Organization on the plasma membrane of the retinitis pigmentosa protein RP2: investigation of association with detergent-resistant membranes and polarized sorting

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Mutations in the retinitis pigmentosa protein gene *RP2* account for up to 15 % of X-linked retinitis pigmentosa. RP2 is a novel protein of unknown function, which is targeted to the plasma membrane by dual N-terminal acyl-modification. Dual-acylated proteins are targeted to lipid rafts, and some are subject to polarized sorting. Therefore we investigated the organization of RP2 on the plasma membrane. Endogenous RP2 protein was predominantly localized at the plasma membrane, and exogenously expressed green-fluorescent-protein-tagged protein was also targeted to the membrane in a wide range of cultured cells. High levels of endogenous RP2 protein were present in HeLa cells and in the retinal pigment epithelium-derived cell line ARPE19. A significant proportion of RP2 in cultured neuroblastoma cells was associated with detergent-resistant membranes (DRMs), but much less than other dually acylated proteins (e.g. Lyn and Fyn). In contrast, the RP2-interacting protein Arl3 (ADP-ribosylation factor-like 3) was not found to be associated with DRMs. The association of RP2 with DRMs was cholesterol-dependent. In polarized epithelial cells in culture and *in vivo*, RP2 was present in both the apical and basolateral domains of the plasma membrane. These data show that RP2 is not specific to either domain, unlike some other dually acylated proteins. Interestingly, the level of RP2 protein increased in the epithelial cell line Caco-2 with differentiation and polarization. These data show that RP2 is present on the membrane of all cell types examined both *in vitro* and *in vivo*, and that RP2 associates with lipid rafts, suggesting a potential role for the protein in signal transduction.

Key words: dual acylation, lipid raft, protein targeting, retinal degeneration, tubulin-folding cofactor.

INTRODUCTION

X-linked retinitis pigmentosa (XLRP) is a severe form of retinal degeneration. Patients in the early stages of disease suffer from night blindness and constricted visual fields as a result of peripheral photoreceptor degeneration. As disease progresses, impairment of central vision occurs, resulting in loss of visual acuity and blindness. Mutations in the *RP2* gene have been shown to account for up to 15 % of XLRP [1–3]. The ubiquitously expressed, 350-amino-acid protein RP2 is a novel protein of unknown function that has sites for dual N-terminal acyl-modification by myristoylation and palmitoylation, which targets RP2 to the plasma membrane [4,5]. We have previously demonstrated that the N-terminal Met-Gly-Cys-Xaa-Phe-Ser-Lys motif (where 'Xaa' represents 'any amino acid') of human RP2 is necessary and sufficient for the protein's acyl modification and subsequent plasma membrane localization [6]. A pathogenic mutation ΔS6 in RP2 [1,7] disrupts the acyl-mediated targeting of RP2 to the plasma membrane [4]. This mutation and the high level of evolutionary conservation at the N-terminus of RP2 suggest that membrane localization is essential for the protein's function in the retina [6]. It has been suggested, however, that RP2 may not be targeted to the plasma membrane in all cell types. RP2 tagged at the C-terminus with green fluorescent protein (GFP) was reported to be predominantly cytoplasmic in two human fibroblast cell lines and in the COS-7 cell line [5].

Proteins that are anchored to the cytosolic surface of the plasma membrane by saturated acyl chains have an affinity for microdomains within the membrane, known as lipid rafts [8–11]. These include dually acylated proteins with myristoyl and palmitoyl moieties that insert into the lipid bilayer [12], such as the Src-related kinase Lyn. Lyn functions in the IgE-signalling process, where it has been hypothesized that its localization in lipid rafts facilitates close proximity to, and subsequent phosphorylation of, the Fc receptor initiating the signalling cascade [13,14]. The thirteen N-terminal residues of Lyn, which contain the sites for myristoylation and palmitoylation, fused to GFP are sufficient for its association with lipid rafts [8].

Polarized epithelial cells show macro-compartmentalization of their plasma membrane into distinct apical and basolateral domains that are maintained by tight junctions [15]. These domains differ in lipid and protein composition. Dually acylated proteins with an asymmetrical distribution in polarized cells include Gtk, a Fyn-related tyrosine kinase from the apical membrane of gut columnar epithelial cells [16]. Another Src family tyrosine kinase, c-Yes, may regulate apical signal-transduction pathways in airway epithelial cells [17], while also being reported to co-localize with occludin at the cell-junction area of

Abbreviations used: Arl2/3, ADP-ribosylation factor-like 2 or 3 protein respectively; DMEM, Dulbecco's modified Eagle's medium; DRM, detergent-resistant membrane; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; MDCK, Madin–Darby canine kidney; PAT, palmitoyl acyltransferase; RPE, retinal pigment epithelium; RPGR, retinitis pigmentosa GTPase regulator; TBS, Tris-buffered saline; XLRP, X-linked retinitis pigmentosa.

