Targeting of the type II inositol polyphosphate 5-phosphatase INPP5B to the early secretory pathway

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Summary

The inositol polyphosphate 5-phosphatase INPP5B is closely related to the Lowe syndrome protein OCRL1, sharing a similar substrate specificity, domain organisation and an ability to compensate for loss of OCRL1 in knockout mice. The cellular localisation and functions of INPP5B have remained poorly defined until recently, when a role within the endocytic pathway was suggested. Here, we report that INPP5B is also localised to the early secretory pathway including the Golgi apparatus and ER-to-Golgi intermediate compartment (ERGIC). Consistent with this localisation, INPP5B binds to specific RAB proteins within the secretory pathway, and mutational analysis indicates that RAB binding is required for efficient Golgi targeting of INPP5B. Unlike OCRL1, INPP5B interacts with neither clathrin nor α-adaptin and is largely absent from clathrin-coated intermediates. Expression of INPP5B but not OCRL1 alters the distribution of the cycling protein ERGIC53 when cells are incubated at low temperature (15°C) or in the presence of brefeldin A, causing ERGIC53 to accumulate in the ERGIC, with a concomitant loss from the ER. Our data suggest a role for INPP5B in retrograde ERGIC-to-ER transport and imply that it has functions distinct from those of OCRL1 within both the secretory and endocytic pathways.

Supplementary material available online at
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Key words: Type II 5-phosphatase, INPP5B, Golgi apparatus, Intermediate compartment, Endosome, RAB

Introduction

Phosphoinositides comprise a family of lipids derived from phosphatidylinositol (PtdIns) by differential phosphorylation of the inositol ring at the D-3, D-4 and D-5 positions, generating seven distinct species. Although minor in terms of abundance, phosphoinositides are key regulators of a variety of cellular processes, including intracellular signalling, actin dynamics and membrane traffic (Cantley, 2002; De Matteis et al., 2005; Di Paolo and De Camilli, 2006; Roth, 2004). This can be achieved by acting as precursors to second messengers or through specific interactions with effector proteins that bind via modular binding domains to target and/or alter the activity of these proteins (Balla, 2005; Carlton and Cullen, 2005). In this way, phosphoinositides help determine the functional identity of intracellular membrane compartments, and, as might be expected, different phosphoinositides appear to be concentrated at distinct locations within the cell (Downes et al., 2005). Interconversion between the various phosphoinositide species is mediated by phosphoinositide kinases and phosphatases, which add or remove phosphate from specific positions on the inositol ring. These enzymes must be tightly regulated both spatially and temporally in order for various phosphoinositide-dependent processes to function correctly, and, for certain phosphoinositide kinases and phosphatases, interactions with other proteins, most notably small GTPases, have been shown to affect their localisation and/or catalytic activity (e.g. Godi et al., 1999; Honda et al., 1999; Malecz et al., 2000).

Type II inositol polyphosphate 5-phosphatase (INPP5B) was first discovered nearly 20 years ago (Mitchell et al., 1989), and subsequent studies have shown that it preferentially hydrolyses the 5-phosphate of both phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] and phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3], as well as displaying activity towards soluble inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] and inositol (1,3,4,5)-tetrakisphosphate [Ins(1,3,4,5)P_4] (Jefferson and Majerus, 1995; Matzaris et al., 1994; Schmid et al., 2004). INPP5B has a central 5-phosphatase domain that contains two conserved motifs shared with other 5-phosphatases, plus a RHO GTPase-activating protein (GAP)-like domain at the C-terminus, and is subjected to prenylation at the cysteine within the extreme C-terminal CNPL sequence (Jefferson and Majerus, 1995; Matzaris et al., 1998). The closest homologue of INPP5B is OCRL1, the protein mutated in the X-linked disorder oculocerebrorenal syndrome of Lowe, characterised by defects in eye, brain and kidney function (Attree et al., 1992). INPP5B and OCRL1 share the same domain architecture, exhibit 45% sequence identity and have similar substrate specificity (Lowe, 2005). INPP5B knockout mice are normal, apart from male sterility that is evident in certain genetic backgrounds, whereas OCRL1 knockout mice have no apparent defects (Hellsten et al., 2001; Janne et al., 1998). However, knockout of both INPP5B and OCRL1 results in embryonic lethality, consistent with these enzymes compensating for loss of each other in the mouse, and suggesting that they have overlapping functions within the cell (Janne et al., 1998).

INPP5B was initially thought to be localised to mitochondria

(Speed et al., 1995), but more recently it has been found within the endocytic pathway (Shin et al., 2005). INPP5B binds to RAB5 and localises to enlarged endosomes generated by overexpression of RAB5 (Shin et al., 2005). When cells are stimulated with growth factors, INPP5B translocates to lamellipodia, where it appears to function in a RAB5-controlled enzymatic cascade for the conversion of PtdIns(3,4,5)P_3 to phosphatidylinositol 3-phosphate (PtdIns3*P*) during endocytosis (Shin et al., 2005). This pathway probably links growth factor signalling with membrane dynamics in the endocytic pathway. Like INPP5B, OCRL1 is found in lamellipodia and endosomes, but it is also abundant at the Golgi apparatus (Choudhury et al., 2005; Faucherre et al., 2005; Olivos-Glander et al., 1995; Ungewickell et al., 2004). It can interact with RAC1, components of the clathrin coat and both secretory and endosomal RAB proteins (Choudhury et al., 2005; Faucherre et al., 2003; Hyvola et al., 2006; Ungewickell et al., 2004). Functional studies suggest that OCRL1 participates in endosome-to-Golgi trafficking, but a role during clathrin-mediated endocytosis also appears likely (Choudhury et al., 2005; Lowe, 2005).

Given the similarities between INPP5B and OCRL1 and the ability of these enzymes to compensate for one another in mouse knockout studies, we decided to perform a more comprehensive analysis of the localisation and interactions of INPP5B. Here, we find that INPP5B localises to the early secretory pathway in addition to endosomes. We also find that INPP5B interacts with secretory RAB proteins and that RAB binding is required for efficient targeting of INPP5B to the Golgi apparatus. Overexpression of INPP5B causes a marked redistribution of the cycling protein ERGIC53 to the ER-to-Golgi intermediate compartment (ERGIC) when cells are incubated at low temperature or treated with brefeldin A. Together, our data support a previously unsuspected role for INPP5B within the early secretory pathway, namely in retrograde ERGIC-to-ER transport.

Results

Localisation of INPP5B to compartments of both the early secretory and endocytic pathways

A recent study has localised INPP5B to the enlarged endosomes seen upon expression of constitutively active RAB5 and to growth-factor-induced plasma membrane ruffles (Shin et al., 2005). To investigate INPP5B localisation in greater detail, we prepared an N-terminally GFP-tagged construct and analysed its distribution in various cell types. As shown in Fig. 1, a significant amount of GFP-INPP5B appeared to be cytosolic in HeLa cells. However, there was also a concentration of the protein on a perinuclear ribbon structure reminiscent of the Golgi apparatus. This structure colocalised with GM130 and TGN46, which label the cis- and trans-Golgi, respectively (Fig. 1A). Localisation of GFP-INPP5B to the Golgi apparatus was confirmed by its presence on Golgi fragments generated by nocodazole treatment (Fig. 1B). There was better overlap between GFP-INPP5B and GM130 or ERGIC53 than with TGN46, suggesting that INPP5B is enriched on the cis-side of the Golgi apparatus and is possibly present on the ERGIC (see also Fig. 1D). By contrast, under similar conditions, GFP-OCRL1 overlapped best with TGN46 (see supplementary material Fig. S1), consistent with an enrichment on the TGN, as suggested by previous work (Dressman et al., 2000). Localisation of INPP5B to the Golgi was not a fixation artefact and was observed using both methanol- and paraformaldehyde-fixed cells (supplementary material Fig. S2). Moreover, this localisation was also seen in the other cell types analysed, including normal rat kidney (NRK) and COS-7 cells (supplementary material Fig. S2, and data not shown) and was independent of the expression level or the type of tag used: FLAG-tagged INPP5B exhibited the same Golgi distribution as the GFP-tagged version (supplementary material Fig. S3).

