The protein deficient in Lowe syndrome is a phosphatidylinositol-4,5-bisphosphate 5-phosphatase

(signal transduction/inherited disease/X chromosome-linked/oculocerebrorenal syndrome)

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ABSTRACT Lowe syndrome, also known as oculocerebrorenal syndrome, is caused by mutations in the X chromosome-encoded *OCRL* gene. The *OCRL* protein is 51% identical to inositol polyphosphate 5-phosphatase II (5-phosphatase II) from human platelets over a span of 744 aa, suggesting that *OCRL* may be a similar enzyme. We engineered a construct of the *OCRL* cDNA that encodes amino acids homologous to the platelet 5-phosphatase for expression in baculovirus-infected Sf9 insect cells. This cDNA encodes aa 264–968 of the *OCRL* protein. The recombinant protein was found to catalyze the reactions also carried out by platelet 5-phosphatase II. Thus *OCRL* converts inositol 1,4,5-trisphosphate to inositol 1,4-bisphosphate, and it converts inositol 1,3,4,5-tetrakisphosphate to inositol 1,3,4-trisphosphate. Most important, the enzyme converts phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 4-phosphate. The relative ability of *OCRL* to catalyze the three reactions is different from that of 5-phosphatase II and from that of another 5-phosphatase isoenzyme from platelets, 5-phosphatase I. The recombinant *OCRL* protein hydrolyzes the phospholipid substrate 10- to 30-fold better than 5-phosphatase II, and 5-phosphatase I does not cleave the lipid at all. We also show that *OCRL* functions as a phosphatidylinositol 4,5-bisphosphate 5-phosphatase in *OCRL*-expressing Sf9 cells. These results suggest that *OCRL* is mainly a lipid phosphatase that may control cellular levels of a critical metabolite, phosphatidylinositol 4,5-bisphosphate. Deficiency of this enzyme apparently causes the protean manifestations of Lowe syndrome.

Lowe syndrome, also known as oculocerebrorenal syndrome, is an X chromosome-linked disorder that is characterized by severe mental retardation, areflexia, hypotonia, a variety of eye abnormalities, and a renal Fanconi syndrome with impaired reabsorption of glucose, amino acids, phosphate urate, and bicarbonate. Patients have renal tubular acidosis, metabolic bone disease, and growth failure. The eye abnormalities include cataracts, decreased acuity, and glaucoma (1, 2). The protean manifestations of the disorder suggest that the defect involves a gene that mediates a critical cellular function shared by many cell types and organs. In 1992 Nussbaum and co-workers (3) identified a candidate Lowe syndrome gene by positional cloning of X chromosome breakpoints in two affected females. The predicted protein, designated *OCRL*, was found to be 51% identical over a span of 744 aa to inositol polyphosphate 5-phosphatase type II (5-phosphatase II) (4), an enzyme of the inositol phosphate signaling pathway that was originally isolated from human platelets (5) and that catalyzes the hydrolysis of the 5-position phosphate from both inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] and inositol 1,3,4,5-tetrakisphosphate [$\text{Ins}(1,3,4,5)\text{P}_4$]. These same substrates are also utilized by a more distantly related platelet isoenzyme

inositol polyphosphate 5-phosphatase type I (5-phosphatase I) (6–9). These soluble inositol phosphates are involved in calcium signaling reactions, and thus the 5-phosphatase enzymes function in signal-terminating reactions (10). Recently it has been shown that 5-phosphatase II also hydrolyzes the 5-phosphate from the phospholipid phosphatidylinositol 4,5-bisphosphate [$\text{PtdIns}(4,5)\text{P}_2$] (9, 11). We now report that *OCRL*, the protein encoded by the Lowe syndrome locus, also is a $\text{PtdIns}(4,5)\text{P}_2$ 5-phosphatase. *OCRL* also hydrolyzes the other 5-phosphatase substrates, albeit less well than the other isoenzymes.

