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Lowe syndrome protein OCRL1 interacts with Rac GTPase in the trans-Golgi network

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The oculocerebrorenal syndrome of Lowe (OCRL) is a rare X-linked disorder characterized by severe mental retardation, congenital cataracts and renal Fanconi syndrome. OCRL1 protein is a phosphatidylinositol 4,5-bisphosphate 5-phosphatase with a C-terminal RhoGAP domain. Considering the pleiotropic cellular functions of Rho GTPases (Rho, Rac and Cdc42) and their dysregulation in several forms of mental retardation, we have investigated the so far unexplored function of the RhoGAP domain of OCRL1. Activated Rac GTPase was found to stably associate with the OCRL1 RhoGAP domain *in vitro* and to co-immunoprecipitate with endogenous OCRL1. Contrasting with other GAPs, OCRL1 RhoGAP exhibited a significant interaction with GDP bound Rac *in vitro*. As compared to Rac, other Rho GTPases tested showed reduced (Cdc42) or no binding (RhoA, RhoG) to OCRL1 RhoGAP. Immunofluorescence studies in HEK and COS7 cells and Golgi perturbation assays with Brefeldin A demonstrated that a fraction of endogenous Rac co-localizes with OCRL1 and γ-adaptin in the trans-Golgi network. The OCRL1 RhoGAP domain showed low Rac GAP activity *in vitro*, and when expressed in Swiss 3T3 cells induced specific inhibition of RacGTP dependent ruffles, consistent with OCRL1 being an active RacGAP. OCRL1 appears to be a bifunctional protein which, in addition to its PIP2 5-phosphatase activity, binds to Rac GTPase. This novel property may play a role in localizing OCRL1 to the trans-Golgi network. Moreover, loss of OCRL1 RhoGAP and the resulting alteration in Rho pathways may contribute to mental retardation in Lowe syndrome, as illustrated in other forms of X-linked mental retardation.

INTRODUCTION

Rho GTPases (Rho, Rac, Cdc42) are intracellular signalling proteins belonging to the family of small G proteins or Ras superfamily. Like all GTPases, Rho GTPases cycle between an active (GTP bound) and an inactive (GDP bound) conformation. In the active state, they interact with one of their target (or effector) proteins to mediate downstream signalling. The GTP/GDP cycle of Rho GTPases is highly regulated by guanine nucleotide exchange factors (RhoGEFs) which catalyse nucleotide exchange and mediate activation, and by GTPase activating proteins (RhoGAPs) which stimulate GTP hydrolysis leading to inactivation (1). In all eucaryotic cells from yeast to mammals, Rho GTPases control multiple cellular functions including actin cytoskeleton organization, vesicular trafficking, transcriptional regulation and cell cycle progression. Rho GTPases are therefore considered as major intracellular relays for regulating cell growth and division, and cell migration and differentiation (2,3). In particular, multiple studies have demonstrated the involvement of Rho GTPases in neuronal morphogenesis and development of the central nervous system (CNS) (4,5).

Consistent with their key role in multiple aspects of physiology, Rho GTPases and Rho-dependent pathways are being implicated in the pathophysiology of a growing number of human diseases. Rho GTPase-dependent pathways appear to be involved in tumorigenesis and metastasis, both in experimental models and in naturally occurring human tumors (6,7). In addition, alterations in genes coding for Rho regulators and effectors are causally involved in several human genetic disorders. In particular, various forms of mental retardation have recently been ascribed to mutations in genes of the Rho pathways (8,9). Three X-linked genes *OPHN1*, *ARHGEF6* and *PAK3* encoding RhoGAP, RhoGEF and Rho effector proteins,

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respectively, have been involved in non-specific mental retardation (10–12). Recently, a newly discovered RhoGAP (MEGAP/srGAP3) has been implicated in a sporadic case of severe mental retardation with hypotonia (13). In addition, complex genetic disorders such as Aarskog-Scott syndrome (14), tuberous Sclerosis (15,16), and Williams syndrome (17), in which cognitive impairments are associated with various clinical symptoms, have also been ascribed to genetic alterations in components of the Rho signalling pathways.

Oculocerebrorenal syndrome of Lowe (OCRL; MIM 309000) is a rare X-linked developmental disorder characterized by a pleitropic phenotype including congenital cataracts, neonatal hypotonia, severe mental retardation, and renal Fanconi syndrome. The gene responsible for Lowe syndrome was identified by positional cloning of X chromosome breakpoints and was found to encode a 105 kDa protein, designated OCRL1 (18). Sequence analysis revealed that OCRL1 contains a central inositol polyphosphate/phophoinositide 5-phosphatase domain and a RhoGAP homology domain in the C-terminal region (19).