In addition to the Golgi staining, GFP-INPP5B was also present on numerous cytoplasmic puncta. Double labelling indicated that some of these structures, most notably those in the cell periphery, contained the transferrin receptor, indicating that they are endocytic intermediates (Fig. 1C, see also Table 1). Overlap with the early endosomal marker EEA1 was less compelling. In addition, there were a substantial number of GFP-INPP5B puncta that lacked both the transferrin receptor and EEA1. Many of these structures overlapped with markers of the ERGIC, including β-COP and ERGIC53 (Fig. 1D), indicating the localisation of GFP-INPP5B to the ERGIC in addition to the Golgi apparatus and endosomes (Fig. 1D). Quantitation revealed that, at steady state, 47% of the GFP-INPP5B punctate structures contained ERGIC53, whereas 50% were positive for the transferrin receptor (Table 1). By contrast, when a similar analysis of GFP-OCRL1 was performed, only 21% of the GFP-OCRL1 puncta contained ERGIC53, whereas 72% were positive for the transferrin receptor.

INPP5B is largely absent from clathrin-coated vesicles and fails to bind to clathrin or AP-2

Previous studies have shown that OCRL1 is enriched in clathrin-coated buds and vesicles, which probably results from its binding to the clathrin heavy chain and to α-adaptin, a subunit of the plasma membrane AP-2 clathrin adaptor complex (Choudhury et al., 2005; Ungewickell et al., 2004). We therefore investigated whether INPP5B is present in clathrin-coated transport intermediates. By immunofluorescence microscopy, we observed a low degree of overlap between GFP-INPP5B puncta and those containing clathrin (Fig. 2A and Table 1). Despite the low level of colocalisation between INPP5B and clathrin, we nevertheless investigated the possible interaction between INPP5B and clathrin or its associated

Table 1. Quantitation of overlap between GFP-INPP5B or GFP-OCRL1 and marker proteins

	GFP-INPP5B		GFP-OCRL1	
	37°C	15°C	37°C	15°C
ERGIC53	47	98	21	24
COPI	50	ND	19	ND
EEA1	23	ND	22	ND
TfR	50	2	72	69
Clathrin	23	ND	ND	ND

In each case, 300 puncta containing either GFP-INPP5B or GFP-OCRL1 were scored for the presence of the indicated marker protein. Results are expressed as the percentage of GFP-INPP5B or GFP-OCRL1 puncta that contained the marker of interest. ND, not determined; TfR, transferrin receptor.

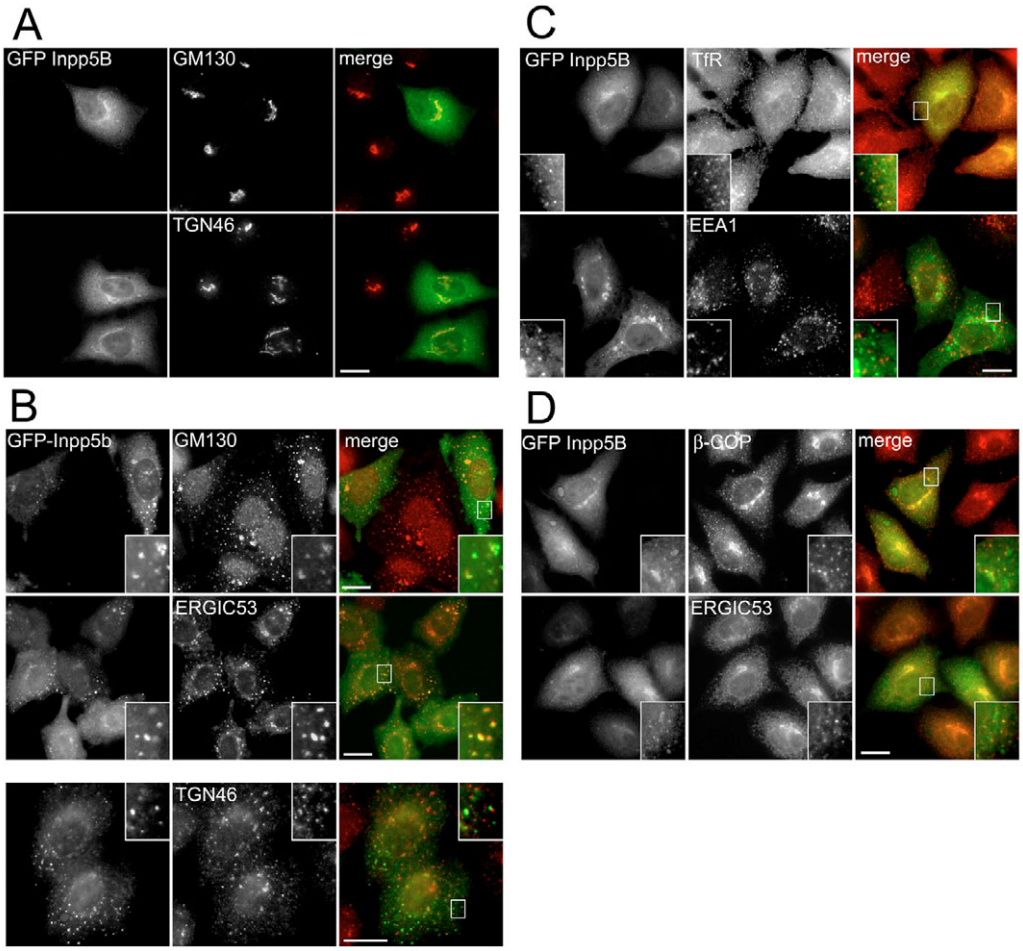

Fig. 1. Cellular localisation of GFP-INPP5B. (A) HeLa cells were transiently transfected with vector encoding GFP-INPP5B, fixed in methanol, labelled with antibodies against GM130 or TGN46 and visualised by epifluorescence microscopy. (B) HeLa cells transiently expressing GFP-INPP5B were treated with 5 μg/ml nocodazole for 2 hours before labelling with antibodies to GM130, ERGIC53 or TGN46. (C,D) HeLa cells transiently expressing GFP-INPP5B were labelled with antibodies against the endosomal markers transferrin receptor (TfR) or EEA1 (C) or the ERGIC-localised proteins β-COP or ERGIC53 (D). GFP-INPP5B appears green and the other markers red in the merged images. Insets show a magnified view of the boxed areas. Bars, 10 μm.

adaptor proteins as there are examples of proteins that interact with the clathrin machinery but are absent from purified vesicle preparations – the GGA proteins, for example (Bonifacino, 2004). It remained possible, therefore, that INPP5B associates with the clathrin machinery but is not packaged efficiently into, or recruited to, vesicles. However, yeast two-hybrid experiments indicated that INPP5B fails to bind to the clathrin heavy chain terminal domain or the α-adaptin appendage domain, which both interact with OCRL1 (Fig. 2B). Pulldown experiments also indicated a lack

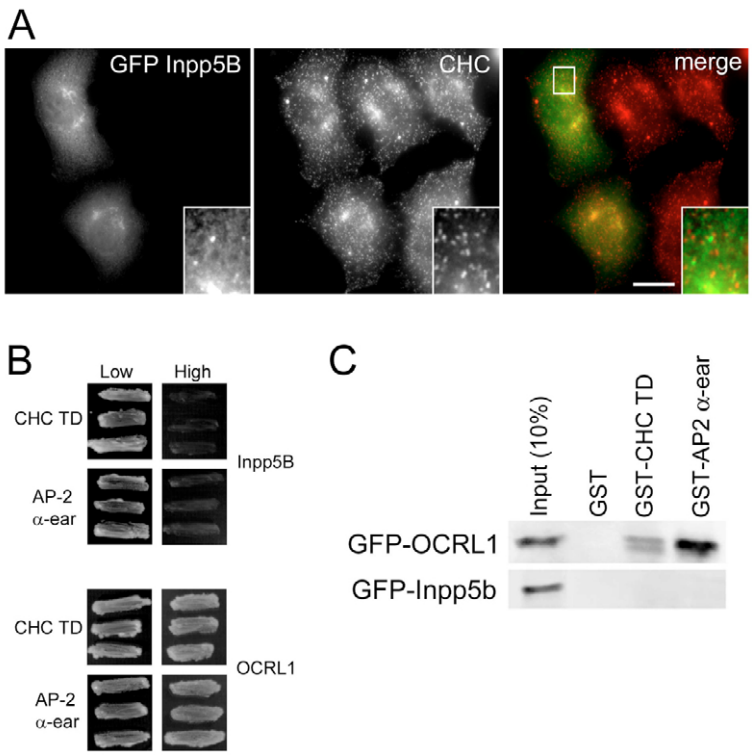

Fig. 2. INPP5B does not colocalise with clathrin or bind to clathrin or AP-2. (A) HeLa cells transiently expressing GFP-INPP5B (green) were labelled with antibodies against the clathrin heavy chain (red). Insets show a magnified view of the boxed areas. Bar, 10 μm. (B) The interaction between OCRL1 or INPP5B and the terminal domain of the clathrin heavy chain (CHC TD) or the α-adaptin appendage domain (AP-2 α-ear) was tested in the yeast two-hybrid system. Growth on high selection indicates an interaction between the proteins. (C) Beads containing immobilised recombinant GST, GST-clathrin terminal domain or GST-α-adaptin appendage domain were incubated with extracts prepared from HeLa cells expressing GFP-OCRL1 or GFP-INPP5B, and the bound proteins detected by western blotting with antibodies against GFP.