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Madin–Darby canine kidney (MDCK) cells [18]. One model of apical sorting to the plasma membrane is on the basis of the formation of these lipid microdomains rich in glycosphingolipids and cholesterol, lipid rafts [10]. In the *trans*-Golgi network, certain transmembrane and glycosylphosphatidylinositol (GPI)-anchored proteins are recruited into lipid microdomains by protein–lipid or protein–protein interactions. These complexes become enriched in raft-based transport containers destined for the apical plasma membrane [19]. It has been suggested that, in polarized epithelial cells and neurons, lipid rafts accumulate in the apical and axonal plasma membrane respectively, although they also exist at reduced levels in the basolateral and somatodendritic membranes [13].

The data presented in the present study demonstrate that the dual-acylation motif of RP2 targets the protein to the plasma membrane in a wide range of cell types, although levels of the protein are variable between different cell types and increase during epithelial cell differentiation. Furthermore, we tested the hypothesis that the RP2, as a dual-acylated protein, associates with detergent-resistant membranes and is sorted to the apical membrane of polarized epithelial cells.

EXPERIMENTAL

Cell culture and transfection

All culture media were from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). SH-SY5Y, CHO, HEK293, ARPE19 and COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12. Lymphoblastoid cells were cultured in RPMI 1640 Glutamax-I medium. HeLa and NIH-3T3 cells were cultured in DMEM. Caco-2 and MDCK cells were cultured in DMEM containing 0.11 $g \cdot l^{-1}$ sodium pyruvate. With the exception of NIH-3T3 cells, which were cultured in 10 % donor calf serum, all cells were cultured in the presence of 10 % (v/v) fetal-calf serum. Lymphoblastoid cell cultures were not treated with antibiotics. Caco-2, MDCK, ARPE19 and NIH-3T3 cells were grown in medium containing 100 units $\cdot ml^{-1}$ penicillin and 100 $\mu g \cdot ml^{-1}$ streptomycin. All other cells were grown in medium containing 50 $\mu g \cdot ml^{-1}$ gentamicin. For maintenance, cells were passaged every 3–4 days or when they were approx. 90 % confluent. For immunofluorescent staining and transfections, cells were cultured in 8-well chamber slides (Life Technologies). Caco-2, ARPE19 and MDCK cells were also grown on cell-culture filters (ICN Biomedicals Inc., Basingstoke, U.K.). Lymphoblastoid cells were cultured in suspension and fixed on slides by cytospin centrifugation [20]. Calcium phosphate transfections with RP2–GFP used 2 μg of plasmid per chamber-slide well. The RP2–GFP construct contained full-length wild-type RP2 in the *Bam*HI–*Age*I site of pEGFP-N1, its construction having been described previously [4]. MDCK cells were micro-injected, using a method described previously [21], with RP2–GFP plasmid DNA when confluent. For cholesterol depletion, SH-SY5Y cells were treated with 10 mM methyl-β-cyclodextrin (Sigma) for 30 min, before cell lysis and detergent-resistant membrane (DRM) fractionation [22,23].

RP2 antisera

Production and characterization of affinity-purified sheep polyclonal antisera S974 has been described previously [4,6]. The anti-peptide RP2 sera hRP2-337-350 were raised against a peptide with the sequence VDSFYNFADIQMGI, corresponding to the C-terminus of RP2, the characterization of which was described by Grayson et al. [24]. S974 was used in Western blot analysis of RP2 in human cell lysates and immunofluorescence staining of human cells. S974 does not cross-react with mouse RP2, whereas hRP2-337-350 shows cross-reactivity with the mouse protein.

Quantification of endogenous RP2 levels in cell lysates

Levels of RP2 protein in lysates generated from cultured human cells were quantified using a Western blotting assay, which we have described previously [20]. Lysates were generated from cells that had been cultured until confluence, except for Caco-2 cells, which were cultured for up to 10 days post-confluence.

Confocal microscopy of cells expressing endogenous RP2 and RP2–GFP chimaeric protein

For immunofluorescence, cells were fixed with 3.7 % (v/v) formaldehyde for 10 min at room temperature, and then permeabilized with 0.1 % (w/v) saponin. RP2 was detected using S974 directly labelled with Alexa Fluor 488 (Molecular Probes, Eugene, OR, U.S.A.). For detection of RP2–GFP, cells were fixed in 3.7 % (v/v) formaldehyde for 10 min at room temperature. Antibodies used for the detection of Na^+/K^+ATPase, sucrase-isomaltase and ZO-1 were as described previously [25,26]. After incubation with primary antibodies for apical and basolateral markers, appropriate Cy3-conjugated secondary antibodies were used. Fluorescence was detected using a Zeiss LSM 510 laser scanning confocal microscope.

