Rab15 Effector Protein: A Novel Protein for Receptor Recycling from the Endocytic Recycling Compartment

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INTRODUCTION

Early endosomes comprise two distinct compartments identified primarily through trafficking studies using the transferrin receptor (TfR). At steady state, internalized TfR resides on sorting endosomes (SEs) and the endocytic recycling compartment (ERC) (Maxfield and McGraw, 2004). In SEs, the TfR is sorted for direct recycling back to the plasma membrane or transported to lysosomes via late endosomes for down-regulation (Yamashiro et al., 1984; Yamashiro and Maxfield, 1987a,b; Schmid et al., 1988; Ghosh et al., 1994). A second slower route for receptor recycling occurs from the ERC, after receptor transit through SEs (Sheff et al., 1999; Maxfield and McGraw, 2004). An emerging model suggests that in addition to sorting desensitized receptors for down-regulation, SEs and the ERC provide a local reserve of intracellular receptors for rapid delivery to the cell surface (Szekeres et al., 1998; Lin et al., 2000; Park et al., 2004).

Rab GTPases are potent regulators of membrane trafficking events (reviewed in Somsel Rodman and Wandinger-Ness, 2000; Stein et al., 2003). They are small monomeric GTPases that cycle between a GTP-bound and GDP-bound state, which is regulated through molecular interactions with guanine nucleotide exchange factors and GTPase activating proteins (reviewed in Pfeffer and Aivazian, 2004). Furthermore, rabs are generally compartment specific, functioning through effector molecules to regulate vesicle budding, receptor sorting, and cytoskeletal interactions (Somsel Rodman and Wandinger-Ness, 2000; Segev, 2001). Distinct endocytic rabs regulate the rapid and slow modes of TfR recycling from SEs and the ERC, respectively. Rab4 preferentially regulates rapid receptor recycling from the SEs (van der Sluijs et al., 1992), although some Rab4 is also associated with the ERC (Sheff et al., 1999; de Renzis et al., 2002). Conversely, Rab11 has been shown to regulate protein transport from the ERC to the trans-Golgi network and the plasma membrane. Moreover, these transport pathways are differentially affected in cells expressing Rab11 mutants with altered guanine nucleotide binding activities (Ullrich et al., 1996; Ren et al., 1998; Wilcke et al., 2000), suggesting that numerous transport pathways could originate from the ERC.

We previously reported that Rab15 is a novel endocytic GTPase that colocalizes with Rab4, Rab5, and the TfR on SEs as well as Rab11 on the ERC (Zuk and Elferink, 1999, 2000), suggesting that Rab15 may link these distinct endosomal compartments. Consistent with this tenet, overexpression of constitutively inactive Rab15 mutants differentially regulated transport through SEs and the ERC. Expression of the GDP-bound mutant Rab15-T22N promoted the recycling of the TfR from both SEs and the ERC. Conversely, overexpression of the guanine nucleotide free mutant Rab15-N121I increased the indirect or slower mode of TfR recycling from the ERC, without affecting rapid recycling from SEs (Zuk and Elferink, 2000). However, the mechanistic differences...
controlling Rab15-mediated receptor trafficking through SEs and the ERC remain poorly understood.

Rabs function via specific effectors to regulate distinct membrane transport steps (Somsel Rodman and Wandinger-Ness, 2000; Pfeffer, 2001; Deneka et al., 2003). Thus, the differential effects of Rab15 mutants on TfR transit through SEs and the ERC could involve unique sets of Rab15–effector interactions or an interacting protein with highly specialized properties. In this study, we identified Rab15 effector protein (REP15) as a novel binding partner for Rab15-GTP. When overexpressed in HeLa cells, REP15 did not localize to SEs; rather, REP15 colocalized with a subset of Rab15 and Rab11 on the ERC. Moreover, REP15 did not interact directly with Rab5 or Rab11, consistent with a role for REP15 as a compartment specific effector for Rab15. Consistent with its subcellular localization, overexpression and small interfering RNA (siRNA)-mediated depletion of REP15 attenuated the recycling of internalized TfR from the ERC without affecting receptor trafficking through SEs or TfR entry into the ERC. Together, our data identify REP15 as a novel component for receptor recycling from the ERC, further supporting that SEs and the ERC are mechanistically distinct endosomal compartments.

MATERIALS AND METHODS

Reagents and Plasmids

Cell culture and general reagents were obtained from Invitrogen (Carlsbad, CA), Fisher Scientific (Hampton, NH), and Sigma-Aldrich (St. Louis, MO) unless specified otherwise. Restriction enzymes were purchased from New England BioLabs (Beverly, MA). Plasmids encoding amino-terminal glutamin (HA)-tagged wild-type and mutant Rab15 in pCI-Neo and pLexA, and a HeLa cell library pretransformed into EGY187 (Mat a) have been described previously (Strick et al., 2002). Plasmids encoding the Rab11 mutants are in pcDNA3.1 and pcDNA3.2, and green fluorescent protein (GFP)-tagged Rab7 were generous gifts from R. Prekeris (University of Colorado Health Sciences Center, Denver, CO) and A. Wandinger-Ness (University of New Mexico, Albuquerque, NM), respectively. For yeast two-hybrid analysis, cDNAs encoding Rab11-G70L and Rab11-S25N were amplified by PCR using specific primer sets described previously and subcloned directly into the EcoRI site of pLexA. Human REP15 lacking an amino-terminal tag was PCR amplified (upper primer, 5'-GAAATGGGGCAGAAAGCATCGCAA-3'; lower primer, 5'-GAAGAAGATCTGGGGCAGAAAGCATCGCAA-3') and the resulting PCR products were subcloned directly into pcR3.1 (Invitrogen). Human REP15 containing an amino-terminal myc (MyeKLISEEDELD) or HA (MYDYVPD/YA) epitope were PCR amplified using the lower primer described above in combination with the appropriate upper primer (myc: 5'-CCGTGAGATCGAAGAAAATTAATCTCAGAAGAAGATCGTGGGCAGAAAGCATCGCAAC-3' or HA: 5'-GATCATACTGTACCTTATATGTACCTCG-3'), and the resulting PCR products were subcloned into pCR3.1 (Invitrogen).

