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All known patient mutations in the ASH-RhoGAP domains of OCRL affect targeting and APPL1 binding

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Abstract

Mutations in the inositol 5-phosphatase OCRL are responsible for Lowe syndrome, an X-linked disorder characterized by bilateral cataracts, mental retardation, neonatal hypotonia, and renal Fanconi syndrome, and for Dent disease, another X-linked condition characterized by kidney reabsorption defects. We have previously described an interaction of OCRL with the endocytic adaptor APPL1 that links OCRL to protein networks involved in the disease phenotype. Here we provide new evidence showing that among the interactions which target OCRL to membranes of the endocytic pathway, binding to APPL1 is the only one abolished by all known disease-causing missense mutations in the ASH-RhoGAP domains of the protein. Furthermore, we demonstrate that APPL1 and rab5 independently contribute to recruit OCRL to enlarged endosomes induced by the expression of constitutively active Rab5. Thus, binding to APPL1 helps localize OCRL at specific cellular sites, and disruption of this interaction may play a role in disease.

Introduction

Lowe Syndrome, also known as Oculocerebrorenal Syndrome of Lowe (OCRL), is an X-linked disorder seen in approximately 1 in 200,000 births. Patients with Lowe Syndrome have bilateral congenital cataracts, mental retardation, neonatal hypotonia, and renal Fanconi syndrome, which is characterized by impaired reabsorption of low molecular weight proteins, ions, and metabolites in the kidney proximal tubule.

Lowe Syndrome is mediated by mutations in the gene encoding a member of the inositol-5-phosphatase protein family, OCRL[1]. Recently, mutations in this gene have also been

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described in patients with Dent disease, a condition involving kidney reabsorption defects similar to those observed in Lowe syndrome [2]. Inositol 5-phosphatases dephosphorylate the five position of inositol polyphosphates and of phosphoinositides [3]. Phosphoinositides are phosphorylated metabolites of phosphatidylinositol and play a key role in cell physiology, including signaling, cytoskeletal regulation, and membrane traffic [4]. The inositol 5-phosphatase gene family includes synaptojanin and INPP5B, which have been implicated in the coupling of PI(4,5)P₂ and PI(3,4,5)P₃ dephosphorylation to endocytosis [5,6]. One role of INPP5B, which is a close homologue of OCRL, is to function in a dephosphorylation cascade along the endocytic pathway leading from PI(3,4,5)P₃ at the cell surface to PI(3)P, the signature phosphoinositide of endosomes [6].

In OCRL and INPP5B the central inositol 5-phosphatase domain, which uses PI(4,5)P₂ and PI(3,4,5)P₃ as the preferred substrates, is flanked at the C-terminal side by an ASH domain and a catalytically inactive RhoGAP-like domain [7,8]. OCRL was initially localized to the Golgi complex [9,10]. Recently, OCRL was also found to have a prominent localization on endosomes and to be important at early stations of the endocytic pathway, including clathrin-coated pits [11], consistent with its ability to bind clathrin, the endocytic clathrin adaptor AP-2, the endosomal protein Rab5, and the plasma membrane associated Rho family GTPases Rac and Cdc42 [12-15]. In addition, OCRL was also found to bind to, and colocalize on endosomes with, APPL1, an endosomal adaptor protein and another Rab5 interactor [11].

Both Lowe Syndrome and Dent disease can be caused by truncating or missense mutations in OCRL. The overwhelming majority of missense mutations are localized to the 5-phosphatase domain (<http://research.nhgri.nih.gov/lowe/>), underscoring the importance of the 5-phosphatase activity of this protein. A small number of missense mutations are located in the COOH-terminal region (ASH and RhoGAP-like domains) (<http://research.nhgri.nih.gov/lowe/>) and thus are not expected to prevent the expression of a catalytically active protein. We have previously shown that three patient mutations in this region abolished binding to APPL1, while preserving the ability of the protein to bind a Rho-GTPase and clathrin, two other COOH-terminal region interactors [11]. We have now determined the impact on protein interactions of the other two reported missense mutations in the COOH-terminal region of OCRL as well as of a new mutation that we report here. We have also further examined the role of mutations in the ASH-RhoGAP-like domain of OCRL on the intracellular targeting of the protein. The results of this analysis provide new evidence for a critical physiological role of the interaction of OCRL with APPL1.