Subsequently, OCRL1 has been shown to be a catalytically active phosphoinositide 5-phosphatase with phosphatidylinositol 4,5-bisphosphate (PIP2) as a preferred substrate (20). OCRL1 was found to localize primarily to the trans-Golgi network (TGN) (21,22) and possibly to lysosomes (23). In Lowe syndrome, enzymatic deficiency, which leads to accumulation of PIP2 in patients cells, is believed to cause the protean manifestations of the syndrome (24). Although several mutations of OCRL1 in Lowe patients have been mapped to the RhoGAP-like region (25–27), the functional role of this domain has not been investigated so far. However, sequence comparisons of OCRL1 orthologues demonstrate that this domain is evolutionary conserved from amoeba to mammals, suggesting that it harbours a unique function (19,28,29).

Considering the pleiotropic functions of Rho dependent pathways and their involvement in CNS developmental defects, we have investigated the function of the RhoGAP domain of OCRL1; we report here that OCRL1 RhoGAP forms stable complexes with Rac GTPase both *in vitro* and within the cell, and that Rac may play a role in the localization of OCRL1 to the trans-Golgi network.

RESULTS

Binding of Rac and Cdc42 GTPases to the RhoGAP domain of OCRL1

The RhoGAP-like C terminal domain of OCRL1 (OCRL1-GAP) (residues 701–901) was first used as a GST fusion protein for *in vitro* binding experiments. As shown in Figure 1, GST pulldown assays in lysates from Rho GTPase-transfected COS7 cells revealed that OCRL1-GAP can form stable complexes with constitutively activated mutants of Rac (RacL61) and to a lesser extent with Cdc42 (Cdc42L61) (Fig. 1A). By comparison, interaction of OCRL1-GAP with the homologous activated form of RhoA (RhoAL63) was found barely detectable in three independent experiments and considered non-significant. An activated form of RhoG was found not to bind OCRL1-GAP either (not shown). Interestingly, a GST pulldown assay using pure recombinant RacL61 also exhibited a clear binding of the activated GTPase to OCRL1, therefore indicating a direct interaction between the two proteins (not shown). Unexpectedly, OCRL1 RhoGAP domain was also found to interact with RacN17, a mutant of Rac GTPase blocked in an inactive GDP like structure (30) (Fig. 1B); this clearly contrasts with the GAP domain of MgcRacGAP, a regular RhoGAP primarily active on Rac and Cdc42 (31), which showed no detectable interaction with RacN17 in a parallel GST pulldown experiment (Fig. 1B). We also observed, in GST pulldown experiments, that OCRL1 RhoGAP domain can bind to Rac-wt (expressed in COS7 cells), which is thought to be predominantly in the GDP bound form (not shown). It seems therefore likely that OCRL1 RhoGAP is capable of binding both active and inactive forms of Rac.

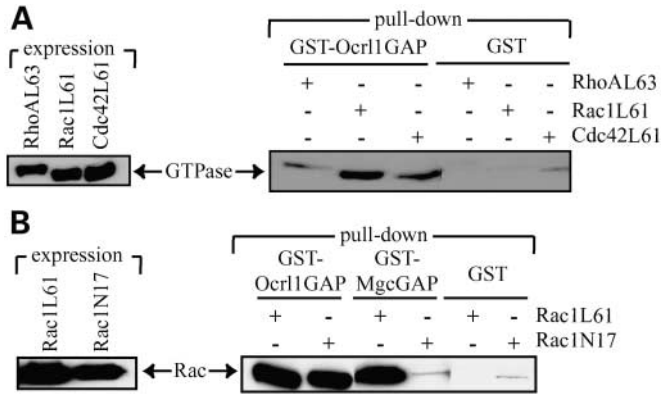

Figure 1. OCRL1 RhoGAP domain interacts preferentially with Rac1 GTPase. GST pulldown assays were conducted by incubating 10 µg of GST or GST fusion proteins with lysate from COS7 cells transiently expressing Myc-tagged Rho GTPases. Presence of GTPase binding to GST proteins was revealed by western blotting using anti-Myc tag antibody (right panels). Level of GTPase expression was controlled in the same way (left panels). Data are representative of at least three independent experiments. (**A**) Comparison of binding ability of GST or GST-OCRL1 GAP domain to constitutively active mutants RhoAL63, Rac1L61 or Cdc42L61. (**B**) Comparison of binding ability of GST, GST-OCRL1 GAP domain or Mgc GAP domain to Rac1L61 or Rac1N17 mutants.

We then tried to demonstrate the binding of Rho GTPases to OCRL1-GAP using a cellular approach: OCRL1-GAP was transiently expressed in HeLa cells and its subcellular localization was analysed by immunofluorescence. As shown in Figure 2, HeLa cells expressing OCRL1-GAP exhibited a nuclear and cytosolic staining. Interestingly, expression in these cells of activated forms of Rac or Cdc42 led to the appearance of a peripheral staining of OCRL1-GAP co-localized with activated forms of Rac or Cdc42, at the plasma membrane. By contrast, OCRL1-GAP domain did not co-localize with activated RhoA when the two proteins were coexpressed in HeLa cells (Fig. 2).