Immunohistochemistry

Segments of the small intestine of adult mice were dissected after cardiac perfusion with 4 % (w/v) paraformaldehyde. After 16 h of fixation, samples were dehydrated with industrial methylated spirits, equilibrated in xylene and embedded in paraffin wax. Cross-sections 8 μm in thickness were cut, and mounted on to electrostatically charged glass slides. After dewaxing, the samples were stained using the streptavidin–biotin method. Endogenous peroxidase activity was blocked by incubating the sections in 0.5 % hydrogen peroxide. Antigen retrieval was achieved by microwaving the gut sections in Tris-buffered saline (TBS) [100 mM Tris/HCl/150 mM NaCl (pH 7.6)] containing 5 % (v/v) urea. Non-specific background was blocked with 10 % normal swine serum (Dako, Cambridge, U.K.) and 2 % BSA in TBS for 45 min, before incubation overnight at 4 °C with hRP2-337-350 (1:100 dilution) in 0.1 % BSA in TBS. The slides were then incubated with biotin-conjugated swine anti-rabbit IgG (1:300 dilution) (Dako) in TBS containing 0.1 % BSA for 45 min, washed 3 × 5 min in TBS containing 0.1 % BSA and incubated for a further 45 min with streptavidin-biotinylated horseradish-peroxidase complex (Dako), and washed 3 × 10 min with TBS. The immunoreaction was visualized using 3′,3′-diaminobenzidine (DAB) in 0.03 % hydrogen peroxide in TBS as a chromogen. The sections were dehydrated, clarified and mounted. To verify the specificity of the immunostaining, sections were also stained with no primary antiserum, pre-immune serum and hRP2-337-350 pre-absorbed with the peptide epitope (20 $\mu g/\mu l$).

DRM isolation

DRMs were isolated using a variation of the method of Madore et al. [27]. Briefly, SH-SY5Y cell pellets were resuspended in

10 mM Tris/HCl, pH 8.2, containing a protease inhibitor cocktail (Sigma, Poole, Dorset, U.K.). Triton X-100 was added to SH-SY5Y samples to a final concentration of 1 % (v/v), and the samples were then incubated for 30 min at 4 °C. The gradient consisted of the sample in a 40 % sucrose cushion with 30 % and 5 % sucrose steps overlaid. 1 % Triton X-100 was present throughout the gradient. Following centrifugation (200 000 ***g*** for 18 h at 4 °C), fractions of the gradient were withdrawn (from the top) and equal fraction volumes were analysed by Western blotting. RP2 was detected in the fractions using antisera S974. Lyn, Fyn, and Rho A were detected using commercially available antibodies from Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.). Transferrin receptor was detected using monoclonal H68.4 from Zymed (Zymed Laboratories Inc., South San Francisco, CA, U.S.A.). Arl3 was detected using a rabbit polyclonal antiserum (kindly given by Dr Sally Lewis and Dr Nicolas Cowan, New York University Medical Center, New York, NY, U.S.A.). For the detection of the ganglioside GM_1, fractions were dot-blotted, and those containing the ganglioside were identified by binding of biotinylated cholera toxin β-subunit (Sigma).

RESULTS

RP2 is localized to the plasma membrane in a wide range of cell types

We have previously shown that endogenous human RP2 is localized predominantly to the plasma membrane in the neuroblastoma-derived cell line SH-SY5Y [4] and in lymphoblastoid cells [20]. In addition, we have shown that RP2 fused at the C-terminus to GFP is targeted to the plasma membrane in CHO cells [6]. Another study has reported that RP2–GFP was localized to the plasma membrane in HeLa cells, but was cytoplasmic in two human fibroblast cell lines and COS-7 cells, and suggested that RP2 may be processed differently in some cell types [5].

To investigate further the intracellular localization of RP2 in cultured human cells, we compared the localization of the endogenous RP2 protein in SH-SY5Y, lymphoblastoid, HeLa, HEK-293T, Caco-2 and ARPE19 cells by immunofluorescence staining using affinity-purified sheep polyclonal anti-RP2, serum S974 [4] (Figure 1A). Consistent with our previous findings, RP2 staining was localized mainly at the plasma membrane in SH-SY5Y and lymphoblastoid cells [4,20]. Plasma membrane staining also dominated in HeLa, HEK-293T and the epithelial cell lines Caco-2 and ARPE19. Not all of the RP2 protein was membrane-associated, since low levels of diffuse cytoplasmic staining were observed in all cell types.