Materials and Methods

Plasmids and antibodies

Wild type OCRL, APPL1, Myc-tagged RacV12A, and Rab5Q79L plasmids in pcDNA3.1 and wild type APPL1 in EGFP-C1 were described in Erdmann et al. 2007 [11]. pGEX-6P-1 plasmids encoding GST-fusions of the COOH-terminal region of OCRL (amino acids 564 to 901 of human OCRL) and of the OCRL binding region of APPL1 (amino acids 403-413 of human APPL1) were also described in Erdmann et al. 2007, while a Rab5a construct in the same vector was a kind gift of Jan Modregger from our lab. Patient mutations were introduced into the OCRL COOH-terminal region or into full length HA-OCRL using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The following antibodies were obtained from commercial sources: rabbit anti-HA (Covance), mouse anti-HA-Alexa 488 (Invitrogen), mouse anti-Myc (Santa Cruz), rabbit anti-Myc (Upstate), goat anti-APPL1 (Abcam). Mouse anti-clathrin heavy chain (TD1) was obtained from ATCC.

Identification of the OCRL mutation

DNA was extracted by standard methods. The 23 coding exons and their flanking intronic sequences of OCRL1 gene were amplified from genomic DNA using forward and reverse primers designed according to nucleotide sequences of OCRL1. PCR amplified fragments were purified and sequenced on an ABI 3100 automated sequencer (Applied Biosystem). We identified a missense mutation in exon 18 due to substitution of T >G at nucleotide 1986 (c. 1986T>G) (based on the cDNA sequence described in the Lowe Syndrome Mutation Database at the NHGRI). This causes a substitution of cysteine to tryptophan at position 662 of OCRL according to the above mentioned sequence (or 679, according to the sequence reported as NP_000267.2).

Cell Culture, Transfection, and Microscopy

Cos-7 cells (ATCC, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were grown at 37°C with 5% CO₂. Transfections were carried out with Fugene (Roche). Fluorescence microscopy was performed by standard epifluorescence (Axioplan 2 Imaging Microscope) on cells fixed in 4% formaldehyde in phosphate buffer.

Pulldown experiments

GST fusion proteins were purified on glutathione sepharose using standard protocols. Adult rat brains were homogenized in lysis buffer (PBS, 0.5% Triton [v/v], protease inhibitor mixture (Roche) followed by ultracentrifugation (100,000 × g, 45 min, 4°C). Cos-7 cells expressing proteins of interest were lysed. The resulting extracts in lysis buffer were incubated on ice for 10 minutes, and centrifuged at 18,000 × g for 10 minutes. GST pull-downs from rat brain or cell extracts were performed using standard protocols. Prior to its use in pulldown assays, GST-Rab5 was converted to its active (GTP bound) form by preincubation with GTPγS as described in Christoforidis and Zerial 2000 [16].

Results

OCRL patient mutations abolish binding to APPL1

The location in the COOH-terminal region of human OCRL (ASH-RhoGAP-like domain) of the five reported mutations and of a novel mutation that we have identified in an Italian family (C679W) are shown in Fig. 1A. We have previously shown that the ΔE585, I768N, and A797P mutations abolish binding to, and co-localization with APPL1, while retaining binding to Rac and clathrin [11]. Mutant forms of the COOH-terminal region of OCRL harboring the three other mutations, V577E [17], L687P (B. Roa unpublished data, <http://research.nhgri.nih.gov/lowe/>), and C679W, were tested for protein interactions in GST pulldown assays. None of these mutant proteins bound to APPL1 (Fig. 1B). In contrast, binding to clathrin and Rac was preserved (Fig. 1B).