These results demonstrate that a fraction of OCRL1-GAP is translocated to the plasma membrane by activated forms of Rac and Cdc42 and therefore confirm that the Rho GAP domain of OCRL1 stably associates to activated Rac (or Cdc42) in HeLa cells. Similar experiments were conducted in COS7 cells with identical results (not shown).

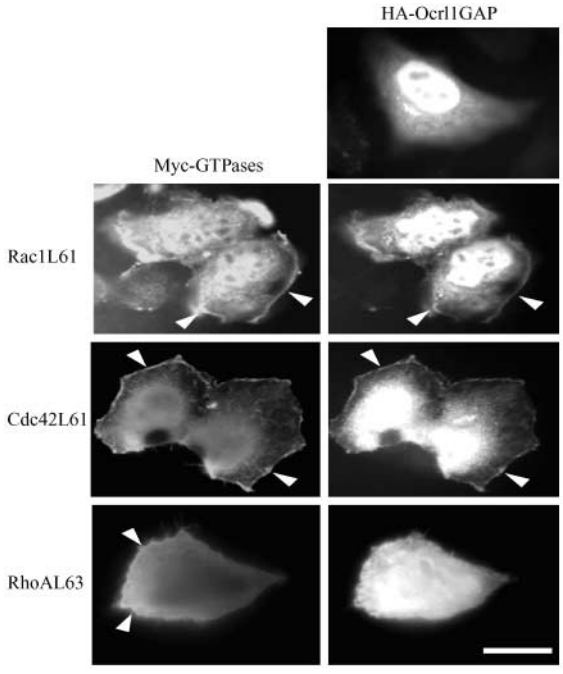

Figure 2. Activated Rac1 translocates OCRL1 RhoGAP domain to the plasma membrane. HeLa cells were transiently co-transfected with a plasmid encoding HA-tagged OCRL1 GAP domain and an empty vector or a vector encoding Myc-tagged Rho mutants. Cellular localization of proteins was examined by immunofluorescence. White arrows show the membranous localization of observed proteins. Bar represents 20 μm.

Co-immunoprecipitation and co-localization of Rac GTPase and endogenous OCRL1

To address the physiological relevance of these interactions, we attempted to demonstrate the formation of a complex between endogenous OCRL1 and Rac GTPase in an intracellular environment. Using an antibody to the N-terminal region of human recombinant OCRL1, we found that the endogenous protein was easily detectable in COS7 cells by immunoblot and could be specifically immunoprecipitated from a cell lysate (Fig. 3). In COS7 cells expressing various Rho GTPases, RacL61 could be co-immunoprecipitated with OCRL1 (Fig. 3); by contrast, nor Rac-wt neither Cdc42L61 could be detected in immunoprecipitates, suggesting a weaker association with OCRL1 (not shown). Our tentative conclusion is that a stable complex between Rac GTPase and endogenous OCRL1 protein can form intracellularly, and that the activated form of Rac is the preferred partner of OCRL1. Therefore, we focused on Rac in the following experiments.

According to previous studies, OCRL1 has been found localized to the TGN (22,32) and lysosomes (23) in fibroblasts and in several other cell types. We have used a kidney-derived epithelial cell line HEK293-T and a kidney fibroblast cell line COS7, both expressing high levels of endogenous OCRL1 (as compared with other cells tested) to reinvestigate the localization of OCRL1 and address the question of Rac co-localization. In COS7 cells (Fig. 4), specific immunodetection of OCRL1 showed a juxta and peri-nuclear staining, colocalizing with the TGN specific marker γ-adaptin, very similar to previously published results (22). To determine the subcellular localization

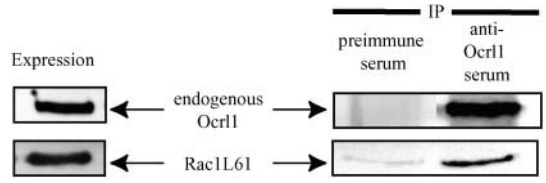

Figure 3. Activated Rac1 co-immunoprecipitates with endogenous OCRL1. COS7 cells were transiently transfected with a vector encoding Myc-tagged Rac1L61. Both proteins can be specifically detected by western blotting (left panels). Immunoprecipitation (IP) of endogenous OCRL1 was done with pre-immune or anti-OCRL1 serum, and analyzed by western blotting (right panels). Co-precipitation of Rac1 was revealed using anti-Myc antibody. Result shown is a representative assay out of three independent experiments.

