The DnaJ-domain Protein RME-8 Functions in Endosomal Trafficking*

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Through a proteomic analysis of clathrin-coated vesicles from rat liver we identified the mammalian homolog of receptor-mediated endocytosis 8 (RME-8), a DnaJ domain-containing protein originally identified in a screen for endocytic defects in Caenorhabditis elegans. Mammalian RME-8 has a broad tissue distribution, and affinity selection assays reveal the ubiquitous chaperone Hsc70, which regulates protein conformation at diverse membrane sites as the major binding partner for its DnaJ domain. RME-8 is tightly associated with microsomal membranes and co-localizes with markers of the endosomal system. Small interfering RNA-mediated knock down of RME-8 has no influence on transferrin endocytosis but causes a reduction in epidermal growth factor internalization. Interestingly, and consistent with a localization to endosomes, knock down of RME-8 also leads to alterations in the trafficking of the cation-independent mannose 6-phosphate receptor and improper sorting of the lysosomal hydrolase cathepsin D. Our data demonstrate that RME-8 functions in intracellular trafficking and provides the first evidence of a functional role for a DnaJ domain-bearing co-chaperone on endosomes.

Molecular chaperones of the heat shock protein 70 family including regulation of endosomal trafficking and morphology.

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5 The abbreviations used are: Hsc70, heat shock cognate 70; CCV, clathrin-coated vesicle; TGN, trans-Golgi network; TD, terminal domain; CHC, clathrin heavy chain; RME-8, receptor-mediated endocytosis 8; CI-MPR, cation-independent mannose 6-phosphate receptor; EGF, epidermal growth factor; GST, glutathione S-transferase; PBS, phosphate-buffered saline; DME, Dulbecco’s modified Eagle’s medium; siRNA, small interfering RNA; EEA1, early endosomal antigen 1; VAMP, vesicle-associated membrane protein.
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7 The on-line version of this article (available at http://www.jbc.org) contains Supplemental Figs. 1 and 2.
8 The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AY779857.
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[Supplemental Figs. 1 and 2.]

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RME-8 Functions at Endosomes

**EXPERIMENTAL PROCEDURES**

**Antibodies and Fluorescent Probes**—A rabbit polyclonal RME-8 serum was raised against a synthetic peptide, SNLPPPDVD-HEAGDLGYQT, containing amino acids 2226–2243 at the C terminus of human RME-8 coupled to keyhole limpet hemocyanin through an added N-terminal cysteine. A rabbit polyclonal serum against human enthoprotin/epsinR was previously described (17). Monoclonal antibodies against CHC used for immunofluorescence was generated from the hybridoma X22 obtained from American Type Culture Collection (Manassas, VA). Mouse monoclonal antibodies against CHC, syntaxin 6, early endosomal antigen 1 (EEA1), Grb2, and AP-1 (γ-adaptin) were purchased from BD Transduction Laboratories. Mouse monoclonal antibodies against the following proteins were from the noted commercial sources: Rab9, Abcam (Cambridge, MA); CI-MPR and AP-2 (α-adaptin), ABR (Golden, CO); Na+/K+ ATPase, Upstate Biotechnology (Lake Placid, NY); auxilin 2/cyclin G-associated kinase, MBL International (Woburn, MA); transferrin receptor, Zymed Laboratories Inc. (South San Francisco, CA). Rat monoclonal anti-Hsc70 was from StressGen (Victoria, BC), and rabbit polyclonal antibodies against cathepsin D and EGF receptor were from DAKOCytomation (Mississauga, ON) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. A pan-cadherin polyclonal antibody was from Sigma. Polyclonal antibodies recognizing VAMP 4 and CI-MPR were generous gifts of Dr. Richard Scheller (Genentech, San Francisco, CA) and Dr. Paul Luzio (University of Cambridge, Cambridge, UK), respectively. Transferrin-Alexa Fluor 647, EGF-Texas Red, EGF-Alexa Fluor 488, and cholera toxin B-Alexa 594 were from Molecular Probes Inc. (Eugene, OR).

