateway compatible open reading frames (ORFs) coding for CRP-1 (Y32F6B.3), CDC-42 (R07G3.1), and RGA-1 (Rho GTPase-activating protein 1, W02B12.8) in pDONR201 vectors were retrieved from the *C. elegans* ORFeome collection version 1.1 (Reboul *et al.*, 2003). Endogenous *crp-1* and *cdc-42* STOP codons, converted to sense codon in the ORFeome clones (pDONRcrp-1(F-), pDONRcdc-42(F-)) were restored in pDONRcrp-1 and pDONRcdc-42 by PCR using specific primers (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGAGAACAAATTGAAATTGGTAG/5'-GGGGACCACTTTGTACAAAGAAAGCTGGG-TATCAAGTATTGTACACAAGGATTGG and 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGCAGACGATCAAGTGCG/5'-GGGGACCACTTTGTACAAAGAAAGCTGGG-TATCAAGTATTGTACACAAGGATTGG, respectively) and pDONRcrp-1(F-), pDONRcdc-42(F-) as templates. Resulting PCR products were inserted in pDONR201 by BP recombination. CRP-1(GAV) mutant was generated by PCR according to the overlap extension method (Horton *et al.*, 1989) using pDONRcrp-1 as template, mutagenic overlapping oligonucleotides (5'-GGATGGCGCCGTCGGTAAACAAGTCTTC/5'-TGTTTTACCGACGGCCATCCCCACGACTACC), and pDONR-specific oligonucleotides (5'-GATCTCGGGCCCCAAATAAT/5'-GGCTCATAACACCCCTTGTA). The resulting PCR products as well as the ORFs were transferred from pDONRcrp-1(F-), pDONRcdc-42(F-), pDONRcrp-1, and pDONRcdc-42 by LR recombination in Gateway compatible pGex2TK and pRK5Myc destination vectors. RGA-1 GAP domain was amplified from the pDONRW02B12.8 ORFeome clone using 5'CGGGATCCGCGGAAACACCACCAGC and 5'CGGGAATTCCTCAATGATCAAATACTG as specific primers. The resulting PCR fragment was then digested by *Bam*HI and *Eco*RI restriction enzymes and ligated in pTrcHisA (Invitrogen). DNA constructs generated by PCR were systematically sequence verified.

Cell Culture, Transfection, and Immunostaining

NIH3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotics and maintained at an atmosphere of 5% CO₂. For transfection, cells were plated on ionized glass coverslips and transfected using Lipofectamine (Invitrogen) following the manufacturer's instructions. Forty-eight hours after transfection, cells were fixed in 3.7% formaldehyde and processed for indirect immunofluorescence as described previously (Jenna *et al.*, 2002). *C. elegans* animal were fixed and stained as described by Finney and Ruvkun (1990).

Expression and Purification of Recombinant Proteins

Recombinant CRP-1, CRP-1(GAV), and CDC-42 were produced in *E. coli* (DH5α) as GST fusion proteins and purified on glutathione-Sepharose beads as described previously (Self and Hall, 1995). GST and GST-fusion proteins were eluted from the beads using 20 mM reduced glutathione in 20 mM Tris, pH 7.5, 25 mM NaCl, 0.1 mM dithiothreitol (DTT). RGA-1 GAP domain was produced in DH5α as a hexahistidine (His6)-fusion protein and purified on Ni-NTA Agarose beads (QIAGEN, Chatsworth, CA) according to the manufacturer's instructions. His6-tagged proteins were eluted from nickel chelate agarose beads using 300 mM imidazole in 20 mM Tris, pH 7.5, and 0.1 mM DTT. Protein concentration was determined using Bradford reagent (Bio-Rad, Richmond, CA) and the quality of purified proteins was assessed by analysis of Coomassie Blue-stained SDS-polyacrylamide gels.

In Vitro GTPase Enzymatic Assays

These assays were similar to previously published procedures (Hussain *et al.*, 2001; Jenna *et al.*, 2002) except that they were adapted to a 96-well format. For nucleotide-exchange activity, 0.2 μM purified, GDP-loaded CRP-1, CRP-1(GAV), and CDC-42 were incubated with 5 μM GTP γS and 0.167 μM of GTP γ [35S] (0.25 mCi.mmol⁻¹) in assay buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 500 ng/μl bovine serum albumin [BSA], 2.5 mM MgCl₂). After 0-, 5-, 10-, 15-, 20-, and 25-min incubation at 37°C, the reaction mix containing 0.1 pmol of GTPase was filtered through nitrocellulose membrane in a 96-well manifold (Bio-Rad) and washed extensively. Radioactivity trapped on the filters was then quantified using a Cyclone phosphorimager (Packard, Meriden, CT). For GTP hydrolysis and dissociation measures, the concentration of active GTPase, corresponding to the amount of GTPase able to bind to GTP, was estimated as described previously (Lamarche-Vane and Hall, 1998). One hundred picomolar active GTPase was loaded with either GTP γ [32P] or GTP γ [35S] and incubated in the presence of 1 mM GTP and 2 mM MgCl₂ for up to 15 min at either 37°C or at 25°C in the presence of 1, 100, or 1000 nM of RGA-1 GAP-His6 domain. At the indicated time, one-sixth of the reaction mix was filtered and washed, and the radioactivity trapped on the filter quantified as described above.

Strains and Genetic Analyses

C. elegans animals were maintained at 23°C under standard conditions (Brenner, 1974). Bristol N2 was used as the reference strain. The allele *crp-1(ok685)* (also named called Y32F6B.3(ok685)V) was provided by the *C. elegans* Knock-out Consortium. This mutant was identified after ultraviolet and trimethyl-psoralen mutagenesis by nested PCR using primers located 2.1-kbp apart within *crp-1*. This allele was outcrossed three times with N2. To position deletion breakpoints in *crp-1(ok685)* mutant, genomic DNA (gDNA) was purified from *crp-1(ok685)* animal using DNazol reagent (Invitrogen) according to the manufacturer's instructions and cDNA was synthesized using thermoscript RT-PCR system (Invitrogen) after purification of total *crp-1(ok685)* RNA using RNazol (Invitrogen). *crp-1* coding sequence was then PCR amplified from both *crp-1(ok685)* gDNA and cDNA using specific primers (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGAGAACAAATTGAAATTGGTAG/5'-GGGGACCACTTTGTACAAAGAAAGCTGGGTATCAAGTATTGTACACAAGGATTGG), inserted into pDONR201 by BP recombination and sequenced.