MATERIALS AND METHODS

[^{32}P]Orthophosphate was from ICN. Horseradish peroxidase-linked anti-rabbit IgG and ECL Western blotting detection reagents were purchased from Amersham Life Sciences. $\text{PtdIns}(4,5)\text{P}_2$ was purchased from Boehringer Mannheim and silica gel 60 TLC plates (20 cm $\times$ 20 cm $\times$ 0.2 mm) were from Merck. The pVL1393 baculoviral expression vector and BaculoGold transfection kit were from Pharmingen. [^{32}P]Ins(1,4,5) P_3 was prepared as described (12). ^3H -labeled Ins(1,4,5) P_3 , Ins(1,3,4,5) P_4 , Ins(1,4) P_2 , and $\text{PtdIns}(4,5)\text{P}_2$ were purchased from DuPont/NEN. The Adsorbosphere SAX HPLC column was purchased from Alltech.

Construction of Recombinant *OCRL* Baculovirus Transfer Vector. The *OCRL* cDNA cloned into the *Bam*HI site of the polylinker region in a pBluescript SK (Stratagene) vector (ps.OCRLB-1) was provided by R. L. Nussbaum (National Institute of Health) (3). Two *OCRL* cDNAs were constructed for subcloning into a baculovirus expression vector by a PCR strategy. A sense PCR primer included a *Bam*HI linker followed by *OCRL* nt 806–828 (5'-CGGGATCCAAAATGCTTCCACGTGAAAAAGAAGC-3'); the antisense primer contained the complement of nt 1530–1550, where an *Sph* I site is located (5'-CCTCATTTGGCATCAGGCATGC-3'). This PCR product encodes amino acids Met²⁶⁴ to Met⁵⁰⁶ after digestion with *Sph* I and *Bam*HI. The second cDNA was constructed by PCR using the same antisense primer and a sense primer that is identical to that shown above with insertion of nucleotides encoding seven histidine residues following nt 806–808. (5'-CGGGATCCAAAATGCACCACCACCACCACCACCTTCCACGTGAAAAAGAAGC-3'). The two PCR products were digested with *Bam*HI/*Sph* I and subcloned into the same region of ps.OCRLB-1 to replace the original aa 1–506 of the OCRLB-1 cDNA. The resulting plasmids ps.OCRL4 and ps.OCRL5 (with histidine tag) were sequenced throughout the PCR region to exclude mutations (Sequenase 2.0; United States Biochemical). ps.OCRL4 and ps.OCRL5 were subsequently subcloned between the *Bam*HI and *Xba* I sites of the baculovirus transfer vector pVL1393 (PharMingen).

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Abbreviation: 5-phosphatases I and II, inositol polyphosphate 5-phosphatases I and II.

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Production of Recombinant Baculovirus Expressing OCRL Protein. Sf9 cells were maintained at 27°C in TNM-FH insect medium supplemented with 10% fetal bovine serum and 100 µg of gentamicin per ml. Recombinant baculoviruses expressing OCRL were produced in infected Sf9 cells. Single plaques were isolated from the primary virus stock, and amplified to a titer of 10^8 plaque-forming units/ml by the standard protocol (PharMingen).

Baculovirus Expression and Labeling. Recombinant baculoviruses containing either 5-phosphatase II cDNA (9), ps.OCRL4, ps.OCRL5, or a control construct encoding protein-tyrosine phosphatase Meg-01 (13) were grown in TNM-FH medium with 10% heat-inactivated fetal bovine serum and 100 µg of gentamicin per ml (14).

For studies on 32 P-labeling of Sf9 cell phospholipids, 3×10^6 Sf9 cells were infected with baculovirus encoding the above-mentioned constructs. Three days after infection the cells were harvested from 60-mm dishes and resuspended in 450 µl of phosphate-free Grace's insect medium containing 1 mCi (37 MBq) of [32 P]phosphate at room temperature. Samples of 100 µl were taken after various times and added to 100 µl of 1 M HCl. 32 P-labeled phosphatidylinositols were further extracted with chloroform/methanol, separated by TLC, and measured as described (15).

