The C2 domains of the class I Rab11 family of interacting proteins target recycling vesicles to the plasma membrane

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Summary
The Rab11 family of interacting proteins (Rab11-FIP) is a recently identified protein family composed of, to date, six members that interact with Rab11. They all share a highly homologous Rab11-binding domain (RBD) at their C-termini. However, apart from the RBD, they vary in their domain organization. Rab11-FIP3 and Rab11-FIP4 possess an ezrin-radixin-moesin (ERM) domain in their C-terminal half and EF hands in their N-terminal region. They have been termed class II Rab11-FIPs. The class I Rab11-FIPs, Rab coupling protein (RCP), Rip11 and Rab11-FIP2, each have a C2 phospholipid-binding domain near their N-termini. Although they are still membrane associated, truncation mutants of the class I Rab11-FIPs that lack their C2 domains display an altered subcellular distribution in vivo, indicating that this domain plays an important role in specifying their correct intracellular localization. To determine the phospholipids to which they bind, a protein phospholipid overlay assay was performed. Our results indicate that the class-I Rab11-FIPs bind preferentially to phosphatidylinositol-(3,4,5)-trisphosphate [PtdIns(3,4,5)\(P_3\)] and the second messenger phosphatidic acid. Stimulation of PtdIns(3,4,5)\(P_3\) or phosphatidic acid synthesis results in the translocation of the Rab11-FIPs from a perinuclear location to the periphery of the cell. By contrast, the transferrin receptor does not translocate to the plasma membrane under these conditions. This translocation is dependent on the presence of the C2 domain, because class I Rab11-FIP green-fluorescent-protein fusions that lack the C2 domain cannot translocate to the plasma membrane. We propose that the C2 domains of the class I Rab11-FIPs function to target these proteins to ‘docking sites’ in the plasma membrane that are enriched in PtdIns(3,4,5)\(P_3\) and phosphatidic acid.

Key words: Rab11-FIPs, C2 domain, phosphatidylinositol (3,4,5)-trisphosphate, endocytic recycling compartment, Phosphatidic acid, Phospholipase D

Introduction
Rab11 is a small GTPase that plays a role in regulating membrane traffic from the endocytic recycling compartment (ERC) to either the plasma membrane or the trans-Golgi network (TGN). Recently, several Rab11-interacting proteins have been identified that share a highly homologous Rab11-binding domain (RBD) (Hales et al., 2001; Lindsay et al., 2002; Prekeris et al., 2000). Owing to the presence of this RBD, these proteins were grouped into the same family, termed the Rab11 family of interacting proteins (Rab11-FIP). However, apart from the RBD, they have distinct domain organizations, allowing them to be subdivided into two classes. The class II Rab11-FIPs include Rab11-FIP3 and Rab11-FIP4, and possess an ezrin-radixin-moesin (ERM) domain, EF hands and a proline-rich domain, and have been localized to the ERC, TGN and centrosome (Hales et al., 2001; Hickson et al., 2003; Wallace et al., 2002). Rab11-FIP3 and Rab11-FIP4 were also independently identified as Arf-GTPase-binding proteins and termed arphiphilin 1 and arphiphilin 2 (Hickson et al., 2003; Shin et al., 2001; Shin et al., 1999). Rab coupling protein (RCP), Rip11 and Rab11-FIP2, comprise the class I Rab11-FIPs. In addition to the C-terminal RBD, they also possess a C2 domain near their N-terminus. They are predominantly membrane bound and localized to the ERC under steady-state conditions.

C2 domains are found in many proteins that are involved in cell signalling [e.g. phosphoinositide-3-kinase (PI-3-kinase) and PTEN] and membrane trafficking (e.g. rabphilin-3A and synaptotagmin). They are approximately 130 amino acid motifs, first identified in protein kinase C (PKC) (Cho, 2001), which serve as protein-phospholipid and protein-protein binding modules. Their phospholipid binding can be Ca\(^{2+}\) dependent or Ca\(^{2+}\) independent. In some cases, the C2 domain is involved in the translocation of the protein to specific regions within the cell. For example, the C2 domains of conventional PKCs, which bind anionic phospholipids, mediate the rapid translocation of PKC to the plasma membrane (Oancea and Meyer, 1998). By contrast, the C2 domain of cytosolic phospholipase A\(_2\) (cPLA\(_2\)) translocates to the nuclear envelope upon Ca\(^{2+}\) influx (Gijon et al., 1999; Perisic et al., 1999).