Generation of CRP-1 Transgenic Animals

The translational GFP fusion of CRP-1 was constructed by amplifying a 4300-base pair DNA fragment from N2 genomic DNA using specific primers (5'-GCTCTAGAGCATGTCACCTTCATTCATACCG and 5'-CGGGGTACCGTACACAAGGATTGGGGTT). The resulting DNA fragment, starting 2264 base pairs upstream of *crp-1* ATG initiation codon and ending one base pair upstream of its stop codon was digested with *Xba*I and *Kpn*I and ligated in pPD117.01 upstream of the GFP(S65C) reporter sequence. The resulting pPD117.01crp-1::GFP construct was then coinjected into young adult N2 hermaphrodites at 40 ng/μl together with 160 ng/μl pRF4 vector containing the *rol-6(su1006)* marker as described elsewhere (Mello *et al.*, 1991). Five independent *rol-6(su1006)* lines were examined by fluorescence microscopy.

crp-1(ok685) Ex[che-14::GFP *rol-6(su1006)*] and *crp-1(ok685)* Ex[crp-1::GFP *rol-6(su1006)*] animals were isolated by mating Ex[che-14::GFP *rol-6(su1006)*] (kindly provided by Dr Michel Labouesse, Institut de Génétique et de Biologie Moléculaire, Illkirch, France) and Ex[crp-1::GFP *rol-6(su1006)*] L4 hermaphrodites with *crp-1(ok685)* males. F2 *rol* animals homozygous for *crp-1(ok685)* were identified by nested PCR using *crp-1*-specific primers (5'-GAAGACAACGCCTCTGGAAG/5'-AGGAAATGGGTGAGCAATG).

RNA Interference

CRP-1 coding sequence was inserted in the Gateway-compatible pL4440 vector and transformed in HT115(DE3) bacteria. Isolated transformant clones were grown in Luria-Bertani (LB) medium supplemented by 100 μg/ml ampicillin for 8 h at 37°C and plated on NGM plates containing 1 mM IPTG and 100 μg/ml ampicillin. Plates were incubated 16 h at room temperature and inoculated either with Ex[che-14::GFP *rol-6(su1006)*] or Ex[crp-1::GFP *rol-6(su1006)*] embryos prepared using the hypochlorite method. Worms were

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Figure 2. Genetic characterization of *crp-1(ok685)* mutant. (A) Localization of *crp-1* transcribed sequence and *crp-1(ok685)* deletion on chromosome V. *crp-1* exons (squares, numbered from 1 to 4) and introns (lines) are indicated as well as translated sequences (black squares) in both N2 and *crp-1(ok685)* containing RB855 strain. Genomic DNA fragment fused to GFP and used to generate CRP-1::GFP expressing animals is indicated by an arrowed line. (B) *crp-1* sequence from ATG to stop codon were amplified from N2 and RB855 genomic DNA (gDNA) and cDNA (cDNA) and separated by electrophoresis in agarose gel. Size of DNA fragments is indicated in base pairs (bp).

shown for other small G proteins of the Ras superfamily. CRP-1 also shows a very atypical acidic hypervariable region (Figure 1C, white box) and, just adjacent to the CAAX motif, a potentially palmitoylated cysteine residue. Unlike most Rho GTPases, CRP-1 lacks the "Rho insert domain" (Wennerberg and Der, 2004; Figure 1C, gray box). Based on this sequence analysis, CRP-1 may be either considered as an atypical Rho GTPase or as a novel unclassified member of the Ras-superfamily of small G proteins.

CRP-1 Is Not Essential for *C. elegans* Embryonic Development and Survival

To investigate the biological function(s) of CRP-1, we used a reverse genetic approach leading to loss of *crp-1* function(s). A *crp-1* deletion (*crp-1(ok685)*) was obtained from the *C. elegans* Knockout Consortium. PCR amplification and sequencing of both genomic (gDNA) and complementary (cDNA) DNAs coding for the mutant CRP-1 revealed that the *ok685* mutation consisted of a 658-base pair deletion in gDNA and 129 base pairs in cDNA (Figure 2B) that caused a frame shift in exon 3 and generated a premature STOP codon 81 nucleotides downstream of the ATG in the cDNA sequence (Figure 2A). This mutation resulted in the expression of a truncated CRP-1 protein containing only 28 amino acids, including 9 missense residues. Given that this deletion led to the removal of all the protein domains required for GTPase function, *crp-1(ok685)* was therefore considered as a null allele.

The anatomical observation of *crp-1(ok685)* embryos, larvae, and adults using DIC microscopy did not reveal any obvious morphological abnormality. As previously reported

for animals subjected to *crp-1(RNAi)* (Jantsch-Plunger *et al.*, 2000; Kamath and Ahringer, 2003), neither embryonic nor larval lethality was observed upon *crp-1* gene silencing and animals presented normal life span and egg laying efficiency (unpublished data). Unlike other Rho GTPases, for which loss of function mutations resulted in obvious morphological and/or behavioral phenotypes because of their critical function in cell and organ morphogenesis, the apparent wild-type phenotype of *crp-1(ok685)* animals suggested that *crp-1* could be either functionally redundant with other proteins or implicated in subtle cellular mechanisms not observable using standard DIC microscopy.

Therefore, to assess the biological function(s) of CRP-1 in *C. elegans*, we further investigated the characteristics of CRP-1 recombinant protein through the integration of in vitro, ex vivo, and in vivo data.