Antibodies

HA monoclonal antibody (mAb) 12CA5 (Roche Diagnostics, Indianapolis, IN), monoclonal human TR H68.4, polyclonal Rab11 antibody (Zymed Laboratories, South San Francisco, CA), C-myc monoclonal 9B11 antibody (Cell Signaling Technology, Beverly, MA), early endosome antigen 1 (EEA1) mAb (BD Transduction Laboratories, San Diego, CA), and heat shock cytosolic 70 protein (Hsc70) polyclonal antibody (Stressgen Biotechnologies, Victoria, British Columbia, Canada) were purchased from sources as indicated. Alexa794- labeled transferrin (Tfn), goat anti-mouse and anti-rabbit secondary antibodies conjugated to Alexa647 or Alexa594, were purchased from Invitrogen. A Rab7 polyclonal antibody was a generous gift from P. van der Stuijs (University Medical Center Utrecht, The Netherlands). A Syntaxin 13 polyclonal antibody was a gift from R. Prekeris (University of Colorado Health Sciences Center). An anti-glutathione S-transferase (GST) polyclonal antibody was a generous gift from Peter McPherson (McGill University, Montreal, Quebec, Canada). Rabbit polyclonal antisera for Rab15 was generated against the synthetic peptide (NH2-CQAHRKELDGLRTC-COOH) (Covance, Denver, PA). To prepare an antibody against REP15, human REP15 (residues 3-256) was cloned directly into pGE-4T-GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) expressed as a GST-fusion and purified by affinity chromatography using glutathione agarose beads (Sigma-Aldrich). When thymol cleavage with S. aureus V8 protease (S. aureus V8 protease (Covance). The resulting REP15 anti-sera was purified by initial passage over GST immobilized on AminoLink coupling gel, and the flow through was subsequently purified against GST-REP15 immobilized against AminoLink coupling gel (Pierce Chemical, Rockville, IL).

Confloc Microscopy and Colocalization Studies

For confocal analysis, HeLa cells were seeded onto Matrigel (BD Biosciences, San Jose, CA) in 6-well coated coverslips and fixed in 4% paraformaldehyde (Ted Pella, Redding, CA) in phosphate-buffered saline (PBS) for 10 min, blocked and permeabilized with 10% goat sera (HyClone Laboratories, Logan, UT), 0.02% saponin, and 1% bovine serum albumin (BSA) in PBS, and then incubated with the appropriate antibody in PBS supplemented with 1% BSA, 0.02% saponin overnight at 4°C. Cells were washed three times with PBS and incubated with the appropriate secondary antibody coupled to Alexa488 or Alexa594 in PBS containing 1% BSA, 0.02% saponin for 1 h at room temperature. The coverglasses were mounted onto glass slides using FluorSave reagent (Calbiochem, San Diego, CA). Images were obtained using a BX50 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a Plan-Apo 100×/1.35 oil immersion objective and argon and krypton lasers with excitations at 488 and 568 nm, respectively. Images were then processed using Fluoview 2.0 imaging software (Olympus).

To avoid unbiased selection, the two channels were imaged separately and not merged until acquisition was complete. Before acquisition, PMT and the Laser power adjustments were optimized for each channel to avoid saturation of a particular channel. Images were processed with the Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA), saved as TIFF files, then imported into MetaMorph 4.6 (Molecular Devices, Sunnyvale, CA) and analyzed using the Measure Colocalization Application as follows. Individual cells were selected using the region of interest selection function in MetaMorph 4.6, and the threshold and areal units were empirically determined to ensure that the background signals were not contributing to the quantification. Values were calculated as the percentage of pixels for signal A colocalizing with the percentage of pixels for signal B, and then normalized to the total area of the given cell. The final values represent the mean ± SE for six to ten randomly selected cells from three independent experiments.

Cell Culture, Transfections, TfR Trafficking Assays, and Organelle Immunolocalizations

All cell culture, transfections, uptake studies, and organelle immunolocalizations have been described previously (Zuk and Elferink, 1999, 2000; Strick et al., 2002). Membrane fractions enriched in early or late endosomes were prepared by sucrose floatation gradient fractionation as described previously (Prekeris et al., 1998; Zuk and Elferink, 1999, 2000; Yan et al., 2004). Crude membrane fractions enriched in early, recycling, and late endosomes were prepared as described previously (Bache et al., 2003). Cell surface biotinylation assays for measuring internalized TfR were performed as described previously (Schmidt et al., 1997). Data points were collected in triplicate and were expressed as the ratio of internalized TfR versus surface TfR. Internalization rate constants for TfR were determined as described previously (Li et al., 2005), and the kinetic parameters were obtained by fitting the data with a linear regression over time. siRNA depletion experiments were performed using commercial control (D-001210-01-2) and REP15 (M-030132-00) siRNAs (Dharmacon, Lafayette, CO) and transfected into cells using LipofectAMINE 2000 reagent (Invitrogen).