of interaction between INPP5B and clathrin or AP-2, whereas OCRL1 could bind to both (Fig. 2C). Together, these data indicate that INPP5B differs from OCRL1 in that it is not enriched into clathrin-coated transport intermediates and interacts with neither clathrin nor AP-2 components of the clathrin coat.

Golgi targeting of INPP5B is mediated by its C-terminus

To understand better how INPP5B is targeted to membranes, various GFP-tagged constructs were expressed in HeLa cells and their localisation monitored by fluorescence microscopy (Fig. 3A). Mutation of the aspartic acid within the conserved sequence PAWCDRIL in the 5-phosphatase domain (D524A), which renders INPP5B catalytically inactive (Jefferson and Majerus, 1996), had no effect on the targeting of INPP5B to the Golgi apparatus, ERGIC or endosomes (Fig. 3B; supplementary material Fig. S4). Similarly, mutation of the C-terminal cysteine (C910A), which abolishes prenylation of INPP5B (Jefferson and Majerus, 1995), did not affect targeting of INPP5B (Fig. 3B). This is in agreement with the lack of effect of deletion of the CAAX box upon membrane association of mouse INPP5B (Matzaris et al., 1998). A construct containing the N-terminus and 5-phosphatase domain (amino acids 1-564) of INPP5B was cytosolic, whereas a construct comprising the region downstream of the catalytic domain, including the RHO GAP-like domain and linker region connecting this to the catalytic domain (residues 564-913), is targeted to the Golgi to an extent similar to that of the wild-type full-length protein (Fig. 3B). Further truncations of this C-terminal region abolished membrane targeting (data not shown), suggesting that both the linker and RHO GAP-like domains are required for this process or that folding of this domain is impaired by further truncation.

INPP5B interacts with both secretory and endosomal RAB proteins

It has recently been shown that INPP5B binds to the endocytic RAB protein RAB5 but not to RAB4 (Shin et al., 2005). Given the localisation of INPP5B to the Golgi apparatus and ERGIC, we tested whether it also binds to RAB proteins localised to these compartments. Yeast two-hybrid analysis indicated that INPP5B can interact with: RAB1A and RAB2A, which are localised to the ERGIC and cis-Golgi, with RAB33B and RAB6A, which are localised to the Golgi stack, with RAB6 also found on the TGN, with RAB8A, which is also on the TGN, with RAB9A, which is involved in trafficking from late endosomes to the TGN, as well as with endosomal RAB5A, as expected (Fig. 4A; each RAB protein shown is isoform A unless indicated otherwise). The interaction in each case was to the GTP-locked version of the RAB protein (Q to L), and did not occur with the inactive GDP-locked forms (S or T to N). No interaction was detected with GTP-locked forms of RAB4A, RAB10, RAB11A or RAB14. To examine further the interactions between INPP5B and RAB proteins, pulldown experiments were performed. As shown in Fig. 4B, GFP-INPP5B expressed in HeLa cells was able to bind to GTP-locked forms of RAB1, RAB2, RAB5, RAB6 and RAB9, in agreement with the yeast two-hybrid experiments. Binding in each case was dependent on the nucleotide status of the RAB protein, only occurring with the GTP- and not GDP-locked form (Fig. 4C, and data not shown). No binding to RAB4, RAB11 or RAB14 was observed, as expected (Fig. 4B). However, contrary to the yeast two-hybrid experiments, there was no detectable interaction with GTP-locked RAB8 or RAB33B (Fig. 4B). This might reflect a weak interaction between these proteins and INPP5B that is sufficient to permit yeast growth in the

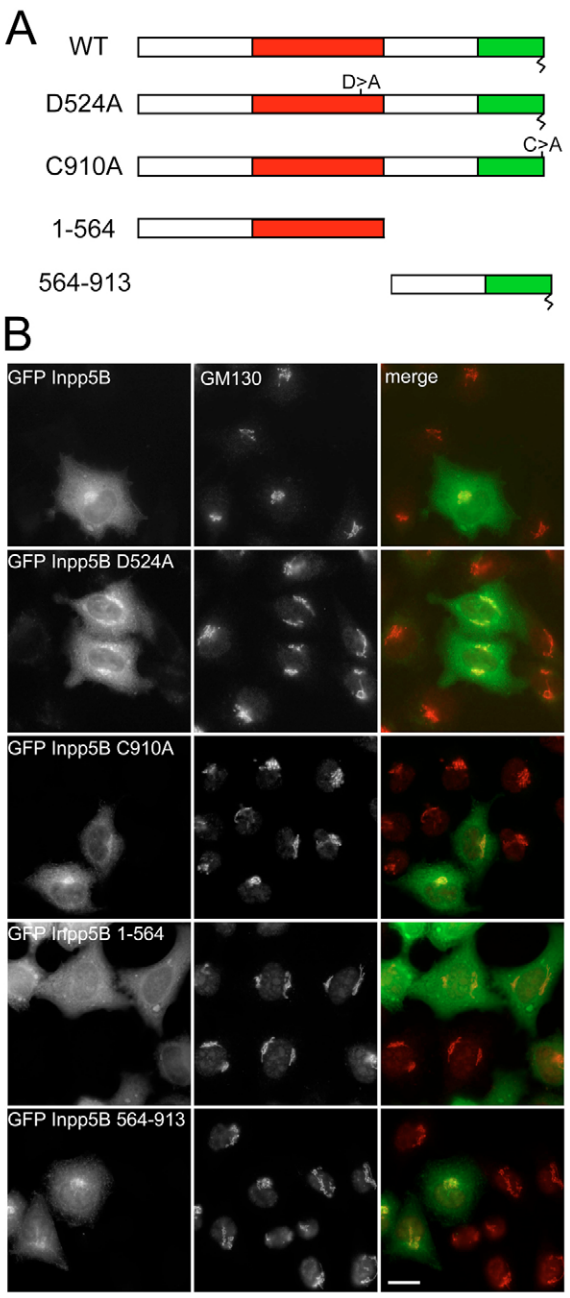

Fig. 3. The C-terminal linker and RHO GAP-like domains are required for the targeting of INPP5B to membranes. (A) Schematic view of INPP5B point mutant and truncation constructs. The 5-phosphatase domain is indicated in red, and the RHO GAP-like domain in green. Prenylation of the C-terminus is indicated by a zigzag line. (B) GFP-tagged INPP5B point mutant and truncation constructs (green) were transiently expressed in HeLa cells, which were fixed in methanol and labelled with antibodies against GM130. Bar, 10 μm.