Recombinant proteins were harvested from Sf9 cells grown for 3 days after infection. Cells were washed with phosphate-buffered saline and then suspended in 50 mM Mes/Tris buffer (pH 6.5) containing 3 mM MgCl₂, 2 mM EGTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 10 µg of aprotinin per ml, 10 µg leupeptin per ml, and 10 µg of pepstatin A per ml. The cells were sonicated on ice twice for 20 sec at 200 W, and the sonicate was centrifuged at $16,000 \times g$ for 20 min. The supernatant was frozen and thawed once and recentrifuged to eliminate a large precipitate. The secondary supernatant was harvested and stored at -20°C. The Sf9 supernatant containing ps.OCRL4 proteins was used in enzyme assays. The supernatant from ps.OCRL5 was affinity purified on a nickel chelating resin (Ni-NTA) by the manufacturer's protocol (QIAexpress purification kit; Qiagen, Chatsworth, CA). Essentially homogeneous OCRL was eluted from the column with 50 mM sodium phosphate, pH 6.0/0.3 M NaCl/10% (vol/vol) glycerol/0.14 M imidazole.

Enzyme Assays and Antibody Production. Assay of 5-phosphatase activity using [32 P]Ins(1,4,5)P₃ and [3 H]Ins(1,3,4,5)P₄ as substrate was as described (5, 6). Assay of 5-phosphatase activity using [3 H]PtdIns(4,5)P₂ was as described (7) except that PtdIns(4,5)P₂ was at a concentration of 50 µM and that the assay was conducted at 37°C. The products formed from incubation of OCRL protein with Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ were separated by HPLC on an Adsorbosphere SAX column equilibrated in 20 mM NH₄H₂PO₄ at pH 3.5. Products were eluted with a linear gradient from 0 to 0.75 M NH₄H₂PO₄ over 100 min. 0.5-ml fractions were collected, and radioactivity was measured in a liquid scintillation counter.

We prepared rabbit polyclonal antibodies to a peptide that represents the carboxyl terminus of OCRL protein (RAIQ-FLLGFLGSEED) (Pocono Rabbit Farm, Cacadenis, PA). For immunoblot analysis, proteins were separated by SDS/PAGE, transferred to nitrocellulose sheets and incubated with the anti-peptide antiserum at 1:3000 dilution. Detection was performed by enhanced chemiluminescence (ECL; Amersham) or by staining with Coomassie brilliant blue. The concentration of affinity-purified OCRL protein was estimated by Bio-Rad Coomassie G200-based protein assay and quantitative immunoblotting of various amounts of protein. The concentration of OCRL protein in crude extracts was estimated by comparison of the immunoblots of purified OCRL with those of crude proteins run in different amounts on SDS/polyacrylamide gels. The crude extracts were used for all subsequent characterization of OCRL, since the activity of

recombinant protein recovered from the nickel affinity column was poor and the purified protein proved to be unstable.

RESULTS

The finding that the protein mutated in Lowe syndrome, OCRL, is homologous to 5-phosphatase II suggested that OCRL might be an isoenzyme of 5-phosphatase. We initially assayed extracts of fibroblast cell lines from patients with Lowe syndrome for 5-phosphatase activity using Ins(1,4,5)P₃ and found no abnormalities. Phosphatidylinositol turnover and levels of inositol phosphates were also measured in fibroblasts from patients, and no differences from control fibroblasts were found (data not shown).

