Rab4 and Rab11 are small GTPases belonging to the Ras superfamily. They both function as regulators along the receptor recycling pathway. We have identified a novel 80-kDa protein that interacts specifically with the GTP-bound conformation of Rab4, and subsequent work has shown that it also interacts strongly with Rab11. We name this protein Rab coupling protein (RCP). RCP is predominantly membrane-bound and is expressed in all cell lines and tissues tested. It colocalizes with early endosomal markers including Rab4 and Rab11 as well as with the transferrin receptor. Overexpression of the carboxyl-terminal region of RCP, which contains the Rab4- and Rab11-interacting domain, results in a dramatic tubulation of the transferrin compartment. Furthermore, expression of this mutant causes a significant reduction in endosomal recycling without affecting ligand uptake or degradation in quantitative assays. RCP is a homologue of Rip11 and therefore belongs to the recently described Rab11-FIP family.

The large number of Rab proteins identified in mammalian cell lines and their localization to specific compartments within the cell suggest that they each play a crucial and specific role in the transport process. Rabs cycle between a membrane-bound state and a cytosolic state. This cycling is guanine nucleotide-dependent and is regulated by various accessory factors. Several Rab proteins including Rab4, -5, -7, -9, and -11 are known to be involved in the process of endocytosis (1). Rab5 is important in the homotypic fusion between early endosomes as well as in the transport to the early endosomal compartment (2, 3). Additionally, it seems to be necessary for the budding of vesicles from the plasma membrane (4). Rab4 has been localized to the early endosomal compartment and affects recycling (5, 6) as well as trafficking to the late endosomal compartment (7). Considering the localization and functional effects of Rab4, it is likely that it controls traffic through/from the early sorting endosomes. Rab11 has been localized to the recycling compartment (8) and to trans-Golgi network (TGN) membranes (9) and controls endosomal recycling (8) as well as traffic to the Golgi apparatus (10). Rab4 and Rab11 have been shown by multicolor imaging (11) and by biochemical experimentation (12) to overlap in their localization along the endocytic pathway. Rab7 and -9 are localized to the late endosomal compartment (13, 14) and control late endocytic trafficking (15–18) and trafficking from late endosomes to the trans-Golgi Network (14), respectively.

An essential task in elucidating the function of Rab proteins is to identify their interacting proteins. Several such proteins have already been identified, including rabaptin 5, a 100-kDa protein with two pairs of coiled-coil regions, which was initially found in a yeast two-hybrid screen to identify effectors of Rab5 (19) and has subsequently been shown to also interact with Rab4 (20). Several other Rab4-interacting proteins have also been identified, including rabaptin 4 (21), syntaxin 4 (22), Rab4-interacting protein (Rab4ip) (23), and the dynein light intermediate chain 1 (dynein LIC-1) (24). Rabphilin 41 (Rab11BP) (25, 26), Rip11 (27), and myosin Vb (28), and the Rab11-FIP family have been identified as interacting targets of Rab11 (29, 30).

To further understand the function of Rab4, a yeast two-hybrid screen was performed to identify proteins that interact with the GTP-bound form of Rab4. Here, we report the identification of a novel, ~80-kDa protein that interacts with Rab4 and Rab11. We localize the endogenous protein to the early endosomal recycling compartment (ERC) and show that overexpression of a deletion mutant of this protein perturbs the morphology of this compartment and strongly inhibits endosomal recycling. Because of its interaction with two Rabs that function in endosomal recycling, its localization to the early endosomal recycling compartment, and its functional effects on the recycling process, we propose to name this the Rab coupling protein (RCP). Interestingly, this protein is related to the Rab11-interacting protein (Rip11) (27) and, therefore, belongs to the recently reported Rab11-FIP protein family (29, 30).

**MATERIALS AND METHODS**

**cDNA Cloning and Plasmid Construction—**The Rab4 two-hybrid constructs have been described previously (24). pLex-Rab5, pLex-Rab11BP, pLex-Rab11BP (25, 26), Rip11/pp75 (27), myosin Vb (28), and the Rab11-FIP family have been identified as interacting targets of Rab11 (29, 30).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF368294.

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1 The abbreviations used are: TGN, trans-Golgi network; ERC, endosomal recycling compartment; Rab, Rab coupling protein; BHK cells, baby hamster kidney cells; WT, wild type; CHAPS, sodium cholate; FITC, fluorescein isothiocyanate; Tfn, transferrin; TfnR, Tfn receptor; EEA1, endosome-associated antigen 1.