**cDNA Constructs**—KIAA0678, encoding a large C-terminal fragment of human rece 1302–1366; Fig. 1) was amplified from KIAA0678 cDNA by PCR and was subcloned in-frame into pGEX-4T1 vector (Amersham Biosciences) adding an N-terminal GST tag. The terminal domain (amino acids 1302–1366; Fig. 1) was amplified from KIAA0678 cDNA by PCR and was subcloned in-frame into pGEX-4T1 vector (Amersham Biosciences) adding an N-terminal GST tag. The terminal domain (amino acids 1302–1366; Fig. 1) was amplified from KIAA0678.

**FIGURE 1. Amino acid sequence of human RME-8.** Dashed line denotes the DnaJ domain, underline denotes the IVM repeats, bold letters denote sequences that match a consensus for binding to the terminal domain of the CHC, and double underline denotes the peptide sequence used to raise the RME-8 antibody.

For affinity selection experiments with the CHC TD, soluble fractions resulting from pH 11.0 extraction of P3 pellets, as described above, were neutralized to pH 7.4 by dilution in buffer A and were then centrifuged at 245,000 × g for 10 min. Triton X–100 was added to the supernatant to 1% final and 2-mg aliquots of the extracts were incubated overnight at 4 °C with GST or GST–CHC–TD precoated to glutathione-Sepharose beads. After incubation, beads were washed with buffer A containing 1% Triton X–100, and proteins specifically bound to the beads were analyzed by SDS-PAGE and Western blot. In other experiments, adult rat kidneys were homogenized in 10 volumes of buffer B (20 mM HEPES, pH 7.4, 25 mM KCl, 2 mM MgCl$_2$, 10 mM NH$_4$SO$_4$, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin) and centrifuged at 800 × g for 10 min. The resulting supernatant were incubated with 10 mM EDTA or varying concentrations of adenine nucleotides for 30 min, and 2-mg aliquots of the resulting supernatant were centrifuged at 245,000 × g for 10 min. The resulting supernatant were centrifuged at 245,000 × g for 30 min, and 2-mg aliquots of the resulting supernatant were incubated with 10 mM EDTA or varying concentrations of adenine nucleotides for 10 min at 25 °C before incubation for 1 h at 25 °C with GST–DnaJ domain fusion protein pre-coated to glutathione-Sepharose beads. Incubations with ATP were performed in the presence of an ATP regenerating system. After incubation, beads were washed with buffer B containing 1% Triton X–100, and proteins specifically bound to the beads were analyzed by SDS-PAGE and Western blot or bands were extracted from Coomassie-blue-stained gels.
stained gels and analyzed by tandem mass spectrometry as described (22).

**Immunofluorescence Analysis of RME-8 Localization**—Cells grown on poly-L-lysine-coated coverslips were washed in PBS (20 mM NaH2PO4, 150 mM NaCl, pH 7.4) and then fixed for 20 min in 2% paraformaldehyde, PBS. In some cases cells were first incubated for 1 h in serum-free DMEM and then incubated with 5 μg/ml Cy3-transferrin or 100 ng/ml Texas Red-EGF for 20 min at 37 °C before fixation. After fixation, cells were permeabilized with 0.2% Triton X-100, PBS (or 0.05% saponin/PBS for AP-2 and EGF receptor) and processed for immunofluorescence with the appropriate primary and secondary antibodies. All immunofluorescence images presented in the study were obtained using a Zeiss 510 laser scanning confocal microscope.