Biotinylated Transferrin Enzyme-Linked Immunosorbent Assays (ELISAs)

Tfn-depleted cells were incubated in DMEM with 5 µg/ml biotinylated Tfn (B-Tfn; Pierce Chemical) in DMEM containing 2 mg/ml BSA for 1 h at 16°C to load B-Tfn into SEs. Noninternalized ligand was removed by three alternative washes with ice-cold DMEM supplemented with 2 mg/ml BSA (2% Adml BSA). The cells were chased at 37°C for 0–10 min in DMEM with a 100-fold excess of unlabeled Tfn, to promote ligand recycling from the SEs. In studies measuring Tfn recycling from the ERC, the cells were subjected to a second 10 min chase at 37°C followed washes with PBS, pH 4.2, and PBS containing 2 mg/ml BSA, to deplete the cell of SE-associated B-Tfn and to promote ligand transport to the ERC. One set of cells was incubated at 4°C and represented the amount of B-Tfn internalized into the ERC. The cells were then incubated at 37°C for increasing time (10–60 min) to promote recycling from the ERC to the cell surface. The amount of B-Tfn recycled into the chase media was quantified by ELISA, using anti-Tfn antibody-coated plates according to manufacturer’s instructions (Bethyl Laboratories, Montgomery, TX). Bound B-Tfn was detected using 0.1 µg/ml streptavidin-horseradish peroxidase followed by Quantum blue fluorochrome (Pierce). Data were then measured in a SpectroMax Gemini fluorescence microplate reader (Molecular Devices) and expressed as relative fluorescent units (RFU) ± SE per microgram of protein.
REP15 and Transferrin Receptor Recycling

RESULTS

REP15, a Novel Effector for Rab15

We previously reported that Rab15 mutants differentially regulated TfR trafficking through SEs and the ERC (Zuk and Elferink, 1999, 2000), raising the possibility that Rab15 function could be regulated in part via interactions with compartment-specific effectors. We recently identified mammalian suppressor of Sec4 (Mss4) as a binding partner for Rab15 in a yeast two-hybrid screen using a HeLa cell cDNA library. Moreover, interactions between Rab15 and Mss4 modulated the inhibitory effect of Rab15 on TfR trafficking in HeLa cells and homotypic SE fusion in vitro (Strick et al., 2002). To identify additional effectors for Rab15, we screened the yeast two-hybrid HeLa cell cDNA library using the GTP-bound mutant Rab15-Q67L as bait. Of the six clones isolated, DNA sequence analysis confirmed that four of the clones encoded novel proteins of unknown function that will be characterized and reported elsewhere. However, two clones encoded an identical open reading frame of 233 amino acids and hence were analyzed further. BLAST searches against the human and mouse genomes revealed that the open reading frame shared 100 and 73.8% identity, respectively, with amino acids 3–236 of a hypothetical protein of unknown function (accession nos. XP370686 and NP079886, respectively) (Figure 1A). Subsequent analysis using the SMART database detected no putative functional

or membrane domains and revealed no similarity with other Rab effectors identified to date. Accordingly, we named the predicted protein Rab15 effector protein or REP15 (GenBank accession no. AY662682). Subsequent reverse transcription-PCR analysis confirmed that the mRNAs for REP15 and Rab15 are coexpressed in HeLa cells (Figure S1A).

As a potential binding partner for Rab15, we predicted that REP15 would also enrich in membrane fractions. Because REP15 did not contain apparent transmembrane or lipid modification motifs for membrane association, we examined the putative membrane binding properties of REP15 using biochemical criteria. A postnuclear supernatant was prepared from HeLa cells transiently expressing HA-tagged REP15, fractionated into membrane and cytosolic fractions by centrifugation and examined by Western analysis (Figure 1B). When overexpressed in HeLa cells, REP15 distributes with the TfR, a marker for endocytic membranes and with Hsc70, a cytosolic marker, consistent with our previous report on Rab15 (Zuk and Elferink, 2000). Subsequent biochemical analysis of the membrane fraction revealed that REP15 was partially extracted from salt-washed membranes (NaCl and PBS) but not from membranes after a high pH wash (Figure 1B). These data suggest that the membrane binding properties of REP15 likely involve protein–protein interactions rather than binding via neutral phospholipids.

**REP15 Specifically Binds to Rab15-GTP**

We next examined the guanine nucleotide dependence of REP15 binding to Rab15 using LacZ and Leu2 as reporter genes in a yeast two-hybrid binding assay. High levels of β-galactosidase activity were detected in strains coexpressing REP15 with GTP-bound forms of Rab15 (wild type or the GTP-bound mutant Q67L) but not the GDP-bound (T22N) or nucleotide-free (N121I) mutants of Rab15 (Figure 2A and Table 1). Western analysis confirmed that comparable levels of Rab15 were expressed in the diploid strains, indicating that the observed differences in binding were not due to altered levels of wild-type or mutant Rab15 (Figure 2A). Similarly, no interaction was observed with REP15 and wild-type or mutant Rab5, a GTPase associated with SEs, confirming the specificity of the results (Table 1).