We also examined the intracellular targeting of RP2–GFP in transfected non-human cell types (Figure 1B). As we have reported previously, the chimaeric protein localized predominantly to the plasma membrane in the fibroblast-like CHO cells [6]. RP2–GFP was also targeted to the plasma membrane in the dog kidney epithelial cell line (MDCK), mouse embryonic fibroblast NIH-3T3 cells and the monkey-kidney-derived, fibroblast-like COS-7 cell line. In COS-7 cells, more cytoplasmic and nuclear staining was observed relative to other cell types. High levels of RP2–GFP overexpression in COS-7 cells caused the plasma membrane component of the staining to be partially obscured by intracellular membrane, cytoplasmic and nuclear staining, as the cells became saturated with heterologous protein. These data indicate that RP2 is targeted to the plasma membrane in a wide range of human and other mammalian cell types, including fibroblasts.

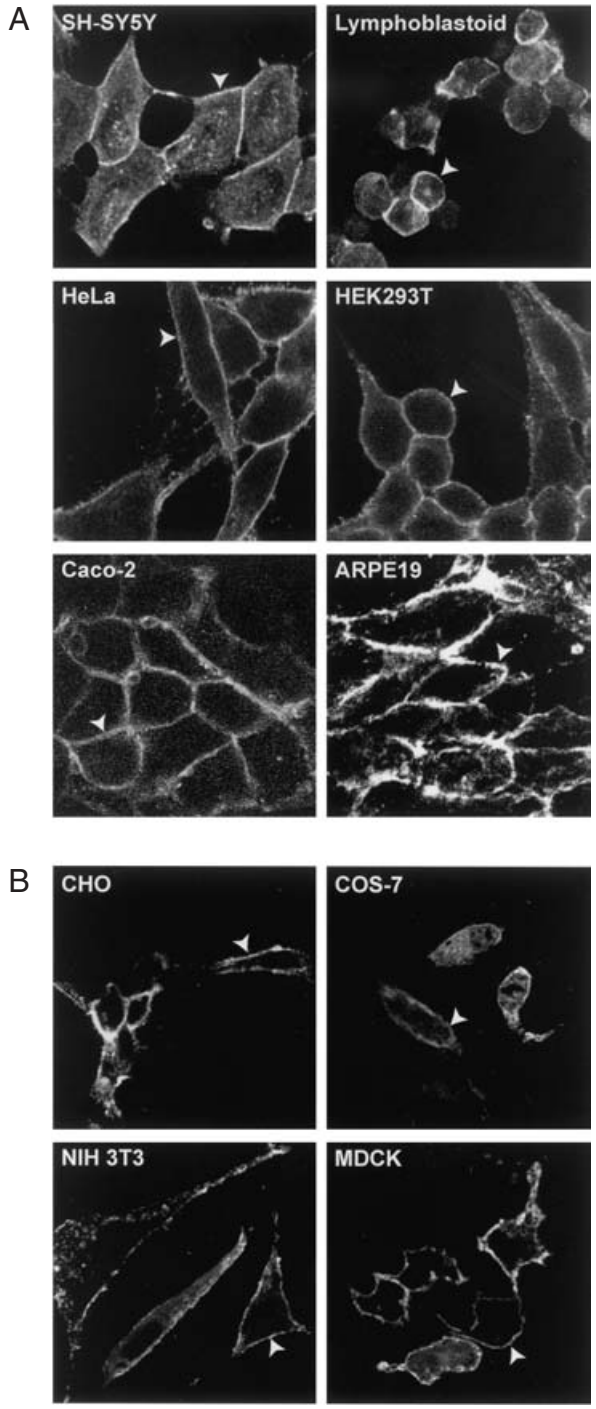

Figure 1 Confocal analysis of the intracellular localization of RP2 protein in a range of cell types

RP2 protein localization at the plasma membrane is highlighted by white arrowheads. (**A**) Localization of endogenous RP2 detected by immunofluorescence in SH-SY5Y, lymphoblastoid, HeLa, HEK-293T, Caco-2 and ARPE19 cells. Confocal images are 55 μm^2. (**B**) Localization of RP2–GFP chimaeric protein in transiently transfected CHO, COS-7, NIH-3T3 and MDCK cells. Confocal images are 80 μm^2.

Levels of RP2 are variable between different cell types

Although RP2 expression and localization at the plasma membrane appear ubiquitous, the level of RP2 in different cultured cell types was highly variable. We quantified relative levels of RP2 in

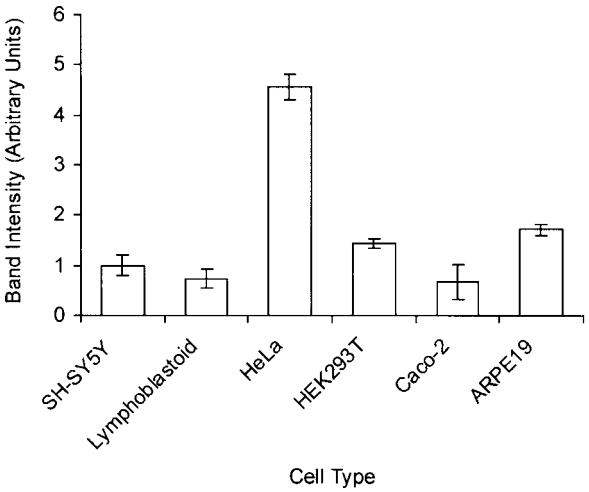

Figure 2 RP2 protein levels in a range of cell types

Relative levels of RP2 in cell lysates were determined from densitometric analysis of Western blots containing recombinant protein standards, and were normalized to the amount of RP2 in SH-SY5Y cells. Error bars represent S.E.M.

