Lowe Syndrome Protein OCRL1 Interacts with Clathrin and Regulates Protein Trafficking between Endosomes and the *Trans*-Golgi Network[□]

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Oculocerebrorenal syndrome of Lowe is caused by mutation of OCRL1, a phosphatidylinositol 4,5-bisphosphate 5-phosphatase localized at the Golgi apparatus. The cellular role of OCRL1 is unknown, and consequently the mechanism by which loss of OCRL1 function leads to disease is ill defined. Here, we show that OCRL1 is associated with clathrin-coated transport intermediates operating between the *trans*-Golgi network (TGN) and endosomes. OCRL1 interacts directly with clathrin heavy chain and promotes clathrin assembly *in vitro*. Interaction with clathrin is not, however, required for membrane association of OCRL1. Overexpression of OCRL1 results in redistribution of clathrin and the cation-independent mannose 6-phosphate receptor (CI-MPR) to enlarged endosomal structures that are defective in retrograde trafficking to the TGN. Depletion of cellular OCRL1 also causes partial redistribution of a CI-MPR reporter to early endosomes. These findings suggest a role for OCRL1 in clathrin-mediated trafficking of proteins from endosomes to the TGN and that defects in this pathway might contribute to the Lowe syndrome phenotype.

INTRODUCTION

Phosphoinositides are a minor but important class of phospholipids that regulate a variety of cellular processes, including intracellular signaling, cytoskeletal dynamics, and membrane trafficking. Although they were originally identified as precursors to signaling molecules in signal transduction pathways, it is now clear that phosphoinositides can act as signals in their own right by recruiting proteins onto membranes (Hurley and Meyer, 2001). This is mediated by modular domains on the proteins that recognize and bind specific phosphoinositide species (Cullen *et al.*, 2001). In this way, phosphoinositides can establish and maintain the iden-

tity of membrane domains and thereby influence events such as transport vesicle budding and fusion (De Matteis and Godi, 2004).

One of the best examples of a phosphoinositide regulating membrane traffic is that of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] in clathrin-mediated endocytosis. This process requires an array of accessory proteins that act in a sequential manner to drive coat assembly, membrane invagination, vesicle scission, and uncoating of the budded vesicle (Evans and Owen, 2002). PI(4,5)P₂ plays a central role in endocytosis by recruiting many of the necessary accessory proteins, including the clathrin adaptor AP2, epsin, AP180, and dynamin. Enzymatic modulation of PI(4,5)P₂ levels would be expected to regulate endocytosis, and consistent with this, PI(4)P 5-kinase I β has recently been shown to regulate both AP2 recruitment to the plasma membrane and the rate of constitutive endocytosis in HeLa and CV-1 cells (Padron *et al.*, 2003). Another important regulator is synaptojanin, which has a PI(4,5)P₂ 5-phosphatase domain in addition to a Sac1p-like polyphosphoinositide phosphatase domain that can hydrolyze PI(3)P, PI(4)P, PI(5)P, and PI(3,5)P₂ to PI (McPherson *et al.*, 1996). This phosphatase is recruited to clathrin-coated pits through interaction with clathrin and AP2 (McPherson *et al.*, 1996; Haffner *et al.*, 2000). Knockout studies in mice suggest synaptojanin is required for uncoating of clathrin-coated vesicles (Cremona *et al.*, 1999), whereas studies in other systems also implicate synaptoja-

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Abbreviations used: CI-MPR, cation-independent mannose 6-phosphate receptor; EEA1, early endosome-associated antigen 1; FRAP, fluorescence recovery after photobleaching; OCRL1, oculocerebrorenal syndrome of Lowe protein 1; siRNA, small interfering RNA; STXB, Shiga toxin B subunit; TfR, transferrin receptor.

nin as a negative regulator of early stages in vesicle formation, probably through dephosphorylation of PI(4,5)P₂ (Hill *et al.*, 2001; Rusk *et al.*, 2003).

Relatively low amounts of PI(4,5)P₂ have been detected at the Golgi apparatus *in vivo* (Watt *et al.*, 2002), but PI(4)P 5-kinase activity has been measured in isolated Golgi membranes, and kinase activity can be stimulated by ADP-ribosylation factor (ARF), suggesting it may regulate vesicular trafficking (Godi *et al.*, 1999; Jones *et al.*, 2000). Analysis of the yeast synaptojanin-like protein Sjl3p/Inp53p has shown that both its Sac1p-like and PI(4,5)P₂ 5-phosphatase activities are required for proper delivery of proteins from the *trans*-Golgi network (TGN) to endosomes (Ha *et al.*, 2003), suggesting PI(4,5)P₂ levels may influence trafficking between these compartments. A role in TGN to plasma membrane traffic also is suggested by the ability of PI(4,5)P₂ to rescue trafficking when shuttled into cells depleted of a Golgi-localized PI 4-kinase (Wang *et al.*, 2003). However, the extent to which PI(4,5)P₂ regulates trafficking events at the Golgi apparatus remains poorly defined and awaits further characterization of PI(4,5)P₂ effectors and enzymes that regulate Golgi levels of this phosphoinositide.

A significant fraction of cellular PI(4)P is localized to the Golgi apparatus, and several PI 4-kinases have been detected there (De Matteis *et al.*, 2002). PI(4)P likely plays a direct role in carrier formation at the TGN. In conjunction with ARF1, it can recruit the clathrin adaptors AP1 (Wang *et al.*, 2003) and epsinR (enthoprotin, CLINT) (Kalthoff *et al.*, 2002; Wasiak *et al.*, 2002; Hirst *et al.*, 2003; Mills *et al.*, 2003) to the TGN membrane. Both proteins regulate clathrin budding at the TGN, and recent studies also suggest a role in retrograde trafficking from endosomes back to the TGN, consistent with the localization of these proteins to clathrin-coated buds on endosomes (Stoorvogel *et al.*, 1996; Mallard *et al.*, 1998; Meyer *et al.*, 2000; Crump *et al.*, 2001; Valdivia *et al.*, 2002; Saint-Pol *et al.*, 2004). Together with ARF1, PI(4)P also recruits the pleckstrin homology domain-containing proteins OSBP and FAPP1 to the TGN (Dowler *et al.*, 2000; Levine and Munro, 2002; Godi *et al.*, 2004), which have recently been implicated in TGN to plasma membrane carrier formation (Godi *et al.*, 2004).