**siRNA-mediated Knock Down of RME-8**—siRNAs matching selected regions of RME-8 sequence were synthesized by Qiagen with dT overhangs already annealed. The sequences were submitted to BLAST search to ensure specificity of the target. Of the four sequences selected, one (RME-8.3) led to a decrease of RME-8 protein expression superior to 90%. The DNA target sequence of RME-8.3 was 5’-AAGCTGCTCAGATATGAAAA-3’. For siRNA transfection, COS-7 cells were plated in DMEM without antibiotics. Cells were plated in 6-well plates or on coverslips in 24-well plates such that they would be 60% confluent 24 h post-plating. At this time, cells were transfected with siRNAs at a final concentration of 80 nM using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, and transfections were repeated 24 h later. For the control, cells were transfected with Lipofectamine 2000 only. Experiments were performed 72 h after the first transfection.

**Analysis of EGF, Transferrin, and Cholera Toxin B Endocytosis**—COS-7 cells, mock-transfected or transfected with RME-8.3 siRNA, were serum-starved for 1 h in DMEM and then incubated for 15 min at 37 °C with transferrin-Cy3 (5 μg/ml), EGF-Texas Red (10 ng/ml), or cholera toxin B-Alexa 594 (5 μg/ml). Cells were processed for immunofluorescence analysis as described previously. Alternatively, cells were treated the same but were incubated with transferrin-Alexa Fluor 647 (5 μg/ml) or EGF-Alexa Fluor 488 (10 ng/ml). In these cases, one set of cells for each ligand was washed twice in PBS and then fixed in 2% paraformaldehyde, PBS. A second set of cells was washed in acidic buffer (0.2 M acetic acid, pH 2.8, containing 0.5 M NaCl) for 15 min at 4 °C to strip cell surface EGF or transferrin, and the cells were then washed twice in PBS and fixed as above. The levels of fluorescent EGF and transferrin were assessed using a FACScan with data analyzed using the CellQuantPro program (BD Biosciences). The percentage of ligand internalized was determined by dividing the amount of ligand in cells (acid-washed samples) versus total ligand (cells washed with PBS alone) for 10,000 cells.

**Cathepsin D Sorting Assay**—COS-7 cells mock-transfected or transfected with RME-8.3 siRNA were washed and placed in cysteine/methionine-free DMEM (Invitrogen) for 1 h at 37 °C. Cells were then pulsed for 10 min with 0.25 mCi/ml Pro-Mix [35S] (Amersham Biosciences), washed, and chased in DMEM with 1% fetal bovine serum, 5 mM mannose-6-phosphate and 1 mM methionine. At 1-, 2-, 3-, and 4-h time points, media were collected, cell lysates were prepared, and cathepsin D was immunoprecipitated as described (23). Immunoprecipitated proteins were separated on SDS-PAGE and processed for autoradiography using a STORM PhosphorImager (Amersham Biosciences) followed by exposure to x-ray film. Quantitation was performed using ImageQuant software.

**RESULTS**

**Identification of Mammalian RME-8**—A proteomic analysis of CCVs isolated from rat liver identified proteins that are predicted products of cDNAs and genomic sequence (24). One such protein, identified in the annotated rat genome under gi27721389, is the rat homolog of RME-8. The rat sequence could be aligned with fly and worm orthologs with the blat algorithm, a series of 56 exons from region q22.1 of chro-
mososome 3 could be annotated to reveal a sequence that was identical to the sequence predicted from the overlapping clones. siRNAs targeting each of the three clones were effective in reducing the expression of the endogenous protein in COS-7 cells as detected with a polyclonal anti-endogenous protein in COS-7 cells as detected with a polyclonal antibody against a C-terminal peptide (see Fig. 6 and data not shown). The human RME-8 sequence (entered into GenBank™ under accession number AY779857) encodes a 2243-amino acid protein that is 46, 43, and 28% identical throughout its length to Drosophila, C. elegans, and Arabidopsis RME-8, respectively. As originally described for the C. elegans protein (14), human RME-8 has a central DnaJ domain flanked on either side by two IWN repeats of unknown function (Fig. 1).