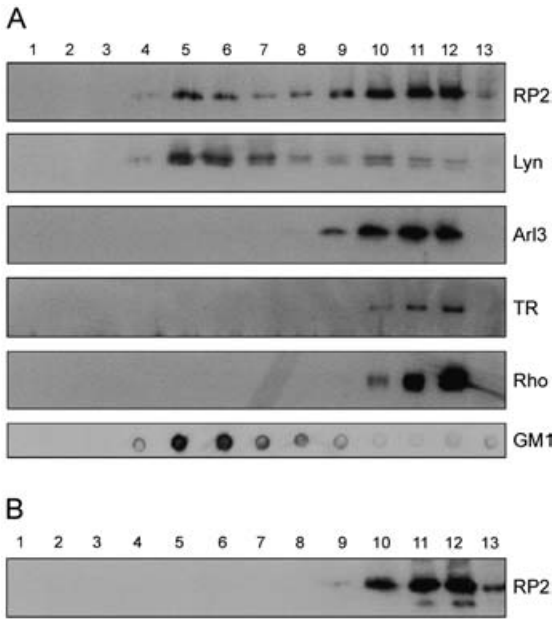

Figure 3 Fractionation of RP2 with detergent-resistant membranes

(**A**) The distribution of RP2, Lyn, Arl3, transferrin receptor (TR), Rho A (Rho) and ganglioside G_{M1} on a sucrose gradient following solubilization of SH-SY5Y cells in 1 % (w/v) Triton X-100 at 4 °C. Equal fraction volumes were loaded on to the gel for electrophoresis. Fractions are numbered from the top of the gradient. (**B**) The distribution of RP2 on a sucrose gradient following solubilization of SH-SY5Y cells in 1 % Triton X-100 at 4 °C after cholesterol depletion with methyl-β-cyclodextrin.

different cell types using a well-characterized semi-quantitative Western blotting assay for RP2 [20], normalized for equal protein loading (Figure 2). The highest levels of RP2 were observed in HeLa cells, which had over 2.5-fold more RP2 than any of the other cell types examined. Interestingly, the retinal pigment epithelium (RPE) cell line, ARPE19, also had relatively high levels of RP2. HEK-293T cells also expressed RP2 at relatively high levels, whereas SH-SY5Y, lymphoblastoid and Caco-2 cells contained comparatively less RP2 protein.

RP2 is associated with DRMs

Dually acylated protein, including Src family tyrosine kinases, can associate with lipid rafts [8,12,28]. As RP2 is a novel protein with N-terminal myristoyl and palmitoyl modification, we tested its possible association with lipid rafts. An established technique for the identification of raft components is detergent solubilization at 4 °C, which leads to the isolation of a light membrane fraction, termed DRMs. This fraction contains the remnants of cellular raft domains aggregated together [29,30]. We purified DRMs by centrifugation to equilibrium on a sucrose-density gradient [27]. In SH-SY5Y cells, the majority of RP2 remained in the region of the gradient containing fully solubilized proteins (fractions 9–12); however, a significant proportion co-purified with DRMs (fractions 5 and 6) as determined by the lipid-raft marker, ganglioside GM_1 (Figure 3A). The dually acylated Src related kinases Lyn (Figure 3A) and Fyn (results not shown) also co-purified with DRMs. Lyn was predominantly in fractions 5 and 6, but a small amount was also present in the region of the gradient containing solubilized proteins. The specificity of the DRM fraction was confirmed by Western blotting for membrane-associated proteins that are not present in lipid rafts. Transferrin receptor, a type II membrane protein, and Rho, a prenylated small GTPase, did not float in the gradient, and both were present only in fractions 10–12 (Figure 3A). The ADP-ribosylation factor-like protein 3 (Arl3), which is known to interact with RP2 [31], was found exclusively in the region of the gradient containing solubilized proteins.

Cholesterol depletion of cells with methyl-β-cyclodextrin disrupts DRMs [22,23]. In SH-SY5Y cells treated with methyl-β-cyclodextrin before DRM fractionation, no RP2 was present in fractions 5 and 6 (Figure 3B), showing that the association of RP2 with DRMs was cholesterol-dependent. After 30 min of methyl-β-cyclodextrin treatment, no change in cell viability was observed, and cholesterol was not enriched in fractions 5 and 6 relative to other fractions of the gradient. These data suggest that a proportion of RP2 is recruited to lipid-raft microdomains at the plasma membrane of cells.