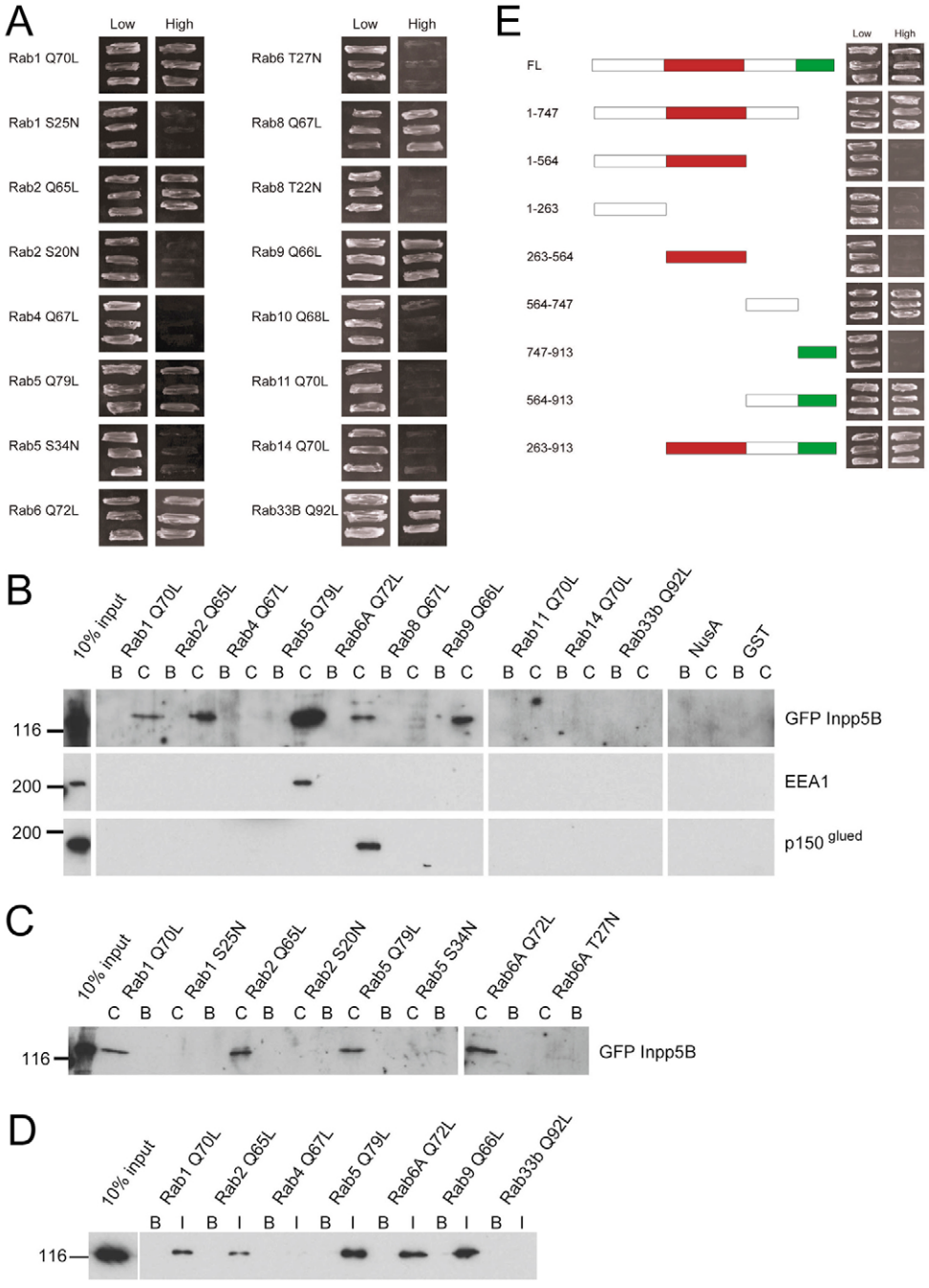

Fig. 4. INPP5B interacts with RAB proteins associated with the secretory and endocytic pathways. (A) Full-length INPP5B was tested for interaction with the indicated GTP-locked (Q to L) or GDP-locked (S or T to N) RAB proteins. Growth on high selection indicates interaction. (B) Beads containing NusA or GST alone or GST-tagged RAB proteins 1, 2, 4, 5, 6, 9, 11, 14 and 33B or NusA-tagged RAB 8 were incubated with buffer alone ('B') or HeLa cell extract containing GFP-INPP5B ('C'), and bound proteins were detected by western blotting. (C) Beads containing GST-tagged versions of the indicated RAB proteins were incubated with buffer alone ('B') or HeLa cell extract containing GFP-INPP5B and bound GFP-INPP5B detected by western blotting. (D) Beads containing GST-tagged versions of the indicated GTP-locked RAB proteins were incubated with buffer alone ('B') or recombinant full-length INPP5B ('I'), and bound protein detected by western blotting. (E) The indicated truncated forms of INPP5B were tested for interaction with RAB5Q79L in the yeast two-hybrid system. Growth on high selection indicates interaction.

two-hybrid system but not enough to enable binding in pulldown experiments.

To determine whether INPP5B binds directly to RAB proteins, pulldown experiments were performed using purified components. As shown in Fig. 4D, purified recombinant INPP5B bound directly to GTP-locked RAB1, RAB2, RAB5, RAB6 and RAB9, but not to RAB4 or RAB33B. We next investigated the location of the RAB-binding site in INPP5B by expressing various INPP5B truncation mutants together with RAB5Q79L in the yeast two-hybrid system. A construct comprising the N-terminus and 5-phosphatase domain failed to interact with RAB5, whereas a construct comprising the linker and RHO GAP-like domains bound strongly (Fig. 4E). Further truncation of this region revealed that the linker region was sufficient for RAB5 binding, at least in the yeast two-hybrid system.

Interaction with RAB proteins is required for Golgi targeting of INPP5B

To determine the functional significance of the interaction between INPP5B and RAB proteins, we decided to generate point mutants that were defective in RAB binding. Previous work has shown that OCRL1, like INPP5B, binds to RAB proteins through its linker domain and identified residues that, when mutated, abrogate RAB binding (Hyvola et al., 2006). Mutation of these residues impairs targeting of OCRL1 to the

Golgi apparatus and endosomes, strongly suggesting that RAB proteins are required for targeting of OCRL1 in vivo (Hyvola et al., 2006). Inspection of the INPP5B linker sequence indicates that the same residues are conserved in this protein (Fig. 5A). Therefore, we generated equivalent mutations in INPP5B and first analysed their effects on RAB binding. As shown in Fig. 5B, the S589P and G688D mutations abolished the interaction of INPP5B with RAB5Q79L and RAB6Q72L. Each construct was then expressed in HeLa cells to monitor the effects on Golgi targeting. Unlike the wild-type protein, which was, as expected, targeted to the Golgi apparatus, both S589P and G688D mutants were exclusively cytosolic (Fig. 5C).

A possible reason for the loss of RAB binding and Golgi targeting in the mutants is that their folding is impaired. Limited proteolysis was therefore performed to assess whether gross misfolding of the mutant proteins had occurred. Wild-type and mutant INPP5B were metabolically labelled and immunoprecipitated from cells before digestion with increasing amounts of trypsin. There were no obvious differences in the digestion patterns of the various constructs, suggesting that the mutant proteins were folding in a manner similar to that of the wild-type protein (Fig. 5D). This suggests that it is the specific loss of RAB interaction, and not misfolding, that causes the mutant proteins to remain in the cytosol, consistent with the hypothesis that RAB binding is required for efficient targeting of INPP5B in vivo.

