Lowe syndrome protein Ocrl1 is translocated to membrane ruffles upon Rac GTPase activation: a new perspective on Lowe syndrome pathophysiology

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INTRODUCTION

Oculocerebrorenal syndrome of Lowe (OCRL, MIM 309000) is a rare X-linked disorder characterized by bilateral cataract, mental retardation and renal Fanconi syndrome. The Lowe syndrome protein Ocrl1 is a PIP2 5-phosphatase, primarily localized to the trans-Golgi network (TGN), which ‘loss of function’ mutations result in PIP2 accumulation in patient’s cells. Although PIP2 is involved in many cell functions including signalling, vesicle trafficking and actin polymerization, it has been difficult so far to decipher molecular/cellular mechanisms responsible for Lowe syndrome phenotype. We have recently shown that, through its C-terminal RhoGAP domain, Ocrl1 forms a stable complex with Rac GTPase within the cell. In line with this finding, we report here that upon epidermal growth factor induced Rac activation in COS-7 cells, a fraction of Ocrl1 translocates from TGN to plasma membrane and concentrates in membrane ruffles. In order to investigate the functionality of Ocrl1 in plasma membrane, we have analysed PIP2 distribution in human dermal fibroblasts (HDFs) from Lowe patients versus control HDFs. As revealed by both immunodetection and green fluorescent protein–PH binding, PIP2 was found strikingly to accumulate in PDGF induced ruffles in Lowe HDFs when compared with control. This suggests that Ocrl1 is active as a PIP2 5-phosphatase in Rac induced membrane ruffles. Cellular properties such as cell migration and establishment of cell–cell contacts, which depend on ruffling and lamellipodia formation, should be further investigated to understand the pathophysiology of Lowe syndrome.

This protein, designated Ocrl1, has been characterized as a catalytically active phosphoinositide 5-phosphatase with phosphatidylinositol 4,5-bisphosphate (PIP2) as a preferred substrate (3,4). Consistent with the in vitro specificity of the enzyme, PIP2 was found to accumulate in cell lines derived from kidney proximal tubules or fibroblasts of patients with Lowe syndrome, i.e. devoid of Ocrl1 (5,6). PIP2 is a major player in phosphoinositide (PI) metabolism and its accumulation may interfere in many cell processes including cell signalling, protein trafficking and actin polymerization (7,8). Ocrl1 has been found to localize primarily to the trans-Golgi network (TGN) (4,9) and possibly to lysosomes (10) and
endosomes (11). So far, the discovery of the enzymatic function of Ocrl1 and its subcellular localization to the Golgi has not revealed how the enzyme defect and PIP2 accumulation cause the protein manifestations of the disease. Recently, however, abnormalities in the actin cytoskeleton of Lowe fibroblasts have been demonstrated including decreased stress fibres, altered response to depolymerizing agents and abnormal distribution of several actin binding proteins regulated by PIP2 (12).

Although several mutations of OCRL1 in Lowe patients map to the RhoGAP like region (13), the functional role of this domain had not been investigated until recently. Yet, sequence comparisons of OCRL1 orthologs demonstrate that this domain has been evolutionarily conserved from amoeba to mammals, suggesting an important function (14).

We have recently reported that the activated GTP bound form of Rac, a member of the Rho GTPase family, forms a stable complex with the RhoGAP domain of Ocrl1 both in vitro and in vivo; in addition, we found that a large fraction of endogenous Rac colocalizes with Ocrl1 in the TGN, consistent with a physiological interaction between the two proteins (15).

Rho GTPases are ubiquitously expressed signalling molecules belonging to the Ras superfamily of small G proteins. They are activated downstream of many types of receptor and control multiple cellular responses including actin cytoskeleton reorganization, transcriptional regulation and cell cycle progression (16,17). Therefore, they are major relays in signal transduction pathways regulating the growth and migration of most cell types. The biosynthetic pathway of Rho GTPases comprises complex post-translational modifications resulting in the prenylation of the C-terminus (usually geranylgeranylation in the case of Rho GTPases); these modifications are necessary for association of Rho GTPases to cell membranes and strictly required for their signalling function (16,18). Together with the fact that Ocrl1 possesses neither intrinsic transmembrane domain nor lipophilic post-translationally acquired moiety, our previous results demonstrating a stable Rac/Ocrl1 interaction suggested that the association of Ocrl1 with endocellular membranes could be mediated by Rac (15). We therefore reasoned that numerous extracellular stimuli, known to induce the activation and recruitment of Rac to the plasma membrane, should result in the simultaneous translocation of Ocrl1. We have investigated this hypothesis using transfection of constitutively activated Rac as well as growth factor stimulated cells; we show here that a fraction of Ocrl1 is efficiently translocated to the plasma membrane upon Rac activation. Consistent with this finding, we also demonstrate that, in Ocrl1 defective dermal fibroblasts from Lowe patients but not in normal dermal fibroblasts, PIP2 accumulates in membrane ruffles after cell stimulation. This unprecedented observation supports the new idea that a dysregulation of plasma membrane PI plays a role in the pathophysiology of Lowe syndrome.