RME-8 is detected as a protein of ~220 kDa in most tissues and cell lines examined (Fig. 2, A and B). A reactive band seen in selective extracts at ~140 kDa may represent a proteolytic fragment or an alternatively spliced form of RME-8 (Fig. 2). Subcellular fractionation of kidney extracts reveals that RME-8 is enriched in P3 microsomes with no protein detectable in a cytosolic (S3) fraction, whereas the plasma membrane protein cadherin is enriched in P1 and P2 fractions that sediment at lower g forces (Fig. 2C). Purified plasma membrane that is highly enriched for cadherin and EGF receptor has a relatively weak RME-8 signal (Fig. 2D). RME-8 does not extract from microsomal fractions after treatment with 1% Triton X-100, 150 mM NaCl, or 500 mM NaCl but is partially extracted by sodium carbonate, pH 11.0 (Fig. 2E). The integral membrane protein Na+/K+ ATPase is resistant to NaCl and pH 11.0 but is extracted with 1% Triton X-100 (Fig. 2E). Thus, RME-8 appears to be an extrinsic membrane protein tightly associated with a Triton X-100-insoluble microsomal compartment.

**RME-8 Functions at Endosomes**

**FIGURE 3. Interaction of RME-8 with Hsc70.** A, GST and a GST fusion protein encoding the DnaJ domain of RME-8 (GST-DnaJ) were coupled to glutathione-Sepharose and used for affinity selection assay from soluble kidney extracts. Kidney extract or buffer alone was incubated with (+) or without (−) 3 mM ATP for 10 min at 25 °C before the addition to the fusion proteins. Proteins specifically bound to the beads along with a 1/10 aliquot of the kidney extract (starting material (SM)) were processed by SDS-PAGE and Coomassie Blue staining. The ~70-kDa protein that bound to the GST-DnaJ domain in the presence of ATP was excised and analyzed by tandem mass spectrometry revealing 33 unique peptides for Hsc70. All other bands detected were present when fusion proteins were incubated with buffer alone, indicating that they are aggregated fusion protein or are proteins co-purifying from bacterial extracts. B, GST and GST-DnaJ domain fusion protein were coupled to glutathione-Sepharose and used for affinity selection assay from soluble kidney extracts. Kidney extracts were first incubated for 10 min at 25 °C with EDTA (nucleotide-free, NF), 3 mM ADP, or 3 mM ATP. Proteins specifically bound to the beads along with a 1/10 aliquot of the kidney extract (starting material (SM)) were processed by SDS-PAGE and Western blot with Hsc70 antibody. C, equal protein aliquots of a soluble kidney extract containing no ATP (O) or increasing concentrations of ATP (1 μM to 5 mM in an ATP regenerating system) were incubated with GST-DnaJ domain pre-coupled to glutathione-Sepharose. Proteins specifically bound to the beads were processed by SDS-PAGE and Western blot with Hsc70 antibody.

**FIGURE 4. Association of RME-8 with CCVs.** A, GST and a GST fusion protein encoding the terminal domain of the CHC (GST-TD) were coupled to glutathione-Sepharose and used for affinity selection assay from soluble kidney extracts. Proteins specifically bound to the beads along with a 1/10 aliquot of the kidney extract (starting material (SM)) were processed by SDS-PAGE and Western blot with antibodies against RME-8, γ-adaptin (AP-1), and enthropin. B, equal protein aliquots of the various fractions of the subcellular fractionation procedure leading to highly enriched CCVs were processed by SDS-PAGE and Western blot with RME-8, auxilin 2, and CHC antibodies. H, homogenate; P, pellet; S, supernatant; SM, homogenate.
ments, and co-immunoprecipitation experiments are complicated by the fact that the endogenous protein can only be solubilized under conditions that are likely to disrupt protein-protein interactions (Fig. 2E).