of Rac GTPase in COS7 cells, we used two different Rac specific antibodies exhibiting no cross reactivity with Cdc42 in western blotting experiments. Interestingly, immunodetection of Rac showed a major peri and juxta-nuclear staining overlapping with γ-adaptin staining (Fig. 4A). Finally, double-label immunofluorescence of Rac and OCRL1 showed an obvious overlapping of the two proteins in COS7 cells (Fig. 4A). Identical results were obtained in HEK293-T cells (not shown). The fungal metabolite Brefeldin A (BFA) has been previously shown to disrupt Golgi stacks and to induce relocalization of the TGN proteins γ-adaptin and OCRL1 to the microtubule organizing center (MTOC) (22). In order to assess more precisely that Rac is associated with the TGN, we performed BFA perturbation assays in COS7 cells in the same conditions as those previously described for OCRL1. Specific MTOC detection was obtained by immunostaining of γ-tubulin which is known to function as the microtubule nucleator at the MTOC (33). Golgi perturbation experiments resulted in a massive dispersion of Rac perinuclear immunostaining and its condensation primarily in two spots strictly overlapping with the γ-tubulin signal (Fig. 4B); this particular pattern could be observed in more than 80% of the observed cells. Moreover, when the same BFA perturbation experiment was performed using a double-label immunofluorescence analysis of Rac and OCRL1, both proteins were found to concentrate principally in the same two juxta nuclear spots (Fig. 4B).

Altogether, these data demonstrate that a large fraction of Rac is, like for OCRL1, associated with the TGN, and therefore support the view that Rac/OCRL1 interaction may take place in this compartment.

Functional outcomes of the binding of Rac GTPase to OCRL1

To get insight into the functional consequences of Rac/OCRL1 interaction, we examined whether OCRL1 can exert a GAP activity towards Rac GTPase. As shown in Figure 5A, recombinant RhoGAP domain of OCRL1 can stimulate *in vitro* GTPase activity of Rac1 in a dose-dependent manner. However, when comparing *in vitro* specific activities of OCRL1 and MgcRacGAP, OCRL1 appears 10 times less active than MgcRacGAP (not shown). Despite the low activity of recombinant OCRL1-GAP measured *in vitro*, expression of the GAP domain in Swiss 3T3 cells leads to a strong inhibition of Rac dependent actin polymerization. As shown in Figure 5B

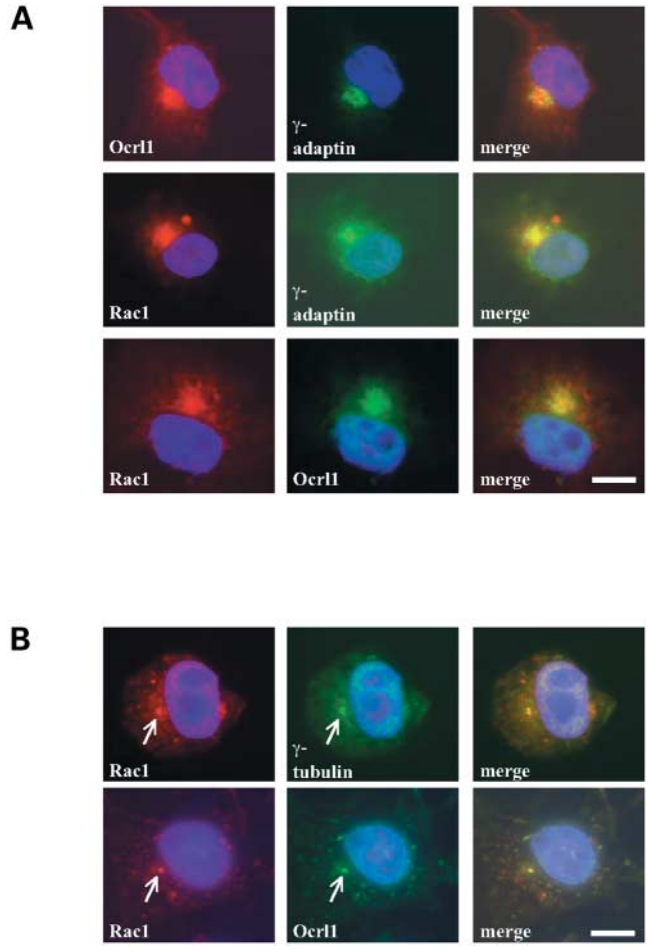

Figure 4. Endogenous Rac1 co-localizes with endogenous OCRL1 to the trans-Golgi network and relocalizes to MTOC after BFA treatment. Cellular localization of endogenous OCRL1, Rac1, γ-adaptin and γ-tubulin was detected by immunofluorescence in kidney fibroblasts (COS7). Nuclear staining was obtained with DAPI. Bar represents 10 μm. (**A**) Untreated cells were double-labelled for OCRL1 or Rac1 with γ-adaptin, a trans-Golgi network marker and signals were merged. Co-localization of Rac1 and OCRL1 was then analyzed in the same way. (**B**) Effect of BFA treatment on the subcellular localization of endogenous Rac1 and OCRL1. After 1 h BFA treatment, immunodetection of Rac and OCRL1 demonstrate that the two proteins co-localize in two concentrated juxta-nuclear spots (arrows) corresponding to the MTOC, as evidenced by γ-tubulin immunostaining.

and C, the formation of EGF induced ruffles which depends on Rac activation was abolished in more than 50% of Swiss3T3 cells expressing OCRL1-GAP; by contrast, LPA-induced stress fibres and bradykinin-induced microspikes (which depend on RhoA and Cdc42, respectively) were not affected by OCRL1-GAP expression. Therefore, it appears that, within the cell, the Rho GAP domain of OCRL1 is capable not only of associating with RacGTP but also specifically inhibiting RacGTP dependent actin reorganization.