Oculocerebrorenal syndrome of Lowe protein 1 (OCRL1) is a Golgi-localized PI(4,5)P₂ 5-phosphatase (Suchy *et al.*, 1995; Zhang *et al.*, 1995). Deficiency of OCRL1 is responsible for Oculocerebrorenal Syndrome of Lowe, a rare X-linked disorder characterized by mental retardation, congenital cataracts, and renal Fanconi Syndrome (Lowe *et al.*, 1952; Attree *et al.*, 1992). Cells from Lowe Syndrome patients have elevated PI(4,5)P₂ levels, suggesting this somehow leads to the above-mentioned symptoms (Zhang *et al.*, 1998; Wenk *et al.*, 2003). One mechanism may be through altering the actin cytoskeleton, because patients' cells seem to have fewer long actin stress fibers, abnormal punctate F-actin staining, and enhanced sensitivity to actin depolymerizing agents (Suchy and Nussbaum, 2002). An alternative mechanism by which disruption of OCRL1 could lead to Lowe syndrome is by altering membrane trafficking, although there is currently no evidence for this. Immunofluorescence microscopy has shown that OCRL1 is present at the Golgi apparatus, with at least some of the protein on the TGN (Olivos-Glander *et al.*, 1995; Dressman *et al.*, 2000). One study also suggested a lysosomal localization (Zhang *et al.*, 1998). Here, we report that OCRL1 is localized to early endosomes and the TGN and enriched in clathrin-coated transport intermediates. OCRL1 interacts directly with clathrin heavy chain and can promote clathrin assembly *in vitro*. We also show that overexpression or depletion of OCRL1 perturbs protein trafficking

at the TGN/endosome interface, suggesting a role in regulating transport between these compartments.

MATERIALS AND METHODS

Materials and Antibodies

All reagents were from Sigma (St. Louis, MO) or Merck (Whitehouse Station, NJ) unless stated otherwise. Protease inhibitors (cocktail set III) were from Calbiochem (San Diego, CA) and used at 1:250. Polyclonal antibodies to OCRL1 were raised in sheep by using glutathione S-transferase (GST)-tagged amino terminus of OCRL1 (residues from 1 to 237) and affinity purified using the fusion protein covalently coupled to glutathione beads (Amersham Biosciences, Piscataway, NJ). X22 clathrin heavy chain antibody was a kind gift from Liz Smythe (University of Sheffield, Sheffield, United Kingdom). Monoclonal antibodies to clathrin heavy chain, γ -adaptin, α -adaptin, and early endosome-associated antigen 1 (EEA1) were from BD Transduction Laboratories (Lexington, KY). Monoclonal anti-transferrin receptor (H68.4), CD8 (UCHT-4) and GFP antibodies were from Zymed (South San Francisco, CA), Sigma, and Molecular Probes (Eugene, OR), respectively. Monoclonal anti-lysosome-associated membrane protein-2 was from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Rabbit and mouse anti-cation-independent mannose 6-phosphate receptor (CI-MPR) antibodies were a gift from Paul Luzio (University of Cambridge, Cambridge, United Kingdom) or purchased from Affinity BioReagents (Golden, CO), respectively. Polyclonal antibodies to TGN46 and TGN38 were kindly provided by Vas Ponnambalam (University of Leeds, Leeds, United Kingdom). Rabbit anti-golgin-97 was a kind gift from Dr. Nobuhiro Nakamura (Kanazawa University, Kanazawa, Japan). Monoclonal anti-Golgi antibody CTR433 was kindly provided by Michel Bornens (Curie Institute, Paris, France). Rabbit polyclonal antibodies to GGA1 and golgin-160 were kindly provided by Margaret Robinson (University of Cambridge, Cambridge, United Kingdom) and Ed Chan (University of Florida, Gainesville, FL), respectively. ML07 anti-GM130 (anti-N73pep) has been described previously (Nakamura *et al.*, 1997). Fluorophore and horseradish peroxidase-conjugated secondary antibodies were purchased from Molecular Probes and Tago Biosource International (Camarillo, CA), respectively.

Molecular Biology and Yeast Two-Hybrid Analysis

All constructs were made using standard molecular biology techniques. A 2.7-kb cDNA encoding full-length OCRL1 (isoform b, accession no. NP_001578) was amplified from a human liver cDNA library by PCR and cloned into the *Bgl*III and *Xba*I sites of the pEGFP-C1 vector (BD Biosciences Clontech, Palo Alto, CA). Deletion of the 5-phosphatase domain (residues 237–539) was carried out by PCR. Full-length OCRL1 was cloned into pGEX 4T (Amersham Biosciences) for expression of GST-tagged protein. Full-length OCRL1 was cloned into the baculovirus transfer plasmid pBAC-2cp (Novagen, Madison, WI) for expression in insect cells. Full-length and truncated versions of human OCRL1 cDNA were inserted into the yeast two-hybrid activation domain vectors pGADT7 or pGBKT7. pGADT7-clathrinTD and pGBKT7-FL-or truncated OCRL1; pGADT7-FL-OCRL1 and pLexA clathrin HD or clathrin DD were cotransformed into the yeast reporter strain AH109 on synthetic medium lacking leucine and tryptophan (low selection) and then restreaked onto synthetic medium lacking leucine, tryptophan, histidine, and adenine with 2% glucose as the carbon source (high selection) according to the BD Biosciences Clontech yeast protocol handbook. Primer sequences for all manipulations are available upon request. All constructs were verified by DNA sequencing using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit, version 2 (Applied Biosystems, Foster City, CA). pGAD-clathrin TD, pLexA-clathrin HD, and pLexA-clathrin DD were kindly provided by Harald Stenmark (Norwegian Radium Hospital, Oslo, Norway).

Cell Culture and Transfection

HeLa, normal rat kidney (NRK), and COS-7 cells were grown at 37°C and 5% CO₂ in DMEM, supplemented with 10% fetal bovine serum (FBS). HeLa cells stably expressing CD8-CIMPR were a kind gift from Matthew Seaman (University of Cambridge) and grown in DMEM containing 10% FBS and 0.5 mg/ml G418 (Seaman, 2004). Suspension HeLa cells were grown at 37°C and 5% CO₂ in RPMI 1640 medium containing 10% FBS. Hypertonic treatment and K⁺ depletion were performed as described previously (Wu *et al.*, 2001). Hypertonic incubations were carried out in 0.2 M sucrose for 45 min at 37°C, whereas K⁺ depletion was achieved by hypotonic swelling of the cells in 50% diluted DMEM containing 1 mM ouabain for 5 min followed by incubation in ice-cold K⁺-free medium (50 mM HEPES, pH 7.3, 100 mM NaCl, 10 mM glucose, 1 mM MgCl₂, 0.1 mM CaCl₂) for 20 min. HeLa and NRK cells were transiently transfected using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA), respectively, according to the manufacturers' instructions and incubated for 16–20 h before fixation. HeLa cells stably-expressing green fluorescent protein (GFP)-tagged wild-type OCRL1 were made using the Tet-ON system from BD Biosciences Clontech according to the manufacturer's instructions. GFP-OCRL1 expres-

sion was induced by adding 1 μ g/ml doxycycline for 20 h. Western blotting and immunofluorescence indicated that GFP-OCRL1 was uniformly expressed in all cells at a two- to threefold higher level than endogenous OCRL1 (Figure S1).

RNA Interference

RNA interference was performed on HeLa cells by using Oligofectamne (Invitrogen) and small interfering RNA (siRNA) oligos (Dharmacon Research, Boulder, CO) as described previously (Diao *et al.*, 2003). Clathrin was targeted with the sequence UAAUCCAAUUCGAAGACCAU and lamin A with AACUGGACUCCAGAGAACA. OCRL1 was targeted with the sequence AAGCUUCUACAAGGAGCAGC or by using the corresponding Smartpool from Dharmacon Research. The Smartpool contained four separate RNA duplexes, all distinct from the OCRL1 sequence mentioned above. Cells were analyzed 72 h after transfection.