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Rab7Q67L, and pLex-Rab534A. These were gifts from M. Zerial (19), and pLex-rasG12V and pLex-A-laminC were from A. Vojeck (37). pLex-Rab6 constructs have been previously described (38), and pLex-Rab11 constructs were made by subcloning the canine Rab11 WT, S25N, and Q70L cDNA as an 855-bp fragment from the previously described pGEMRab11 construct (50). pLex-Rab11Q70L/144E was generated by two-step site-directed mutagenesis as follows. pLex-Rab11Q70L was used as the template in the first step with SeqF (5′-CTTCTGTCAGCAGAGCTCTC-3′) and the 144E primer (5′-TGCAAATTCT-TACTCCCTCGGTGCTCCT-3′). The first-round product was used as the primer in a second-step PCR with the BonR reverse primer (5′-AATTCCGGCCGGAATTC-3′). The resulting second-round PCR product was subcloned into pVJL10 (pLex). pTrcHisC-H13 and pcDNA3.1/HisB-H13 were obtained by subcloning the 1-kilobase H13 cDNA fragment from pGADGH into the EcoRI site of pTrcHisC (Invitrogen) and pcDNA3.1/HisB (Invitrogen), respectively. pTrcHisA-Rab4Q67L was generated by subcloning the BamHI fragment from pBlueBacHis-Rab4Q67L (Invitrogen) into the BamHI site of pTrcHisA (Invitrogen). ΔCIP was amplified from a plasmid cDNA library (MARATHON, CLONTECH) using the upstream primer, GSP1 (5′-CGGATCCGACAGAGGCTATGATAAG-3′), and downstream primer, GSP6 (5′-GGATCCGACAGGATCTAAGCAGAGATT-3′). The product was digested with BamHI and subcloned into pcDNA3.1/HisB, pGADGH, and pGEX6P-1 (Amersham Biosciences, Inc.). A 1-kilobase EcoRI restriction fragment that contained the full RCP open reading frame was subcloned into the pGEMI vector. All constructs were verified by double strand sequencing using the dyeoxynucleotide chain termination method (39).

Two-hybrid Screening—The yeast two-hybrid screen was performed as previously described (40). Briefly, the yeast reporter strain L40 (MATa trpl1 leu2 his3A-lexA-His3 URA3/exa-la2X) was transformed with pVJL10-Rab4Q67L-CXC and grown on synthetic medium lacking tryptophan. This transformant was then transformed with an oligo-dT-primed HeLa cDNA library (MATCHMAKER, CLONTECH) using the upstream primer, GSP1 (5′-CGGATCCGACAGAGGCTATGATAAG-3′), and downstream primer, GSP6 (5′-GGATCCGACAGGATCTAAGCAGAGATT-3′). The product was digested with BamHI and subcloned into pcDNA3.1/HisB, pGADGH, and pGEX6P-1 (Amersham Biosciences, Inc.). A 1-kilobase EcoRI restriction fragment that contained the full RCP open reading frame was subcloned into the pGEMI vector. All constructs were verified by double strand sequencing using the dyeoxynucleotide chain termination method (39).

Gene Expression Panel—1 μl of first strand cDNA from each tissue of a Rapid Scan Gene Expression Panel (Origene) was used as template in a 40-cycle PCR reaction with the RCP gene-specific primers GSP-1 (5′-GAAAATTTGGGAGTCCGCGGTCGTTCA-3′) and GSP-2 (5′-GCTTCCAGGACTCCCTTTGA-3′). The primers were designed to amplify the region of RCP corresponding to amino acids 384–529, yielding a product of 435 base pairs. An annealing temperature of 60 °C and an elongation time of 1 min were used. Clone H13 cDNA was used as template in PCR reactions with first-round PCR reactions. Clone H13 cDNA was used as template in the positive control reaction, and the negative control contained 1 μl of distilled H2O instead of template.

Recombinant Proteins—The H13 polypeptide and Rab4Q67L protein were purified as follows. BL21 (DE3) E. coli cells were transformed with pTrcHisC-H13 and pGEX4D-1. Transformants were grown for 8 h in medium lacking saponin and incubated with the primary antibodies diluted in 5% fetal bovine serum, phosphate-buffered saline. The secondary antibodies were verified by double strand sequencing using the dyeoxynucleotide chain termination method. The yeast two-hybrid screen was performed as previously described (40). Briefly, the yeast reporter strain L40 (MATa trpl1 leu2 his3A-lexA-His3 URA3/exa-la2X) was transformed with pVJL10-Rab4Q67L-CXC and grown on synthetic medium lacking tryptophan. This transformant was then transformed with an oligo-dT-primed HeLa cDNA library (MATCHMAKER, CLONTECH) using the upstream primer, GSP1 (5′-CGGATCCGACAGAGGCTATGATAAG-3′), and downstream primer, GSP6 (5′-GGATCCGACAGGATCTAAGCAGAGATT-3′). The product was digested with BamHI and subcloned into pcDNA3.1/HisB, pGADGH, and pGEX6P-1 (Amersham Biosciences, Inc.). A 1-kilobase EcoRI restriction fragment that contained the full RCP open reading frame was subcloned into the pGEMI vector. All constructs were verified by double strand sequencing using the dyeoxynucleotide chain termination method (39).