DISCUSSION

So far, all studies of OCRL1 have focused on its phosphoinositide 5-phosphatase domain and convincingly demonstrated its enzymatic activity towards phosphatidylinositol 4,5-bisphosphate and its crucial role in regulating the level of this metabolite within the cell. We have addressed for the first time the properties and possible functions of the C-terminal RhoGAP domain of OCRL1. While numerous mutations (including missense mutations) found in Lowe syndrome patients affect this region of OCRL1, most of these mutations seem to result in the absence of OCRL1 protein in patients cells, thus precluding any firm conclusion about the intrinsic function of OCRL1 C-terminus (our unpublished data). However, sequence comparisons of OCRL1 orthologs demonstrate that this RhoGAP domain is evolutionary conserved (19,28,29). Moreover, recent studies have identified four inositol 5-phosphatases in *Dictyostelium discoideum*, one of which, Dd5P4, is homologous to OCRL1 with a RhoGAP domain in its C-terminus. Inactivation of the gene coding for Dd5P4 leads to severe defects in growth and development; interestingly, these defects can be restored by expression of the complete protein but not by the 5-phosphatase catalytic domain alone, therefore demonstrating the critical role of the RhoGAP with respect to Dd5P4 function (28).

The results reported here clearly indicate that OCRL1 interacts with Rho GTPases through its RhoGAP C-terminus, raising the question of the function of this interaction. While the localization of OCRL1 to the TGN has been documented in several cell types, it is not clear so far how OCRL1 associates with or is targeted to endocellular membranes. Sequence analysis of OCRL1 indicates that it has no transmembrane domain nor does it contain a consensus sequence for myristoylation or prenylation. By contrast, Rho proteins are post-translationally modified at their carboxy-terminus by prenylation of a conserved cysteine allowing their interaction with membranes (1); they could therefore mediate the association of OCRL1 with endocellular membranes. Cdc42 was a prime candidate owing to its reported localization at the Golgi apparatus (34,35). Although Cdc42 associates significantly with OCRL1 RhoGAP in GST pulldown and membrane translocation assays, no interaction between endogenous OCRL1 and Cdc42 could be detected in co-immunoprecipitation experiments. By contrast, by all criteria studied including co-immunoprecipitation, Rac appears to be the preferred partner of OCRL1. Although the role of activated Rac in triggering ruffle formation at the plasma membrane has been extensively documented (36,37), recent studies have reported that, in different cell types, a large fraction of Rac protein is localized to perinuclear endomembranes (38,39). In agreement with these observations, we found that in both HEK293-T and COS7 cells, the bulk of endogenous Rac co-localizes with γ-adaptin and OCRL1 in the TGN. This observation was confirmed by the results of BFA perturbation asssays, showing that, similar to γ-adaptin and OCRL1, Rac relocalizes to MTOC, an additional feature of TGN-associated proteins. These data therefore strongly support the idea that OCRL1/Rac interaction could take place physiologically in the TGN.

Therefore, we propose that OCRL1 associates with TGN through the binding of its C-terminus RhoGAP domain to Rac GTPase. The ensuing question which will have to be addressed in future studies is whether OCRL1 PIP2 5-phosphatase activity may be regulated by Rac, which would establish a novel connection between phosphoinositide metabolism and Rho signalling.