RESULTS

Activated Rac mutant recruits Ocrl1 to membrane ruffles in COS-7 cells

It has long been demonstrated that constitutively activated Rac mutants localize primarily to the plasma membrane and stimulate the polymerization of cortical actin; this results in specific actin dependent structures known as ruffles and lamellipodia, where the bulk of activated Rac is located (data not shown) (19,20). We have previously shown that in Hela or in COS-7 cells co-expressing Ocrl1 RhoGAP domain and a constitutively activated mutant of Rac (Rac1L61), Ocrl1 RhoGAP colocalizes with activated Rac at the plasma membrane suggesting that this domain does interact with activated Rac (15).

In the present work, we performed similar experiments to analyse the subcellular localization of full-length green fluorescent protein (GFP) ‘tagged’ Ocrl1 (GFP–Ocrl1) expressed in COS-7 cells. As presented in Figure 1A, transient expression of GFP–Ocrl1 alone induces a strong juxta-nuclear staining that colocalizes with TGN markers (data not shown), with little or no peripheral staining. In contrast, coexpression of Rac1L61 and GFP–Ocrl1 results in a significant fraction of Ocrl1 being translocated to membrane ruffles where it colocalizes with Rac1L61 (Fig. 1B, C and D).

Similar observations could be made when analysing the subcellular localization of endogenous Ocrl1 in COS-7 cells. As described in previous studies (4,15) and in agreement with the pattern shown in Figure 1, immunodetection of Ocrl1 in COS-7 cells revealed a juxta-nuclear labelling corresponding to TGN. Transient expression of Rac1L61 in those cells resulted in the translocation of a fraction of Ocrl1 to the plasma membrane. Moreover, as can be seen in Figure 2,
Ocrl1 specific immunostaining shows a striking colocalization with Rac labelling, in membrane ruffles. Collectively, these results indicate that upon expression of activated Rac, Ocrl1 is translocated to membrane ruffles. EGF induces a Rac-dependent translocation of Ocrl1 to membrane ruffles in COS-7 cells

In many cell types, epidermal growth factor (EGF) has been shown to specifically activate Rac and Rac-dependent actin polymerization, whereas lysophosphatidic acid (LPA) and bradykinin are known specific activators of RhoA and Cdc42 pathways, respectively (20). Treatment of serum-starved COS-7 cells with EGF does induce the recruitment of Rac to plasma membrane and the formation of actin dependent membrane ruffles (data not shown). We therefore utilized this cellular model to analyse the effects of growth factor induced Rac activation on Ocrl1. As expected from previous results (Fig. 1), immunodetection of Ocrl1 in serum-starved COS-7 cells did not reveal significant membrane staining. In contrast, after 10 min of EGF treatment, we observed a strong Ocrl1 specific immunostaining that colocalized with polymerized actin in membrane ruffles (Fig. 3A); this pattern is clearly Rac dependent, as it is completely absent in cells expressing RacN17, a dominant negative mutant form of Rac (Fig. 3A). Membrane translocation of Ocrl1 also appears to be Rac specific because LPA (Fig. 3B) or bradykinin (data not shown) does not induce any strong translocation. Moreover, it is worth noting that Ocrl1 does not colocalize with LPA-induced stress fibres, indicating that Rac-dependent translocation of Ocrl1 does not rely on non-specific binding to polymerized actin.

Taken together, these data demonstrate that upon Rac activation, a fraction of Ocrl1 is translocated from TGN to the plasma membrane. Consistent with Ocrl1 being a specific partner of activated Rac, it appears preferentially associated with Rac-dependent membrane ruffles. This result therefore suggests that Ocrl1 may contribute to PI regulation at the plasma membrane. Because PIP2 is believed to be the preferential substrate of Ocrl1, we next...
examined whether the loss of Ocrl1 in Lowe patients’ cells may result in an excess of PIP2 in membrane ruffles.