Antibodies—Antibodies used included mouse monoclonal β-Actin (Sigma), monoclonal anti-transferrin receptor antibody (TfnR) (B3/25, Boehringer Mannheim), sheep anti-TGN46 (Serotec), and anti-Xpress (Invitrogen). Anti-Xpress was used to detect H13 and ΔCIP expressed as fusion proteins in the pcDNA3/HisB vector. Monoclonal anti-lysozyme-phosphatidic acid was a kind gift from J. Gruenberg. The affinity-purified polyclonal Rab11 antibody has been described elsewhere (10). Anti-H13 rabbit affinity-purified antibody was generated as follows. Serum from a H13-inoculated rabbit was affinity-purified on nitrocellulose (Schleicher & Schuell) and then exposed on a PhosphorImager (Molecular Dynamics).

Cell Lines and Transfection—HeLa and HeLa Rab4GFP cells were maintained in culture as previously described (24). BHK cells were cultured in Glasgow’s minimal essential medium supplemented with 10% tryptophosphate broth, 5% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 μM L-glutamine. For overexpression studies, HeLa cells were infected for 45 min with either the attenuated MVA T7 RNA polymerase recombinant vaccinia virus (a kind gift from G. Sutter) or the V77 vaccinia virus and then transfected with the appropriate plasmids using either DOPSER or DOTAP (Roche Molecular Biochemicals) according to the manufacturer’s instructions. For colocalization with Rab11, ΔCIP was transfected into HeLa cells using the Effectene transfection reagent (Qiagen) according to the manufacturer’s instructions. Six hours after the addition of the DNA/Effectene complex, the cells were fixed and processed for immunofluorescence microscopy. For simultaneous staining of Rab4 and Rab11, ΔCIP was transfected into HeLa cells using the Effectene transfection reagent (Qiagen) according to the manufacturer’s instructions. Six hours after the addition of the DNA/Effectene complex, the cells were fixed and processed for immunofluorescence microscopy.
used were donkey anti-mouse conjugated to fluorescein isothiocyanate (FITC) and donkey anti-rabbit conjugated to Texas Red or donkey anti-rabbit conjugated to 9-aminocarbonyl-2-methoxycarbonyl (Jackson Immunoresearch). The coverslips were mounted onto slides with Mowiol (Sigma). Immunofluorescence images were recorded on a Leica TCS confocal microscope as previously described (42) or by conventional epifluorescence microscopy using a NIKON E-600 microscope fitted with a Hamamatsu chilled CCD C5985 camera. All images were processed using Adobe Photoshop and Adobe Illustrator software.

**Ligand Internalization**—Six hours post-transfection, serum-starved cells grown on glass coverslips were allowed to internalize FITC-coupled iron-saturated holotransferrin (33 μg/ml; Sigma) for 1 h at 37°C. The cells were then briefly rinsed with ice-cold phosphate-buffered saline and fixed.

Subcellular Fractionation and Membrane Washing—HeLa cells were resuspended in 250 mM sucrose, 90 mM KCl plus protease inhibitors, buffered saline and fixed.

**RESULTS**

**Identification of Full-length Rab Coupling Protein**—Clone H13 contains the full-length human RCP, and we report here its localization (11, 12) and function sequentially (5–8) in the regulation of receptor recycling.

Furthermore, in a previously reported independent Rab4 two-hybrid screen (24), we isolated a mouse clone named M25 that comprises the extreme carboxy-terminal 65 amino acids of H13 and retains the ability to interact with Rab4 and Rab11.2 Thus, we have been able to localize the Rab4/Rab11-interacting motif of this polypeptide to 65 amino acids.

**Localization of Rab4-interacting Clones Using the Yeast Two-hybrid System**—We utilized the yeast two-hybrid technique to identify proteins that interact with Rab4. A GTPase-deficient, therefore constitutively active, mutant of Rab4 (Rab4Q67L) was used as “bait” to screen an oligo-dT-primed HeLa cDNA library. Approximately $3 \times 10^6$ transformants were screened, of which 44 grew on selective medium lacking histidine and activated the $\beta$-galactosidase reporter gene. Fourteen of these displayed a specific, nucleotide-dependent interaction with Rab4. By hybridization and sequencing analysis, five of the clones were determined to be rabaptin 5, a Rab5 effector that also interacts with Rab4 (20). Four clones, represented by clone H13, expressed an identical 1.9-kilobase cDNA. The remaining five clones from the screen have yet to be characterized. Clone H13 was chosen for this study, and we demonstrate here that it interacts strongly with Rab4Q67L and Rab4WT, but weakly with the GDP-locked mutant (Rab4S22N) (Fig. 1). No interaction was observed with a mutant that is GTP-locked but has an amino acid change in the putative effector domain (Rab4Q67L/I41E), suggesting that the interaction is mediated through this domain. No reporter gene activation was observed with the GTPase-deficient forms of Rab3, Rab5, Rab6, or Rab7 (Fig. 1B).