RP2 is present in the apical and basolateral domains of polarized epithelial cells

Polarized targeting of peripheral membrane proteins has been reported in epithelial cells and neurons. Dual-acylated proteins and proteins that are present in lipid rafts have been reported to be targeted to the apical membrane [13]. Therefore we investigated whether RP2 was preferentially targeted to the apical or basolateral domains of the plasma membrane in polarized epithelial cells by confocal microscopy. Endogenous RP2 localization was compared with that of apical and basolateral markers of polarized Caco-2 cells in XZ section (Figure 4A). RP2 staining closely overlapped with staining of both Na^+/K^+ATPase in the basolateral domain and brush-border enzyme sucrase-isomaltase in the apical domain of the plasma membrane. To support these observations in Caco-2 cells, we examined the localization of RP2–GFP on the plasma membrane of polarized MDCK cells (Figure 4B). RP2–GFP was present in the basolateral and the apical domain, as demonstrated by its distribution relative to the tight junction marker ZO-1. Polarization of RP2 was not observed in Caco-2 or MDCK cells under any growth conditions, including culture for 5 weeks on membrane filters.

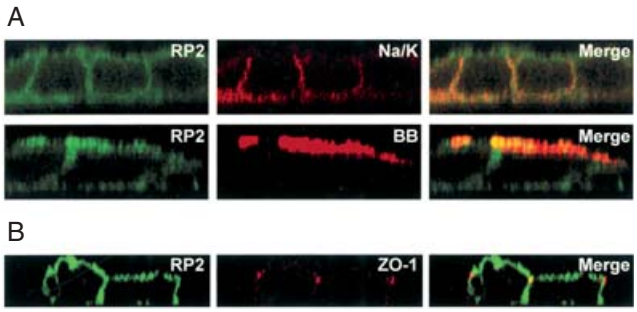

Figure 4 RP2 localization in polarized epithelial cell in culture

(**A**) Double immunofluorescent confocal microscopy XZ sections showing RP2 staining in Caco-2 cells overlapping with both Na^+/K^+ATPase (Na/K) localization in basolateral plasma membrane and brush-border enzyme sucrase-isomaltase (BB) localization in the apical plasma membrane. Confocal images are 55 × 20 μm. (**B**) Confocal microscopy XZ section showing RP2–GFP chimaeric protein fluorescence and endogenous ZO-1 tight-junction protein immunofluorescent staining in micro-injected MDCK cells. Confocal images are 60 × 16 μm.

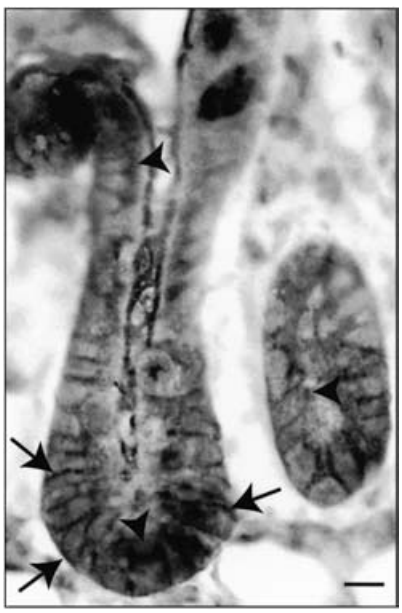

Figure 5 RP2 localization in polarized epithelial cells *in vivo*

RP2 immunohistochemical staining in mouse small intestine. Arrows indicate basolateral membrane staining in columnar epithelium cells of the crypts of Lieberkühn. Apical staining is indicated by arrowheads. The luminal staining is non-specific. The scale bar is 10 μm.

To complement our study in cultured epithelial cells we also examined the localization of RP2 *in vivo*. RP2 expression was analysed in the columnar epithelium cells of the crypts of Lieberkühn of the mouse small intestine (Figure 5). Immunohistochemical staining with a rabbit peptide antibody raised against the C-terminus of RP2 [24] was most intense at the plasma membrane of epithelial cells. Immunolabelling was of similar intensity in the basolateral and apical regions of the plasma membrane. RP2 distribution appeared identical in epithelial cells in both the crypt and the upper region of the villus. RP2 staining was most intense in the crypts and decreased along the crypt–villus axis. Pre-immune serum and peptide competition controls demonstrated the specificity of the plasma membrane staining pattern (results not shown). These results indicate that, even though RP2 is DRM-associated and dually acylated, it is not targeted to specific membrane macrodomains in polarized epithelial cells.