Shiga Toxin B (STxB) Trafficking and Transferrin Uptake

Shiga toxin trafficking experiments were performed as described previously (Saint-Pol *et al.*, 2004). HeLa cells were transiently transfected with GFP-OCRL1 or GFP-OCRL1 Δ 237-539 and incubated in ice-cold culture medium containing 1 μ g/ml Cy3-labeled STxB for 30 min. Cells were then shifted to 37°C for 45 min before fixation in paraformaldehyde and processing for immunofluorescence microscopy. For transferrin uptake, transiently transfected cells were incubated with 5 μ g/ml Alexa-594-conjugated holo-transferrin (Molecular Probes) in binding medium (Liebowicz's medium containing 2 mg/ml bovine serum albumin [BSA]) for 1 h on ice. The cells were then washed three times with ice-cold phosphate-buffered saline (PBS) and incubated in warm binding medium containing 0.1 mg/ml unlabeled holo-transferrin for 5 or 15 min before fixation in paraformaldehyde and processing for fluorescence microscopy.

Immunofluorescence Microscopy and Fluorescence Recovery after Photobleaching (FRAP)

For immunofluorescence microscopy, cells were grown on coverslips and fixed in 3% (wt/vol) paraformaldehyde (PFA) in PBS at room temperature (RT) for 20 min. PFA-fixed cells were quenched with 10 mM glycine, pH 8.5 (in PBS), and permeabilized with 0.1% Triton X-100 (in PBS) for 4 min at RT. In some cases, cells were fixed in 100% methanol at -20°C, as indicated in the figure legends. Coverslips were incubated for 20 min at RT with primary antibodies diluted into PBS containing 0.5 mg/ml BSA. Cells were washed three times with PBS and incubated for a further 20 min at RT with fluorophore-conjugated secondary antibodies diluted in PBS/bovine serum albumin. The DNA dye Hoechst 33342 (200 ng/ml) was included in the second incubation. Coverslips were mounted in Mowiol, allowed to dry, and analyzed using an Olympus BX60 upright microscope equipped with a MicroMax cooled, slow-scan charge-coupled device camera (Roper Scientific, Trenton, NJ) driven by MetaMorph software (University Imaging, Downingtown, PA). FRAP was performed as described previously (Wu *et al.*, 2001). For each experimental condition, a minimum of 10 data sets was averaged to get the mean and SD for each time point. Each data set refers to the fluorescence measured in a defined region of a single cell before and after photobleaching of that region. Data were normalized so that the maximum fluorescence was set to 100% and the minimum to 0%. There was no significant loss in fluorescence from the repeated scanning of the cell at low levels. There was only ~80% recovery of the GFP-OCRL1 after photobleaching because the Golgi pool of OCRL1 was ~20% of the total fluorescence in the cell.

Electron Microscopy

Cells were fixed in 4% PFA overnight or 2% PFA and 0.2% glutaraldehyde for 3 h at room temperature in 0.2 M HEPES, pH 7.4, and processed for ultrathin frozen sectioning as described previously (Liou *et al.*, 1996).

Coimmunoprecipitation

Doxycycline-treated parental HeLa cells or cells stably expressing GFP-OCRL1 were lysed in HKM buffer (20 mM HEPES, pH 7.4, 0.1 M KCl, 1 mM MgCl₂, 1 mM DTT) containing 0.5% (wt/vol) Triton X-100 and protease inhibitors for 15 min on ice. Lysates were clarified by centrifugation and incubated with appropriate antibodies for 1 h at 4°C. Immune complexes were collected by incubating with 10 μ l of protein G-Sepharose (Amersham Biosciences) for a further 1 h at 4°C and eluted from the beads by boiling in SDS-PAGE sample buffer. Bound and unbound fractions were analyzed by Western blotting with appropriate antibodies.

Protein Preparation

Plasmids encoding GST, GST-OCRL1 full-length, GST-golgin-84 head region (Diao *et al.*, 2003), and GST-clathrin terminal domain (TD, residues 1-579; kindly provided by Harald Stenmark) were transformed into *Escherichia coli* BL21 (DE3) cells. Cells were induced with 0.1 mM isopropyl β -D-thiogalactoside for 3 h at 37°C (GST, GST-clathrin), 3 h at 30°C (GST-golgin-84), or 18 h at 18°C (GST-OCRL1); lysed in Bugbuster HT (Novagen) containing protease

inhibitors; and recombinant proteins were purified on glutathione-Sepharose beads (Amersham Biosciences). Proteins were eluted from the beads with reduced glutathione and desalted into HKM containing 10% sucrose before snap freezing and storage at -80°C. Recombinant his-tagged OCRL1 was made in insect High Five cells (Invitrogen) by using the Bacvector system (Novagen) according to the manufacturer's instructions. Cells were lysed in TNM buffer (50 mM Tris, pH 8, 150 mM NaCl, 5 mM MgCl₂, 5 mM β -mercaptoethanol) containing 1% NP-40 and protease inhibitors and purified on Ni²⁺-NTA beads (Novagen). Bound proteins were eluted in TNM buffer containing 250 mM imidazole, desalted into HKM containing 10% sucrose, snap frozen in liquid nitrogen, and stored at -80°C. Clathrin was purified from bovine brain as described previously (Ma *et al.*, 2002). Bovine brain clathrin-coated vesicles were extracted in 0.5 M Tris, pH 7.0, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM DTT on ice for 30 min, and the released proteins were concentrated using 50% ammonium sulfate before fractionation on a Superose 6 column run in 0.5 M Tris, pH 7.0, 1 mM DTT. Fractions containing clathrin were pooled, snap frozen, and stored at -80°C.