Interestingly, there was also a strong interaction with all Rab11 constructs (Fig. 1). These results demonstrate that clone H13 encodes a polypeptide that interacts with two early endosomal Rab GTPases that have been shown to overlap in their regulation of receptor recycling.

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RCP Interacts with Two Early Endosomal Rabs

FIG. 2—continued

C

D

CC-1

CC-2

H13

85

379

δRC

649

M25

649

649

649

FIG. 2—continued
RCP Interacts with Two Early Endosomal Rabbs

Fig. 3. RCP is a ubiquitously expressed 80-kDa protein. A, a cDNA expression panel (Origene) was used as template in PCR reactions with two internal RCP gene-specific primers. I, no template (negative control); 2, brain; 3, heart; 4, testis; 5, lung; 6, spleen; 7, ovary; 8, small intestine; 9, liver; 10, H13 (positive control). B, postnuclear supernatants (100 μg) of the following human cell lines were analyzed by SDS-PAGE and immunoblotting with anti-H13 antibody. BeWo, placental trophoblastic choriocarcinoma; HL60, promyelocytic monocyte; A431, epidermoid carcinoma; HeLa, cervical carcinoma. A single 80-kDa band was seen in all of the cell lysates.

Fig. 4. RCP is a predominantly membrane-bound protein. A, total cell extract, postnuclear supernatant (PNS), high speed supernatant (Cytosol), and high speed pellet (Membranes) were prepared from HeLa cells and analyzed by SDS-PAGE and immunoblotting with anti-H13 and anti-β-actin. B, HeLa postnuclear supernatant was treated on ice for 90 min under the conditions indicated before centrifugation. RCP was identified in the 100,000 × g pellet (P) or supernatant (S). The accuracy of the fractionation was controlled by probing with anti-TfnR and anti-β-actin. C, H13 polypeptide expressed in E. coli BL21 cells was resolved on a polyacrylamide gel and transferred to nitrocellulose (in triplicate). After renaturation, the blots were incubated with 10 μg of [α-32P]GTP-labeled Rab5WT or Rab4WT or Rab11WT. The blots were washed several times and exposed on a PhosphorImager.

 Trafficking via the apical recycling compartment (27). The remaining three members (FIP1, FIP2/nRip11, and FIP3/Eferin) of the family have been reported as Rab11/Rab25-interacting proteins localizing to the endosomal recycling compartment in polarized and non-polarized cells. However, while these proteins share a number of similar features, RCP is the only member reported to interact with Rab4. RCP is therefore a new member of a family of proteins that interact with Rab11/Rab25 and/or Rab4 and function along the endosomal recycling pathway.

RCP Is a Widely Expressed, Predominantly Membrane-bound Protein—We investigated the expression pattern of RCP by a PCR approach utilizing, as template, the first strand cDNA generated from a panel of human tissues and RCP gene-specific primers to amplify a product of known size (435 base pairs) corresponding to RCP amino acids 384–529. Similar amounts of the expected 435-base pair product were generated from all eight human tissues tested (Fig. 3A), suggesting the presence of equivalent amounts of RCP mRNA in these tissues. To confirm PCR specificity, we included reactions for a positive control (clone H13 cDNA as template) in addition to a negative (no template) control.

To study the biological function and intracellular localization of RCP, antisera was raised against bacterially expressed and purified H13 polypeptide (H13). Affinity-purified antisera identified a protein migrating with an apparent molecular mass of ~80 kDa (Fig. 3B) in all cell lines tested. The RCP full-length open reading frame was subcloned into a mammalian expression vector and expressed in HeLa cells. The molecular weight of the recombinant protein corresponded to that of endogenous RCP (data not shown).

Fractionation of HeLa cells into total cellular lysate, postnuclear supernatant, high speed pellet (membrane fraction), and high speed supernatant (cytosol fraction) demonstrated that RCP is a predominantly membrane-associated protein (Fig. 4A). We investigated the stability of RCP membrane association by detergent, high salt, and high pH treatments. RCP was totally membrane-extracted by 1% Triton X-100, whereas it was partially extracted by either 1 M NaCl or 0.1 M Na2CO3 (pH 11) (Fig. 4B). The localization of the TfnR and β-actin was monitored as fractionation controls.