**PIP2 accumulates in membrane ruffles in dermal fibroblasts from Lowe patients**

Patients were diagnosed for Lowe syndrome on the basis of clinical and genetic evidences. To confirm the diagnosis and to evaluate the degree of the enzymatic defect, human dermal fibroblasts (HDFs) obtained from skin biopsies were analysed for the amount of Ocrl1 protein by western blot and assayed for PIP2 5-phosphatase activity. As previously observed in many cases in our series, including patients bearing missense mutations (21), HDFs from Lowe patients were found, in most cases, strongly depleted of Ocrl1 protein and enzymatic activity (Fig. 4). Enzymatic activities were 1.3 +/− 0.6 and 1.8 +/− 0.4 nmol/min.mg in patients’ cells versus 8.7 +/− 1.4 and 8.6 +/− 1.2 nmol/min mg in control HDFs.

In order to evaluate the PIP2 content in membrane ruffles, cells cultured in the presence of fetal calf serum (FCS), were stained for polymerized actin with phalloidin and PIP2 was detected using two independent tools, i.e. specific anti-PIP2 antibodies and a GFP tagged PH domain derived from PLC delta. Both probes gave essentially the same results showing a clear accumulation of PIP2 in plasma membrane ruffles of patients’ cells when compared with control cells (Fig. 5A and B) (data not shown). In the case of GFP-transfected cells, the fraction of cells exhibiting GFP−PH positive or GFP−PH negative ruffles was determined by categorizing cells for PIP2 content at membrane ruffles among three classes of intensity: high (positive), medium and low (negative). Counting more than 100 FCS responsive cells of each cell type revealed 52% of GFP positive and 15% of GFP negative cells among Lowe HDFs (33% with intermediate intensity) and an opposite proportion of 52% GFP negative and 14% GFP positive cells among control HDFs (34% with intermediate intensity). Of note, PIP2, which appears largely colocalized with polymerized actin in the ruffles, is mostly not detected over stress fibres. This is in full agreement with the preferential association of Ocrl1 with membrane ruffles, which we mentioned earlier.

HDFs play an essential role in cutaneous wound repair and remodelling. A number of growth factors and cytokines have been reported to affect HDF proliferation and mobility (22). Among them, platelet derived growth factor (PDGF) appears as the major promotility factor for HDFs in human serum and is known to specifically induce ruffling in this cell type (22,23). PDGF elicits its biological responses via binding to its specific cell surface receptors and subsequent activation of multiple downstream pathways which have been extensively characterized; these include activation of PKC, Akt, Ras and Rac GTPase (24).
We therefore analysed PIP2 content in PDGF stimulated HDF from patients and controls. Serum-starved HDF from either normal controls or Lowe patients showed the same flattened and smooth morphology and a similar PIP2 immunostaining pattern with no noticeable difference at cell periphery. As expected, PDGF treatment provoked in both normal and patients’ cells, a profound remodelling of actin cytoskeleton including the formation of spectacular membrane ruffles. As shown in Figure 6, control and Lowe patients’ HDF showed the same type of polymerized actin structures as revealed by phalloidin treatment; in contrast, PIP2 as detected by immunolabelling (Fig. 6A) or GFP–PH staining (Fig. 6B) revealed a striking accumulation of PIP2 in Lowe HDF ruffles, whereas little or no PIP2 staining was apparent in control HDF membrane ruffles. In the case of GFP-transfected cells, the fraction of cells exhibiting GFP–PH positive or GFP–PH negative ruffles was determined by categorizing phenotype of PIP2 at membrane ruffles among three classes of intensity: high (positive), medium and low (negative). Counting more than 100 PDGF responsive cells, we found 60% of positive, 31% of medium level and 9% of negative among Lowe HDFs versus 14% of positive, 47% of medium level and 39% of negative among control HDFs. Similar numbers were obtained when PIP2 measurement at membrane ruffles was made with antibody. Again, more than 100 cells of each type were examined. These results therefore demonstrate an abnormal accumulation of PIP2 in PDGF induced ruffles in Lowe-derived HDFs. This is in full agreement with the

![Figure 6](https://academic.oup.com/hmg/article/14/11/1441/605453/1445)