Several studies have demonstrated that expression of truncation mutants of the Rab11-FIPs that lack their C2 domains results in a significant inhibition in the rate of endosomal recycling (Lindsay et al., 2002; Lindsay and McCaffrey, 2002; Prekeris et al., 2000). Transferrin is retained in an intricate tubular network within the cell and its recycling...
back to the plasma membrane is inhibited. This indicates that the C2 domain of the class I Rab11-FIPs is likely to play an important role in regulating the transport of ligands and their receptors back to the plasma membrane. We show here that the in vivo localization of the C2 domain truncation mutants of the class I Rab11-FIPs, while membrane associated, is different from that of the wild-type proteins.

We have previously demonstrated that the C-terminal 65 amino acids of RCP and Rab11-FIP2, which encompass their α-helical coil and RBD, are sufficient for membrane binding (Lindsay et al., 2002; Lindsay and McCaffrey, 2002). This suggests that the C2 domain is not required for the recruitment of the Rab11-FIPs from the cytosol to intracellular membranes. Given this observation, there are several possible roles that the C2 domains of the class I Rab11-FIPs might play. One hypothesis is that the three C2 domains bind different phospholipid(s) and thus provide a means by which each Rab11-FIP can sense a specific membrane microdomain and thus provide a mechanism for ‘fine-tuning’ their subcellular localization. Data from the Scheller group revealed that treatment of MDCK cells with kinase inhibitors results in the translocation of Rip11 from the ERC to the plasma membrane. Thus, it was proposed that Rip11 traffics between these two locations (Prekeris et al., 2000). It is therefore possible that the C2 domains function to target vesicles carrying the Rab11-FIPs to the plasma membrane. A third possibility is that the C2 domains function as protein-protein interaction modules, in a mode similar to synaptotagmin, which binds syntaxin at the plasma membrane in a calcium-dependent manner (Katan and Allen, 1999).

With these possibilities in mind, we used a protein-phospholipid overlay technique and determined that all three C2 domains bind preferentially to PtdIns(3,4,5)P3 and phosphatidic acid (PA). We found that synthesis of either PtdIns(3,4,5)P3 or PA by stimulating A431 cells with epidermal growth factor (EGF) or a phorbol ester resulted in the translocation of endogenous CRC and Rab11-FIP2 from the ERC to the plasma membrane. Treatment of cells with wortmannin before stimulation abolished this translocation. Plasma membrane translocation was dependent on the presence of the C2 domain because truncation mutants lacking this domain did not translocate in treated cells. Each of the class-I Rab11-FIPs localized with Akt/PKB in cells stimulated with EGF. Akt/PKB has a pleckstrin homology (PH) domain that binds PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (Watton and Downward, 1999). These results confirm that the class I Rab11-FIPs traffic from the ERC to the plasma membrane and that their C2 domains target them to the plasma membrane.

Materials and Methods
Antibodies
The anti-CRC affinity-purified antibody has been described previously (Lindsay et al., 2002). The Rab11 and Rab11-FIP2 antibodies were raised in rabbits against Escherichia-coli-purified 6xHis-Rab11 and 6xHis-Rab11-FIP2(1-277). The resulting antisera were affinity purified and used at dilutions of 1:250 and 1:25, respectively. The anti-haemagglutinin (anti-HA) mouse monoclonal antibody was from Roche and used at a 1:1000 dilution. Mouse monoclonal anti-ZO1 antibody was purchased from BD Transduction Laboratories and used at a 1:150 dilution.