We therefore turned to a direct approach to define the function of the OCRL protein. An OCRL cDNA expression vector was constructed so that translation started at Met²⁶⁴. This choice was based on our previous experience with the homologous platelet 5-phosphatase II. The 5-phosphatase II cDNA encodes a protein of 942 aa but the amino-terminal sequence of the isolated platelet protein begins at aa 270. This finding suggests that the platelet enzyme may be processed from a larger precursor. A construct of the 5-phosphatase II (aa 250–942) expressed in Sf9 cells yielded a recombinant protein with an enzyme activity that was identical to that of the protein isolated from platelets (9). Also, the homology between OCRL and 5-phosphatase II begins at the region of the amino terminus of the mature platelet enzyme. The long open reading frame 5' of this region has no homology to 5-phosphatase II. Because the conserved sequence in OCRL coincided with the mature processed form of platelet 5-phosphatase II, we assumed that the OCRL protein might also be processed, and therefore we engineered the expression construct to begin at the same region. In initial experiments we infected Sf9 cells with recombinant baculovirus harboring ps.OCRL4 and ps.OCRL5. After 3 days of infection, cells were harvested and disrupted by sonication and extracts of the ps.OCRL5-infected cells were affinity purified. The crude extracts and purified protein were run in SDS/polyacrylamide gels, transferred to nitrocellulose membranes, stained with Coomassie Blue, and immunoblotted with anti-peptide antibodies. The affinity-purified OCRL migrated as a single band (Fig. 1, lane 4) at an apparent molecular mass larger than that

FIG. 1. SDS/PAGE and immunoblotting of OCRL protein. Soluble extracts from Sf9 cells infected with baculovirus constructs expressing both histidine-tagged and untagged OCRL recombinant proteins were analyzed by SDS/8% PAGE. The histidine-tagged OCRL was affinity purified by Ni-NTA affinity chromatography (see *Materials and Methods*). Lanes 1–4 were stained with Coomassie blue. Lane 1, high-range molecular weight markers (Bio-Rad); lane 2, prestained low-range molecular weight markers (Bio-Rad); lane 3, untagged OCRL-expressing Sf9 cell extract containing 30 ng of OCRL protein; lane 4, 0.9 µg of affinity-purified OCRL protein; lanes 5 and 6, same as lanes 3 and 4, but analyzed by immunoblotting with a rabbit polyclonal antibody directed against a carboxyl-terminal peptide of OCRL.

predicted from the cDNA (82 kDa). The OCRL protein was barely visible in the Coomassie-stained gel (lane 3) but was readily apparent upon immunoblotting (lane 5).

Enzyme Activity of OCRL. We assayed recombinant OCRL for activity with both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ and found that it hydrolyzed both substrates (Fig. 2). Thus $[4,5\text{-}^{32}\text{P}_2]\text{Ins}(1,4,5)\text{P}_3$ was converted to an InsP_2 that was coeluted with a standard of $\text{Ins}(1,4)\text{P}_2$ and $[^{32}\text{P}]\text{phosphate}$ (Fig. 2A). $\text{Ins}(1,3,4,5)\text{P}_4$ was converted mainly to an inositol trisphosphate that was coeluted with a standard of $\text{Ins}(1,3,4)\text{P}_3$ (Fig. 2B) with a small amount of an InsP_2 product (fraction 91) that represented action of a phosphatase present in Sf9 extracts. We also tested $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$, $[^3\text{H}]\text{Ins}(3,4)\text{P}_2$, $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$, and $[^3\text{H}]\text{InsP}_6$ as substrates, and no hydrolysis was found. Thus the OCRL protein hydrolyzes the same inositol phosphate substrates as 5-phosphatase I and II isoenzymes.

We also tested the phospholipid $\text{PtdIns}(4,5)\text{P}_2$ as substrate for OCRL protein as compared with a known $\text{PtdIns}(4,5)\text{P}_2$ 5-phosphatase, 5-phosphatase II (Fig. 3). Under these conditions there was no detectable hydrolysis with extracts from control cells infected with virus expressing Meg-01 recombinant protein (data not shown). Remarkably, OCRL had tremendous activity in this assay, with kinetics that were distinct from those of platelet 5-phosphatase II. The reaction of the latter enzyme, when assayed at 37°C , was not linear with time after a few seconds, whereas OCRL hydrolysis of the lipid was essentially linear for 2 min. When 5-phosphatase II was assayed at room temperature under conditions where the assay was linear with time, a specific activity similar to that found at 20 sec and 37°C was observed (9). At 20 sec OCRL was 8-fold more active than 5-phosphatase II and at 2 min the difference