To identify the Rab15 binding domain(s) in REP15, we prepared different amino- and carboxy-terminal deletion mutants of REP15 and examined their ability to interact with Rab15-Q67L in a yeast two-hybrid binding assay. No interaction was observed in diploid strains coexpressing comparable levels of Rab15-Q67L and REP15 lacking the amino terminal (residues 151–236) or carboxy-terminal (residues 3–151) domains (Figure 2B and Table 1). Similarly, no interaction was detected between Rab15-Q67L and a REP15 mutant lacking 100 amino acids from the amino terminus. Thus, Rab15-GTP likely interacts through multiple sites of REP15 or via a highly ordered structural motif requiring full-length REP15.

We verified the guanine nucleotide dependence of Rab15 binding to REP15 by performing pull-down studies. We previously reported that endogenous Rab15 was not readily detected in baby hamster kidney, Chinese hamster ovary (CHO), Trvb-1, or HeLa cells using our existing Rab15 antibodies (Zuk and Elferink, 1999; Strick et al., 2002). Thus, we performed binding studies using cell lysates prepared from HeLa cells transiently overexpressing wild-type, HA-tagged Rab15 (HArab15) and full-length REP15 expressed as a recombinant GST-fusion protein. Before incubation with recombinant REP15, cell lysates were then incubated in the absence or presence of guanosine 5’-O-(2-thio)diphosphate (GDPβS) or the nonhydrolysable analog GTPγS, to lock wild
type Rab15 into a GDP or GTP-bound state respectively. The lysates were incubated with GST-REP15 immobilized on glutathione agarose beads and the resulting REP15-HArab15 complexes isolated and examined by Western analysis (Figure 2C). REP15 bound efficiently to wild type Rab15 pre-loaded with guanosine 5'-O-(3-thio)triphosphate. Conversely, no REP15 binding was detected with wild-type Rab15 in the presence or absence of GDP/S, or beads containing GST only. Thus, consistent with our yeast two-hybrid data, REP15 preferentially interacts with GTP-bound Rab15.

**REP15 Localizes with Rab15 on Endosomal Membranes**

To determine the subcellular localization of REP15, we prepared a polyclonal antibody against recombinant REP15 and confirmed its specificity by Western analysis. As shown in Figure 3A, the anti-REP15 antibody reacted with GST-REP15, but not with GST only. Conversely, Western analysis using the REP15 antibody detected overexpressed cMycREP15, but not endogenous REP15 protein in HeLa cell lysates (Figure 3B) under these conditions. Similarly, endogenous REP15 expression in untransfected or mock-transfected HeLa cells was not readily detected using the REP15 polyclonal antibody in immunofluorescence microscopy studies (our unpublished data).

To confirm that REP15 is endogenously expressed in HeLa cells, a crude membrane fraction enriched in SEs and the ERC was prepared from untransfected HeLa cells and cells overexpressing cMycREP15. Before fractionation, the cell lysates were incubated with 1 mM GTP/S for 30 min at 4°C.

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**Table 1.** REP15 interacts with GTP-bound Rab15 but not Rab5 or Rab11. β-Galactosidase activity in Rfu was determined in yeast diploid strains coexpressing the indicated bait constructs in pLexA and prey constructs in pB42AD

<table>
<thead>
<tr>
<th>pLexA Construct</th>
<th>pB42AD:REP15 Construct</th>
<th>Rfu</th>
<th>Interaction</th>
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</thead>
<tbody>
<tr>
<td>Rab15 WT</td>
<td>REP15 (3-236)</td>
<td>121,884.6 ± 6679.9</td>
<td>+</td>
</tr>
<tr>
<td>Rab15 Q67L</td>
<td>REP15 (3-236)</td>
<td>113,834.0 ± 6361.9</td>
<td>+</td>
</tr>
<tr>
<td>Rab15 T22N</td>
<td>REP15 (3-236)</td>
<td>8629.2 ± 180.7</td>
<td>–</td>
</tr>
<tr>
<td>Rab15 N121I</td>
<td>REP15 (3-236)</td>
<td>17,743.8 ± 2410.8</td>
<td>–</td>
</tr>
<tr>
<td>Rab15 Q67L</td>
<td>REP15 (3-151)</td>
<td>20,697.3 ± 342.9</td>
<td>–</td>
</tr>
<tr>
<td>Rab15 Q67L</td>
<td>REP15 (151-236)</td>
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<td>–</td>
</tr>
<tr>
<td>Rab15 Q67L</td>
<td>REP15 (100-236)</td>
<td>4072.0 ± 623.7</td>
<td>–</td>
</tr>
<tr>
<td>Rab5 WT</td>
<td>REP15 (3-236)</td>
<td>3240.0 ± 1015.2</td>
<td>–</td>
</tr>
<tr>
<td>Rab5 Q79L</td>
<td>REP15 (3-236)</td>
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</tr>
<tr>
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<td>9503.7 ± 1333.2</td>
<td>–</td>
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<tr>
<td>Rab5 N133I</td>
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<td>26,407.3 ± 1193.1</td>
<td>–</td>
</tr>
<tr>
<td>Rab5 S25N</td>
<td>REP15 (3-236)</td>
<td>1825.3 ± 563.6</td>
<td>–</td>
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</tbody>
</table>

ND, not determined.