Plasmid constructs
Fusions between green fluorescent protein (GFP) and RCP and Rab11-FIP2 have been described previously (Lindsay et al., 2002; Lindsay and McCaffrey, 2002). GFP-Rip11 was generated by polymerase chain reaction (PCR) using pBlueScriptIIK S KIAA0857 (Kazusa DNA Research Institute) as template and the primers Rip11 FWD (5’-CGGAATTCCTGCGGCGCCAGG-3’) and Rip11 REV (5’-CGGAATTCGACACAGGACTGATGCGTCATCAGG-3’). The product was digested and ligated into the EcoRI site of pEGFP-C1 (Clontech). GFP-RCP (199) was generated by ligating the ~600 bp SalI fragment from pEGFP-C3 RCC into the SalI site of pEGFP-C3. GFP-RCP (200-649) was constructed by ligating the SalI-BamHI fragment from pEGFP-C3 RCC into the SalI-BamHI site of pEGFP-C1. GFP-Rab11-FIP2(1-284) was generated by PCR using Rab11-FIP2FWD (5’-CGGAATTCCTGCGGCGCCAGGACCCAAGAAG-3’) and Rab11-FIP2REV (5’-CGGAATTCGACACAGGACTGATGCGTCATCAGG-3’) and ligating into the EcoRI site of pEGFP-C1. GFP-Rab11-FIP2(277-512) was generated by ligating the HindIII-BamHI fragment from pEGFP-C1 Rab11-FIP2 into the HindIII-BamHI site of pEGFP-C2 (Clontech). GFP-Rip1(1-218) was generated by digesting pEGFP-C1 Rip1 with BamHI and religating the backbone. GFP-Rip1p(219-653) was constructed by ligating the ~2.2 kb BamHI fragment from pBSKII KIAA0857 into pEGFP-C1. RCP(1-199) was subcloned into pGEX2T (Amersham Biosciences). Rip1(1-218) was subcloned into the BamHI site of pGEX3X and Rab11-FIP2(1-284) was subcloned into the EcoRI site of pGEX3X (Amersham Biosciences). All constructs generated by PCR were confirmed by sequencing.

Expression and purification of GST fusion proteins
Glutathione-S-transferase (GST) fusion-protein expression was induced in E. coli XL-1 Blue cells with 0.1 mM isopropyl-thiogalactoside (IPTG) at 30°C for 4 hours. The bacterial cells were resuspended in 1x PBS and lysed with 1% Triton X-100 plus 5 mM dithiothreitol. The lysate was clarified by centrifugation at 12,000 g and the protein was bound to glutathione-agarose (Sigma), which was washed twice with PBS. The protein was eluted with 10 mM glutathione and subsequently dialysed overnight in PBS.

Protein-phospholipid assays
The protein-phospholipid overlay assays were carried out according to the manufacturers instructions. Briefly, PipArrays™ (Echelon Biosciences) were blocked in 3% bovine serum albumin (BSA) in Tris-buffered saline-0.1% Tween-20 (TBST) and then incubated overnight with 0.5 μg ml−1 GST fusion protein. The membranes were then washed extensively with 3% BSA in TBST and probed with anti-GST antibody (Amersham Biosciences). After washing, the membranes were incubated with horseradish peroxidase (HRP)-coupled anti-rabbit IgG and then visualized by autoradiography. Densitometry was performed using GeneTools analysis software (Syngene). The intensity of each phosphoinositide spot, at a concentration at which the signal was not saturated, was measured. The spot with the highest intensity was set at 100 [this was PtdIns(3,4,5)P3 in every case]. The intensity of the other phosphoinositide signals, at the same concentration, were normalized against PtdIns(3,4,5)P3. The results presented are the mean of three independent experiments. PipArrays with PA, phosphatidylethanolamine (PE) (Sigma) were made as described previously (Dowler et al., 2002).

Cell culture and immunofluorescence
HeLa and A431 cells were maintained in culture in Dulbecco’s modified Eagle’s medium (DMEM) (BioWhittaker) supplemented
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with 10% foetal bovine serum, 100 units ml\(^{-1}\) penicillin, 100 \(\mu\)g ml\(^{-1}\) streptomycin and 2 mM glutamine. Transfections were carried out using Effectene transfection reagent (Qiagen) according to the manufacturer's instructions. 8 hours after transfection, the medium was replaced with fresh medium and, 16 hours later, the cells were fixed with 3% parafomaldehyde and mounted with Mowiol. For antibody labelling, the cells were permeabilized with 0.05% saponin supplemented with 0.2% BSA. Images were acquired on a confocal microscope (Zeiss LSM 510) using a PlanApo 63\(\times\) 1.4 NA oil-immersion objective. Images were processed using Image Examiner software (Carl Zeiss) and imported into Adobe Illustrator.