CRP-1 Is an Enzymatically Atypical Small G Protein

The ORFs encoding CRP-1 and CDC-42 were obtained from the *C. elegans* ORFeome (Reboul *et al.*, 2003) and transferred to prokaryotic and eukaryotic expression vectors. CRP-1 and CDC-42 GST-fusion proteins were expressed in *E. coli* and purified as described in *Materials and Methods*. CRP-1 and CDC-42 nucleotide-exchange and GTP hydrolysis activities were measured in vitro using 96-well adapted filtration assays. Nucleotide-exchange activities were measured by following the time-dependent binding of GTP γ [35 S] to GDP-preloaded GTPases, whereas GTP hydrolysis activities were measured by following the time-dependent hydrolysis of the 32 P-phosphate by the GTPases preloaded with GTP γ [32 P] (Hussain *et al.*, 2001; Jenna *et al.*, 2002). In comparison to CDC-42, CRP-1 showed a very weak nucleotide-exchange activity as well as a reduced dissociation of radioisotope from GTP γ [32 P]-loaded GTPase over time (Figure 3, A and B). To investigate whether the radioisotope release resulted from GTP γ [32 P] hydrolysis or from nucleotide dissociation from the protein, similar assays were carried out using the nonhydrolysable GTP γ [35 S]. This revealed that whereas GTP γ [35 S] formed a very stable complex with CDC-42, it dissociated rapidly from CRP-1 (Figure 3C). Hence, the unstable complex between GTP γ [32 P] and CRP-1 is caused by GTP dissociation from the protein rather than elevated hydrolysis activity.

GTPase-activating proteins (GAPs) are known to regulate Rho GTPases by enhancing their intrinsic GTP hydrolysis activity (Jenna and Lamarche-Vane, 2003). To test whether incubation of CRP-1 with a GAP protein would enhance an existing but undetectable GTP hydrolysis activity, we retrieved the cDNA encoding RGA-1 from the *C. elegans* ORFeome. This protein is the ortholog of the Cdc42- and RhoA-specific mammalian GAP, p50-RhoGAP. We amplified RGA-1 GAP domain and generated a RGA1-GAP domain fused to His6-tag. The percentage of GTP γ [32 P] bound on CDC-42 or CRP-1 after a 5-min incubation at 25°C in the presence of increasing concentration of RGA-1 GAP domain was then measured using the filtration assay described above. We demonstrated that, as expected, RGA-1 increased CDC-42 hydrolysis activity (Figure 3D), but no obvious effect was observed on CRP-1 (Figure 3D).

The fact that CRP-1 did not show any significant GTP hydrolysis activity was consistent with its nonconventional P-loop sequence. Indeed, the CDC-42 highly conserved glycine-12 was replaced by a threonine residue in CRP-1 sequence (Figure 1C). Interestingly, conservation of this residue has been shown to be critical for GTPase-dependent GTP hydrolysis activity of all members of the Ras superfamily of small G protein (Sprang, 1997). To investigate whether the restoration of a CDC-42-like P-loop could partially reverse CRP-1 deficiency, the TYT residues (12–14) (Figure 1C, circled) in

Figure 3. CRP-1 is deficient in GTP hydrolysis and shows a low nucleotide-exchange activity. (A) GDP-loaded CRP-1 (○), CRP-1(GAV) (●) and CDC-42 (□) were incubated in presence of GTP γ [35 S] and incubated at 37°C. (B) GTPases, loaded with either GTP γ [32 P] or GTP γ [35 S] (C) were incubated at 37°C in presence of 1 mM GTP. (D) GTPases, loaded with GTP γ [32 P] were incubated for 5 min at 25°C in the presence of 1 mM GTP and 1, 100, or 1000 nM of RGA-1 GAP domain. For all assays, GST (Δ) is used as a negative control, and the radioactivity associated with proteins at indicated times measured after filtration through a nitrocellulose membrane.

CRP-1 amino acid sequence were replaced by GAV (CDC-42 sequence). The enzymatic characterization of the resulting CRP-1(GAV) mutant revealed a nucleotide-exchange activity (Figure 3A) and instability of GTP γ [35 S]/GTPase complex (Figure 3C) similar to those observed for CRP-1. However, as predicted, the hydrolysis activity of CRP-1(GAV) was significantly increased compared with CRP-1 (Figure 3B). These data indicate that CRP-1 is an atypical small G protein, which displays a very low affinity for GTP and is deficient for GTP hydrolysis in vitro.

CRP-1 Does Not Reorganize the Actin Cytoskeleton in NIH3T3 Cells

To investigate whether CRP-1, like most Rho GTPases, could be targeted to cellular membranes and regulate actin cytoskeleton remodeling, nonprenylated mutants of CRP-1 and CDC-42 were generated (CRP-1(F-) and CDC-42(F-), respectively) by fusion of nine C-terminal extraamino-acids residues. N-terminal Myc-tagged fusions of CDC-42, CDC-42(F-), CRP-1, CRP-1(F-), and CRP-1(GAV) were then expressed in NIH3T3 cells. Twenty-four hours after transfection, cells were fixed and stained for filamentous actin using TRITC-conjugated phalloidin. CDC-42 and CRP-1 were detected by indirect immunofluorescence using anti-Myc antibodies. Consistent with the data obtained for its mammalian ortholog, CDC-42 expressed in mouse fibroblasts is properly targeted to cel-

lular membranes and induces robust filopodia formation (Gibson and Wilson-Delfosse, 2001), whereas a nonfarnesylated CDC-42 mutant, CDC-42(F-), is not (Figure 4). In contrast, neither wild-type CRP-1, nonfarnesylated CRP-1(F-) nor CRP-1(GAV) induced any significant remodeling of the actin cytoskeleton (Figure 4). This indicates that sequence homology between CRP-1 and CDC-42 is not sufficient for CRP-1 to achieve CDC-42 function on actin cytoskeleton remodeling when ectopically expressed in NIH3T3 cells. In addition, these data also suggest that CRP-1 inability to induce formation of actin structures is independent of CRP-1 GTP-hydrolysis deficiency.