Preparation of Cell Extracts

S3 HeLa cells from a 2-liter suspension culture were washed with PBS and lysed in lysis buffer (20 mM HEPES, pH 7.4, 0.2 M sucrose, 0.1 M KCl, 1 mM DTT) containing protease inhibitors (Calbiochem) by using a ball-bearing homogenizer with an 8.02-mm bore and 8.01-mm diameter ball. The broken cells were spun at 1000 $\times$ g to make a postnuclear supernatant (PNS) that was centrifuged at 100,000 $\times$ g for 45 min at 4°C. The supernatant was removed (cytosol) and the pellet (membranes) resuspended in lysis buffer. Both fractions were snap frozen and stored at -80°C. Pig brain cytosol was prepared by homogenizing pig brain in 2 volumes of 20 mM HEPES, pH 7.4, 0.1 M K acetate, 2.5 mM Mg acetate, 1 mM DTT containing protease inhibitors by using a Waring blender. Homogenates were centrifuged twice at 1000 $\times$ g to generate a PNS that was subsequently centrifuged at 100,000 $\times$ g for 1 h. The supernatant (cytosol) was snap frozen and stored at -80°C. Human placenta crude membranes and clathrin-coated vesicles were prepared using a modification of the method described by Pearse (1982). Placental tissue was homogenized in 2 volumes of vesicle buffer (140 mM sucrose, 20 mM MES, pH 6.6, 70 mM K acetate, 0.5 mM Mg acetate, 1 mM EGTA, 1 mM DTT) containing protease inhibitors by using a Waring blender and centrifuged in a Beckman JA-10 rotor at 5000 rpm for 30 min at 4°C. The supernatant was incubated with 10 U/ml pancreatic RNase for 30 min at room temperature before centrifugation in a Beckman type 19 rotor for 3 h at 19,000 rpm at 4°C to generate a crude membrane pellet. To prepare coated vesicles, the pellet was resuspended in vesicle buffer and layered onto a 10-90% (wt/vol) ²H₂O gradient (in vesicle buffer) and centrifuged at 45,000 $\times$ g for 30 min. The total supernatant was diluted 4 times in vesicle buffer, centrifuged at 100,000 $\times$ g for 2 h through a cushion of 8% (wt/vol) sucrose/90% (wt/vol) ²H₂O (in vesicle buffer). The pellet was resuspended in vesicle buffer and layered onto a 9% (wt/vol) ²H₂O/2% (wt/vol) Ficoll to 90% (wt/vol) ²H₂O/20% (wt/vol) Ficoll gradient and centrifuged at 80,000 $\times$ g for 16 h. The coated vesicles were recovered from a broad zone near the bottom of the gradient, pelleted, resuspended in vesicle buffer, snap frozen, and stored at -80°C. HeLa Tet-ON cell extracts were prepared by incubating cells in HKM containing 0.5% Triton X-100 and protease inhibitors for 15 min on ice and clarifying the lysates by centrifugation at 15,000 $\times$ g for 10 min.

GST Pull-Down Experiments

HeLa membranes were extracted in HKM containing 0.5% Triton X-100 and protease inhibitors for 30 min on ice and clarified by centrifugation at 45,000 rpm for 15 min in a TLA55 rotor. Pig brain cytosols were diluted to 10 mg/ml with HKM containing Triton X-100 (0.1% final concentration) and clarified as described above. HeLa membrane extract (0.5 mg) or pig brain cytosol (1 mg) was incubated for 3 h or overnight at 4°C with 5-20 μ g of GST fusion protein coupled to glutathione-Sepharose beads. Beads were washed four times with HKM containing 0.1% Triton X-100 and bound proteins eluted with SDS-PAGE sample buffer. Bound and unbound proteins were subjected to SDS-PAGE followed by Western blotting or Coomassie Blue staining.

Clathrin Cage Binding and Assembly

For binding to cages, purified clathrin was dialyzed overnight against 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES), 1 mM DTT, pH 6.0, followed by centrifugation for 1 h at 150,000 $\times$ g. The pellet was resuspended in 20 mM MES, 150 mM NaCl, 1 mM DTT, pH 6.0, and the concentration of pure clathrin baskets was determined by measuring absorbance at 280 nm. Clathrin baskets were incubated with OCRL1 at 25°C in 20 mM MES, 150 mM NaCl, 1 mM DTT, pH 6.0, and then centrifuged at 380,000 $\times$ g for 6 min at 4°C. The amount of OCRL1 in the supernatant was quantified by SDS-PAGE and gel scanning by using Chemilmager (Alpha Innotech, San Leandro, CA). Clathrin assembly was performed by mixing clathrin triskelia (0.3 μ M) without OCRL1 or with different concentrations of OCRL1 and dialyzing overnight against 0.1 M MES, 1 mM DTT, pH 6.5, at 4°C. To measure clathrin polymerization, aliquots of the samples after dialysis were centrifuged at 380,000 $\times$ g for 6 min at 4°C. The supernatant were analyzed for free clathrin and free OCRL1 by SDS-PAGE followed by gel scanning by using Chemilmager.

Figure 1. Immunofluorescence localization of OCRL1. Immunofluorescence microscopy of COS-7 cells double labeled with antibodies to OCRL1 (red) and either golgin-97, clathrin heavy chain (CHC), γ -adaptin, or CI-MPR (green). Insets show magnified views of the perinuclear regions indicated by boxes in the merges and indicate puncta that contain both markers. Arrowheads indicate additional puncta outside this region that lack golgin-97 (a–c) or contain CHC (d–f), γ -adaptin (g–i), or CI-MPR (j–l). Bar, 20 μ m.

RESULTS

OCRL1 Is Localized to the Golgi Apparatus and Endosomes

OCRL1 has previously been localized to the TGN (Dressman *et al.*, 2000), suggesting it may play a role in vesicular trafficking there. To analyze this possibility we first sought to compare the localization of OCRL1 with various proteins involved in trafficking at the TGN. For this purpose, we prepared an affinity-purified antibody to localize endogenous OCRL1 and also transiently expressed a GFP-tagged version of the protein into cells. As expected, labeling of COS-7 cells for endogenous OCRL1 revealed significant overlap in the perinuclear region with the TGN marker golgin-97 (Figure 1, a–c). We also observed cytoplasmic puncta that did not contain golgin-97 (Figure 1, a–c, arrowheads). The specificity of this staining was confirmed by the loss of both punctate and perinuclear Golgi staining upon RNA interference (RNAi)-mediated depletion of OCRL1 (Figure S2). Because the punctate structures were reminiscent of clathrin-coated transport intermediates, we performed double labeling with antibodies to clathrin and the γ -adaptin subunit of the TGN-associated AP1 clathrin adaptor. In both cases, there was a high degree of colocalization with OCRL1 in the puncta (Figure 1, d–f and g–i). The relative amounts of OCRL1 and clathrin or γ -adaptin varied between different puncta, and often the labeling seemed to be next to each other on the same structure. Many of the

OCRL1 puncta also contained the CI-MPR, a cargo receptor that cycles between the TGN and endosomes in clathrin-coated intermediates (Figure 1, j–l). Similar results were obtained upon transient expression of GFP-tagged OCRL1 in NRK cells (Figure S3).

Because clathrin, AP1 and CI-MPR also localize to endosomes, we performed double labeling with the early endosomal marker EEA1. As shown in Figure 2, a–c, a significant proportion of the OCRL1 puncta contained EEA1, identifying them as early endosomes. We also observed colocalization with the transferrin receptor (TfR) in both the peripheral puncta and larger perinuclear structures that may correspond to recycling endosomes (Figure 2, d–f), but little overlap was seen with the late endosome/lysosome marker CD63 (Figure 2, g–h). Similar results were found with GFP-OCRL1 transiently expressed in HeLa cells (Figure S4). Together, these results suggest OCRL1 is present on the TGN and early endosomes and possibly on intermediates moving between these compartments, but not on late endosomes or lysosomes.