The auxilins, which bind to Hsc70 via a DnaJ domain, also bind to the TD of the CHC and function in the uncoating of CCVs by recruiting Hsc70 to clathrin coats (4). The identification of mammalian RME-8 on CCVs and the presence within the protein of four potential type II clathrin boxes (LLDFL, LLEMV, LLEFL, LLDYI; Fig. 1), interaction motifs for the CHC TD (25–27), suggest that RME-8 could function analogously to auxilins. To address this issue, we first tested if RME-8 binds to the TD. Proteins were extracted from kidney P3 microsomes with NaCO₃ buffer, pH 11.0, and the extracts were subsequently adjusted to pH 7.4 and incubated with a GST-CHC-TD. RME-8 failed to bind the TD, whereas enthoprotin/epsinR and the clathrin adaptor protein 1, which bind to the TD through type II clathrin boxes in whole or in part, respectively (28, 29), both bound in the same experiment (Fig. 4A). Moreover, whereas RME-8 is present on CCVs purified from liver, consistent with its identification in this organelle, it was not enriched (Fig. 4B). Neuron-specific auxilin 1 (30) and ubiquitously distributed auxilin 2/cyclin-G-associated kinase (Fig. 4B) are both highly enriched on CCVs. Thus, RME-8 is unlikely to function analogously to auxilins in the uncoating of CCVs.

Localization of Endogenous RME-8 in Mammalian Cells—Studies in invertebrates demonstrated that GFP-RME-8 was localized to endosomal structures in coelomocytes of C. elegans (14) and that RFP-RME-8 partially overlaps with GFP-tagged Rab5 and Rab7 in Garland cells of Drosophila (15). Confocal immunofluorescence analysis of COS-7 cells (Fig. 5) and HeLa cells (data not shown) reveals that endogenous mammalian RME-8 has a distributed punctate pattern with accumulation of larger puncta in a perinuclear region. RME-8 puncta are partially co-localized with EEA1 (Fig. 5A) and transferrin that has been endocytosed for 20 min (Fig. 5B), indicating that pools of the protein are present on early and recycling endosomes. RME-8 also co-localizes with the CI-MPR (Fig. 5C) and is found in puncta surrounding Texas Red-EGF after 20 min of endocytic uptake (Fig. 5D), suggesting that pools of RME-8 are present on late endosomes. That RME-8 puncta surround EGF may reflect the presence of EGF within the lumen of the late endosome with RME-8 on the limiting membrane, and in fact, endocytosed EGF can even be seen in many cases to be adjacent to or surrounded by the EGF receptor (Fig. 5E). Little co-localization is seen between RME-8 and syntaxin 6, a TGN marker, and no co-localization is seen with LAMP1, a marker of lysosomes (data not shown). Moreover, RME-8 does not co-localize with AP-2, indicating that it is not a component of clathrin-coated
pits on the plasma membrane (Supplemental Fig. 1). Together, these results demonstrate that RME-8 is found predominantly on endosomes.

RME-8 Loss of Function Causes Defects in EGF Endocytosis—To explore the functional roles of RME-8, we took a loss of function approach. Four siRNAs were designed to sequences from the coding region of the mRNA of the human protein. Upon transfection into COS-7 and HeLa cells, three of these siRNAs suppressed RME-8 protein expression by greater than 50% (data not shown). The most effective, RME-8.3, reduced RME-8 expression in COS-7 cells to near undetectable levels based on immunofluorescence microscopy (Fig. 6A) and Western blot (Fig. 6B). Quantification of the Western blots revealed that RME-8 expression in RME-8.3-treated cells was 6 ± 0.7% of that seen in mock-transfected cells (Fig. 6B). Depletion of RME-8 did not affect the expression levels of CHC, Hsc70, EEA1, Na+/K+-ATPase, syntaxin 6, Rab9, or Grb2 (Fig. 6B).