CRP-1 Localizes in trans-Golgi Network and Recycling Endosomes in NIH3T3 Cells

When expressed in NIH3T3 cells CRP-1 localized mainly in perinuclear punctuated structures. This subcellular localization was independent of CRP-1 GTP hydrolysis activity because identical staining was observed with CRP-1(GAV) (Figure 4). However, it was dependent on CRP-1 prenylation because cellular localization of CRP-1(F-) mutant was mainly cytosolic (Figure 4). In addition, cell fractionation studies carried out with CRP-1-expressing NIH3T3 cells revealed that CRP-1 partitioned with cellular membranes (unpublished data). This indicates that CRP-1 is likely to be anchored to intracellular membrane compartments through its prenyl group.

Figure 4. CRP-1 does not reorganize the actin cytoskeleton in NIH3T3 cells. NIH3T3 cells were transfected with pRK5Myc-CRP-1, -CRP-1(F-), -CRP-1(GAV), -CDC-42, or -CDC-42(F-) constructs. Twenty-four hours after transfection cells were fixed, permeabilized, and immunostained using TRITC-coupled Phalloidin (left panel, Actin) and anti-Myc/FITC-coupled anti-mouse antibodies (right panel). Scale bar, 5 μ m. For each construct at least 100 transfected cells were observed and the percentages of cells presenting lamellipodia (white bar), filopodia (black bar), or stress fibers (dashed bar) are represented in a bar graph.

The subcellular localization of Myc-tagged CRP-1 was then assessed either by coexpression with GFP-tagged compartment specific markers or by staining CRP-1-expressing cells using compartment marker specific antibodies. Colocalization of CRP-1 with these markers was analyzed using confocal microscopy (Figure 5). CRP-1 did not significantly colocalize with BiP (Figure 5, A, F, and K), ERGICp58 (Figure 5, B, G, and L), or α -mannosidase II (Figure 5, C, H, and M) used, respectively, as markers for the endoplasmic reticulum, the ER-Golgi intermediate compartment or the Golgi apparatus. However, a subtle perinuclear colocalization of CRP-1 was observed with clathrin (Figure 5, D, I, and N), as well as a very clear colocalization with transferrin receptors (Figure 5, E, J, and O). These data indicate that CRP-1 most likely localizes to transferrin receptor-enriched recycling endosomes and, to a lesser extent, to the *trans*-Golgi network (TGN) when expressed in NIH3T3 cells.

CRP-1 Is Expressed in a Subset of Epithelial-like Cells in *C. elegans*

The spatio-temporal regulation of CRP-1 expression in *C. elegans* was then investigated by generating transgenic ani-

mals that expressed a CRP-1::GFP translational fusion under the control of the endogenous *crp-1* promoter. CRP-1 expression pattern was observed by epifluorescence microscopy on living animals. CRP-1::GFP fusion protein was first expressed in pharyngeal cells at the early comma stage of embryogenesis (Figure 6A). At the twofold stage, CRP-1 was also detected in the rectum (unpublished data) and later, at the threefold stage throughout the entire digestive tract (Figure 6B). After hatching, CRP-1 expression was extended to the head and tail support cells and to the excretory cell (Figure 6C). This expression pattern was maintained throughout larval stages (unpublished data) except in dauer larvae where CRP-1 expression was restricted to the excretory system (Figure 6D). In young adult, CRP-1::GFP expression decreased in the pharynx and appeared in the vulval epithelium (Figure 6H). CRP-1 expression in intestinal and rectal valve (Figure 6, C and J), in intestinal cells (Figure 6J), in head and tail support cells (Figure 6, E–G), in excretory cell (Figure 6I), and in vulval and rectal epithelia (Figure 6, H, J, and K) was then maintained throughout adulthood in both males and hermaphrodites.

Figure 5. CRP-1 colocalizes with clathrin and transferrin receptor in NIH3T3 cells. NIH3T3 cells were transfected with pRK5Myc-CRP-1 alone (A, C, D, F, H, I, K, M, and N) or together with pEGFP-ERGICp58 (B, G, and L) or pEGFP-TransferrinR (E, J, and O). Twenty-four hours after transfection cells were fixed, permeabilized, and immunostained using anti-Myc antibodies (A–E; green, K–O), anti-BiP (F; red, K), anti- α -mannosidase II (H; red, M), and anticlathrin (I; red, N). ERGIC-p58 and TransferrinR were localized by observation of EGFP epifluorescence (G and J; red, L and O). Colocalization of CRP-1 and compartment markers was assessed by confocal microscopy. Picture merges were carried out using Northern Eclipse (K–O). Scale bar, 5 μ m.

These data demonstrate that *crp-1* expression is tightly regulated during *C. elegans* development both spatially and temporally. Interestingly, CRP-1 was expressed exclusively in a subset of epithelial cells and myoepithelial cells (pharyngeal muscles), suggesting that this protein may have some epithelial cell specific function(s). In addition, CRP-1 expression was detectable after epithelial morphogenesis, except in the pharynx muscle where its expression was detectable at very early stage of pharynx development.

CRP-1 Regulates the Apical Trafficking of CHE-14 and C_6 -NBD-ceramide in a Subset of Epithelial Cells