Values represent the means ± SE of three independent studies. Statistically significant interactions (+) between proteins are indicated (p < 0.001; analysis of variance [ANOVA]).

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**Figure 2.** REP15 binding to Rab15 is guanine nucleotide dependent. (A) Yeast strains expressing REP15 fused to the activation domain of B42 (pB42AD:REP15) were mated with strains expressing wild type (wt) or the indicated mutants of Rab15 fused to LexA (pLexA:Rab15), and the resulting diploids were examined for protein interactions. Blue colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactoside or growth on Leu- plates indicate specific interactions between REP15 and Rab15. Western analysis (Wn) confirmed comparable levels of Rab15 expression in all diploid strains. (B) The indicated amino- and carboxy-terminal truncations of REP15 were fused to the activation domain of B42, mated with a yeast strain expressing Rab15-Q67L, and the resulting diploids were examined for the presence (+) or absence (−) of protein interactions using a yeast two-hybrid approach as described above. (C) A postnuclear supernatant (PNS) prepared from HeLa cells expressing wild-type HA-tagged Rab15 (HArab15) was incubated in the absence (−) or presence (+) of GTP/S or GDP/S before incubation with GST-REP15. Complexes were isolated and examined by Western analysis using antibodies for HArab15 and GST-REP15.
REP15 preferentially binds to GTP-bound Rab15. Under these conditions, Western analysis of the endosomal membrane fractions detected low levels of REP15 in untransfected control HeLa cells (Figure S1B). Conversely, high levels of REP15 and cMyc immunoreactivity were detected in fractions prepared from cells overexpressing cMycREP15. REP15 was detected in early endosomal membranes enriched in EEA1, the TIR, and Syntaxin 13, a t-SNARE that resides on SE membrane (Figure 3D) (Trischler et al., 1999). Conversely, REP15 immunoreactivity was not detected with down-regulated TIR in late endosomal membrane fractions. Thus, REP15 localizes to early endosomal membranes, consistent with a role as a Rab15 binding protein.

**REP15 Localizes to the ERC**

We previously reported that Rab15 localized to both peripheral SEs as well as the ERC (Zuk and Elferink, 1999), where it functioned to differentially regulate TIR transport (Zuk and Elferink, 2000). Our studies showing that REP15 colocalized with Rab15 in a perinuclear distribution in HeLa cells suggested that REP15 might be a compartment specific effector for Rab15 at the ERC. To address this issue, we examined the localization of transiently expressed cMycREP15 on early endosomal membranes using confocal microscopy. No significant overlap in cMycREP15 staining was observed with the SE-specific markers EEA1 or Rab4 (Figure 4) or the late endosomal marker Rab7 (our unpublished data) (Barbiert et al., 1994; Bottger et al., 1996; Rybin et al., 1996) (our unpublished data). Conversely, cMycREP15 staining overlapped with Calnexin or mannosidase II, markers for the endoplasmic reticulum and the cis/medial-Golgi, respectively (Lippincott-Schwartz et al., 1991; Wada et al., 1991) (our unpublished data). Conversely, cMycREP15 staining overlapped with endogenous Rab11, an established marker for the ERC (Ullrich et al., 1996) (Figure 4). Quantitative analysis of the confocal micrographs showed that 56 ± 7.1% of cMycREP15 overlapped with endogenous Rab11 in the perinuclear region of the cell.

To determine whether the perinuclear distribution of REP15 corresponded to the ERC, we performed organelle immunoprecipitation studies. Postnuclear supernatants were prepared from HeLa cells transiently expressing cMycREP15, and immunoprecipitated under nondenaturing conditions using an antibody against the ERC marker Rab11. The resulting immune complexes were examined by Western analysis.
REP15 comigrated with Rab11 and the TfR but not with the early endosomal marker EEA1 (Figure 5A). To verify the association of REP15 with the ERC, we examined the colocalization of REP15 with internalized TfN under chase conditions that promoted TfN transport from SEs to the ERC. Duplicate sets of HeLa cells stably expressing REP15 were allowed to internalize Alexa594-labeled transferrin (Alexa-Tfn) at 16°C for 1 h, conditions that specifically load Alexa-Tfn into the SEs and decrease transport to subsequent endosomal compartments, including the ERC (Ren et al., 1998; Zuk and Elferink, 2000). Control studies confirmed that the intracellular distribution of REP15 in cells loaded at 16°C was indistinguishable from that detected in cells loaded at 37°C, indicating that incubation at 16°C does not alter the expression and/or targeting of REP15 (our unpublished data). After internalization, the cells were washed and then chased at 37°C for 1 or 30 min in media containing unlabeled TfN to promote Alexa-Tfn recycling from SEs and transport to the ERC. The cells were then fixed, stained for cMycREP15 with endogenous EEA1 or Rab4, and examined by confocal microscopy (Figure 5B). After a 1-min chase, high amounts of Alexa-Tfn colocalized with EEA1 in SEs (57.9 ± 13.6%). The lower levels of ligand colocalizing with Rab11 and REP15 (11.1 ± 3.0 and 23.8 ± 3.8%, respectively), likely represent an incomplete block in TfR transport from SEs to the ERC in HeLa cells under these conditions. Conversely, a 30-min chase at 37°C resulted in decreased colocalization of Alexa-Tfn with EEA1, and a concomitant increase in Alexa-Tfn colocalizing with REP15 and Rab11 (54.4 ± 1.8 and 68.1 ± 4.3%, respectively), consistent with ligand transport to the ERC. The increased colocalization of REP15 with internalized Alexa-Tfn at longer chase periods, confirms that REP15 associates with the ERC rather than SEs.