FIG. 2. Adsorbosphere Sax HPLC of the products of $[^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$ and $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ after incubation with OCRL recombinant protein. (A) Seventeen nanomoles (20,000 cpm) of $[4,5\text{-}^{32}\text{P}_2]\text{Ins}(1,4,5)\text{P}_3$ was incubated with 110 ng of Sf9-expressed OCRL protein in a final volume of 100 μl at 37°C for 10 min. The reaction was quenched with 500 μl of cold water, and 2000 cpm of $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$ was added as internal standard (std, marked by arrow) for analysis by Adsorbosphere Sax HPLC. (B) One picomole (20,000 cpm) of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ was incubated with 110 ng of the same OCRL protein in 100 μl at 37°C for 10 min. The reaction was quenched with 500 μl cold water, and 4000 cpm of $[^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$ was added as internal standard for analysis by the same method as in A. Elution positions of $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ were determined by a separate run of standards.

FIG. 3. $\text{PtdIns}(4,5)\text{P}_2$ 5-phosphatase (5-ptase) activity of recombinant OCRL and 5-phosphatase II. OCRL protein (1 ng) or 5-phosphatase II (10 ng) was incubated with 50 μM $\text{PtdIns}(4,5)\text{P}_2$ (5760 cpm/nmol) in a 50- μl reaction mixtures at 37°C for various times. The product of the reaction $\text{PtdIns}(4)\text{P}$ was separated from $\text{PtdIns}(4,5)\text{P}_2$ by TLC. The results are from one of three experiments with similar results.

was 33-fold. A comparison of the kinetics of OCRL vs. platelet 5-phosphatase is shown in Table 1. OCRL is mainly a $\text{PtdIns}(4,5)\text{P}_2$ 5-phosphatase under the conditions used. Thus, even at 20 sec, OCRL hydrolyzed the lipid substrate 590- and 1080-fold better than it hydrolyzed the water-soluble substrates [dividing product formed at 20 sec by V_m/K_m for $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$]. By comparison this same calculation for 5-phosphatase II yields ratios of 8 for $\text{Ins}(1,4,5)\text{P}_3$ and 42 for $\text{Ins}(1,3,4,5)\text{P}_4$ (Table 1).

We also determined that OCRL functions as a $\text{PtdIns}(4,5)\text{P}_2$ 5-phosphatase in Sf9 cells infected with either ps.OCRL4 or a baculoviral construct encoding a control protein, Meg-01. We measured the turnover of cellular phospholipid after 3 days of infection by pulse-labeling with $[^{32}\text{P}]\text{phosphate}$ for 1–30 min (Fig. 4). In Sf9 cells expressing the control protein (as well as in uninfected Sf9 cells; data not shown) more ^{32}P was incorporated into $\text{PtdIns}(4,5)\text{P}_2$ than into $\text{PtdIns}(4)\text{P}$ at all times. In contrast, cells expressing OCRL had a decreased incorporation of ^{32}P into $\text{PtdIns}(4,5)\text{P}_2$, due to hydrolysis of the 5-phosphate group with a relative increased incorporation into $\text{PtdIns}(4)\text{P}$. The incorporation of ^{32}P into total cell phospholipids was the same in both infections (14 cpm/min per nmol of phospholipid for the control and 11 cpm/min per nmol of phospholipid for OCRL).

DISCUSSION

Lowe syndrome is the first human disorder described resulting from a defect in the inositol phosphate signaling pathway. It is clear that deficiency of the OCRL protein is the cause of Lowe syndrome, as shown by analysis of numerous patients with either deletions of the OCRL locus, absence of mRNA, or nonsense mutations (2, 3, 16). The homology of OCRL to platelet 5-phosphatase II suggested that OCRL is an isoenzyme of 5-phosphatase. The findings in this report indicate that the OCRL protein is mainly a lipid 5-phosphatase. The fact that OCRL has substrate preference quite different from the platelet 5-phosphatase isoenzymes may explain why these other enzymes do not compensate for the deficiency of OCRL in patients with Lowe syndrome. Thus OCRL is only 10–15% as active toward $\text{Ins}(1,4,5)\text{P}_3$, and 15–30% as active toward $\text{Ins}(1,3,4,5)\text{P}_4$, as 5-phosphatase I and II, as judged from the relative catalytic efficiencies as shown in Table 1. In contrast, OCRL has much greater activity toward the lipid substrate than the previously described enzymes.