Taken together, the data described above indicate that the expression levels of RCP, when tested both by reverse transcriptase-PCR and immunoblotting, appeared to be relatively similar in all cell types tested. Furthermore, RCP encodes an 80-kDa protein that is predominantly membrane-associated and is partially resistant to high salt and high pH treatments but is completely solubilized from membranes by Triton X-100.

The Carboxy Terminus of RCP Binds Directly to Rab4 and Rab11 in Vitro—The findings of the yeast two-hybrid system that H13 interacts with Rab4 and Rab11 were confirmed biochemically. H13, expressed in E. coli, was resolved by SDS-
RCP Interacts with Two Early Endosomal Rabs

Not with Markers of the Degradative Pathway

To further understand the biological function of RCP, we examined the localization of endogenous RCP in HeLa cells by immunofluorescence. It displays a punctate vesicular pattern in the cytoplasm that colocalizes partially with the TfnR in the perinuclear region of the cell (Fig. 5A). Since the available Rab4 antibodies do not detect endogenous Rab4 by immunofluorescence, we compared endogenous RCP localization with Rab4, utilizing a previously described stable GFP-Rab4 HeLa cell line (24). Endogenous RCP again partially colocalized with GFP-Rab4 in the perinuclear area (Fig. 5B). To investigate colocalization with Rab11, endogenous Rab11 was visualized with an affinity-purified rabbit antibody. Since the RCP antibody is also rabbit, HeLa cells were transfected with an epitope-tagged RCP construct (ΔRCP) (see Fig. 2D). ΔRCP was visualized with a mouse monoclonal antibody directed against the epitope. The recombinant protein was expressed at a low level such that its pattern closely resembled the endogenous RCP pattern. We observed extensive colocalization with Rab11. Furthermore, when ΔRCP is expressed, Rab11 is more distinctly membrane-associated, appearing to be either recruited or retained on the ΔRCP-positive structures (Fig. 5C). RCP does not colocalize with lysobisphosphatidic acid, a phospholipid found on late endosomes and, thus, a marker of the degradative pathway (32, 33) (Fig. 5D). We also compared the localization of transiently overexpressed ΔRCP with endogenous early endosome-associated antigen 1 (EEA1) by immunofluorescence and find that these two markers display minor co-localization (data not shown). EEA1 is a Rab5 effector protein that appears to poorly localize to the perinuclear recycling compartment (11, 12, 34). Since RCP and EEA1 display very little co-localization, it is likely that RCP distribution is to a post-EEA1/Rab5, ERC location.

Overexpression of H13 Tubulates the Transferrin Compartment—To determine any morphological effects of H13 on the early endosomal compartment, we transiently transfected HeLa cells with a plasmid that expresses this truncated RCP polypeptide. After transfection, the cells were allowed to continuously internalize FITC-labeled Tfn at 37 °C. As expected, in the mock transfected (control) cells, the FITC-Tfn accumulated in distinct endosomal structures in the perinuclear area of the cytoplasm as well as to some more peripheral vesicles (Fig. 6A). In cells expressing H13, the morphology of the Tfn compartment changed dramatically, displaying FITC-Tfn-labeled membrane tubular structures that extend throughout the cytoplasm (Fig. 6B). In transfected cells, which display this morphology but express H13 at a lower level, it is possible to visualize H13 on these transferrin-positive membranes (Fig. 6C). Since H13 is likely to be nonfunctional, it is probably acting in a dominant-negative manner with respect to Rab4 and/or Rab11 by sequestering them in a nonfunctional complex, thus preventing their interaction with endogenous RCP. It is interesting to note that the abnormal Tfn compartment generated on H13 expression is similar to that observed when the dominant-negative Rab11S25N mutant is expressed (10).

To investigate the identity of the tubulated Tfn compartment, we examined the structures labeled by FITC-Tfn after internalization at 18 °C for 45 min, or uptake at 18 °C followed by a chase at 37 °C for 10 min. A temperature block of 18 °C permits endosomal internalization but inhibits passage from early/sorting endosomes. When H13-expressing cells were incubated at 18 °C, only vesicular structures were labeled by FITC-Tfn (Fig. 7A). However, when the temperature block was lifted, we observed FITC-Tfn labeling a tubulated compartment (Fig. 7B). Since the H13-induced tubules are inaccessible to FITC-Tfn at 18 °C, it is likely that these tubules represent an abnormal endosomal recycling compartment.