**Figure 6.** PIP2 accumulates in PDGF-induced ruffles from Lowe dermal fibroblasts. Normal and Lowe dermal fibroblasts were serum-starved for 24 h, stimulated or not for 10 min by PDGF and processed for immunofluorescence. Ruffles are detected using actin filaments visualized by Alexa fluor 594-phalloidin. (A) Pl(4,5)P2 detected with anti-Pl(4,5)P2 antibody, is visualized with FITC. (B) Cells were transfected with the PLCδ PH domain fused to GFP in order to visualize Pl(4,5)P2 localization. Scale bar represents 20 μm.
observation made in FCS cultured Lowe HDFs and confirms that a major perturbation of PIP2 metabolism takes place in plasma membrane and more specifically in membrane ruffles of Lowe patients’ cells.

DISCUSSION

Ocrl1 has long been characterized as a PI 5-phosphatase with PIP2 as a preferred substrate (3,4). As expected, ‘loss of function’ mutations in the OCRL1 gene as characterized in Lowe syndrome patients, have been shown to result in elevation of PIP2 in cell lines derived from patients (5,6). Since Ocrl1 is mainly localized to the TGN, it may act primarily on the pool of PIP2 associated with TGN membranes. Ocrl1 hydrolyzes PIP2, forming PI 4P, a lipid with a recognized role in the vesicular trafficking from TGN to plasma membrane (25). Therefore, it is generally accepted that the defect in Ocrl1 results in accumulation of PIP2, the main substrate of Ocrl1 phosphatase activity, in membrane ruffles. To address the issue of the functionality of Ocrl1 in membrane ruffles, we asked whether a defect in Ocrl1 as observed in cells from Lowe patients, might result in perturbing the PIP2 metabolism in membrane ruffles. The analysis of PIP2 showed a clear accumulation in the plasma membrane of Lowe HDF when compared with control HDF. Interestingly, PIP2 accumulation was found particularly evident in ruffles from Lowe HDF, whether induced by FCS or PDGF.

In previous studies, PIP2 cellular content has been found clearly increased in cell lines derived from kidney proximal tubules and fibroblasts of Lowe patients, suggesting that Ocrl1 is a major PIP2 5-phosphatase at least in these cell types. Our results demonstrate for the first time that Ocrl1 defect in skin fibroblasts from Lowe patients, induces PIP2 accumulation in a particular cell compartment, i.e. plasma membrane. Therefore, although the function of Golgi/TGN where the bulk of Ocrl1 is located may be affected in Lowe syndrome cells, our observation support the new idea that structural and functional alterations in plasma membrane ruffles may contribute to cellular defects in Lowe patients.

Why PIP2 accumulates particularly in the ruffles of Lowe patients HDF and what are the possible consequences of PIP2 accumulation in these structures are among the multiple questions raised by our results.

While PIP2 content is much higher in plasma membrane than in any other cell compartment (27), it is also noteworthy that several distinct enzymes with PIP2 5-phosphatase activity are translocated to membrane ruffles upon growth factor stimulation. These include SKIP and PIPP, two PIP2 5-phosphatases (28,29), which according to their in vitro PIP2 hydrolyzing activity and their wide expression spectrum could compensate for Ocrl1 deficiency in Lowe patients’ skin fibroblasts. At a first glance, the finding that PIP2 accumulates in the ruffles of Lowe-derived HDF would suggest that Ocrl1 is the most active PIP2 5-phosphatase in these cells. However, additional factors such as the ‘true’ substrate specificity of these dephosphorylating enzymes in living cells, their relative expression in various cell types and the kinetics of their recruitment to membrane ruffles following growth factor stimulation should be considered in future studies.

The consequences of PIP2 accumulation in membrane ruffles also constitute a complicated issue. PIP2 is involved in many cell processes including cell signalling, protein trafficking and actin polymerization (8,30) and it has multiple potential fates depending on which metabolic pathway (PLC, PI3K, PI 5-phosphatase or PH-containing proteins) is favoured in a particular cell or cell compartment.

An impairment of these processes in epithelial cells would possibly explain the alteration in kidney tubules and lens from Lowe patients.

In summary, we have reported previously that, following Rac activation in cultured cells, Ocrl1 is translocated to the plasma membrane and accumulates specifically in membrane ruffles. As a consequence, the lack of Ocrl1 in HDF from Lowe patients’ cells results in accumulation of PIP2, the main substrate of Ocrl1 phosphatase activity, in membrane ruffles. This unprecedented observation demonstrates that the Golgi/TGN is not the sole cell organelle affected by Ocrl1 loss of function and raises the question of the role of plasma membrane perturbation in the pathophysiology of Lowe syndrome.