The localization of CRP-1 in TGN and recycling endosomes as observed in NIH3T3 cells as well as its restricted expression in a subset of epithelial-like cells in animal suggested that CRP-1 may play a role in polarized membrane trafficking events. To address this possibility, we analyzed the effect of the *crp-1(ok685)* deletion on the trafficking of two transmembrane proteins, CHE-14 and myotactin shown to be both expressed in a subset of epithelial cells in *C. elegans* and to be respectively targeted to the apical and the basolateral membranes (Hresko *et al.*, 1999; Michaux *et al.*, 2000). Although some differences in total fluorescence intensity, due to variability in staining procedures, were detected from one animal to another, no significant difference in the basolateral distribution of myotactin in pharyngeal cells was observed in *crp-1(ok685)* when compared

with wild-type (WT) animals (Figure 7, E and F). In contrast, the distribution of CHE-14::GFP was altered in vulval and rectal epithelia in *crp-1(ok685)* animals as it distributed mainly in intracellular punctuated structures (Figure 7, A–D). The efficiency of CHE-14 trafficking was measured by calculating the percentage of CHE-14::GFP detected at the apical membrane in vulval and rectal epithelia for 6–13 *crp-1(ok685)* and wild-type animals (Figure 7G). CHE-14 trafficking appeared to be altered significantly in *crp-1(ok685)* animals in both vulval ($p = 5.48E-04$) and rectal ($p = 9.7E-05$) epithelia when compared with wild-type animals. To confirm that the trafficking alteration observed in *crp-1(ok685)* animals was due to the disruption of CRP-1 expression, we subjected CHE-14::GFP-expressing wild-type animals to *crp-1(RNAi)* using a feeding procedure. As described above, the alteration of CHE-14 trafficking was also significantly observed in both vulval ($p = 0.0050$) and rectal ($p = 0.0379$) epithelia of *crp-1(RNAi)* animals when compared with wild-type but demonstrated a reduced penetrance when compared with *crp-1(ok685)*, probably due to residual CRP-1 activity after RNAi.

We also investigated the subcellular distribution of cytosolic apical markers of intestinal cells, namely the intermediate filament IFB-2 and the adherent junction component AJM-1. IFB-2 and AJM-1 distribution was assessed by immunostaining on fixed animals. No obvious difference in

Figure 6. CRP-1 is expressed in a subset of epithelial and myoepithelial cells. Cellular localization of CRP-1::GFP was assessed by observation of GFP epifluorescence in embryos at early comma (A) and threefold (B) stages, in L1 (C) and dauer (D) larvae, as well as in hermaphrodite and male adults (E–K). The Pharynx (Ph.), the intestinal valve (Int. V.), the intestinal epithelium (Int.), the excretory cell (Exc.), the socket cells (So.), the sheath cells (Sh), the vulva epithelium (V. ep.), the rectum epithelium (R. ep.) and the stroma epithelium (Str.) are indicated. Scale bars, 20 μ m.

IFB-2 and AJM-1 distribution was observed in *crp-1(ok685)* when compared with wild-type animals (Figure 8, A–D).

To investigate whether the role of CRP-1 in the trafficking of apical membrane proteins may also affect lipid distribution, we assessed the localization exogenous C₆-NBD-ceramide, a fluorescently labeled sphingolipid, in intestinal cells. The cellular distribution of C₆-NBD-ceramide was observed by direct fluorescence microscopy after ingestion of exogenous C₆-NBD-ceramide/bovine serum albumin complex by young adults for 2 h, followed by a 2-h washing with M9 buffer. The distribution of exogenous C₆-NBD-ceramide was then studied in the intestinal epithelial cells of wild-type and *crp-1(ok685)* animals. In mutant animals, a significant alteration ($p = 3.40E-07$) of the distribution of C₆-NBD-ceramide and/or its potential byproducts was observed, as reflected by the accumulation the fluorescent probe(s) at the apical membrane of intestinal cells (Figure 8, E–G).

Together these data indicate that CRP-1 expression is required for an efficient apical trafficking of CHE-14::GFP and uptake/trafficking of C₆-NBD-ceramide (and/or its downstream by-products) in a subset of epithelial cells. In addition, the alteration of CRP-1 expression did not appear to be detrimental for myotactin basolateral trafficking in pharyngeal cells and for apical targeting of IFB-2 and AJM-1 in intestine, thus indicating that CRP-1 function would be restricted to apical membrane trafficking events.

DISCUSSION

The regulation of polarized membrane trafficking is critical for epithelia to ensure their barrier and signaling functions

and consequently to control body homeostasis. These mechanisms were shown to be largely regulated by members of the Rho family of GTPases in mammals (reviewed in Van Aelst and Symons, 2002). However, their function in *C. elegans* epithelial cells has not been clearly demonstrated yet.

In this study, we have characterized a novel *C. elegans* CDC-42-related GTP-binding protein CRP-1. This protein shows atypical enzymatic characteristics, is exclusively expressed in terminally polarized epithelial-like cells in *C. elegans*, and localizes to the *trans*-Golgi network (TGN) and recycling endosomes when ectopically expressed in mouse fibroblasts. The integration of these functional characteristics allowed us to hypothesize a function for CRP-1 in polarized membrane trafficking in epithelial cells. Consistent with this hypothesis, *crp-1* null mutant animals revealed an altered apical trafficking of CHE-14 in vulval and rectal epithelial cells and of C₆-NBD-ceramide in intestinal cells while displaying an efficient basolateral trafficking of myotactin in the pharynx and an efficient apical targeting of soluble IFB-2 and AJM-1 in intestinal cells. Our data suggest therefore a function for CRP-1 in apical membrane trafficking in a subset of epithelial cells. However, we cannot yet exclude an epithelial cell-type-specific role of CRP-1 which would lead to the regulation of CHE-14 trafficking in vulval and rectal cells and the nonalteration of myotactin trafficking in pharyngeal cells.