Since Rab11 also regulates transport to the TGN, we investigated the localization of TGN46 in cells expressing either H13 or Rab11S25N. TGN46 is a glycoprotein that cycles between the plasma membrane and the TGN. As previously reported (10), we observe a proportion of TGN46 localizing to the Rab11S25N-generated tubules. H13-induced tubules similarly display TGN46 on the transferrin receptor positive tubules (data not shown).
Increased Rab11 Function Reverses H13 Tubulation of the Tfn Compartment—As already indicated, H13 expression produces an early endosomal phenotype reminiscent of that generated by Rab11GDP (10). With this in mind, we were interested in determining whether the H13-induced tubulation of the Tfn compartment could be reversed by excess wild-type or dominant-positive Rab11. We examined this by co-transfecting clone H13 with either Rab11WT or Rab11Q70L and allowing cells to internalize FITC-Tfn. In cells that co-overexpressed H13 and active Rab11, the Tfn compartment phenotype was reversed (Fig. 8). This is consistent with the proposal that H13 sequesters endogenous Rab11 in a nonfunctional complex and the addition of active Rab11 bypasses this block. We also investigated the effect of H13 co-expression with Rab4 WT or Rab4Q67L. As previously reported, increased Rab4 function leads to tubulation of the Tfn compartment (7). Thus, as expected, co-expression of H13 with Rab4 WT or Q67L also resulted in tubulation of the Tfn compartment (data not shown).

Overexpression of H13 Inhibits Recycling—To investigate the effect that H13 or full-length RCP has on the kinetics of the receptor recycling pathway, the trafficking of 125I-Tfn was analyzed. BHK cells cotransfected with H13 and active Rab11, the Tfn compartment phenotype was reversed (Fig. 8). This is consistent with the proposal that H13 sequesters endogenous Rab11 in a nonfunctional complex and the addition of active Rab11 bypasses this block. We also investigated the effect of H13 co-expression with Rab4 WT or Rab4Q67L. As previously reported, increased Rab4 function leads to tubulation of the Tfn compartment (7). Thus, as expected, co-expression of H13 with Rab4 WT or Q67L also resulted in tubulation of the Tfn compartment (data not shown).

Overexpression of H13 Inhibits Recycling—To investigate the effect that H13 or full-length RCP has on the kinetics of the receptor recycling pathway, the trafficking of 125I-Tfn was analyzed. BHK cells cotransfected with H13 or full-length RCP and the human TfnR were incubated with 125I-Tfn on ice. Excess 125I-Tfn was washed off, and the cells were allowed to internalize and recycle the ligand at 37 °C. The amount of 125I-Tfn in the culture medium, and therefore recycled, was measured at different time points (Fig. 9A) and compared in each case to the amount of recycled Tfn in control cells (cells transfected with the human TfnR alone). Overexpression of H13 resulted in a dramatic inhibition in the rate of recycling after ~10 min. In contrast, full-length RCP slightly stimulated recycling when compared with the control. Dominant-negative

**FIG. 6.** Overexpression of clone H13 causes a perturbation of the transferrin compartment. HeLa cells were infected with the modified vaccinia virus and transfected with either pcDNA3.1/HisB-H13 or mock-transfected with the empty pcDNA3.1/HisB empty vector for 6 h. FITC-Tfn (green) was then continuously internalized at 37 °C for 45 min before fixation. Cells overexpressing H13 were identified with anti-H13 (red). A, mock-transfected cells displayed a vesicular FITC-Tfn pattern with a concentration in the perinuclear region. B, cells expressing H13 displayed a striking FITC-Tfn-positive tubular network. C, some cells that displayed the H13 tubular phenotype expressed the recombinant protein at a lower level such that it was possible to see it localizing to the FITC-Tfn-positive tubules.

**FIG. 7.** The H13-induced tubules are derived from the recycling compartment. HeLa cells were transfected with pcDNA3.1/HisB-H13. Six hours after transfection FITC-Tfn was internalized at 18 °C for 45 min. The cells were either washed with ice-cold phosphate-buffered saline and fixed immediately or chased at 37 °C for 10 min before fixation. A, cells fixed after the 18 °C uptake, FITC-Tfn displayed a vesicular pattern, with no tubules labeled. B, in cells that were chased at 37 °C for 10 min, the FITC-Tfn was able to access the H13-induced tubules.