When HA-tagged wild type OCRL and Myc-tagged APPL1 were cotransfected in COS-7 cells, the two proteins colocalized on a subset of peripheral endosomes as reported. However, HA-tagged OCRL harboring the three patient mutations not previously tested did not colocalize with APPL1, despite the continued punctate distribution of APPL1 at the periphery and the residual punctate distribution of OCRL, at least in the case of the L687P and C679W mutations (Fig. 2). Thus, all six disease-causing mutations in the COOH-terminal region of OCRL abolish binding to APPL1 and disrupt co-localization with this protein.

Binding to Rab5 is preserved

Rab5 is an important interactor of OCRL [14]. Rab5 binding was shown to involve the COOH-terminal portion of the inositol-5-phosphatase domain of OCRL as well as downstream sequences, but the effect of patient mutations on this interaction has not been examined. We used GST-pulldowns to test the binding of immobilized Rab5-GTP to full length OCRL expressed in COS-7 cells. Both wild type OCRL and mutant OCRL harboring three of the patient mutations shown in Fig. 1A (Δ E585, I768N, and A797P) were analyzed. The I768N and A797P mutants retained the ability to bind Rab5, while the Δ E585 mutant revealed decreased Rab5 binding (Fig. 3A). Thus, a defect in Rab5 binding could not explain the disease phenotype since it is not a common feature of OCRL with patient mutations.

Hyvola et al. introduced targeted mutations into OCRL which partially disrupted interaction with Rab5 in vitro, including Rab5 [14], a finding which we revisited for the G664D mutant and confirmed (Fig. 3A). The Rab5 G664D mutant was reported to have reduced, but not absent, colocalization with enlarged endosomes induced by transfection of constitutively active Rab5 (Rab5Q79L). Residual targeting could be due to a preserved interaction with APPL1. Thus, we assessed the binding of the G664D mutant to APPL1. A GST-fusion of the 11-mer minimal binding sequence of APPL1 was indeed able to pull down both wild type- and G664D mutant- HA-tagged OCRL (Fig. 3B).

Independent roles of APPL1 and Rab5 in the localization of OCRL

The findings described above are consistent with a scenario in which interactions with APPL1 and Rab5 independently contribute to OCRL localization. This possibility was further assessed by cell cotransfection experiments. Both EGFP-APPL1 (Fig. 4A) and HA-tagged wild type OCRL (Fig. 4B), accumulated on the enlarged endosomes induced by transfection of constitutively active Rab5 (Rab5Q79L) as reported previously [11,14,18]. OCRL G664D, i.e. the Rab5 binding OCRL mutant, also partially localized to these structures (Fig. 4B) as reported [14]. Similarly, HA-OCRL with patient mutations I768N or A797P, which abolish binding to APPL1, but retain the ability to bind Rab5, co-localized with Rab5 on these enlarged endosomes (Fig. 4B). In contrast, HA-OCRL with patient mutation Δ E585, which abolishes binding to APPL1 and shows a decrease in Rab5 binding comparable to the decrease observed for Rab5 binding mutant G664D, was not present on the enlarged endosomes (Fig. 4B). These results strongly support the hypothesis that APPL1 and Rab5 play independent roles in the recruitment of OCRL to Rab5 positive organelles.

Discussion

Together with our previous report [11], the present study indicates that a common characteristic shared by all six known disease-causing missense mutations in the COOH-terminal region of OCRL is the disruption of the interaction of this protein with APPL1. Binding to Rac and clathrin is preserved by all six patient mutations while the interaction with Rab5 is preserved by at least two of the mutant OCRLs. Therefore, APPL1 is the only protein whose binding has now been shown to be consistently disrupted by patient missense mutations in the COOH-terminal region of OCRL. Of note, many OCRL mutations that result in a truncation of the COOH-terminal region, i.e. the ASH-RhoGAP domain region, also produce disease (<http://research.nhgri.nih.gov/lowe/>). Since we have shown that even very short truncations of this region of OCRL/INPP5B disrupt APPL1 binding [11], we conclude that lack of APPL1 binding is a common feature of all OCRL mutations in this region.