CRP-1 Is Implicated in Apical Membrane Trafficking in Epithelial Cells

Polarized membrane trafficking in epithelial cells consists of either a direct sorting of biosynthetic proteins and sphingo-

Figure 7. CRP-1 expression is required for an efficient apical trafficking of CHE-14::GFP in vulva and rectum. Subcellular distribution of CHE-14::GFP in vulva (A and B) and rectal (C and D) epithelia was observed in wild-type (wt), *crp-1(ok685)* and *crp-1(RNAi)* animals by confocal microscopy. The percentage of CHE-14::GFP at the apical membrane of vulva and rectum (G) epithelia was measured in three-dimensional stacks of images from 6 to 13 different wild-type (black bar), *crp-1(ok685)* (dashed bar), and *crp-1(RNAi)* (white bar) animals. Subcellular distribution of myotactin in the pharynx was observed by indirect fluorescence microscopy after fixation and immunostaining of wild-type (E), *crp-1(ok685)* (F) animals with MH46 antibodies. Scale bars, 20 μ m. * $p < 0.05$, as determined by Student's *t* test compared with control.

lipids to the apical or basolateral membranes, or an indirect routing of membrane components by endocytic/recycling or transcytic processes (Van IJzendoorn and Hoekstra, 1998; Ait Slimane and Hoekstra, 2002; Maier and Hoekstra, 2003; Mostov *et al.*, 2003). These sorting routes are organized, respectively, from the TGN and the subapical compartment (SAC), the epithelial equivalent of recycling endosomes (Van IJzendoorn *et al.*, 2000; Ait Slimane and Hoekstra, 2002). CRP-1 localization to the TGN and recycling endosomes in fibroblasts as well as its exclusive expression in *C. elegans* epithelial and myoepithelial cells strongly suggested the involvement of this protein in polarized membrane trafficking. This hypothesis was confirmed by gene deletion and/or silencing of *crp-1*, which resulted in the alteration of CHE-14 trafficking and C₆-NBD-ceramides uptake and/or trafficking at the apical membrane of vulval/rectal epithelial and intestinal cells, respectively. Interestingly, the trafficking of myotactin, a basolateral transmembrane protein, was not altered in *crp-1(ok685)* mutant, indicating that CRP-1 may be specific of apical membrane trafficking. In addition, the proper apical targeting of IFB-2 and AJM-1 in *crp-1(ok685)* intestinal cells suggests that CRP-1 function would be restricted to integral membrane components.

The role of Rho GTPases in membrane trafficking is thought to be mainly mediated through actin cytoskeleton remodeling (Symons and Rusk, 2003). However, some examples have shown that Rho GTPases could also control membrane trafficking through actin-independent manners by modulating phosphatidylinositol lipid metabolism (Ren and Schwartz, 1998; Malecz *et al.*, 2000) or regulating clathrin-coat assembly (Yang *et al.*, 2001; Symons and Rusk, 2003). Because ectopic expression of CRP-1 in mouse fibroblasts

did not induce any significant remodeling of the filamentous actin structures, we postulated that CRP-1 may regulate membrane trafficking events in such manners. In addition, CRP-1 inability to reorganize the actin cytoskeleton in fibroblast, suggested that it may not play a critical role in cell morphogenesis. This hypothesis is consistent with the normal anatomy of *crp-1(ok685)* animals as observed by DIC microscopy and unaltered immunolocalization of adherent junction (AJM-1) and subapical cytoskeleton markers (IFB-2) in epithelial cells.

Potential Role of CRP-1 in the Maintenance of Epithelial Functions in Postdevelopmental Stages

Sphingolipids have been clearly established to play a major role in the control of apical trafficking and transcytosis of proteins in mammalian polarized epithelial cells (Ait Slimane and Hoekstra, 2002). In addition, it has been clearly established that small GTP-binding proteins regulated the trafficking of these lipids (Choudhury *et al.*, 2002). Although such a function has not yet been confirmed in *C. elegans*, we believe that in *crp-1(ok685)* animals, C₆-NBD-ceramides may not be properly up-taken or may not follow their normal trafficking routes. In addition, neuronal abnormalities caused by mutations in human and *C. elegans* homologues of the Niemann-pick type C1 disease protein, a protein whose function is critical for the intracellular distribution of cholesterol and sphingolipids (Li *et al.*, 2004), indicate that those trafficking events may be conserved from worm to mammals. The potential consequences of an alteration of the apical uptake and/or trafficking of sphingolipids and CHE-14, which regulates the exocytosis of lipid modified proteins at the apical surface of *C. elegans* epithelial cells (Michaux *et*

Figure 8. CRP-1 expression is required for an efficient trafficking of C₆-NBD-ceramide in intestinal cells. Subcellular distribution of IFB-2 (A and B) and AJM-1 (C and D) in intestinal cells was observed by indirect fluorescence microscopy after fixation and immunostaining of wild-type (wt) and *crp-1(ok685)* animals with MH33 and MH27 antibodies, respectively. Subcellular distribution of exogenous C₆-NBD-ceramide (E and F) was observed by direct fluorescence microscopy after a 2-h incubation of wild-type (wt) and *crp-1(ok685)* animals in 10 mM BSA adsorbed C₆-NBD-ceramide and a 2-h washing in M9 buffer. The percentage of C₆-NBD-ceramide at the apical membrane of intestinal cells (G) was measured digital images from 20 different wild-type (black bar) and *crp-1(ok685)* (dashed bar) animals. Scale bars, 20 μ m. * $p < 0.05$, as determined by Student's *t* test compared with control.

al., 2000), would suggest broadened consequences of *crp-1* knockout than those observed in our study. *Che-14* loss of function mutants and mutant altering cholesterol and presumably sphingolipids trafficking such as the *C. elegans* Niemann-pick type C1 disease proteins, NCR-1 and NCR-2 and the gp330/megalin-related proteins, LRP-1, show obvious morphological phenotypes such as protruding vulva (Puv) or rectum and excretory system malformation (Yochem *et al.*, 1999; Michaux *et al.*, 2000; Li *et al.*, 2004). In contrast, *crp-1(ok685)* mutants did not display any visible morphological differences when compared with wild-type animals. This observation suggests that the alteration of CHE-14 apical trafficking and sphingolipids uptake and/or trafficking induced by *crp-1* deletion did not result in any significant modification of CHE-14 function and membrane composition at critical development stages. This hypothesis correlates well with the occurrence of CRP-1 expression in vulval, rectal, and intestinal epithelial cells after epithelium morphogenesis. The integration of CRP-1 expression patterns and *crp-1* null-phenotypes suggests therefore a role for CRP-1 in epithelial function rather than in cell morphogenesis. This model is also supported by CRP-1 expression pattern in dauer larvae. Indeed, at this stage many tissues and organs such as the intestinal track and sensory apparatus have their morphology modified in response to dauer signals, thus resulting in interruption or alteration of their function(s) (Popham and Webster, 1979; Albert and Riddle, 1983). Interestingly, from all epithelial cells expressing CRP-1, only the excretory cell maintains its proper morphology and function in dauer larvae (Nelson *et al.*, 1983) and is shown in our study to continuously express CRP-1::GFP at detectable levels (Figure 6D).