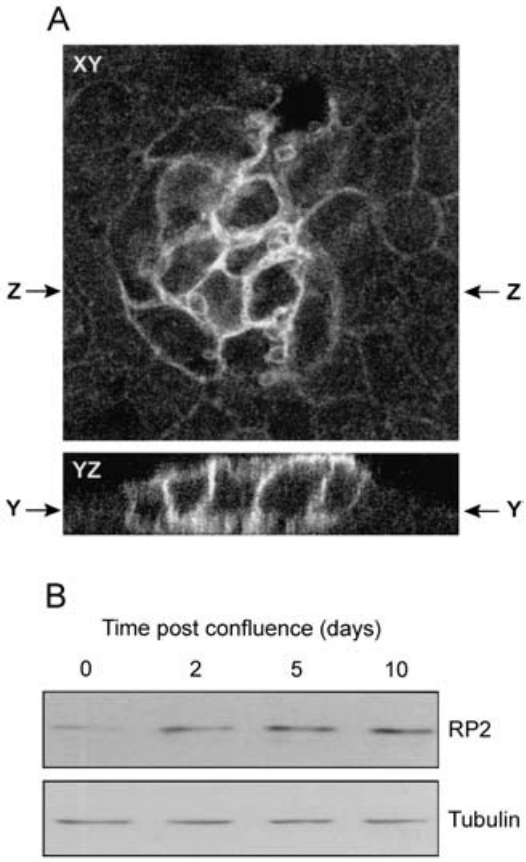

Figure 6 Levels of RP2 increase in differentiating Caco-2 cells

(**A**) XY and YZ section confocal immunofluorescent images showing a monolayer of Caco-2 cells 5 days post-confluence, containing an island of cells with more intense RP2 staining. In the XY section, arrows mark the position of the YZ section, and in the YZ section, arrows mark the position of the XY section. The XY image is 75 μm^2 and the YZ image is 75 × 16 μm. (**B**) A representative Western blot showing levels of RP2 in Caco-2 cell lysates 0, 2, 5 and 10 days post-confluence. Levels of β-tubulin are shown as an internal standard for total protein loading.

Levels of RP2 in Caco-2 cells increase with differentiation

We observed that the intensity of RP2 plasma membrane staining in Caco-2 cells increased with the degree of cellular differentiation. In a post-confluent monolayer of Caco-2 cells, 'islands', with more intense RP2 plasma membrane staining, were detected (Figure 6A). Cells within these islands expressed marker proteins for cell differentiation and polarization, such as the brush-border enzyme sucrase-isomaltase, a protein that is up-regulated in differentiating intestinal epithelial cells [25] (Figure 4). The cells were also taller and more cuboidal in appearance when imaged in YZ section, indicating a higher degree of cellular differentiation than surrounding cells. RP2 staining was much lower in the undifferentiated Caco-2 cells, but the strongest component of the staining was still plasma-membrane-localized. To investigate further the increase in RP2 levels in Caco-2 cells as they differentiated, we compared levels of the protein by Western blot analysis in cells grown for increasing periods of time post-confluence (Figure 6B). Levels of RP2 protein doubled from 0–10 days post-confluence, correlating with Caco-2 cell differentiation. Despite this doubling in the level of RP2

after differentiation, the level of RP2 was still low compared with ARPE19 or HeLa cells, and was equivalent to that in SH-SY5Y cells (Figure 2). Collectively, these results indicate that levels of RP2 increase in Caco-2 cells with the degree of polarized differentiation. In contrast, the level of RP2 did not significantly change between ARPE19 cells that had just reached confluence, and those that were cultured for 5 weeks post-confluence (results not shown). Long-term ARPE19 cultures expressing the RPE-differentiation marker RPE65, however, do not exhibit a strong polarized sorting phenotype of apical and basolateral markers when grown on cell-culture plastic matrix. This observation suggests that these cells do not fully differentiate over this timescale and may, therefore, not stimulate an increase in RP2 expression.

DISCUSSION

We have previously demonstrated that RP2 is targeted to the plasma membrane by an N-terminal Met-Gly-Cys-Xaa-Phe-Ser-Lys motif, which mediates dual acylation of the protein [6]. It has been suggested, however, that RP2 may not be targeted to the plasma membrane in all cell types [5]. To address this issue, we investigated the localization of both endogenous and GFP-tagged RP2 in a wide range of cell types. We observed that RP2 was efficiently targeted to the plasma membrane in each of the cell types examined. In COS-7 cells expressing high levels of the RP2–GFP protein, however, the chimaeric protein also accumulated on intracellular membranes and in the cytoplasm, suggesting a potential saturation of the dual acyl-modification machinery. The ubiquitous expression of the acyl-modification machinery predicts that RP2 should be targeted to the plasma membrane in most cell types, and our data confirm this hypothesis. Possible reasons for the reported differences in localization seem likely to reflect variations in experimental conditions, such as high levels of heterologous protein expression or the level of endogenous acylated proteins, rather than cell-type variation in the myristoylation and palmitoylation machinery. The association of RP2 with the plasma membrane may be dynamic and, therefore, one should consider the possibility that RP2 localization could be regulated by other cellular factors in addition to N-myristoyl transferase and palmitoyl acyltransferase (PAT).