**FIG. 8.** Overexpression of active Rab11 rescues HeLa cells from the H13 phenotype. HeLa cells were cotransfected with pcDNA3.1/HisB-H13 and either Rab11WT, Rab11Q70L, Rab11S25N, or the pGEM1 empty vector. FITC-Tfn (green) was continuously internalized at 37 °C for 45 min before fixation. Overexpressed H13 was detected with anti-H13 (red), and overexpressed Rab11 was detected with anti-Rab11 (cyan). A, H13-induced FITC-Tfn tubules were observed in the mock-transfected cells. B, co-transfection of Rab11WT rescued the H13 phenotype. FITC-Tfn was seen in vesicular structures, similar to the pattern observed in non-transfected cells. C, Rab11Q70L also had the ability to rescue the H13 phenotype. D, dominant-negative Rab11, Rab11S25N, as expected did not rescue the phenotype.
RCP Interacts with Two Early Endosomal Rabs

**FIG. 9.** H13 inhibits $^{125}$I-Tfn recycling. BHK cells were co-transfected with the human transferrin receptor and either H13 (○), full-length RCP (○), Rab4S22N (●), or Rab11S25N (□). Control cells (■) were transfected with the human TfnR alone. A, $^{125}$I-Tfn was bound to the cell surface on ice for 1 h, excess ligand was washed off, and then the cells were allowed to internalize the $^{125}$I-Tfn at 37°C. The amount of recycled $^{125}$I-Tfn in the culture medium, expressed as a percentage of the total $^{125}$I-Tfn, was measured at the indicated time points. B, to analyze the kinetics of recycling specifically from the ERC, the transfected BHK cells were pulsed with $^{125}$I-Tfn for 5 min at 37°C, excess ligand was removed, and the cells were chased for a further 30 min at 37°C. The amount of $^{125}$I-Tfn in the culture medium was then measured at the indicated times after the chase and expressed as a percentage of the total Tfn (i.e., cell associated Tfn and Tfn in the culture medium). For both assays, the effects on recycling were compared with the control cells. The data represent the average of a minimum of three experiments (in triplicate) with S.E.
Rab4, Rab4S22N, also inhibited recycling but to a lesser extent than H13.

To specifically examine the effects on recycling from the ERC, the assay was modified to allow the kinetics of the “slow/long” pathway to be analyzed (8). BHK cells transfected with the human transferrin receptor and the indicated constructs were pulsed with 125I-Tfn for 5 min, excess ligand was washed off, and the cells were chased for a further 30 min at 37 °C. This allows the Tfn following the “fast/short” pathway to cycle out of the cell and the Tfn trafficking along the slow pathway to load the ERC. After the chase, the amount of 125I-Tfn in the culture medium was measured after various time points. Overexpression of H13 resulted in a strong inhibition of recycling from the ERC, whereas full-length RCP showed a slight stimulation of recycling (Fig. 9B). Rab11S25N also inhibited recycling, as previously reported (8), but to a lesser extent than H13.

Furthermore, because Rab4 affects late endosomal trafficking (7) as well as recycling (5), we investigated whether RCP may also play a role in regulating transport along the degradative pathway. Overexpression of H13 did not affect the uptake or degradation of 125I-EGF but inhibited EGF recycling (data not shown). Taken together, the data from the quantitative assays demonstrate that RCP functions in endosomal recycling, most likely through the ERC.

DISCUSSION

Rab proteins serve a critical role in the regulation of intracellular transport events. With the deposition of the human genome sequence, it is now known that there are 60 different human Rab proteins (35), and it is expected that each member controls a specific event in a transport pathway. The receptor recycling pathway is under the control of several Rabs, including Rab5, Rab4, and Rab11, which function in a sequential fashion. The current view proposes that Rab5 controls transport from the plasma membrane and early endosomal fusion, Rab4 regulates transport events from the sorting endosomes, and Rab11 regulates transport through the perinuclear recycling compartment.

Here, we report the identity and characterization of Rab coupling protein, a novel ~80-kDa membrane-associated protein that interacts with both Rab4 and Rab11. We demonstrate that the carboxy-terminal 65 amino acids of RCP are sufficient for Rab4 and Rab11 binding. The RCP/Rab4 interaction is strongly nucleotide-dependent, with RCP interacting preferentially with the GTP bound form of Rab4. Furthermore, the interaction with Rab4 is abolished when the GTP-locked form carries a mutation in its putative effector domain. The situation with Rab11 appears somewhat different, in that the RCP/Rab11 interaction appears stronger (two-hybrid and transient expression/immunofluorescence data) than that of RCP with Rab4. However, the RCP/Rab11 interaction is less dependent on the Rab11 nucleotide-bound state. RCP is homologous to pp75, a phosphoprotein that has been implicated in neonatal and systemic lupus erythematosus (36). While our work was in progress, a study from Scheller and co-workers (27) reported pp75 as a Rab11-interacting protein (Rip11) that functions in the regulation of apical membrane trafficking. More recently, work from two groups has described other members of this family of Rab-interacting proteins under various names, Eferin or Rip proteins (30) and Rab11-family interacting proteins (Rab11-FIPs 1–3) (29). RCP is distinct from, but related to, these recently described Rab11/Rab25-interacting proteins and is therefore a new member of this novel Rab-interacting protein family.