However, the fact that alteration of CRP-1 expression did not result in any obvious anatomical change or life span reduction may indicate that CRP-1 expression is not required for epithelial function in normal culture conditions. We cannot exclude, however, the possibility that *crp-1* null animals could display specific phenotypes under stress conditions because of the modification of epithelial membrane protein and lipid composition. This is consistent with the established function of sphingolipids in cellular response to environmental stresses in mammals (Cutler and Mattson, 2001; Hannun and Obeid, 2002; Jenkins, 2003).

In conclusion, our study demonstrates that CRP-1 is a very specialized small G protein involved in membrane trafficking in *C. elegans* epithelial cells. Its restrictive epithelial expression in *C. elegans* and subcellular localization together with its unique enzymatic activity and inability to reorganize the actin cytoskeleton in fibroblasts, constitute a large panel of differences to other members of the Rho GTPase family. These functional differences are also consistent with the analysis of the predicted CRP-1 protein structure, which shows as much homology for Rab as for Rho GTPases (unpublished data). CRP-1 may therefore represent a nematode specific evolutionary functional link between Rho and Rab GTPases.

ACKNOWLEDGMENTS

We are grateful to the *C. elegans* Knockout consortium and participating groups for providing of *crp-1(ok685)* animals. We also thank Dr. Joe D. Schrag (BRI, Montreal) for preliminary structure analysis, Dr. John Presley and Archana Srivastava for assistance with confocal microscopy, as well as to the members of the Chevet's laboratory for their critical comments. This work was supported by a grant from the Canadian Institutes for Health Research to

E.C. (MOP53357). S.J. was supported in part by a CIHR postdoctoral fellowship and a grant from Génome Québec to the Cell Map project. M.E.C. was supported by a fellowship from the Fonds de la Recherche en Santé du Québec. E.C. is a Junior scholar from the Fonds de la Recherche en Santé du Québec.