OCRL1 Is Present in Clathrin-coated Vesicles

To determine whether OCRL1 is present in clathrin-coated transport intermediates, cryoelectron microscopy was performed on HeLa cells stably expressing low levels of GFP-OCRL1 (Figure S1). As shown in Figure 3, a–c, GFP-OCRL1 was present in buds and vesicles with an electron-dense

Figure 2. Colocalization of OCRL1 with endosomal markers. Immunofluorescence microscopy of COS-7 cells double labeled with antibodies to OCRL1 (green) and either EEA1, TfR, or CD63 (red). Insets show magnified views of the perinuclear regions indicated by boxes in the merges. Arrowheads indicate puncta outside this region that contain EEA1 (a–c) or TfR (d–f) or that lack CD63 (g–i). Bar, 20 μ m.

appearance that also labeled with antibodies to clathrin. Buds positive for both OCRL1 and clathrin were found at the Golgi apparatus, consistent with the idea that OCRL1 is present in vesicles transporting material between the TGN and endosomes. Quantitation revealed that 25% of the clathrin-positive bud and vesicle profiles in the Golgi region contained labeling for OCRL1. We next wanted to assess the extent of OCRL1 enrichment in clathrin-coated vesicles. For this purpose, clathrin-coated vesicles were purified from human placenta and analyzed by Western blotting. Comparison of purified vesicles with a crude placental membrane fraction revealed that OCRL1 is enriched in the vesicle frac-

tion (Figure 3d). This was not due to contamination of the vesicle preparation because the Golgi proteins TGN46 and GM130 were significantly depleted or absent. Interestingly, OCRL1 was enriched in the vesicles to a greater extent than CI-MPR, suggesting it may function as part of the vesicle trafficking machinery.

OCRL1 Binds Clathrin Heavy Chain and Promotes Clathrin Assembly In Vitro

Perhaps the simplest mechanism for OCRL1 recruitment into clathrin-coated buds and vesicles is physical association with the coat. Many components of the clathrin trafficking machinery have been shown to interact with clathrin itself, and we therefore decided to test whether OCRL1 has this property. We initially tested whether full-length OCRL1 can interact with different domains of clathrin heavy chain in the yeast two-hybrid system (Figure 4a). Although there was no significant interaction between OCRL1 and the distal domain (DD, residues 493–1072) or hub domain (HD, residues 1066–1675) of clathrin heavy chain, we observed a strong interaction between OCRL1 and the clathrin heavy chain terminal domain (TD, residues 1–579). These results suggest OCRL1 binds specifically to the terminal domain of clathrin heavy chain.

This was tested further using pull-down experiments. GST-tagged clathrin TD was coupled to glutathione-Sepharose and incubated with either pig brain cytosol or a HeLa membrane extract and bound proteins detected by Western blotting. OCRL1 bound to GST-clathrin TD but not GST alone (Figure 4b). As expected, the positive control γ -adaptin bound only to GST-clathrin TD, whereas golgin-160, another peripheral Golgi protein, failed to interact with either recombinant protein. Reciprocal pull-downs were performed with beads containing GST-tagged full-length OCRL1. Endogenous clathrin bound to GST-OCRL1, but not

Figure 3. OCRL1 is enriched in clathrin-coated vesicles. (a–c) HeLa cells stably expressing GFP-OCRL1 were processed for cryo-electron microscopy and labeled with antibodies to GFP (15-nm gold) and clathrin heavy chain (10-nm gold). Arrowheads indicate coated buds and vesicle profiles labeled for both OCRL1 and clathrin. Bar, 200 nm. (d) Crude membranes (CM) and purified clathrin-coated vesicles (CCV) from human placenta were analyzed by SDS-PAGE and Coomassie Blue staining (CBB) or Western blotting (WB) with antibodies to clathrin heavy chain (CHC), γ -adaptin, CI-MPR, GM130, TGN46, or OCRL1. Equal protein (5 μ g) was loaded in each lane.

Figure 4. OCRL1 interacts directly with clathrin heavy chain. (a) Full-length OCRL1 or lamin C (control) was tested for interaction with the HD, DD, or TD of clathrin heavy chain in the yeast two-hybrid system. Interaction results in growth on high selection. (b) Pig brain cytosol (PBC) or HeLa membrane extract (HME) were incubated with glutathione beads coupled to GST or GST-clathrin TD and recovered proteins analyzed by Western blotting. (c) PBC or HME was incubated with glutathione beads coupled to GST, GST-golgin-84 head domain (g84), or GST-OCRL1 and recovered proteins analyzed by Western blotting. (d) Purified clathrin was incubated with glutathione beads coupled to GST, GST-golgin-84 head, or GST-OCRL1 and bound proteins analyzed by Coomassie Blue staining. (e) Extracts prepared from parental HeLa cells or cells stably expressing GFP-OCRL1 were incubated with either control antibodies (nonimmune IgG) or antibodies to GFP and bound proteins analyzed by Western blotting with antibodies to clathrin heavy chain (CHC) or OCRL1 (GFP-OCRL1). (f) OCRL1 constructs were tested for interaction with clathrin TD in the yeast two-hybrid system. Growth on high selection indicates interaction.

to GST-golgin-84 head domain or GST alone (Figure 4c). In contrast, neither γ -adaptin or golgin-160 bound any of the recombinant proteins. To test whether the interaction between clathrin and OCRL1 is direct, binding experiments were performed with purified proteins. As shown in Figure 4d, purified clathrin bound to immobilized GST-OCRL1 but not to GST-golgin-84 or GST alone, showing a direct interaction. To confirm that OCRL1 binds clathrin *in vivo*, coimmunoprecipitations were performed. Antibodies to GFP immunoprecipitated clathrin from cells stably expressing GFP-tagged OCRL1, but not from control cells (Figure 4e), and neither protein was precipitated by control IgG. Further evidence for an interaction *in vivo* comes from the ability of overexpressed OCRL1 to redistribute clathrin to cytoplasmic OCRL1-containing structures (Figures 7, d–f, and S8, d–f).

The clathrin binding site in OCRL1 was mapped using the yeast two-hybrid system to the region C-terminal of the 5-phosphatase domain (Figure 4f). Within this region is a sequence (LIDLE) matching the “clathrin box” consensus of L[L/I][D/E/N][L/F][D/E] responsible for binding to the terminal domain of clathrin heavy chain (Dell’Angelica, 2001). However, deletion of this sequence in OCRL1 did not abolish clathrin binding, suggesting the presence of another clathrin binding site. In support of this, further truncation

analysis showed that the region between amino acids 539–600 is required for clathrin binding (Figure 4f).