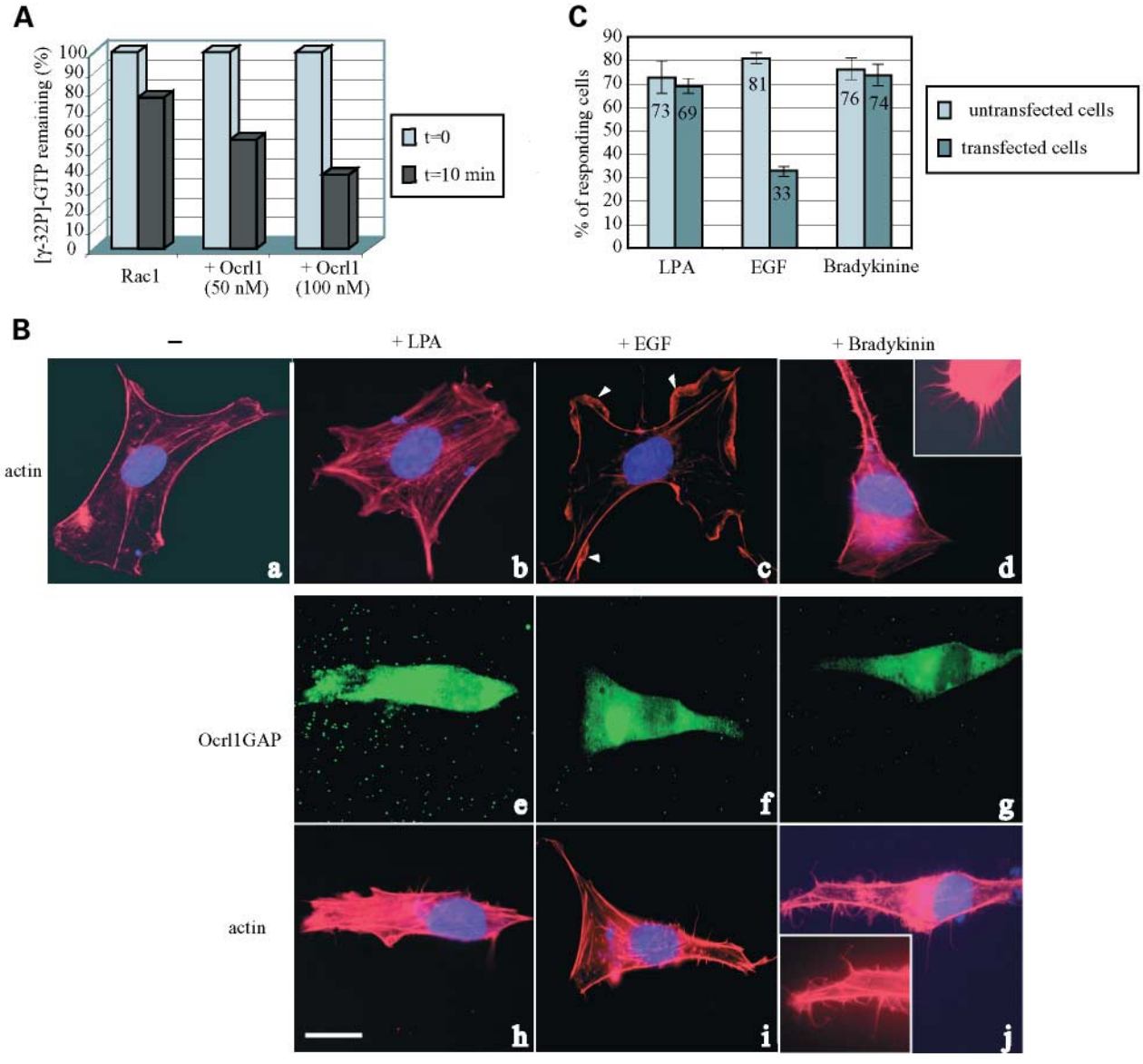

Figure 5. OCRL1 RhoGAP domain shows a GAP activity towards Rac1 GTPase. (**A**) *In vitro* GAP activity of OCRL1 RhoGAP domain. GTPase activity of recombinant Rac1 was measured alone or in the presence of OCRL1 RhoGAP domain (50 or 100 nM). Amount of [γ-^{32}P]GTP remaining bound to Rac1 after 10 min was determined and expressed as a percentage. Data shown are representative of at least three independent experiments. Average effects of 50 nM and 100 nM OCRL1 GAP domain are: +85% ± 11% (SEM) and +170 ± 17% (SEM) respectively. (**B**) Inhibitory effect of OCRL1 RhoGAP domain on growth factor-induced actin reorganization. Swiss 3T3 cells, mock-transfected (a–d) or transiently expressing OCRL1 GAP domain (h–j), were serum-starved, stimulated for 10 min with LPA, EGF or bradykinin. Actin filaments were visualized with Alexa fluor 594-phalloidin. OCRL1 GAP domain is detected by immunfluorescence with FITC (e–g). Nuclear staining was obtained with DAPI. Arrows in panel c indicate typical membrane ruffle structures and the inset in panels d and j shows typical microspike structures. The bar represents 20 μm. (**C**) Counting of cells exhibiting growth factor-induced actin changes after expression of OCRL1 GAP domain. More than 100 cells were observed in each case and results are expressed as a percentage of responding cells. Error bars represent SEM calculated from six independent experiments.

Another issue raised by our results is whether OCRL1/Rac interaction may have additional outcomes in term of Rac function. Specifically, according to *in vitro* measurements, OCRL1 RhoGAP domain exhibits a low but significant GAP activity towards GTPase Rac, which raises the question of its physiological relevance.