We first examined for alterations in clathrin-mediated endocytosis in COS-7 cells depleted of RME-8. RME-8 knock down caused no apparent defect in uptake of fluorescent transferrin as determined by immunofluorescence microscopy, but there was a noticeable decrease in intracellular EGF (Fig. 7A). To quantify this result, we used fluorescence-activated cell sorting. Control and knock-down cells were incubated with fluorescent ligands for 15 min to allow internalization and were then fixed after a PBS wash (total ligand) or acid wash (internalized ligand) before fluorescence-activated cell sorting analysis. Interestingly, we noticed a decrease in the total amount of EGF associated with knock-down cells compared with control, whereas transferrin levels were not affected. Western blots revealed that this result reflected a decrease in EGF receptor expression levels (Fig. 7B). In addition, the percentage of total EGF that was internalized was decreased by ~50%, indicating that the ability of the remaining pool of receptor to undergo endocytosis was compromised (Fig. 7C). No effect of RME-8 knock down was observed on the endocytosis of cholera toxin B (Fig. 7D), a marker of the caveolae-mediated endocytic pathway (31).
The enzyme is subsequently targeted to lysosomes where it is cleaved into mature cathepsin D, whereas the MPR escapes degradation by recycling back to the TGN (32–34). Interestingly, RME-8-depleted cells show a striking clustering of the CI-MPR in the perinuclear region, whereas mock-transfected cells demonstrate a more distributed pattern of CI-MPR staining (Fig. 9A). The clustered receptor is co-localized with syntaxin 6 and VAMP 4, markers of the TGN (Fig. 9B), suggesting that the perinuclear compartment is the TGN or is a compartment that clusters near to the TGN. Pro-cathepsin D trafficking was monitored using an assay that examines processing of pro-cathepsin D to its mature form in pulse-chase experiments (23). Mock-transfected cells show a time-dependent accumulation of mature cathepsin D at 31 kDa, whereas RME-8 knock-down cells accumulate less of the mature form (Fig. 9C). A portion of pro-cathepsin D normally escapes transport to endosomes and is instead secreted (Fig. 9C and see, for example, Ref. 35). Interestingly, the secretion of pro-cathepsin D is also reduced after RME-8 knock down (Fig. 9C). Averaging over multiple experiments reveals that the percentage of cathepsin D that is sorted (released and processed relative to total) is significantly reduced from 52.2 to 34.6% (n = 9) at the 3-h time point in mock- versus RME-8.3-transfected cells, respectively (Fig. 9D). The percentage sorted in mock-transfected cells increased to 69.6% at 4 h, where again a significant decrease (to 52.9%, n = 11) is seen after RME-8 knock down (Fig. 9D). Thus, loss of RME-8 function leads to defects in the trafficking of cathepsin D via the MPR.

DISCUSSION

RME-8 was originally identified as a mutant defective in yolk protein uptake in C. elegans (14) and was subsequently shown to function in ligand-stimulated and constitutive endocytosis in Drosophila (15). In both invertebrate systems, RME-8 was found to partially co-localize with endosomal vacuoles (14, 15). Here we demonstrate that mammalian RME-8 is localized throughout the endosomal system and is enriched on microsomal membranes spanning at 205,000 × g. It is not, however co-localized with AP-2, a marker of clathrin-coated structures at the plasma membrane, and its knock down does not affect AP-2 distribution (Supplemental Fig. 1). Moreover, RME-8 is expressed at relatively low levels in highly enriched plasma membrane preparations. Thus, although it is not possible to definitively rule out that a small pool of RME-8 may be present at the plasma membrane, the protein appears to be predominantly endosomal.