**Results**

**Rab11-FIPs require their C2 domain for correct subcellular localization**

RCP, Rip11 and Rab11-FIP2 comprise the class I Rab11-FIPs. They share the same overall domain organization – an \(\alpha\)-helical coil region encompassing the Rab11-binding domain (RBD) at their C-terminus and a highly homologous region of ~200 amino acids at their N-terminus, known as the N-terminal homology domain (NHD). The NHD encompasses their conserved C2 domain (Fig. 1A). They share no significant homology in the region that separates these two domains. A series of GFP-tagged fusions were constructed for each of the class I Rab11-FIPs. These were either the full-length wild-type proteins or truncation mutants that lacked the N-terminal domain. When these fusion proteins were expressed in HeLa cells, it was observed that the truncation mutants displayed an altered subcellular distribution. The full-length proteins all displayed a fine vesicular pattern throughout the cytoplasm (Fig. 1B). By contrast, the truncation mutants that lacked the NHD localized to much fewer but larger vesicular structures. This suggests that the C2 domain plays an important role in the localization of the class I Rab11-FIPs. The fewer, larger vesicular structures to which they localize indicate that the truncation mutants are possibly trapped in vesicular aggregates.

**Class I Rab11-FIPs preferentially bind PtdIns(3,4,5)P\(_3\)**

As C2 domains have been described as phospholipid-binding modules, we set out to determine whether this was a function of the class I Rab11-FIP C2 domains and, if so, to which phospholipids they bound. For this purpose, we used a protein-lipid overlay assay (Dowler et al., 2002). The NHDs of each of the class I Rab11-FIPs were fused to GST and expressed in E. coli. The purified GST fusion proteins were then overlayed onto nitrocellulose membranes on which increasing concentrations of various phospholipids had been spotted. The interaction of the C2 domains with phospholipid was determined by immunoblotting with an anti-GST antibody. All three C2 domains showed their highest binding affinity for PtdIns(3,4,5)P\(_3\) (Fig. 2A). Quantitation of the PipArrays revealed that the Rab11-FIP C2 domains displayed at least a 30% greater affinity for PtdIns(3,4,5)P\(_3\) than to the other phosphoinositides tested (Fig. 2B).

Although binding of the Rip11 C2 domain to PE-containing liposomes has been reported (Prekeris et al., 2000), its ability to bind PA or any of the phosphoinositides had not been investigated. To analyse the relative binding affinities of the class I Rab11-FIP C2 domains for the phospholipids PE and PA, we made arrays spotted with various concentrations of PA, PE, PC and PS. In the case of RCP and Rip11, we observed a very strong preference for PA compared with the other phosphoinositides (Fig. 2C,D). Rab11-FIP2 was unusual in that it consistently showed the strongest binding to the highest concentration of PS but displayed greater affinity for the lower concentrations of PA than for PS. We believe that the interaction with PS is not physiologically relevant because it is only observed at the highest concentration.

Taken together, these results indicate that the class I Rab11-FIPs bind preferentially to the phospholipids PtdIns(3,4,5)P\(_3\) and PA. Because the three C2 domains display similar phospholipid binding profiles, it suggests that they target the class-I Rab11-FIPs to the same membrane environment, making it unlikely that their role is to sense a unique membrane microdomain for each of the Rab11-FIPs. Alignment of their C2 domains reveals a high degree of homology, consistent with the observation that they have a similar phospholipid preference (Fig. 3).
Fig. 2. The C2 domains of the class I Rab11-FIPs bind PtdIns(3,4,5)P$_3$ and PA. (A) PipArrays™ were overlayed with the indicated GST fusion proteins and immunoblotted with anti-GST antibody. (B) Densitometry of the PipArrays indicating that the class I Rab11-FIPs bind preferentially to PtdIns(3,4,5)P$_3$. The results are the mean of three independent experiments. (C) Nitrocellulose was spotted with serial dilutions of PS, PA, PC and PE. They were subsequently overlayed with the indicated GST fusion proteins and immunoblotted with anti-GST antibody. (D) Densitometry of the protein-lipid assay, indicating a preference for PA. The results are representative of three independent experiments.
Class I Rab11-FIPs translocate to the plasma membrane from the ERC

We analysed the in vivo localization of endogenous RCP and Rab11-FIP2 in cells under conditions that stimulate the synthesis of PtdIns(3,4,5)P3 or PA. In the A431 epidermal carcinoma cell line, both RCP and Rab11-FIP2 localized to vesicles throughout the cytoplasm, with strong labelling of the ERC, seen as a tight dot in the perinuclear region. Treatment of serum starved A431 cells with EGF resulted in the translocation of a large proportion of RCP and Rab11-FIP2 to the plasma membrane (Fig. 4A). This translocation was abolished by pretreatment of the cells with the PI-3-kinase inhibitor wortmannin. In the wortmannin-treated cells, the two class I Rab11-FIPs localized to larger cytoplasmic vesicles but showed no plasma membrane staining (Fig. 4A). Thus, upregulation of PtdIns(3,4,5)P3 synthesis results in the translocation of RCP- and Rab11-FIP2-containing vesicles to the plasma membrane, and inhibition of PI-3-kinase, which is responsible for synthesizing PtdIns(3,4,5)P3, abolishes this translocation.