OCRL1 RhoGAP domain shows a relatively low degree of identity with RhoGAP consensus sequence as compared to most other RhoGAPs (19); thus, it contains a glutamine instead of the arginine found in the active site of most GAPs for Ras like GTPases. It is to be noticed however that several active GAPs do not use the so-called 'arginine finger' for GTP hydrolysis, suggesting that alternative GAP mechanisms may operate in these cases (40,41). Also in contrast with other RhoGAPs, OCRL1 RhoGAP domain was found in *in vitro* experiments (GST pulldown assays) to bind efficiently to the GDP bound form of Rac which is the product of the GAP induced catalysis; this unexpected property may contribute to a low turn-over rate of the substrate, resulting in a reduced overall GAP activity observed *in vitro*. However, we have no

evidence that full-length OCRL1 can bind the GDP bound form of Rac in co-immunoprecipitation assays, suggesting that this effect may not be relevant within the cell.

Taken together, these unusual features support the view that OCRL1 is not an archetypal RhoGAP and raise the question of its capacity to down-regulate Rho GTPases inside the cell. It has been shown in several instances that actin-based morphological effects of Rho GTPases can be antagonized by transient expression of specific RhoGAPs; interestingly, in all cases, GAP defective mutants had no effects on cell morphology. Therefore, our observation that OCRL1-GAP specifically inhibits EGF-induced ruffles in Swiss 3T3 cells is consistent with OCRL1 being an active GAP towards Rac GTPase.

This novel enzymatic activity associated with OCRL1 could provide new trails in understanding some of the defects observed in Lowe patients cells.

Thus, it has been proposed that there might be a defect in lysosomal membrane trafficking in Lowe patients cells (23,24). Since Rac has been shown to cooperate with Cdc42 and Rho in regulating several aspects of vesicle traffic (37,42), it will be interesting to address the role of Rac/OCRL1 interaction in mediating these functions.

In another connection, alterations of actin cytoskeleton have been recently described in fibroblasts from Lowe patients consisting of a decrease in long actin stress fibres, enhanced sensitivity to depolymerizing agents and abnormal punctuate F actin staining (21). Interestingly, while this abnormality of the actin cytoskeleton may be related to the complex effects of PIP2 on actin polymerization, it is also noteworthy that a similar phenotype can be achieved through a disbalanced activation of Rac and Rho GTPases. Specifically, it has been shown in fibroblasts and other cell types that Rac activation down-regulates Rho activity which in turn leads to altered stress fibre polymerization (43,44). As a consequence, a loss of function of RacGAP in OCRL1-deficient cells would possibly induce a hyperactivation of Rac, resulting in down-regulation of RhoA and inhibition of RhoA dependent actin organization. In this context, it would be of interest to analyse the balance of Rho and Rac activities in Lowe patients cells.

In summary, the data presented in this report bring evidence that, in addition to its PIP_2 5-phosphatase activity, OCRL1 possesses the ability to bind to Rac GTPase and possibly to interfere with its activation status. This novel property may play a role in localizing OCRL1 to the TGN. In addition, as mentionned above, Rho GTPase signalling pathways are critical to neuronal morphogenesis and functions. As a consequence, alteration in genes coding for Rho regulators and effectors have been causally involved in various forms of mental retardation. Through a similar mechanism, loss of the RhoGAP function of OCRL1 could contribute some of the clinical symptoms in Lowe syndrome and may be specifically involved in mental retardation, thus suggesting an unexpected connection with several other forms of X-linked mental retardation.

MATERIALS AND METHODS

Protein purification

The cDNA encoding OCRL1-GAP domain (residues 701–901 according to GenBank entry U57627) was PCR amplified, cloned in pGex-4T2 vector (Amersham Biosciences) and sequenced. Purification of GST and GST fusion forms of OCRL1(GAP domain), Mgc(GAP domain) (residues 238–513), Rac1 and Rac1L61 was done from *E. coli* following the manufacturer's procedure (Amersham Pharmacia Biotech). Fusion proteins were digested with thrombin when necessary. All buffers contained 10 mM $MgCl_2$. Protein purity, as estimated by Coomassie blue staining, was more than 90%. Protein concentration was determined by colorimetric method using BC assay kit (Uptima).

In vitro binding assays

Lysates of COS7 cells, transiently expressing myc-GTPases, were generated with Hepes 50 mM pH 7.5, NaCl 150 mM, $MgCl_2$ 10 mM, TritonX-100 1%, NP40 0.5% and protease inhibitor cocktail (Amersham). Ten micrograms of GST, GST-OCRL1(GAP domain) or GST-Mgc(GAP domain) fusion proteins were coupled to 20 µl of glutathione-sepharose beads and incubated for 2 h at 4°C with 300 µg of COS7 cell lysate, in lysis buffer. Pelleted beads were washed three times with the same buffer containing 1 mM dithiothreitol. Bound proteins were recovered by boiling beads in Laemmli sample buffer (Sigma) and analysed by western blotting.

Immunoprecipitation of endogenous OCRL1

Cell lysis was conducted with Hepes 50 mM pH 7.5, NaCl 150 mM, $MgCl_2$ 10 mM and TritonX-100 0.1% for COS7 cells. A 700 µg aliquot of cell extract was incubated with 20 µl of immune or pre-immune OCRL1 serum for 1.5 h at 4°C, followed by 2 h incubation with Protein G-Agarose (Roche) at 4°C. After centrifugation, supernatant was collected as the depleted lysate and pelleted beads, washed three times with lysis buffer, represented the immunoprecipitate.