REFERENCES

- Ait Slimane, T., and Hoekstra, D. (2002). Sphingolipid trafficking and protein sorting in epithelial cells. *FEBS Lett.* 529, 54–59.
- Albert, P. S., and Riddle, D. L. (1983). Developmental alterations in sensory neuroanatomy of the *Caenorhabditis elegans* dauer larva. *J. Comp. Neurol.* 219, 461–481.
- Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71–94.
- Choudhury, A., Dominguez, M., Puri, V., Sharma, D. K., Narita, K., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (2002). Rab proteins mediate Golgi transport of caveola-internalized glycosphingolipids and correct lipid trafficking in Niemann-Pick C cells. *J. Clin. Invest.* 109, 1541–1550.
- Cutler, R. G., and Mattson, M. P. (2001). Sphingomyelin and ceramide as regulators of development and lifespan. *Mech. Ageing Dev.* 122, 895–908.
- Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. *Nature* 420, 629–635.
- Finney, M., and Ruvkun, G. (1990). The unc-86 gene product couples cell lineage and cell identity in *C. elegans*. *Cell* 63, 895–905.
- Fransson, A., Ruusala, A., and Aspenstrom, P. (2003). Atypical Rho GTPases have roles in mitochondrial homeostasis and apoptosis. *J. Biol. Chem.* 278, 6495–6502.
- Gibson, R. M., and Wilson-Delfosse, A. L. (2001). RhoGDI-binding-defective mutant of Cdc42Hs targets to membranes and activates filopodia formation but does not cycle with the cytosol of mammalian cells. *Biochem. J.* 359, 285–294.
- Gotta, M., Abraham, M. C., and Ahringer, J. (2001). CDC-42 controls early cell polarity and spindle orientation in *C. elegans*. *Curr. Biol.* 11, 482–488.
- Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* 279, 509–514.
- Hannun, Y. A., and Obeid, L. M. (2002). The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. *J. Biol. Chem.* 277, 25847–25850.
- Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* 77, 61–68.
- Hresko, M. C., Schrieffer, L. A., Shrimankar, P., and Waterston, R. H. (1999). myotactin, a novel hypodermal protein involved in muscle-cell adhesion in *Caenorhabditis elegans*. *J. Cell Biol.* 146, 659–672.
- Hussain, N. K. *et al.* (2001). Endocytic protein intersectin-1 regulates actin assembly via Cdc42 and N-WASP. *Nat. Cell Biol.* 3, 927–932.
- Jantsch-Plunger, V., Gonczy, P., Romano, A., Schnabel, H., Hamill, D., Schnabel, R., Hyman, A. A., and Glotzer, M. (2000). CYK-4, A Rho family gtpase activating protein (GAP) required for central spindle formation and cytokinesis. *J. Cell Biol.* 149, 1391–1404.
- Jenkins, G. M. (2003). The emerging role for sphingolipids in the eukaryotic heat shock response. *Cell Mol. Life Sci.* 60, 701–710.
- Jenna, S., Hussain, N. K., Danek, E. I., Triki, I., Wasiaik, S., McPherson, P. S., and Lamarche-Vane, N. (2002). The activity of the GTPase-activating protein CdGAP is regulated by the endocytic protein intersectin. *J. Biol. Chem.* 277, 6366–6373.
- Jenna, S., and Lamarche-Vane, N. (2003). The superfamily of Rho GTPase-activating proteins. In: *Rho GTPases*. ed. M. Symons, New York: Kluwer Academic, 68–95.
- Kamath, R. S., and Ahringer, J. (2003). Genome-wide RNAi screening in *Caenorhabditis elegans*. *Methods* 30, 313–321.
- Kay, A. J., and Hunter, C. P. (2001). CDC-42 regulates PAR protein localization and function to control cellular and embryonic polarity in *C. elegans*. *Curr. Biol.* 11, 474–481.
- Lamarche-Vane, N., and Hall, A. (1998). CdGAP, a novel proline-rich GTPase-activating protein for Cdc42 and Rac. *J. Biol. Chem.* 273, 29172–29177.
- Li, J., Brown, G., Ailion, M., Lee, S., and Thomas, J. H. (2004). NCR-1 and NCR-2, the *C. elegans* homologs of the human Niemann-Pick type C1 disease protein, function upstream of DAF-9 in the dauer formation pathways. *Development* 131, 5741–5752.
- Lundquist, E. A., Reddien, P. W., Hartwig, E., Horvitz, H. R., and Bargmann, C. I. (2001). Three *C. elegans* Rac proteins and several alternative Rac regulators control axon guidance, cell migration and apoptotic cell phagocytosis. *Development* 128, 4475–4488.
- Maier, O., and Hoekstra, D. (2003). Trans-Golgi network and subapical compartment of HepG2 cells display different properties in sorting and exiting of sphingolipids. *J. Biol. Chem.* 278, 164–173.
- Malecz, N., McCabe, P. C., Spaargaren, C., Qiu, R., Chuang, Y., and Symons, M. (2000). Synaptojanin 2, a novel Rac1 effector that regulates clathrin-mediated endocytosis. *Curr. Biol.* 10, 1383–1386.
- Martin, O. C., and Pagano, R. E. (1994). Internalization and sorting of a fluorescent analogue of glucosylceramide to the Golgi apparatus of human skin fibroblasts: utilization of endocytic and nonendocytic transport mechanisms. *J. Cell Biol.* 125, 769–781.
- Mello, C. C., Kramer, J. M., Stinchcomb, D., and Ambros, V. (1991). Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* 10, 3959–3970.
- Michaux, G., Gansmuller, A., Hindelang, C., and Labouesse, M. (2000). CHE-14, a protein with a sterol-sensing domain, is required for apical sorting in *C. elegans* ectodermal epithelial cells. *Curr. Biol.* 10, 1098–1107.
- Mostov, K., Su, T., and ter Beest, M. (2003). Polarized epithelial membrane traffic: conservation and plasticity. *Nat. Cell Biol.* 5, 287–293.
- Nelson, F. K., Albert, P. S., and Riddle, D. L. (1983). Fine structure of the *Caenorhabditis elegans* secretory-excretory system. *J. Ultrastruct. Res.* 82, 156–171.
- Popham, J. D., and Webster, J. M. (1979). Aspect of the fine structure of the basal zone of the dauer larva of the nematode *Caenorhabditis elegans*. *Can. J. Zool.* 57, 794–800.
- Reboul, J. *et al.* (2003). *C. elegans* ORFeome version 1.1, experimental verification of the genome annotation and resource for proteome-scale protein expression. *Nat. Genet.* 34, 35–41.
- Reddien, P. W., and Horvitz, H. R. (2000). CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in *Caenorhabditis elegans*. *Nat. Cell Biol.* 2, 131–136.
- Ren, X. D., and Schwartz, M. A. (1998). Regulation of inositol lipid kinases by Rho and Rac. *Curr. Opin. Genet. Dev.* 8, 63–67.
- Ridley, A. J. (2001). Rho family proteins: coordinating cell responses. *Trends Cell Biol.* 11, 471–477.
- Rivero, F., Dislich, H., Glockner, G., and Noegel, A. A. (2001). The *Dictyostelium discoideum* family of Rho-related proteins. *Nucleic Acids Res.* 29, 1068–1079.
- Self, A. J., and Hall, A. (1995). Purification of recombinant Rho/Rac/G25K from *Escherichia coli*. *Methods Enzymol.* 256, 3–10.
- Spencer, A. G., Orita, S., Malone, C. J., and Han, M. (2001). A RHO GTPase-mediated pathway is required during P cell migration in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* 98, 13132–13137.
- Sprang, S. R. (1997). G protein mechanisms: insights from structural analysis. *Annu. Rev. Biochem.* 66, 639–678.
- Symons, M., and Rusk, N. (2003). Control of vesicular trafficking by rho GTPases. *Curr. Biol.* 13, R409–R418.
- Van IJzendoorn, S. C., and Hoekstra, D. (1998). (Glyco)sphingolipids are sorted in sub-apical compartments in HepG2 cells: a role for non-Golgi-related intracellular sites in the polarized distribution of (glyco)sphingolipids. *J. Cell Biol.* 142, 683–696.
- Van IJzendoorn, S. C., Maier, O., Van Der Wouden, J. M., and Hoekstra, D. (2000). The subapical compartment and its role in intracellular trafficking and cell polarity. *J. Cell. Physiol.* 184, 151–160.
- Wennerberg, K., and Der, C. J. (2004). Rho-family GTPases: it's not only Rac and Rho (and I like it). *J. Cell Sci.* 117, 1301–1312.
- Wherlock, M., and Mellor, H. (2002). The Rho GTPase family: a Rac to Wrchs story. *J. Cell Sci.* 115, 239–240.
- Wu, Y. C., Cheng, T. W., Lee, M. C., and Weng, N. Y. (2002). Distinct Rac activation pathways control *Caenorhabditis elegans* cell migration and axon outgrowth. *Dev. Biol.* 250, 145–155.
- Yang, W., Lin, Q., Zhao, J., Guan, J. L., and Cerione, R. A. (2001). The nonreceptor tyrosine kinase ACK2, a specific target for Cdc42 and a negative regulator of cell growth and focal adhesion complexes. *J. Biol. Chem.* 276, 43987–43993.
- Yochem, J., Tuck, S., Greenwald, I., and Han, M. (1999). A gp330/megalin-related protein is required in the major epidermis of *Caenorhabditis elegans* for completion of molting. *Development* 126, 597–606.
- Zipkin, I. D., Kindt, R. M., and Kenyon, C. J. (1997). Role of a new Rho family member in cell migration and axon guidance in *C. elegans*. *Cell* 90, 883–894.