Our immunofluorescence data indicates that RCP colocalizes with a small pool of Rab4, whereas there is almost complete colocalization with Rab11. Additionally, there is only minor colocalization with EEA1 (data not shown). Altogether, this suggests that RCP is predominantly an ERC protein and, therefore, is likely to interact with the pool of Rab4 that is destined for transport to this compartment. Furthermore, overexpression of H13 (a truncated form of RCP containing the Rab binding domain) results in the formation of an extensive tubular network that can be labeled with FITC-Tfn. These tubules appear to be derived from the recycling compartment since they are inaccessible to Tfn internalized at 18 °C. This phenotype is very similar to that seen upon overexpression of the dominant-negative mutant of Rab11, Rab11S25N (10). Because, as indicated earlier, the interaction between RCP and Rab11 appears stronger than the RCP/Rab4 interaction, it is likely that excess H13 will generate a Rab11 dominant-negative phenotype by preferentially sequestering endogenous Rab11. Consistent with this view, expression of active Rab11 rescues the H13 phenotype. These data support our view that the H13-induced tubulation is generated as a result of Rab11 being sequestered in a non-functional complex.

RCP is the second example of a putative effector protein that interacts with more than one RabGTPase subfamily. Rabaptin 5 interacts with both Rab5 and Rab4 (20). Since Rab4 functions after Rab5 in early endosomal trafficking, it is suspected that rabaptin 5 acts as an intermediate between these two GTPase proteins. Likewise, it is probable that RCP is an intermediate between Rab4 and Rab11, since Rab11 acts after Rab4 in the recycling pathway. Rab11 appears to function exclusively on trafficking through the perinuclear ERC. Rab4, on the other hand, functions primarily at the level of the sorting endosome and affects both recycling and degradation (7), probably by interacting with different sets of effectors. A number of putative Rab4 effectors have already been reported such as rabaptin 5 (20), rabaptin 4 (21), syntaxin 4 (22), Rab4-interacting protein (Rab4ip) (23), and the dynine light intermediate chain-1 (dynine LIC-1) (24).

Our quantitative data demonstrates that RCP functions in the endosomal recycling process and is not involved in the late endosomal pathway. Overexpression of the carboxy-terminal region of RCP, which contains the Rab4 and Rab11 binding sites, results in a dominant-negative effect on Tfn-recycling kinetics. Analysis reveals that this inhibition begins after 10 min of ligand internalization. Further analysis that examined the rate of recycling specifically from the ERC revealed that the RCP dominant-negative mutant inhibited recycling from this compartment. This inhibition was stronger than that observed with the dominant-negative Rab mutants. This may be due to the cumulative effect of the sequestration of Rab4 and Rab11 in an inactive complex and/or the probability that the RCP mutant also sequesters other members of the Rab11 family.

RCP, as a predominantly membrane-associated protein, may be involved in the targeting of a Rab4- or Rab11-mediated complex to the correct compartment. Considering the localization of RCP, which appears predominantly on the Rab11-positive perinuclear ERC, we suggest that it may serve as a target for Rab4 donor vesicles arriving at the acceptor ERC membrane. Another possibility is that RCP interacts with Rab4 at a sorting endosome location and participates in Rab4-mediated trafficking to, or generation of, the ERC. RCP may then serve to recruit Rab11 onto the ERC membrane or, alternatively, interact with Rab11 already associated with the ERC membrane. In fact, when H13 or S6RCP are overexpressed, the membrane localization of Rab11 increases consistent with either of these possibilities. Further studies will be necessary to clarify these issues. It will also be important to investigate the precise events leading to RCP membrane localization after its biosynthesis as well as whether it subsequently cycles on/off mem-
branes. Pertinent to this will be an investigation of whether Rabs are involved in this process and the role of the RCP C2 domain in lipid binding.

The identification of RCP as an interacting partner of Rab4 and Rab11, with many analogies to its related proteins, pp75/Rip11 and the Rab-FIPs (27, 29, 30), contributes further to our understanding of the eukaryotic membrane-trafficking machinery. Furthermore, the similarity between the Rab5/rabaptin 5/Rab4 molecular network and the Rab4/RCP/Rab11 network serve to link-up or couple the endosomal-trafficking machinery, involving several RabGTPases sequentially linking endosomal sub-domains/sub-compartments, both in terms of their overlapping distribution as well as by their interaction with common sub-groups of effectors. Future studies to decipher the molecular details of such interactions and to understand the precise molecular functions of this novel Rab-interacting protein family will be crucial toward our better understanding of the fundamental cellular process of endosomal sorting, recycling, and transcytosis with respect to the events controlled by the Rab GTPases.

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