The majority of Rab-GTP effectors identified to date bind specifically to their cognate Rab. However, some Rab effec-
tors have been shown to be multivalent, interacting with Rabs involved in sequential transport steps. For example, Rabaptin-5 and Rabenosyn-5 interact with GTP-bound Rabs 4 and 5 on SEs (Vitale et al., 1998; de Renzis et al., 2002). The Rab11 effector FIP2 binds Rabs 11 and 8, possibly linking membrane transport between the ERC and the cis-Golgi (Hattula and Peranen, 2000; Lindsay and McCaffrey, 2002). Because REP15 colocalized with Rab11 and Rab15 on the ERC, we used a yeast two-hybrid binding assay to determine whether REP15 is a shared effector for these endosomal Rabs. No direct interaction was observed between REP15 and constitutively active, GTP-bound Rab11 (Q70L) or the GDP-bound mutant Rab11-S25N (Figure 5C and Table 1). Western analysis confirmed that the absence of binding was not due to differences in the expression levels of REP15 or the Rab11 mutants (Figure 5C). These data indicate that REP15 colocalizes with Rab11 and Rab15 on the ERC and not on SEs.

**Effects of REP15 Overexpression on TfR Trafficking**

Since REP15 colocalizes with Rab15 and Rab11 on the ERC, we examined the effect of REP15 overexpression on TfR trafficking through this compartment. Cell surface proteins were biotinylated at 4°C with sulfo-NHS-SS-biotin, which is cleaved by washing the cells with cell-impermeable reducing agents such as sodium 2-mercaptoethanesulfonate (MesNa) (Schmidt et al., 1997) (Figure 6, A and B). After biotinylation, the cells were incubated at 37°C for 2, 4, or 6 min, and the amount of internalized TfR quantified by streptavidin-agarose pull-downs and Western analysis. The internalization rate constants were calculated as a linear regression coefficient and graphed. K_e values of 0.2630 ± 0.035 and 0.2435 ± 0.052 min⁻¹ were determined for HeLa cells overexpressing cMycREP15 and mock-transfected (Con) cells, respectively.
of internalized TfR was maximal at 30 min, decreasing to lower levels at longer chase times (45 and 60 min), consistent with internalized TfR recycling back to the cell surface from peripheral SEs and the ERC (Figure 6B). Conversely, higher levels of internalized TfR were detected in cells stably expressing REP15, reaching maximal levels at 45–60 min. Therefore, overexpression of REP15 in HeLa cells regulates the trafficking of internalized TfR.

Given the accumulation of TfR in REP15-expressing cells, we proposed that REP15 overexpression would result in reduced levels of TfR on the cell surface. To test this, intact mock-transfected HeLa cells and cells overexpressing REP15 were biotinylated with NHS-SS-biotin at 4°C for 30 min. Biotinylated cell surface proteins were isolated from cell lysates using streptavidin-agarose, and the level of biotinylated TfR was examined by Western analysis and spot densitometry. Under these conditions, we routinely detected a 50% reduction in the amount of biotinylated TfR on the surface of REP15-overexpressing cells relative to mock-transfected cells (Figure 6C). Western analysis of the cell lysates confirmed that the differences in surface levels of TfR were not a consequence of discernible differences in the expression level of the receptor (Figure 6C). Thus, the accumulation of internalized TfR in REP15 overexpressing cells corresponded to decreased cell surface levels of TfR.

Using surface biotinylation assays, we next examined whether REP15 overexpression altered the rate of TfR internalization from the cell surface. As shown in Figure 6D, the first order rate constants for TfR internalization were directly comparable in cells stably overexpressing REP15 and mock-transfected control cells, indicating that REP15 does not regulate the internalization of the TfR from the cell surface.

**REP15 Regulates TfR Recycling from the ERC**

The observed decrease in cell surface TfR levels coupled with the intracellular retention of TfR in REP15-overexpressing cells, suggested a role for REP15 in receptor recycling. To test this directly, we developed an ELISA using B-Tfn to measure the recycling of internalized B-Tfn into the cell media. In these studies, mock-transfected HeLa cells and HeLa cells stably expressing REP15 were incubated with B-Tfn for 1 h at 16°C to load the SEs. The cells were shifted to 37°C in media containing a 100-fold excess of unlabeled Tfn for 10 min to promote B-Tfn recycling from SEs and to chase the B-Tfn into the ERC. The cells were then acid washed to remove any recycled B-Tfn that could associate with receptor on the cell surface. One set of cells was retained at 4°C as a measure of B-Tfn internalized into the ERC (Figure 7A). Under these conditions, comparable levels of B-Tfn were loaded into the ERC of control cells versus REP15-expressing HeLa cells (Figure S3). The acid washed cells were then chased for increasing times to promote B-Tfn recycling from the ERC. The medium was collected and the amount of recycled B-Tfn in the chase media measured by ELISA assay (see Materials and Methods). Under these conditions, increasing levels of recycled B-Tfn were detected in the media of control and REP15-expressing cells, reaching a maximum at 40 min (Figure 7B). However, we detected a 47–51% decrease in the relative amount of B-Tfn recycled into the chase media of REP15-expressing cells at 40 and 60 min, respectively, relative to control cells, consistent with a role for REP15 in TfR recycling.