Overexpression of INPP5B alters the distribution of ERGIC53 at 15°C

The localisation of INPP5B to the ERGIC and Golgi apparatus suggested that it participates in membrane trafficking at these compartments. To investigate this possibility, we first analysed the effects of overexpression of INPP5B upon various markers localised to the ERGIC and Golgi apparatus. Cells were analysed both at 37°C and at 15°C, which blocks delivery of proteins from the ERGIC to the Golgi apparatus (Saraste and Kuismanen, 1984). At 37°C, there were no effects of wild-type or catalytically inactive (D524A) INPP5B upon any of the ERGIC or Golgi markers studied (Fig. 6A,B; supplementary material Fig. S4; and data not shown). However, at 15°C, there was an increased accumulation of GFP-INPP5B at the Golgi apparatus and ERGIC (Fig. 6A,B). Quantitation revealed that

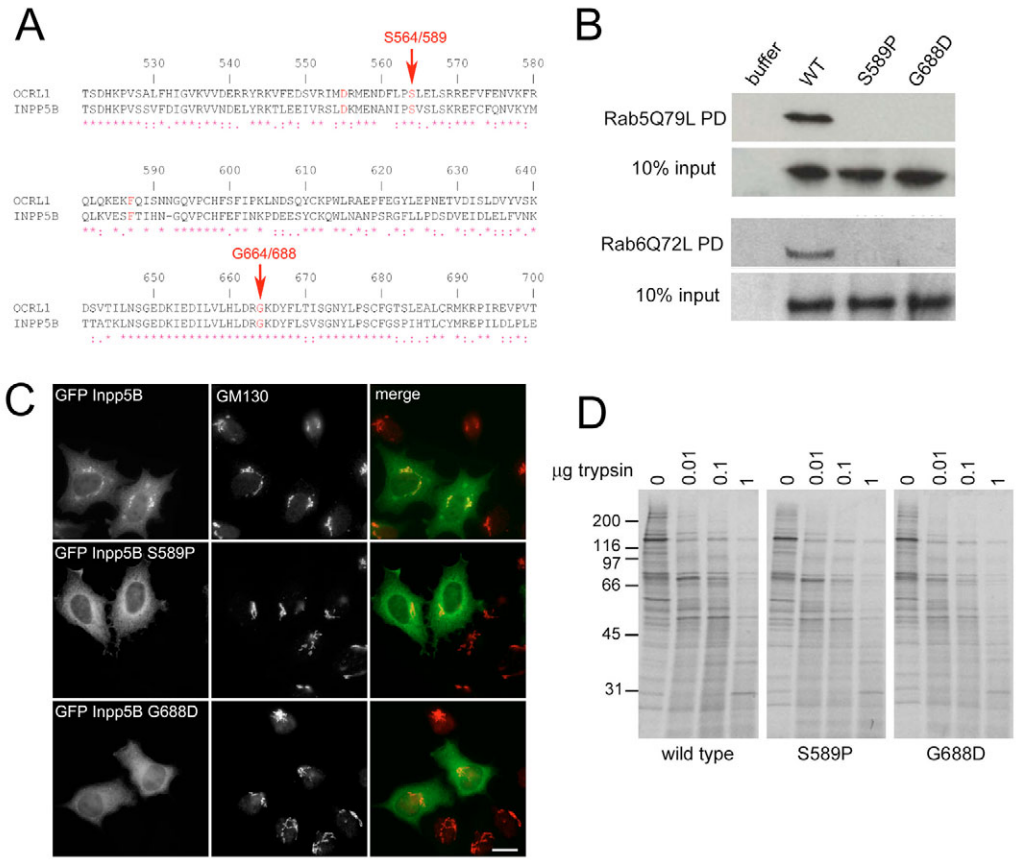

Fig. 5. Golgi targeting of INPP5B requires RAB binding. (A) Sequence alignment of the OCRL1 and INPP5B linker regions showing the conservation of residues whose mutation in OCRL1 reduces RAB binding and membrane targeting (red arrows). (B) Beads containing GST-RAB5Q79L or RAB6Q72L were incubated with buffer alone or HeLa cell extract containing full-length wild-type GFP-INPP5B or the indicated point mutants and bound protein detected by western blotting with antibodies against GFP. (C) Localisation of the indicated GFP-tagged INPP5B proteins (green) in HeLa cells double-labelled with antibodies to GM130 (red). Bar, 10 µm. (D) The indicated GFP-tagged INPP5B proteins were expressed in metabolically labelled HeLa cells and immunoprecipitated with antibodies against GFP before limited digestion with the indicated amounts of trypsin. Digested proteins were analysed by SDS-PAGE and autoradiography. Markers indicate the molecular mass (kDa).

practically all of the punctate structures containing GFP-INPP5B also contained ERGIC53 (Table 1). Interestingly, in cells expressing GFP-INPP5B, ERGIC53 became concentrated at the ERGIC, with a corresponding depletion from the ER at 15°C (Fig. 6A). Similarly, β-COP, a component of the COPI coat that binds to ERGIC53, was recruited onto the INPP5B-containing structures (Fig. 6A). These effects were not a consequence of altered ERGIC morphology as both Sec23 and p115, which label ER exit sites and the ERGIC, respectively, were unaffected by expression of INPP5B (Fig. 6B). There were also no effects on the Golgi markers GM130 and GalNacT2 at 15°C (data not shown). Similar effects upon ERGIC markers were also observed with FLAG-tagged INPP5B (supplementary material Fig. S3).

To understand the mechanism by which INPP5B causes redistribution of ERGIC53 and β-COP at 15°C, various point-mutant and deletion constructs were expressed in cells. Expression of catalytically inactive INPP5B (D524A) induced the same changes in β-COP and ERGIC53 distribution as the wild-type protein (Fig. 6C; only ERGIC53 is shown for each construct, but the same effects were also seen with β-COP). This suggests that the observed changes in distribution of β-COP and ERGIC53 are not due to changes in lipid levels. The construct comprising the N-terminal and 5-phosphatase domains (residues 1-564) was only weakly recruited to the Golgi region at 15°C and failed to redistribute ERGIC53 (Fig. 6C). By contrast, the C-terminal region encompassing the linker and RHO GAP-like domains (residues 564-913) was strongly targeted to the membrane and induced redistribution of ERGIC53. The RAB-binding-deficient INPP5B point mutants S589P and G688D were weakly targeted to the Golgi region at 15°C and failed to alter the distribution of ERGIC53, even at the highest expression levels, suggesting that the interaction with RAB proteins is required for the observed changes in distribution of ERGIC53 (and β-COP) (Fig. 6C, and data not shown).

The accumulation of INPP5B, β-COP and ERGIC53 at the ERGIC upon incubation at 15°C could reflect an aggregation of these proteins on the membrane. To exclude this possibility, cells were incubated at 15°C for 3 hours and then warmed up to 37°C for 10 minutes before fixation. As shown in Fig. 6D, ERGIC53 (and β-COP; data not shown) returned to the same location as that of control cells upon warming, indicating that the redistribution of these proteins induced by INPP5B expression at 15°C is reversible.