RP2 is expressed ubiquitously, but there are significant variations in levels of the protein between cell types and during cellular differentiation. With the exception of HeLa cells, the highest levels of RP2 were observed in ARPE19 cells. These cells have been used extensively as a model for studies of the RPE [32]. The RPE fulfils many essential functions in the retina, including trophic support, metabolite delivery and the phagocytosis of photoreceptor outer segments. The site of the primary pathological deficit for retinitis pigmentosa caused by mutations in RP2 is not clear from the protein's localization in the retina. Therefore it remains possible that mutations in *RP2* could cause retinal disease through a disruption of RPE function, and at least seven genes expressed in the RPE have been associated with photoreceptor degeneration to date [33].

Polarity is a fundamental property of eukaryotic cells that requires the asymmetrical distribution of many plasma-membrane-associated proteins. Dual-acylated proteins and proteins that are present in lipid rafts have been reported to be targeted to the apical membrane [13]. RP2 did not show partitioning into either the apical or basolateral domains of polarized epithelial cells, either *in vitro* or *in vivo*. Raft association alone, however, may not be sufficient for apical sorting. It has been demonstrated that a GPI-anchored form of rat growth hormone was raft-associated, but only apically sorted if glycosylated with an N-glycan, suggesting N-glycans may also act as apical sorting signals for GPI-anchored proteins [34]. RP2 could have a cell-type-specific sorting signal, but our results indicate the compartmentalization of RP2 to a specific membrane macrodomain does not occur in epithelial cells or in the retina [24]. Interestingly, we did observe an increase in levels of RP2 in Caco-2 cells as they polarized, which may suggest a function for RP2 related to cellular differentiation, such as specialized protein transport or signalling.

We have demonstrated that a proportion of RP2 co-fractionates with DRM markers. Cytoplasmic proteins found in the DRM fraction are associated with raft domains via the cytoplasmic leaflet of the lipid bilayer. These include several signalling molecules such as the Gα subunit of heterotrimeric G-proteins, and the Src-like protein kinases Lck, Fyn and Lyn, which depend on multiple acylation for raft association [35–38]. A smaller proportion of RP2 fractionated into DRMs than Fyn and Lyn. However, a greater proportion of RP2 could be raft-associated than our data suggest. Solubilization of membranes with 1% Triton X-100 is a relatively stringent extraction of DRMs [22], isolating ordered sphingolipid/cholesterol domains. It is possible that significantly more RP2 could be raft-associated to semi-ordered lipid domains that would float in less stringent detergents [27]. It has also been reported that raft proteins connected to the cytoskeleton may not float after detergent extraction [13]. This may be particularly relevant to RP2, given its association with the microtubule-associated protein Arl3 [24], and its functional relationship with tubulin [31]. Palmitoylation of RP2 may occur in rafts, as it has been suggested that PAT activity is enriched in these microdomains [39]. PAT activity could also have a role in the rapid repalmitoylation of caveolar-localized signalling proteins that undergo cycles of palmitoylation and depalmitoylation [40]. Lipid rafts provide microenvironments on the cell surface, which concentrate receptors and effectors on both sides of the membrane. This favours specific protein–protein interactions, and can result in the activation of signalling cascades [13]. Our data suggest that RP2 is targeted to the plasma membrane in all cell types, and within the plasma membrane to microdomains, where it may have a role in such a signalling cascade.

RP2 has sequence similarity to the tubulin-specific chaperone, cofactor C [1], which has recently been shown to extend to a functional overlap [31]. Together with cofactors D and E, cofactor C stimulates the GTPase activity of native tubulin in a reaction regulated by Arl2 [41]. RP2 can replace cofactor C in this *in vitro* assay and stimulate the GTPase activity of tubulin in the presence of cofactor D. RP2 does not interact with Arl2; instead, RP2 binds the GTP-bound form of the related protein, Arl3. RP2 does not appear to interact with Arl3 in lipid rafts, because Arl3 was not present in DRMs. This would agree with the observation that the binding between Arl3 and RP2 is diminished when RP2 is myristoylated [31], and suggests that the proteins may interact at a site not on the membrane.

It has been noted that Arl3 also interacts with phosphodiesterase δ ('PDEδ'), which in turn interacts with retinitis pigmentosa GTPase regulator (RPGR) [31]. Mutations in RPGR are another major cause of XLRP [3], and it is tempting to speculate that these proteins function in a common pathway that is essential for photoreceptor viability. Nevertheless, the function of all these proteins in the retina remains to be fully determined. The major challenges now are to delineate the dynamics of RP2 membrane association, identify the interacting partners of RP2 on the membrane and within lipid rafts, and determine the cellular pathways under the control of these proteins in the retina.

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