In agreement with biochemical data, patient missense mutations in the COOH-terminal region of OCRL impair colocalization of OCRL with APPL1 on endosomes. These mutations also prevent targeting of OCRL to the enlarged endosomes induced by constitutively active Rab5,

if Rab5 binding is also impaired. Thus, such mutations have an important effect on the precise localization of OCRL in the endocytic pathway.

The physiological function of phosphoinositide metabolizing enzymes requires the activity of their catalytic domains and targeting/regulatory actions of flanking regions. This also applies to OCRL, whose action on its preferred substrates, PI(4,5)P₂ and PI(3,4,5)P₃, requires targeting to specific sites of action. Many OCRL mutations produce disease by impairing enzymatic activity or reduced or absent protein level. We suggest that mutations in the COOH-terminal portion of OCRL produce disease by preventing correct localization of the protein and that disruption of the interaction with APPL1 plays a key role in pathogenesis.

A disruption in APPL1 binding would also disconnect OCRL from a protein network potentially linked to the disease phenotype [11]. Through APPL1, OCRL can be linked to the small scaffold protein GIPC and thus to megalin [11], the scavenger receptor which in kidney mediates the uptake of low molecular weight proteins [19]. Genetic disruption of both GIPC and megalin in mice yields low molecular weight proteinuria, a defect also seen in Lowe Syndrome and Dent Disease [20,21]. Additionally, APPL1 could link OCRL to TrkA, which could help explain the cognitive manifestations of Lowe Syndrome [11,22,23].

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Figure 1. Patient mutations in OCRL specifically abolish binding to APPL1

(A) Location of patient mutations in OCRL. The crystal structure of the COOH-terminus of OCRL (ASH and RhoGAP domains) is from Erdmann et al 2007 [11]. (B and C) Lysates of rat brain (for APPL1 and clathrin heavy chain) or Cos-7 cells transfected with Myc-tagged RacV12A were incubated with GST, or GST-fusions of wild type or mutant COOH-terminal region of OCRL. Affinity-purified material was analyzed by western blotting for the presence of APPL1, clathrin heavy chain, or Myc-Rac.

Figure 2. Patient mutations in OCRL abolish colocalization with APPL1

Dual color immunofluorescence of cells expressing Myc-APPL1 (red) and wild type HA-OCRL or HA-OCRL with patient mutations (green). Wild type OCRL is localized on spots throughout the cell and co-localizes with APPL1 on peripheral spots. Each of the patient mutations abolishes co-localization with APPL1 though APPL1-positive spots persist in the periphery. Both low and high power views are shown for wild type and for the L687P mutation. Only high power views are shown for the V577E and for the C679W mutation.

Figure 3. Effect of patient OCRL mutations on Rab5 binding and of a Rab5 binding mutation on APPL1 binding

GST pulldowns from lysates of Cos-7 cells transfected with wild type or mutant HA-OCRL. **(A)** Anti-HA western blotting of material affinity purified on GST or GST-Rab5-GTP, showing that only one of three patient mutants tested has reduced Rab5 binding and confirming the reduced Rab5 binding of the G664D mutation, an experimental targeted mutation [14]. **(B)** Anti-HA western blotting of material affinity purified on GST or on a GST fusion of the OCRL minimal binding region of APPL1 (amino acid 403-413 of human APPL1), demonstrating that the experimentally generated Rab5-binding OCRL mutant still binds APPL1.

Figure 4. Only a mutation which impairs both APPL1 and Rab5 binding prevents targeting of OCRL to the enlarged endosomes induced by constitutively active Rab5
 (A) EGFP-APPL1 and Myc-Rab5Q79L co-localize at enlarged endosomes. (B) Wild type HA-OCRL, HA-OCRL with the Rab5 binding mutation (G664D), and HA-OCRL with patient mutations (I768N and A797P) which abolish APPL1 binding co-localize with Myc-Rab5Q79L on enlarged endosomes. HA-OCRL Δ E585, which lacks APPL1 binding and has much reduced Rab5 binding, does not co-localize with Myc-Rab5Q79L on enlarged endosomes.