To further characterize the interaction of OCRL1 with clathrin, we decided to test whether OCRL1 can interact with preformed clathrin cages and also whether it can promote assembly of such cages *in vitro*. OCRL1 bound to preformed clathrin cages, although the binding was weak (Figure 5a). The majority of OCRL1 remained in the unbound fraction (0.65 μ M) when equimolar amounts of OCRL1 and clathrin cages were mixed (0.9 μ M each). This contrasts to the clathrin assembly protein AP180, which binds completely under these conditions (Zhang and Greene, unpublished data). Despite its poor binding to preformed clathrin cages, OCRL1 significantly stimulates the polymerization of clathrin triskelia into cages (Figure 5, b and c). Addition of increasing amounts of OCRL1 to assembly reactions shifted clathrin from the supernatant to the pellet, suggesting assembly of cages (Figure 5b), which was confirmed by electron microscopy (Figure 5c). Densitometry analysis showed that OCRL1 and clathrin were present in the cages at a stoichiometry of one OCRL1 per clathrin heavy chain (Figure 5b, pellet). Thus, OCRL1 promotes clathrin cage assembly *in vitro* and incorporates into cages

Figure 5. OCRL1 promotes clathrin cage assembly in vitro. (a) OCRL1 ($0.9 \mu\text{M}$) was incubated with or without pure clathrin baskets ($0.9 \mu\text{M}$, equivalent to $2.7 \mu\text{M}$ clathrin heavy chains) as indicated. S and T show the high-speed supernatant and total for each sample, respectively. (b) Purified clathrin ($1.45 \mu\text{M}$) was incubated with increasing concentrations of OCRL1 (0, 0.1, 0.3, 0.6, 1, 1.5, and $2 \mu\text{M}$) and dialyzed overnight at pH 6.0 followed by high-speed centrifugation. The supernatant (unassembled) and pellet (assembled) fractions were analyzed by Coomassie Blue staining. (c) Electron microscopy of OCRL1-clathrin baskets formed at $2 \mu\text{M}$ OCRL1. Bar, 100 nm.

with one molecule of OCRL1 bound per clathrin heavy chain.

Membrane Association of OCRL1 Is Independent of Clathrin

The mechanism by which OCRL1 is targeted to membranes is not known. To determine whether clathrin is required for membrane targeting, we depleted clathrin heavy chain by RNAi and analyzed the distribution of endogenous OCRL1 by immunofluorescence microscopy. As can be seen in Figure 6a, OCRL1 was still localized at the Golgi apparatus in cells lacking detectable clathrin heavy chain. FRAP analysis confirmed that the rate of Golgi membrane association of GFP-OCRL1 was not affected by clathrin depletion (Figure 6b; see also Figure S5). FRAP analysis of GFP-OCRL1 also was performed on cells depleted of potassium or incubated in hypertonic sucrose, conditions that lock the clathrin coat onto the membrane. Neither treatment affected the rate of GFP-OCRL1 recovery after photobleaching (Figure 6c), suggesting that clathrin does not play a role in the association of

OCRL1 with Golgi membranes. This is similar to AP1 and AP2, whose recruitment to the membrane also is clathrin independent (Wu *et al.*, 2003).

Overexpression of OCRL1 Disrupts Clathrin-mediated Trafficking between Endosomes and the TGN

The above-mentioned results implicate OCRL1 in clathrin-mediated trafficking at the TGN/endosome interface. To analyze this, we first transiently overexpressed full-length GFP-tagged OCRL1 and a deletion mutant lacking the entire 5-phosphatase domain (GFP-OCRL1 Δ 237-539) in HeLa and NRK cells and studied effects upon various proteins involved in trafficking at the TGN and endosomes. The 5-phosphatase deletion mutant was used as previous experiments in our laboratory had shown it affects Golgi morphology when expressed at moderate-to-high levels (expression level was determined by the relative fluorescence intensity of transfected cells), suggesting it may act as a dominant mutant (Figure 7). At low expression levels, the deletion mutant is correctly targeted to the Golgi apparatus, which retains its normal morphology, indicating that the 5-phosphatase domain is dispensable for membrane targeting (Figure S6). At higher levels of expression, the mutant accumulates in enlarged cytoplasmic structures that sometimes have a vacuolar appearance (Figures 7, 8, and S8). These structures are also seen with the wild-type protein (Figure S7), but in fewer cells compared with the mutant. Thus, both the wild-type and deletion mutant give the same morphological effects, but the mutant does so more readily. To avoid repetition, only the results obtained with the mutant are shown below.

Some of the mutant OCRL1-containing cytoplasmic structures in transfected cells contained the Golgi marker GM130 (Figure 7, a–c, arrows) and represent fragmented Golgi. The Golgi fragments were found predominantly in the perinuclear region of the cell. However, many of the OCRL1 punctate structures lacked Golgi markers (Figures 7, a–c, S8, a–c, arrowheads), indicating they are not Golgi fragments. Furthermore, the OCRL1-containing structures could be seen in cells with an intact Golgi (Figure S8, a–c), indicating they are not derived from Golgi fragments. Nearly all OCRL1-containing punctate structures did however contain clathrin heavy chain (Figures 7, d–f, and S8, d–f, arrowheads), which was redistributed to them. We also observed effects upon the distribution of clathrin adaptors. The TGN/endosome adap-

Figure 6. Clathrin is not required for association of OCRL1 with Golgi membranes. (a) Immunofluorescence microscopy of HeLa cells treated with control (lamin A) or clathrin heavy chain (CHC) siRNA for 72 h and labeled with antibodies to CHC and OCRL1. Bar, $20 \mu\text{m}$. (b) HeLa cells treated with control (lamin A) or CHC siRNA and transiently transfected with GFP-OCRL1 were analyzed by FRAP at 37°C , and the kinetics of recovery of Golgi-associated GFP-OCRL1 was plotted. (c) FRAP of GFP-OCRL1 at 37°C in transiently transfected HeLa cells depleted of K^+ or treated with hypertonic sucrose.

Figure 7. Overexpression of GFP-OCRL1 Δ 237-539 in HeLa cells. Immunofluorescence microscopy of HeLa cells transiently transfected with GFP-OCRL1 Δ 237-539 (green) and labeled with antibodies to GM130, clathrin heavy chain (CHC) or the CI-MPR (red). Arrows indicate structures containing both OCRL1 and GM130. Arrowheads indicate OCRL1 structures that either lack GM130 (a–c) or contain CHC (d–f) or CI-MPR (g–i). Transfected cells are indicated with asterisks. Bar, 20 μ m.

tors AP1 and GGA1 exhibited much-reduced perinuclear labeling in cells expressing high levels of mutant OCRL1 (Figures 8, a–c, and S8, j–o). Most of the AP1 and GGA1 seemed cytosolic, but there was some in the OCRL1-containing structures, although this varied between cells. In contrast to AP1 and GGA1, there was no effect of mutant OCRL1 expression upon AP2 distribution (Figure 8, d–f).

Overexpression of mutant OCRL1 resulted in redistribution of the CI-MPR from its typical perinuclear location into the OCRL1-containing structures (Figures 7, g–i, and S8, j–l, arrowheads). Because CI-MPR normally cycles between the TGN and endosomes, we reasoned that the GFP-OCRL1- and CI-MPR-containing structures correspond to enlarged endosomes defective in transport back to the TGN. To test this possibility, cells were first labeled with antibodies to the transferrin receptor and EEA1. As shown in Figure 8, g–i, the majority of the OCRL1 structures contain transferrin receptor, suggesting they are endosome-derived, and consistent with this, many of the structures also contain EEA1 (Figure 8, j–l). The overlap with EEA1 was less than that seen with TfR, suggesting that some of the structures may represent recycling endosomes. Interestingly, EEA1 was concentrated into fewer, larger structures in GFP-OCRL1-expressing cells compared with nontransfected cells, suggesting that overexpression of mutant OCRL1 affects the morphology of early endosomes. The endosomal nature of these structures was further confirmed in experiments where transfected cells were allowed to internalize fluorescently labeled transferrin. The internalized transferrin occurred in the OCRL1 structures after 5–15 min (Figure S9), indicating not only that they are endosomes but also that although morphologically altered, they retain the ability to receive material internalized from the cell surface.