We verified that REP15 overexpression decreased TfR recycling by examining recycling of the TfR from the ERC in transiently transfected cells using a cell surface biotinylation assay. In biotinylated, mock-transfected control cells, the amount of internalized TfR decreased during successive recycling incubations (Figure S4, A and B), consistent with efficient receptor recycling from the ERC to the cell surface. Conversely, the level of TfR in the ERC was unaltered in cells transiently overexpressing REP15, consistent with a block in receptor recycling. Therefore, our ligand and receptor recycling studies indicate that REP15 overexpression results in decreased TfR recycling from the ERC.

We next verified that the effect of REP15 overexpression was specific for TfR recycling from the ERC and not fast TfR recycling from the SE. In these studies, cells were labeled with B-Tfn at 16°C for 1 h to load B-Tfn into the SEs, acid washed to remove surface-bound B-Tfn, and then chased for 5 and 10 min at 37°C in media containing unlabeled Tfn. The amount of B-Tfn recycled into the media from the SEs was quantified using a B-Tfn ELISA. Comparable levels of B-Tfn were detected in the chase media from REP15-expressing and untransfected control HeLa cells, confirming that REP15 does not regulate B-Tfn recycling from the SEs (Figure 7C).

To confirm the effect of REP15 on TfR recycling from the ERC, we used siRNAs to deplete HeLa cells of endogenous REP15. Given the difficulties detecting endogenous REP15 using our REP15 antibody (Figures 3 and S2), the specificity of the REP15 siRNA was verified using HeLa cells stably expressing cMycREP15. Western analysis confirmed that transfection with REP15 siRNA depleted 80–90% of cMycREP15, with no effect on the level of endogenous TfR, β-actin, EEA1, and the endocytic GTPases Rab11 and Rab5. Comparable levels of REP15, TfR, β-Actin, EEA1, Rab11, and Rab5 were detected in untransfected cells and cells transfected with a control siRNA, confirming the specificity of the REP15 siRNA (Figure 7A). We next examined the effect of depleting endogenous REP15 on TfR recycling from the ERC. HeLa cells were transfected with a control siRNA or REP15 siRNA. Seventy-two hours post-transfection, the cells were incubated with B-Tfn for 1 h at 16°C to load the SEs, washed, and chased for 20 min to promote recycling from the SEs and ligand transport to the ERCs. The cells were then washed again and incubated for 10 or 20 min at 37°C to promote recycling from the ERC. The relative amount of recycled B-Tfn in the media was measured using a B-Tfn ELISA (see Materials and Methods). B-Tfn recycling from the ERC in REP15-depleted cells was blocked after a 20-min chase, consistent with our REP15 overexpression studies (Figure 6B). Conversely, B-Tfn recycling from the ERC was unaffected in HeLa cells transfected with a control siRNA (Figure 8B). Additional recycling studies confirmed that siRNA-mediated depletion of REP15 had no effect on B-Tfn recycling from SEs (Figure 8C). Together, these data identify REP15 as a novel Rab15 effector important for TfR recycling from the ERC.

**DISCUSSION**

In this study, we present functional evidence that REP15 is a novel protein important for TfR recycling from the ERC. When overexpressed in HeLa cells, REP15 is compartment specific, colocalizing with Rab15 and Rab11 on the ERC but not with Rab15, Rab4, or EEA1 on SEs. Consistent with its localization, siRNA-mediated depletion of REP15 resulted in retention of the TfR in the ERC, without affecting receptor transport to the ERC or fast receptor recycling from SEs. TfR recycling from the ERC was also defective in cells overexpressing wild-type REP15. Similar inhibitory phenotypes have been reported in overexpression and siRNA-mediated knockdown studies on other endocytic proteins. For example, overexpression and siRNA silencing of the EH domain (EHD) protein has also been reported to inhibit TfR endocytosis (Guilherme et al., 2004). Similarly, epidermal growth...
factor receptor down-regulation was disrupted in HeLa cells overexpressing full-length Hrs, and in cells transfected with siRNA specific for Hrs (Bache et al., 2003; Morino et al., 2004). Thus, the inhibition in TIR recycling detected in cells overexpressing REP15, could be due to the titration of partner proteins involved in this endocytic step. Our data showing that REP15 overexpression did not alter the delivery of internalized TIR to SEs, receptor access to the ERC, or the fast mode of TIR recycling from the SEs further supports our contention that REP15 specifically regulates TIR recycling from the ERC.

Several lines of evidence indicate that REP15 is a binding partner for GTP-bound Rab15 at the ERC. First, REP15 interacts with Rab15 in a guanine nucleotide manner. Second, consistent with our localization data, REP15 did not interact with Rab5, an SE-specific GTPase (Barbieri et al., 1994; Rybin et al., 1996; Roberts et al., 1999). Moreover, despite their colocalization to the ERC, we could not detect an interaction between REP15 and Rab11 in a yeast two-hybrid binding assay. Finally, REP15 colocalized preferentially with a pool of Rab15 on the ERC of HeLa cells, rather than with Rab15 on EEA1-positive peripheral SEs. Although we cannot rule out the possibility that small amounts of REP15 transiently reside on SEs, REP15 overexpression and siRNA-mediated depletion did not affect TIR trafficking through SEs, suggesting that REP15 primarily associates with and regulates TIR recycling from the ERC.