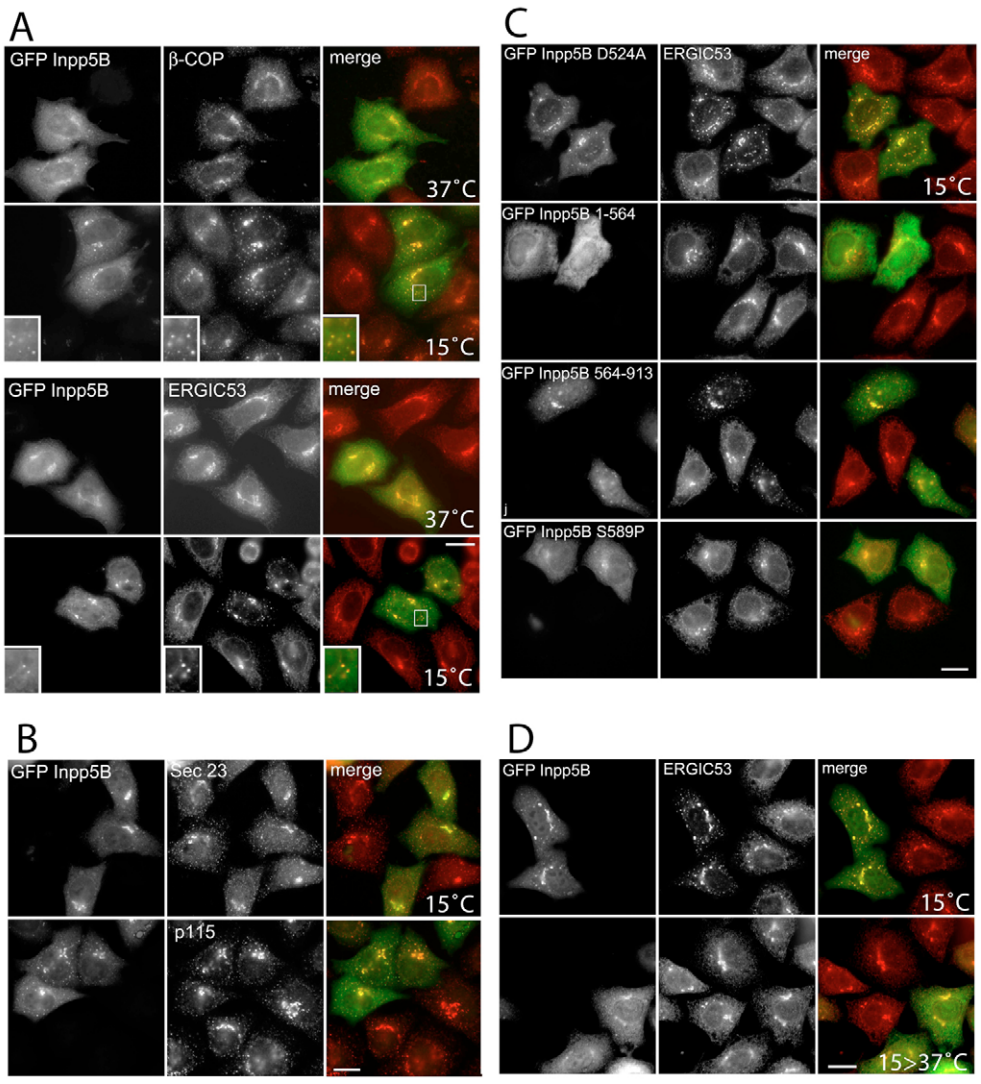

Fig. 6. Redistribution of ERGIC53 and COPI upon expression of INPP5B at 15°C. (A) HeLa cells transiently expressing GFP-INPP5B (green) were incubated at 37°C or 15°C for 3 hours, as indicated, before fixation in methanol and labelling with antibodies against β-COP or ERGIC53 (red). Insets show a magnified view of the boxed area. (B) HeLa cells expressing GFP-INPP5B (green) were incubated at 15°C for 3 hours before fixation in methanol and labelling with antibodies against Sec23 or p115 (red). (C) HeLa cells transiently expressing GFP-tagged wild-type INPP5B or the indicated point or truncation mutants (green) were incubated at 15°C for 3 hours, fixed in methanol and labelled for ERGIC53 (red). (D) HeLa cells transiently expressing GFP-INPP5B (green) were incubated at 15°C for 3 hours and either fixed immediately or shifted to 37°C for 10 minutes before fixation and labelling for ERGIC53 (red). Bars, 10 μm.

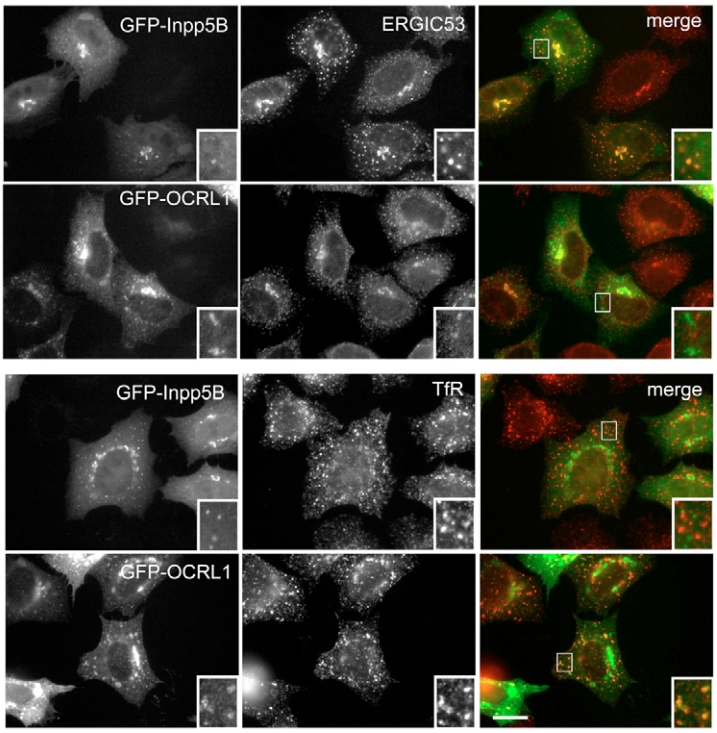

Fig. 7. Comparison of INPP5B and OCRL1 at 15°C. HeLa cells transiently expressing GFP-INPP5B or GFP-OCRL1 (green) were incubated at 15°C for 3 hours before fixation in methanol and labelling with antibodies against ERGIC53 or the transferrin receptor (TfR) (red). Bar, 10 μm. Insets show a magnified view of the boxed area.

To determine whether the redistributions of ERGIC53 and COPI were specific for INPP5B, we studied the effects of GFP-OCRL1 expression upon these markers at 15°C. Unlike INPP5B, GFP-OCRL1 failed to accumulate on the ERGIC at 15°C and had no effect upon the distribution of either ERGIC53 or COPI (Fig. 7; Table 1). Instead, OCRL1 was abundant on endosomes containing the transferrin receptor that appeared larger and more distinct than at 37°C. Thus, expression of INPP5B causes a specific and reversible redistribution of the cycling protein ERGIC53 and associated COPI machinery at 15°C.

Overexpression of INPP5B alters trafficking of ERGIC53 upon treatment with brefeldin A

Our results suggest that expression of INPP5B affects the cycling of ERGIC53 between the ERGIC and ER, at least at 15°C when anterograde transport from the ERGIC to the Golgi apparatus is arrested. The accumulation of ERGIC53 in the ERGIC and its loss from the ER are consistent with an inhibition of retrograde transport from the ERGIC to the ER. To test further whether INPP5B plays a role in retrograde transport, we treated cells expressing INPP5B with brefeldin A (BFA). BFA is a drug that dissociates ADP ribosylation factor (ARF) effectors, including COPI, from Golgi and ERGIC membranes, leading to tubulation of the Golgi and its subsequent fusion with the ER, with an accompanying block in anterograde transport (Lippincott-Schwartz et al., 1990; Orci et al., 1991). Golgi enzymes relocate to the ER upon BFA treatment, whereas Golgi matrix components such as GM130 and cycling proteins such as ERGIC53 appear in structures that are found close to ER exit sites (Lippincott-Schwartz et al., 1990; Mardones et al., 2006). When cells were treated with BFA, GFP-INPP5B accumulated in cytoplasmic puncta containing ERGIC53 (Fig. 8A). There was a dramatic accumulation of ERGIC53 in these structures and a corresponding loss of the protein from the ER, consistent with an inhibition of trafficking from the ERGIC to the ER. GM130 was also present in these structures, where it appeared more concentrated in cells expressing GFP-INPP5B (Fig. 8A). By contrast, there was no effect of expression of INPP5B upon the retrograde delivery of the Golgi enzyme GalNacT2 or Golgi membrane protein Golgin-84 to the ER (Fig. 8A; and data not shown).

The redistribution of ERGIC53 in cells expressing INPP5B was rapid, being detectable as early as 1 minute, and more clearly at 2 minutes, after addition of BFA (Fig. 8B). ERGIC53 remained concentrated in the ERGIC for up to 1 hour in the presence of BFA, with the ERGIC occasionally developing a more tubular appearance at later times. At all times, ERGIC53 was depleted from the ER. The effects on ERGIC distribution were also observed with FLAG-tagged INPP5B and with the catalytically inactive D524A mutant, but not with the RAB-binding mutants S589P and G688D when expressed at comparable levels (data not shown). A possible explanation for the effects upon ERGIC53 is that INPP5B stabilises COPI on the ERGIC membrane, preventing BFA from exerting its effects at this compartment. However, expression of INPP5B did not alter the behaviour of COPI upon BFA treatment, with β-COP redistributing to the cytosol to the same extent as in control cells (Fig. 8C). As a control, we also investigated whether GFP-OCRL1 could alter the distribution of ERGIC53 upon BFA treatment. As shown in Fig. 8D, ERGIC53 was unaffected by expression of OCRL1. These results suggest a specific role for INPP5B in retrograde ERGIC-to-ER transport and indicate that perturbation of this trafficking by overexpression of INPP5B requires interaction with RAB proteins but does not require the catalytic activity of INPP5B or association with COPI.