Overexpression of OCRL1 Blocks Shiga Toxin Trafficking from Early Endosomes to the TGN

The redistribution of CI-MPR into enlarged early endosomes upon GFP-OCRL1 Δ 237-539 overexpression suggests a defect

in trafficking from early endosomes to the TGN. To directly test this possibility, trafficking of STxB subunit was analyzed in OCRL1-expressing cells. This protein can be internalized in a clathrin-independent manner and delivered to early endosomes, from where it is transported to the TGN (Mallard *et al.*, 1998). TGN delivery is dependent upon clathrin and epsinR, but independent of AP1 (Saint-Pol *et al.*, 2004). In nontransfected cells, STxB was internalized and transported efficiently to the TGN as expected (Figure 9a). In contrast, in cells expressing high levels of wild-type or mutant OCRL1, STxB was not delivered to the TGN and instead accumulated in cytoplasmic punctate structures (Figure 9a). Most of these contained GFP-OCRL1 and could be identified as endosomes by labeling with antibodies to the transferrin receptor (Figure 9b). Importantly, STxB trafficking to the TGN was blocked in cells with a normal Golgi morphology (Figure 9a, asterisk), suggesting that the trafficking defect is not a consequence of Golgi fragmentation. These results suggest that expression of OCRL1 at high levels blocks STxB trafficking from early endosomes to the TGN.

Depletion of OCRL1 Causes a Partial Redistribution of CI-MPR and TGN46 to Endosomes

To further assess the role of OCRL1 in trafficking between endosomes and the TGN, we used RNA interference to deplete the protein. For these experiments, we used a HeLa cell line stably expressing a CD8-CI-MPR reporter that has previously proven useful for the *in vivo* analysis of CI-MPR trafficking (Seaman, 2004). As shown in Figure 10a, cellular levels of OCRL1 were depleted by ~90% in RNAi-treated cells, but not in mock-transfected cells or cells treated with a control RNA duplex targeting lamin A. RNAi treatment had no effect upon levels of other proteins analyzed, including clathrin heavy chain (Figure 10a). Depletion of OCRL1 had little discernible effect upon Golgi structure because golgin-97 (Figure 10b) and GM130 (our unpublished data) looked normal in RNAi-treated cells. There was, however,

Figure 8. Overexpression of GFP-OCRL1 Δ 237-539 affects the distribution of γ -adaptin and early endosomal markers. Immunofluorescence microscopy of HeLa cells transiently transfected with GFP-OCRL1 Δ 237-539 (green) and labeled with antibodies to γ -adaptin, α -adaptin, TfR, or EEA1 (red). Arrowheads indicate OCRL1 structures that contain TfR (g-i) or EEA1 (j-l). Transfected cells are indicated with asterisks. Bar, 20 μ m.

an effect upon the distribution of both TGN46 and CD8-CI-MPR, which occurred in numerous puncta in addition to the perinuclear Golgi staining seen in control cells (Figure 10b, arrows). This was due to loss of OCRL1 as the same results were obtained with a separate Smartpool RNA duplex set (Dharmacon Research) that also depleted OCRL1 by $\sim$ 90% (our unpublished data). Double-labeling confirmed both TGN46 and CD8-CI-MPR were in the same puncta (Figure 10c). As shown in Figure 10d, many of the TGN46-positive puncta contained EEA1, identifying them as early endosomes. Consistent with this, there was also a high degree of overlap with transferrin receptor (Figure 10d). Colocalization of TGN46 with the transferrin receptor was more extensive than with EEA1, suggesting some of the puncta may correspond to recycling endosomes (Figure 10d). These results suggest that recycling of TGN46 and CI-MPR from endosomes to the TGN is reduced in OCRL1-depleted cells, consistent with a role for OCRL1 in endosome to Golgi trafficking.

DISCUSSION

The underlying mechanism by which loss of OCRL1 function causes Lowe syndrome is unknown, primarily due to our poor understanding of the cellular role of the protein. Previous work has revealed a Golgi localization for OCRL1,

although it also has been reported on lysosomes (Zhang *et al.*, 1998; Dressman *et al.*, 2000). We localized endogenous OCRL1 to the Golgi apparatus and early endosomes, but failed to detect significant amounts on lysosomes. This is in agreement with Nussbaum and colleagues (Dressman *et al.*, 2000) and supports the recent findings of Ungewickell *et al.* (2004) who reported the presence of GFP-tagged OCRL1 on early endosomal structures. Our results indicate that the localization of GFP-tagged OCRL1 to endosomes is not an artifact of overexpression. Importantly, using both electron microscopy and subcellular fractionation, we also found that OCRL1 is present in clathrin-coated transport intermediates, again consistent with the recent findings of Ungewickell *et al.* (2004). Together, these results suggest OCRL1 may regulate clathrin-mediated trafficking between the TGN and endosomes.

To determine whether OCRL1 regulates TGN/endosome trafficking, we overexpressed GFP-tagged wild-type OCRL1 and a mutant lacking the 5-phosphatase domain in mammalian cells. Both constructs gave similar morphological effects, although the mutant was more potent at inducing them. OCRL1 overexpression resulted in a redistribution of clathrin and the CI-MPR to large cytoplasmic structures containing endosomal markers, most likely corresponding to enlarged endosomes defective in retrograde transport of CI-MPR back to the TGN. Consistent with this, we see a block in delivery of

Figure 9. GFP-OCRL1 overexpression blocks Shiga toxin trafficking from early endosomes to the TGN. HeLa cells were either not transfected (control) or transiently transfected with GFP-tagged OCRL1 wild-type (WT) or the $\Delta 237-539$ mutant (green) and allowed to internalize STxB for 45 min at 37°C and processed for immunofluorescence microscopy. (a) Cells were labeled with antibodies to STxB (red) and the Golgi marker CTR433 (blue). The asterisk indicates a transfected cell with normal Golgi morphology that is defective in STxB transport to the Golgi. (b) Cells were labeled with antibodies to STxB (red) and TfR (blue). Insets show the merge of the boxed regions between STxB (red) and TfR (blue) with regions of overlap in pink. Bar, 20 μm .

STxB from early endosomes to the TGN at high levels of OCRL1 expression. What is the mechanism underlying these effects? Changes in lipid levels are unlikely to be entirely responsible because the wild-type gives similar effects to the mutant, albeit in fewer cells compared with the mutant. An alternative explanation is that overexpression interferes with another aspect of OCRL1 function, such as association with trafficking components. We also cannot exclude the possibility that the changes are brought about indirectly, either through titrating components such as clathrin away from their normal

site of action or through blocking interactions between other trafficking components, for example, association of clathrin with its various accessory proteins.