Antibodies and western blotting

Antibodies to OCRL1 were raised in rabbit against the N-terminal region (amino acids 1–200) produced in *E. coli*. These antibodies were found to detect, as expected, a 105 kDa protein (consistent with the predicted size of OCRL1) in many human cell types including normal human skin fibroblasts; by contrast, the 105 kDa protein was found decreased or completely absent in skin fibroblasts from Lowe syndrome patients. Interestingly, absence of OCRL1 on western blots was correlated in all cases with a complete loss of OCRL1 5 phosphatase activity in the corresponding cells, thus validating the specificity of OCRL1 immunodetection. Other antibodies used in this study were mouse monoclonal anti-Rac1 antibody (Upstate), rabbit polyclonal anti-Rac1 antibody to human recombinant protein, mouse monoclonal anti-Cdc42 antibody (Santa Cruz), mouse monoclonal anti-RhoA antibody (Transduction Laboratories), 9E10 mouse monoclonal antibody to Myc-tag (Roche), 3F10 rat monoclonal antibody to HA-tag (Roche). Golgi apparatus was labelled with mouse monoclonal anti-γ-adaptin antibody (Transduction Laboratories). Secondary antibodies used in immunofluorescence studies were Texas red-conjugated horse anti-mouse IgG (Vector Laboratories), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse

IgG (Sigma), FITC-conjugated rabbit anti-rat IgG (Jackson ImmunoResearch Laboratories), FITC-conjugated goat anti-rabbit IgG (Vector Laboratories) and Texas red-conjugated goat anti-rabbit IgG (Vector Laboratories) antibodies. Cross hybridization of secondary antibodies was checked in each assay with co-staining. For western blotting, proteins were resolved on 12% SDS–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. Signals were revealed using peroxidase-labelled swine anti-rabbit (Dako) or rabbit anti-mouse immunoglobulins (Dako) and ECL detection system (Amersham Biosciences).

Cell culture, transfections and stimulation by growth factors

HeLa, COS7, HEK 293-T or Swiss 3T3 cells were grown in Dulbecco modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% fetal calf serum and penicillin–streptomycin at 37°C with 5% CO_2. COS7 and HeLa cells were transiently transfected (24 h) with expression vectors pRK5-Myc-GTPase or pcDNA-HA-OCRL1(GAP domain) using FuGENE 6 reagent (Roche) following the manufacturer's procedure. Swiss 3T3 cells were transfected using Lipofectamine Plus method (0.5 µg of plasmid DNA per 22 mm well containing glass coverslips) as described by the supplier (Invitrogen). For Swiss 3T3 stimulation assays by growth factors, cells were transfected for 5 h, washed twice and serum-starved for 24 h in 1 ml of DMEM. They were then stimulated by addition of 50 ng of lysophosphatidic acid (LPA; Sigma), 25 ng of EGF (Roche) or 100 ng of bradykinin (Sigma) and were incubated at 37°C for 10 min.

Immunofluorescence and Golgi perturbation assay

HeLa, COS7 or HEK 293-T cells were plated on 18 mm diameter glass coverslips 16–48 h before transfection, transfected with plasmids and observed 16–40 h later. To detect localization of endogenous and transfected GTPases, 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. 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. Endogenous OCRL1 was detected as previously described (32). Coverslips were mounted using Vectashield (Vector Laboratories) with DAPI at 0.5 µg/ml. Cell preparations were observed under a Zeiss Axiophot epifluorescence microscope; images were digitally acquired and processed using Adobe Photoshop 6.0.

Golgi perturbation assays on COS7 cells using Brefeldin A were conducted as described previously by Dressman *et al.* (22) with 5 µg/ml of BFA for 1 h at 37°C.

In vitro GTPase activity assays

They were conducted as previously described (31). [γ^{32}P]GTP-bound form of Rac1 was prepared by incubating 100 ng of protein with 2 µCi of [γ-^{32}P]GTP (NEN Life Science Products) for 15 min. GTP hydrolysis was initiated by raising $MgCl_2$ to 20 mM, and was stopped after 10 min by dilution into ice-cold, 20 mM $MgCl_2$ buffer. [γ-^{32}P]GTP bound to GTPases was quantified, after sample filtration on BA 85 nitrocellulose (Schleicher and Schuell). GAP assays were conducted in the presence of 2.5–15 pmol of GAP domains of OCRL1 or MgcRacGAP during the GTP hydrolysis step. Measurements were done in duplicate. GTP exchange reaction during GTP hydrolysis step was checked using [α^{32}P]GTP-preloaded GTPases under similar conditions and was found not to be significantly affected by the presence of OCRL1-GAP.

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