Discussion

The results of this study indicate that INPP5B localises to the Golgi apparatus and ERGIC in addition to the plasma membrane ruffles and endosomes that were reported previously (Shin et al., 2005). Despite several attempts, we were unable to raise antibodies to endogenous INPP5B that were suitable for microscopy. However, we believe that our localisation of ectopically expressed INPP5B reflects that of the endogenous protein for the following reasons: the localisation of INPP5B was independent of the level of expression, giving a clear Golgi staining even at the lowest detectable levels of expression; the staining was independent of the type of tag used; the same staining pattern was observed in all cell types analysed; and INPP5B bound strongly to RAB proteins specifically localised to the ERGIC and Golgi apparatus. Moreover, the localisation of INPP5B was distinct from its close relative OCRL1 when both proteins were expressed under the same conditions.

Mutational analysis strongly suggests that RAB binding is

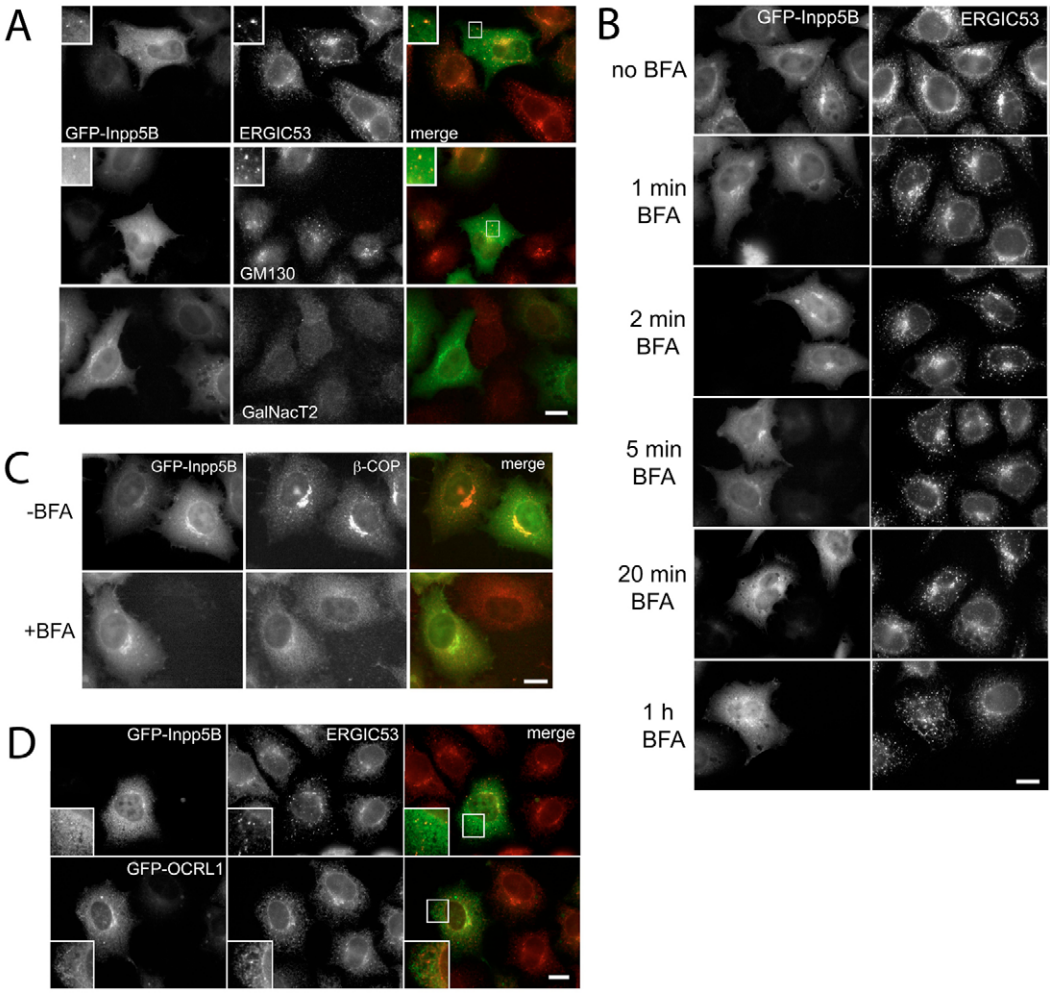

Fig. 8. Expression of INPP5B alters trafficking of ERGIC53 in BFA-treated cells. (A) HeLa cells transiently expressing GFP-INPP5B (green) were incubated with 5 μg/ml BFA at 37°C for 20 minutes before fixation in paraformaldehyde and labelling with antibodies against ERGIC53, GM130 or GalNacT2 (red). (B) HeLa cells expressing GFP-INPP5B (green) were incubated with 5 μg/ml BFA for the indicated times and labelled with an antibody against ERGIC53 (red). (C) HeLa cells expressing GFP-INPP5B were incubated with or without 5 μg/ml BFA for 5 minutes and labelled with antibody against β-COP (red). (D) HeLa cells transiently expressing GFP-INPP5B or GFP-OCRL1 (green) were incubated with 5 μg/ml BFA for 20 minutes and labelled with antibody against ERGIC53. Bars, 10 μm.

required for efficient targeting of INPP5B, and previous work has indicated that RAB5 stimulates the catalytic activity of the enzyme (Shin et al., 2005), arguing for a dual mode of regulation by RAB proteins. This is similar to the situation with OCRL1, where interaction with RAB proteins is important both for membrane recruitment and for stimulation of lipid phosphatase activity (Hyvola et al., 2006). It appears that the mechanisms by which INPP5B and OCRL1 bind to RAB proteins are conserved, with both proteins sharing a high degree of sequence conservation in the RAB-binding region, including those residues that abrogate RAB binding when mutated. However, it is likely that subtle differences also exist as INPP5B can bind to RAB2 and RAB9, whereas OCRL1 does not. Such differences probably contribute to the differential localisation of the two phosphatases. A better appreciation of the mode of interaction between INPP5B or OCRL1 and RAB proteins awaits the resolution of the three-dimensional structure of the C-termini of these phosphatases. While RAB binding is required for efficient targeting of INPP5B and OCRL1 in vivo, it is unlikely to be the only important factor. For example, INPP5B appears largely absent from clathrin-coated intermediates, whereas OCRL1 is enriched in such structures, most likely owing to binding to the coat proteins clathrin and α-adaptin (Choudhury et al., 2005; Ungewickell et al., 2004). Similarly, interactions between the OCRL1 and INPP5B RHO GAP-like domains and other proteins are also likely to play a role in localising these phosphatases. OCRL1 interacts with RAC1, and it has been proposed that this interaction is important for recruitment of OCRL1 to membrane ruffles (Faucherre et al., 2005; Faucherre et al., 2003). A similar interaction between INPP5B and a RHO protein is also likely to occur, which would fit with the requirement for the RHO GAP-like domain in addition to the linker region for the efficient membrane localisation of INPP5B in vivo.

Our data suggest a role for INPP5B in retrograde trafficking between the ERGIC and ER. This would explain the redistribution of ERGIC53 to the ERGIC and its loss from the ER at low temperature or in the presence of BFA. A role in retrograde transport would also be consistent with binding to RAB2, which regulates the formation of retrograde carriers at the ERGIC (Tisdale, 2000). Further analysis using RNA interference to deplete INPP5B failed to alter the distribution of proteins cycling within the early secretory pathway (C.W. et al., unpublished). This might be attributable to insufficient depletion of INPP5B, which is a catalytic enzyme, or compensation by OCRL1, although double depletion of OCRL1 and INPP5B also failed to elicit effects upon the distribution of cycling proteins (C.W. et al., unpublished). An alternative possibility, and one that appears most consistent with our data, is that INPP5B has a minor or regulatory role in

retrograde traffic, which only becomes apparent when it is overexpressed and the cycling of proteins at the ERGIC-ER interface is slowed by low temperature or BFA treatment (Lippincott-Schwartz et al., 1990). Whether INPP5B participates in other trafficking steps within the secretory pathway is currently unclear. We have failed to observe effects of expression or depletion of INPP5B on anterograde trafficking of membrane and soluble cargo, as well as on retrograde transport of the Shiga toxin B subunit from the Golgi apparatus to the ER. Either INPP5B does not play a role in these trafficking pathways, or, if it does, it is a minor one. Further work will be required to determine the extent to which other pathways are regulated by INPP5B, and the mechanisms involved.