A role for OCRL1 in endosome to TGN transport is further supported by our RNAi results. OCRL1 depletion resulted in a partial redistribution of CD8-CI-MPR and TGN46 to early endosomes, suggesting a change in the rate of cycling between endosomes and the TGN. This effect is likely due to changes in phosphoinositides, because both Golgi and endosome levels of PI(4,5)P₂ are elevated and PI(4)P is reduced

Figure 10. RNAi-mediated depletion of OCRL1 redistributes CI-MPR and TGN46 to endosomes. HeLa cells stably expressing CD8-CI-MPR were treated with no siRNA, control siRNA (lamin A), or OCRL1 siRNA for 72 h and analyzed by Western blotting (a) or immunofluorescence microscopy (b–d). (b) Cells were labeled with antibodies to OCRL1 (our unpublished data) and golgin-97, TGN46, or CD8 (CD8-CI-MPR). Images are of cells with no detectable OCRL1 staining. (c) Cells were labeled with antibodies to TGN46 and CD8 (CD8-CI-MPR). (d) Cells were double labeled with antibodies to TGN46 and either EEA1 (top) or TfR (bottom). Bar, 20 μ m.

in OCRL1-depleted cells (Konstantakopoulos, unpublished observations). This supports a role for PI(4,5)P₂ metabolism in TGN/endosome trafficking. This was suggested by previous work on *Saccharomyces cerevisiae* Sjl3p/Inp53p (Ha *et al.*, 2003). Although more related to mammalian synaptojanin than to OCRL1, Sjl3p/Inp53p seems to act at the TGN/endosome interface rather than at the plasma membrane. Mutation of the Sjl3p/Inp53p PI(4,5)P₂ 5-phosphatase domain affects the rate of delivery of certain proteins from the TGN to endosomes, implicating this activity in TGN/endosome traffic (Ha *et al.*, 2003). Interestingly, like OCRL1, Sjl3/Inp53p also binds clathrin, and binding is not entirely dependent on a type I clathrin box (Ha *et al.*, 2003). How might PI(4,5)P₂ regulate trafficking at the TGN/endosome interface? This could occur through PI(4,5)P₂-binding proteins such as dynamin 2 that have been shown to participate in formation of TGN-derived clathrin-coated transport intermediates (Jones *et al.*, 1998). Alternatively, it may act as a precursor for generation of PI(4)P, which in turn will recruit the clathrin adaptors AP1 and epsinR (Kalthoff *et al.*, 2002; Wasiak *et al.*, 2002; Hirst *et al.*, 2003; Mills *et al.*, 2003; Wang *et al.*, 2003). OCRL1 could therefore play a positive or negative role in formation of clathrin-coated intermediates. It also may regulate downstream events such as vesicle uncoating, as has been reported for synaptojanin (Cremona *et al.*, 1999), or in coordination of trafficking with the actin cytoskeleton (Stamnes, 2002). Further work is required to distinguish between these possibilities.

More direct analysis of endosome to TGN trafficking in the RNAi-treated cells by using StxB or CD8 antibody bound to the CD8-CI-MPR reporter failed to reveal a significant effect (Choudhury and Lowe, unpublished observations).

This suggests a mild inhibition of endosome to TGN trafficking such that in single round trafficking assays, there is little discernible difference, but over longer times the inhibition manifests itself as a partial redistribution of cycling proteins such as TGN46 and CD8-CI-MPR to endosomes. This could reflect a minor role for OCRL1 in this trafficking step, alternative trafficking pathways that are independent of OCRL1, or functional compensation by a related enzyme(s). The PI(4,5)P₂ 5-phosphatase Inpp5b shares ~45% sequence identity with OCRL1 (Jefferson and Majerus, 1995). Single OCRL1 or Inpp5b knockouts in mice are viable with little detectable phenotype, yet the double knockout results in embryonic lethality (Janne *et al.*, 1998), suggesting these enzymes may functionally compensate for each other under certain conditions. Thus Inpp5b may be able to partially compensate for loss of OCRL1 in HeLa cells, yet not in the tissues affected in Lowe syndrome. This explanation also would be compatible with the observation that overexpressed OCRL1 blocks StxB trafficking, because the mutant likely acts in a dominant manner that cannot be compensated for by other PI(4,5)P₂ 5-phosphatases. Similarly, we also failed to notice major changes in TGN to endosome transport in OCRL1-depleted cells (measured using cathepsin-D maturation; Choudhury and Lowe, unpublished observations). However, the large amount of OCRL1 at the TGN and its presence in TGN-derived clathrin buds/vesicles suggests it does have a function there. Effects upon TGN to endosome trafficking would be difficult to detect by studying distribution of cycling proteins because these can bypass the TGN-to-endosome route by traveling via the plasma membrane. This ability to bypass the TGN-to-endosome route also could account for the previous observations

that depletion of epsinR does not affect cathepsin-D maturation yet causes redistribution of the CI-MPR to early endosomes (Hirst *et al.*, 2003; Saint-Pol *et al.*, 2004) and depletion of PI4KII α results in CI-MPR redistribution to early endosomes (Wang *et al.*, 2003).

We have identified OCRL1 as a new binding partner for the terminal domain of clathrin heavy chain. During the preparation of this article, Ungewickell *et al.* reported a similar finding (Ungewickell *et al.*, 2004), although in this article a direct interaction was not formally shown. OCRL1 possesses a putative type I clathrin box near its C terminus. Mutation of this sequence reduced clathrin binding by only ~50% in pull-down experiments (Ungewickell *et al.*, 2004). This is in agreement with our finding that an additional clathrin binding site exists in OCRL1. We could map this site to the region between residues 539–600 of OCRL1. This region is devoid of other known clathrin-binding signals such as the DLL or PWXXW motifs (Morgan *et al.*, 2000; Miele *et al.*, 2004), suggesting the existence of a novel binding site. Although OCRL1 bound weakly to preformed clathrin cages, it was able to promote clathrin assembly into cages *in vitro*. This raises the possibility that OCRL1 may have a dual role in clathrin bud formation. It may modulate phosphoinositide levels to regulate association of accessory factors and at the same time promote assembly of clathrin into baskets. At present, it is not clear whether OCRL1 has significant clathrin assembly properties *in vivo*. It may contribute to the process, but it is unlikely to be essential because both AP1 (Pearse, 1989) and epsinR (Wasiak *et al.*, 2002) also have clathrin assembly activity.

How a defective OCRL1 protein causes Lowe syndrome is unknown. In this article, we have shown data supporting a role for OCRL1 in clathrin-mediated transport between endosomes and the TGN. This raises the possibility that trafficking between these compartments is defective in the tissues affected in Lowe syndrome and that defective trafficking may be responsible for the clinical symptoms of the disease. Interestingly, Lowe syndrome patients have elevated levels of lysosomal enzymes in their serum (Ungewickell and Majerus, 1999), consistent with the possibility of defects in CI-MPR trafficking. The priority now is to determine whether such trafficking is indeed perturbed in Lowe syndrome patients.

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