Another factor that may contribute to the inability of 5-phosphatase I and II to compensate for the defect in Lowe syndrome is that the tissue distribution and subcellular local-

Table 1. Kinetic properties of 5-phosphatase isoenzymes and OCRL

Enzyme	Ins(1,4,5)P ₃ substrate (A)			Ins(1,3,4,5)P ₄ substrate (B)			PtdIns(4,5)P ₂ substrate (C)		Ratios*	
	K _m	V _m	V _m /K _m	K _m	V _m	V _m /K _m	20 sec	2 min	C/A	C/B
OCRL	70	8	0.11	30	1.8	0.06	65	290	590	1080
5-Phosphatase II	24	25	1.04	7.5	1.4	0.19	8	8.7	8	42
5-Phosphatase I	7.5	5.3	0.71	0.5	0.2	0.4	ND	ND	—	—

Enzyme assays for OCRL were as described in *Materials and Methods*. Data for 5-phosphatase I (6) and 5-phosphatase II (5) were as reported. We also assayed recombinant 5-phosphatase II in this study and obtained values similar to those listed above. The background activity in baculovirus extracts from control cells infected with baculovirus expressing Meg-01 was 1 nmol/min per mg of protein for Ins(1,4,5)P₃ and 1.2 nmol/min per mg of protein for Ins(1,3,4,5)P₄. Protein concentrations were estimated from immunoblots of various amounts of recombinant proteins compared with those of affinity-purified recombinant protein standards. Units for kinetic values: K_m, μM; V_m, μmol/min per mg of homogeneous protein; PtdIns(4,5)P₂ 5-phosphatase, μmol/mg of homogeneous protein. ND, none detected.

*Ratios of PtdIns(4,5)P₂ 5-phosphatase activity at 20 sec to V_m/K_m for Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄, respectively.

ization of these enzymes may be different. The OCRL transcript is expressed in brain and kidney and in several other organs (3). 5-Phosphatase I is expressed in brain but not in kidney. 5-Phosphatase II is expressed in kidney (unpublished results) but apparently not in brain (2). Which cells in kidney express these enzymes is unknown and the pattern of expression in eye is also yet to be determined.

The cellular consequences of absence of OCRL are not known, although the findings in pulse-labeled Sf9 cells expressing OCRL indicate that deficient cells will have altered ratios of PtdIns(4,5)P₂ to PtdIns(4)P. If cellular levels of PtdIns(4,5)P₂ are in part controlled by this enzyme, it is likely that cells deficient in OCRL will have increased levels of PtdIns(4,5)P₂. Whether this results in altered calcium signaling reactions or has other consequences is yet to be determined. Several studies suggest that PtdIns(4,5)P₂ is important in reactions involving actin assembly (17–21), secretion (22), protein kinase C action (23–25), and ATPases (26, 27), one or more of which could be important in the pathogenesis of Lowe syndrome. All of the studies in the current report were carried out with a recombinant OCRL construct that encodes aa 264–978. The function of the amino-terminal region of the molecule that is not homologous to 5-phosphatase II remains unknown. Whether this region has some other function or

whether the full-length OCRL protein will have the same properties as those reported here awaits further study.

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FIG. 4. Incorporation of [³²P]phosphate into phosphatidylinositols in Sf9 cells. Sf9 cells infected with baculovirus encoding OCRL4 or control Meg-01 protein-tyrosine phosphatase were labeled with [³²P]phosphate for the indicated times. Radioactivity incorporated into PtdIns(4)P and PtdIns(4,5)P₂ was determined as described in *Materials and Methods*. (Inset) Ratio of PtdIns(4,5)P₂ to PtdIns(4)P at 1, 3, 10, and 30 min.