Given the seeming discrepancy between the localization of RME-8 to intracellular endosomes and its role in entry of endocytic tracers, we sought to examine for endocytic defects in mammalian systems. Interestingly, we found no effect of RME-8 knock down on endocytosis of transferrin, a cargo of the constitutive endocytic pathway. However, we did observe a reduction in endocytosis of the EGF receptor. EGF receptor can enter cells via clathrin-mediated mechanisms and via caveolae, and the use of these two pathways is very sensitive to the concentration of EGF (36). We, thus, tested for a potential effect of RME-8 knock down on endocytosis of cholera toxin B, a marker of the caveolae-mediated endocytic pathway (31). Cholera toxin B endocytosis was normal, suggesting that disruption of the caveolae pathway is not responsible for the defect in EGF endocytosis. However, we did make the surprising observation that the level of EGF receptor was significantly reduced in RME-8 knock-down cells. In contrast, the levels of transferrin receptor as well as those of multiple signaling and vesicle trafficking proteins were unaffected. The EGF receptor displays significant rates of constitutive endocytosis (1–2%/min) with trafficking through early and late endosomes (37). Enhancing transport from early to late endosomes or decreasing EGF receptor recycling to the plasma membrane from late endosomes can decrease EGF receptor levels (37). Thus, RME-8 knock down could
disrupt the steady state levels of EGF receptor by disrupting endosomal trafficking. The precise mechanism causing loss of EGF receptor remains under investigation.

RME-8 knock down was also seen to alter the distribution of CHC with ~30% of knock-down cells demonstrating reduced cytosolic staining. This is reminiscent of the depletion of cytosolic clathrin that is seen after overexpression of dominant-negative forms of Hsc70 in mammalian cells (12). Moreover, it is consistent with results in Garland cells in Drosophila in which RME-8 mutants demonstrate a redistribution of GFP-tagged clathrin light chain from peripheral puncta to larger intracellular puncta, a phenotype mimicked by loss of Hsc70 function (13, 15). Thus, in both mammalian and invertebrate systems, RME-8 and Hsc70 are likely to function in a common pathway. It is unlikely, however, that RME-8 function is directly analogous to that of auxilins in uncoating CCVs as RME-8 does not bind directly to CHC and is not enriched on CCVs to the same extent as auxilin 1 and 2. This is consistent with the observation in Drosophila that overexpressed auxilin and RME-8 exhibit different genetic interactions with Hsc70 (15). Regardless, disruption of CHC distribution after RME-8 knock down may contribute to endocytic defects, as cells with the strongest CHC redistribution phenotype appear to have the most severe impairment of EGF endocytosis. In fact, the selective defect in endocytosis of EGF versus transferrin may result from a combination of this clathrin redistribution phenotype and the reduced levels of EGF receptor.

Despite the localization of RME-8 to intracellular membranes, no defect in intracellular trafficking has been described upon loss of RME-8 function. We noticed an accumulation of the CI-MPR in a perinuclear pool after RME-8 knock down. The CI-MPR along with the cation-dependent MPR carry out the delivery of newly synthesized acid hydrolases from the TGN to early and late endosomes for their subsequent transfer to lysosomes (34). To avoid degradation, MPRs recycle back to the TGN before reaching the lysosomal compartment (38). Our data demonstrate that RME-8 is necessary for the normal activity of this trafficking pathway. The receptor accumulates in the vicinity of the TGN based on co-localization studies with TGN markers syntaxin 6 and VAMP 4. This accumulation may represent trapping of the receptor in the TGN itself or an accumulation in a membrane compartment that accumulates near the TGN. However, when we examined the trafficking of cathepsin D, a lysosomal hydrolase, we noticed a decrease in the processing of cathepsin D to its mature form as well as a decrease in the normal basal rate of cathepsin D secretion. The former observation suggests that the MPR does not reach the lysosome, where processing of the pro form of cathepsin D occurs. However, if the CI-MPR was trapped in the TGN, one might expect to see an increase in pro-cathe-
psin D that escaped the TGN in constitutive vesicles. Thus, we favor a
scenario in which cathepsin D can traffic with the MPR from the TGN
but is unable to properly traffic through the endosomal pathway to
reach lysosomes. Thus, in addition to providing the first characterization
of mammalian RME-8, our data provide the first evidence of a role
for the protein in intracellular trafficking. Given its broad tissue distribution and evolutionary conservation, RME-8 is likely to contribute to
such trafficking events in the context of many cell types.

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