MATERIALS AND METHODS

Antibodies and western blotting

Primary antibodies used were rabbit anti-Ocrl1 serum (15), mouse monoclonal anti-Rac1 antibody (Upstate), 9E10 mouse monoclonal antibody to Myc-tag (Roche) and mouse monoclonal anti-PI(4,5)P2 antibody (Assay Designs, Inc.). Secondary antibodies used in immunofluorescence studies were Texas red-conjugated horse anti-mouse IgG (Vector Laboratories), Fluorescein isothiocyanate (FITC)-conjugated

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goat anti-mouse IgG (Sigma), FITC-conjugated goat anti-rabbit IgG (Vector Laboratories) and Alexa Fluor 350 goat anti-mouse IgG (Molecular Probes) antibodies. Cross hybridization of secondary antibodies was checked in each type of co-staining experiments. For western blotting, 30 µg of proteins were resolved on 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotted onto nitrocellulose. Filters were incubated sequentially with primary antibodies and peroxidase-labelled swine antirabbit (Dako). Signals were revealed using the ECL detection system (Amersham Biosciences).

GFP–Ocrl1 expression plasmid
A 2.8 kb cDNA encoding the OCRL1 ORF (isoform b, GenBank® accession no. NM_001587) was amplified from HEK-293T cells by RT–PCR and cloned into the pE GFP-C3 vector (Clontech Inc.) at BamHI sites. The construct was checked for the absence of any mutation by DNA sequencing.

Cell culture, transfections and stimulation by growth factors
COS-7 cells and dermal fibroblasts from two control children and two Lowe affected boys (patient 1 harbours a 2530 G→A splicing mutation and patient 2 harbours a deletion of exon 19–23 of the OCRL1 gene) were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Life technologies) supplemented with 10% FCS and penicillin–streptomycin at 37°C with 5% CO₂. COS-7 cells were transiently transfected (24 h) with expression vectors pRK5-myc-GTPase or pEGFP-full-length Ocrl1 using FuGENE 6 reagent (Roche) following manufacturer’s procedure. Dermal fibroblasts were transfected with expression vectors pE-GFP-PLCδ domain (27) with the Amaxa Biosystems NHDF Nucleofector system (Amersham Biosciences).

Immunofluorescence
COS-7 cells or HDF were plated on 18 mm diameter glass coverslips 16–72 h before transfection, transfected with plasmids and observed 16–40 h later. To detect localization of endogenous and transfected proteins, cells were fixed in 4% PFA for 20 min, permeabilized with 0.2% Triton X-100 for 5 min and blocked with PBS, 1% BSA for 1 h at room temperature. Cells were then incubated in the same solution with primary antibodies for 1 h followed by fluorescein- or Texas red-secondary antibodies and Alexa Fluor 594-phalloidin (Molecular Probes) 0.25 units/ml for 30 min. Labelling technique was optimized in order to get similar signal intensities for the various fluorophores when making co-stainings. Coverslips were mounted on glass slides using Vectashield (Vector Laboratories) with DAPI at 0.5 µg/ml. Cell preparations were observed under a Zeiss Axioptip epifluorescence microscope. Filters used in the fluorescence experiments were band pass for DAPI, FITC and Texas red, with excitation wavelength of 360/51, 485/17 and 560/18 nm, respectively, and with emission wavelength of 460/10, 520/30 and 600/40 nm, respectively. Images were digitally acquired with a cooled CCD camera (Hamamatsu) for each fluorophore separately and processed using Adobe Photoshop software. More than 100 cells of each type were counted for each experiment.

Ocrl1 PI(4,5)P₂ 5-phosphatase activity assay
Ocrl1 enzymatic activity was assayed as previously described (4) from 50 µg of dermal fibroblasts lysate. Briefly, each measurement of PIP2 hydrolysis to PIP was conducted by incubating lysate for 0–15 min at 37°C with 12.5 nmol of PIP2 and 0.1 µCi of [3H]-PIP2 with a CTAB/PIP2 ratio of 10/1. Lipids were then extracted by solvent, resolved by thin layer chromatography and visualized by autoradiography. Lipid spots were then scraped into scintillation vials and radioactivity was counted.

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Conflict of Interest statement. None declared.

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