How can we reconcile the inhibitory effect of REP15 overexpression and siRNA-depletion of REP15 on TIR recycling from the ERC with Rab15-GTP function? We previously reported that Rab15 distributes between SEs and the ERC in a variety of cell types and that it differentially regulates TIR transport through these endosomal compartments (Zuk and Elferink, 1999, 2000). In these studies, expression of the guanine nucleotide-deficient Rab15 mutant N121I, stimulated the slow recycling of TIR from the ERC, without affecting rapid receptor recycling from SEs. Conversely, overexpression of Rab15-GTP had no effect on TIR recycling from the SEs or the ERCs, indicating that Rab15-Q67L does not phenocopy the inhibitory effect of REP15 on TIR recycling. However, in these studies wild-type Rab15 and Rab15-Q67L expression inhibited TIR entry into SEs, primarily at the level of SE fusion (Zuk and Elferink, 2000; Strick et al., 2002). Thus, the inhibition in TIR trafficking through SEs caused by wild-type Rab15 and Rab15-Q67L would be expected to mask any potential effects of Rab15-GTP on trafficking events downstream of SEs, including TIR recycling from the RE. Thus, given the opposing effects of REP15 and the nucleotide deficient Rab15 mutant N121I expression on TIR recycling from the ERC, it seems likely that REP15 could functionally interact with Rab15 to regulate this transport step. The ultimate test for the involvement of this interaction would be to examine the effect of REP15 mutants deficient in Rab15 binding on TIR recycling. However, low transfection levels and toxic effects of existing truncated REP15 mutants were routinely observed in our experiments, thus limiting their use in these types of studies.

Our results demonstrating that overexpression of REP15 resulted in TIR accumulation in the ERC are similar to previous functional studies on Rab11 and receptor trafficking through this compartment. Overexpression of the GTP-bound mutant Rab11-Q70L causes accumulation of the β2-adrenergic receptor as well as the TIR in the ERC (Moore et al., 2004). Interestingly, Rab11-induced accumulation of the β2-adrenergic receptor was associated with impaired β2-adrenergic receptor trafficking to lysosomes. Similarly, interactions between Rab11 and the Rab coupling protein were recently reported to regulate TIR sorting from the degradative pathway to the slow recycling pathway via the ERC (Peden et al., 2004), suggesting a role for Rab11 and possibly the ERC in receptor sorting, rather than receptor recycling per se. In this context, the slow recycling pathway and the degradative pathway would be competitive, such that a reduction in receptor down-regulation could result in receptor accumulating in the ERC.

A key finding presented here is the identification of REP15 as a novel regulator of TIR recycling from the ERC. To date, Rabs 11, 15, and 25a represent the only endocytic GTPases...
with roles for regulating receptor transport through the ERC (Ullrich et al., 1996; Wang et al., 2000; Zuk and Elferink, 2000; Lindsay and McCaffrey, 2002). In nonpolarized cells, Rab11 is associated with the ERC and plays a role in slow TfR recycling. Conversely in polarized Madin-Darby canine kidney (MDCK) cells, Rab11 has been shown to be a marker for apical recycling and does not seem to regulate TfR recycling from the ERC (Wang et al., 2000). Moreover, protein transport to the trans-Golgi network and recycling to the plasma membrane is affected in cells expressing Rab11 mutants (Ullrich et al., 1996; Ren et al., 1998; Wilcke et al., 2000), suggesting a role for this GTPase in multiple transport steps from the ERC. Thus, how could Rab15 and Rab11 function in nonpolarized cells to regulate TfR recycling from the ERC? Rab15 and REP15 could act in concert with Rab11 via yet unidentified partner proteins. In this model, our data showing that REP15 partially colocalizes with Rab15 and Rab11 on the ERC could denote a distinct sorting domain or sub-compartment within the ERC that regulates TfR availability for recycling. Alternatively, this region may correspond to specific domains or regions linking two distinct and differentially regulated recycling pathways in the ERC. Two kinetically distinct recycling routes leading from the ERC to the plasma membrane were recently characterized in CHO cells (Lampson et al., 2001). In these studies, the insulin-regulated amino peptidase is sorted from the TfR in the ERC and is returned to the plasma membrane via distinct transport vesicles, consistent with the existence of different recycling routes from the ERC. Similarly, Rab11 and Rab25a have been shown to regulate distinct recycling pathways through the apical recycling system in MDCK cells (Wang et al., 2000), supporting a multilevel organization for receptor recycling from the ERC in multiple cell types. Future experiments will be required to distinguish which recycling pathway from the ERC is regulated by REP15.

Our studies are consistent with a role for REP15 regulating the exit of the TfR from the ERC and not receptor delivery to this compartment. Overexpression of REP15 resulted in decreased amounts of TfR on the cell surface when measured using a cell surface biotinylation assay. However, comparable levels of internalized TfR were detected in control and cells stably expressing REP15 when the ERC of the cells were loaded at 16°C. Therefore, REP15 overexpressing cells recycled less TfR than control cells, yet with similar kinetics, consistent with a model in which REP15 functions to determine the size of the TfR recycling pool. In this context, REP15 could function to regulate either TfR sorting within the ERC or the generation/maturing of recycling vesicles emanating from the ERC. Notwithstanding, the above-mentioned results identifying REP15 as an ERC-specific regulator of TfR recycling make a significant step toward our understanding of the mechanistic components essential for receptor recycling from this unique endosomal compartment.

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