The basis of INPP5B compensation for OCRL1 loss (and vice versa) in mice is unknown (Janne et al., 1998). Both proteins bind to RAB5 and localise to membrane ruffles and endosomes, as shown in this study and those of others (Choudhury et al., 2005; Faucherre et al., 2005; Hyvola et al., 2006; Shin et al., 2005; Ungewickell et al., 2004). However, INPP5B does not interact with clathrin and appears to be largely absent from clathrin-coated transport intermediates. This argues that INPP5B and OCRL1 participate in different steps during endocytosis, with INPP5B acting either upstream or downstream of clathrin, or in different uptake pathways, with INPP5B participating in non-clathrin mediated uptake. The finding that INPP5B binds to RAB9 also suggests involvement later in the endocytic pathway, which again is different to OCRL1, which does not interact with this RAB protein (Hyvola et al., 2006). Thus, while INPP5B and OCRL1 might have some overlapping functionality, which is sufficient to allow compensation in the knockout mice, it is likely that they also have distinct roles within the endosomal system.

This study argues for a similar relationship between INPP5B and OCRL1 in the secretory pathway. Both proteins localise to the Golgi apparatus and bind to largely similar sets of secretory RAB proteins. However, again, there are notable differences. INPP5B binds to RAB2, whereas OCRL1 does not, and our immunofluorescence data suggest that INPP5B is enriched towards the cis-side of the Golgi apparatus, with OCRL1 more abundant at the trans side (see also Dressman et al., 2000). INPP5B is more abundant on the ERGIC than OCRL1, and its expression inhibits retrograde ERGIC-to-ER trafficking of ERGIC53, whereas expression of OCRL1 does not. Thus, despite their ability to compensate for one another in knockout studies, it appears that INPP5B and OCRL1 have distinct roles within the secretory pathway. A better appreciation of these roles will require a more detailed analysis, particularly in other cell types, including polarised epithelial and neuronal cells similar to those affected in Lowe syndrome, or in appropriate animal cell models.

Materials and Methods

Materials and antibodies

All reagents were from Sigma or Merck unless stated otherwise. Protease inhibitors (cocktail set III) were from Calbiochem and used at a dilution of 1:250. Polyclonal antibodies to INPP5B were raised in rabbit and sheep against amino acids 1-229 fused to the C-terminus of GST and affinity purified using the fusion protein covalently coupled to glutathione-sepharose beads (Amersham Biosciences). Sheep antibody against OCRL1 has been reported previously (Choudhury et al., 2005). Antibody MLO7 against GM130 (anti-N73pep) and antibody 4H1 against p115 were described previously (Nakamura et al., 1997). Affinity purified sheep antibodies against GFP were described previously (Diao et al., 2003). Polyclonal antibodies against TGN46 were acquired from Vas Ponnambalam (University of Leeds, UK). Monoclonal antibody UH-4 against GalNAcT2 was a kind gift from Henrik Clausen (University of Copenhagen, Denmark). Monoclonal mAD (anti-β-COP) was acquired from Rainer Pepperkok (EMBL, Heidelberg, Germany). Monoclonal anti-ERGIC53 (G1/93) was purchased from Alexis Biochemicals. Mouse antibody against FLAG (M2) was purchased from Sigma. Monoclonal antibodies against the clathrin heavy chain, p150^{glued} and EEA1 were purchased from Transduction Labs. Antibody against the transferrin receptor (H68.4) was from Zymed. Fluorophore-conjugated (Alexa 594 and Alexa 488) and HRP-conjugated secondary antibodies were purchased from Molecular Probes and Tago Immunologicals, respectively.

Molecular biology and yeast two-hybrid experiments

All constructs were made using standard molecular biology techniques. Full-length human INPP5B cDNA (accession number: NM_005540) was cloned from a human liver cDNA library and cloned into pEGFP-C3 (Clontech) or a modified version of pcDNA3.1 (Stratagene) with an N-terminal FLAG tag for in vivo expression. INPP5B cDNA was cloned into pGBKT7 (Stratagene) for yeast two-hybrid experiments and pBAC2 (Novagen) for expression of recombinant protein in insect cells. DNA encoding the N-terminal 229 amino acids of INPP5B was cloned into pET41EK-LIC (Novagen) for expression of GST-tagged protein in *Escherichia coli*. Point mutations were generated by PCR using the Quikchange method (Stratagene, La Jolla, CA). All constructs were verified by DNA sequencing using the ABI Prism Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Primer sequences are available upon request. Full-length cDNA encoding GFP-OCRL1 (isoform B, accession number NP_001578) was described previously (Choudhury et al., 2005). Yeast two-hybrid analysis was performed as described previously according to the BD Biosciences Clontech manual (Choudhury et al., 2005). pGADT7-clathrin terminal domain (TD, residues 1-579) was a gift from Harald Stenmark (Norwegian Radium Hospital, Oslo, Norway). The α-adaptin appendage domain was cloned into pGADT7 from a pGEX α-adaptin appendage domain construct kindly provided by Liz Smythe (University of Sheffield, UK).

Cell culture and transfection

Adherent HeLa and NRK cells were grown at 37°C and 5% CO_2 in DMEM containing 10% foetal calf serum (FCS). For nocodazole treatment, cells were first placed on ice for 5 minutes before incubation at 37°C for 2 hours in the presence of 5 μg/ml nocodazole. Brefeldin A was added at 5 μg/ml and cells incubated at 37°C for the indicated time. Adherent cells were transiently transfected with FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions and incubated for 16-20 hours before fixation or lysis. Metabolic labelling was performed in labelling medium (nine parts met/cys-free DMEM containing 10% dialysed FCS mixed with one part met/cys-containing DMEM) containing 50 μCi/ml ^{35}S-met/cys (NEN Life Sciences, UK) for 18-22 hours at 37°C.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously (Choudhury et al., 2005). Quantitation of GFP-INPP5B and GFP-OCRL1 colocalisation with ERGIC and endosome markers was performed by locating GFP-INPP5B-positive puncta, then switching channels to determine whether the relevant marker of interest was present in the same structure. 300 puncta were counted for each marker, and the results expressed as the percentage of GFP-INPP5B or GFP-OCRL1 puncta containing the marker of interest.

Preparation of cell extracts and immunoprecipitation

HeLa cell extracts were prepared by washing adherent cells twice in ice-cold PBS followed by extraction in 1 ml HKMT (20 mM Hepes pH 7.4, 0.1 M KCl, 5 mM $MgCl_2$, 0.5% Triton X-100) (1 ml per 10 cm dish) for 15 minutes on ice. Extracts were clarified before use by spinning at 15,000 ***g*** in a microfuge. INPP5B was immunoprecipitated by incubating the HeLa extract with 2 μg sheep antibody against INPP5B overnight at 4°C, followed by 10 μl protein-G-sepharose. After washing three times with HKMT, proteins were eluted by boiling SDS sample buffer and analysed by western blotting with a rabbit antibody against INPP5B.

Protein binding experiments

Recombinant GST, GST-clathrin terminal domain and GST-AP-2 α-ear were expressed in *E. coli* and purified on GSH-sepharose. A HeLa cell extract (250 μl) was incubated with 10 μg of bait GST fusion protein immobilised on GSH-sepharose beads for 4 hours at 4°C. After washing three times with HKMT, bound proteins were eluted in SDS sample buffer and analysed by western blotting. Recombinant RAB proteins were prepared as described previously (Hyvola et al., 2006). His/S-tagged INPP5B was prepared from insect cells using the same procedure as that for OCRL1 (Choudhury et al., 2005). Binding of GFP-tagged INPP5B (in HeLa cell extracts) and recombinant INPP5B to GST- or NusA-tagged-RAB beads was performed as described previously (Hyvola et al., 2006) except that INPP5B was used instead of OCRL1.

Limited proteolysis

Cells expressing GFP-INPP5B constructs were lysed in HKMT (500 μl per 6 cm dish), and the lysates clarified by centrifugation before immunoprecipitation with 2 μg sheep antibody against GFP and protein G-sepharose. After washing with HKMT followed by trypsin buffer (TB; 20 mM Hepes pH 7.4, 0.1 M KCl, 5 mM $MgCl_2$), beads were split into four equal aliquots and incubated for 20 minutes at 25°C in TB containing 0, 0.01, 0.1 or 1 μg trypsin. Reactions were stopped by addition of SDS sample buffer containing protease inhibitors and boiling before SDS-PAGE.

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