Treatment of cells with the phorbol ester phorbol 12-myristate 13-acetate (PMA), stimulates phospholipase D (PLD) to synthesize PA. There is a dramatic shift of RCP and Rab11-FIP2 to the plasma membrane in PMA-treated A431 cells (Fig. 4B). Wortmannin pretreatment also inhibits plasma-membrane translocation in PMA-stimulated cells. These results provide evidence that agrees with the in vitro phospholipid-binding assays. Treatment of cells under conditions that stimulate the production of either PtdIns(3,4,5)P3 or PA result in a dramatic alteration of the localization of RCP and Rab11-FIP2.

To confirm that the plasma membrane is the site of translocation, control and stimulated cells were co-labelled with antibodies to RCP and ZO-1, the tight junction protein. In cells treated with DMSO alone, RCP was predominantly perinuclear, with very few RCP-positive vesicles near the plasma membrane. However, in cells stimulated with PMA, RCP-positive vesicles shifted away from the perinuclear region and line up along the plasma membrane, as seen with ZO-1 (Fig. 4C). Similar results were observed for endogenous Rab11-FIP2 (data not shown). Together, these results indicate that the class I Rab11-FIPs traffic between the ERC and the plasma membrane. Synthesis of the phospholipids PtdIns(3,4,5)P3 and/or PA is likely to recruit these Rab11-FIP containing vesicles from their intracellular location to the plasma membrane.

C2 domain of the class I Rab11-FIPs is essential for their plasma-membrane translocation

To confirm that the C2 domain of the class I Rab11-FIPs mediates their plasma-membrane translocation, A431 cells were transfected with GFP fusions of the wild type or NHD truncation mutants of each of the Rab11-FIPs. 24 hours after transfection, the cells were treated with 1 µM PMA, or DMSO as a control, for 30 minutes. In control cells, both the wild-type proteins exhibited significant plasma membrane labelling. By contrast, the C2 domain truncation mutants in PMA-treated cells resembled control cells and exhibited no prominent plasma-membrane labelling (Fig. 5). This result indicates that the C2 domain is essential for class I Rab11-FIP plasma membrane translocation. Because PA and PtdIns(3,4,5)P3 levels are tightly regulated, it is possible that Rab11-FIP-mediated transport to the plasma membrane is also regulated.

Class I Rab11-FIP NHD GFP fusions localize to the plasma membrane

To determine whether the N-terminal homology domain (NHD) regions of the class I Rab11-FIPs, alone, have the ability to localize to the plasma membrane, GFP fusions of these domains were expressed in A431 cells. In untreated cells, these fusion proteins were predominantly cytosolic (Fig. 6). We reasoned that this might be due to the low basal level of PtdIns(3,4,5)P3, and so we stimulated PI-3-kinase with EGF. For each of the GFP fusions, we observed a substantial shift to the plasma membrane, in particular to regions of the plasma membrane that form junctions with neighbouring cells (Fig. 6). In cells stimulated with PMA to produce PA, the NHD fusions also localized to the plasma membrane, but less dramatically. They were also found labelling intracellular membrane structures (Fig. 6). These structures are likely to be intracellular compartments such as Golgi, endosomes and endoplasmic reticulum that are also enriched in PA upon phorbol-ester stimulation.
Rab11 translocates to the plasma membrane with the class I Rab11-FIPs

Rab11 binds with high affinity to the Rab11-FIPs in in vitro interaction studies. The Rab11-FIPs also display extensive localization with Rab11 on vesicles and at the ERC. This raises the question of whether this interaction also results in Rab11 trafficking to the plasma membrane upon class I Rab11-FIP translocation, or whether the Rab11-FIPs dissociate from Rab11 before translocation. To examine this, a GFP fusion of wild-type Rip11 expressed in A431 cells was induced to translocate to the plasma membrane by treatment with PMA. The cells were then fixed and labelled for endogenous Rab11. In control cells, Rab11 localized with GFP-Rip11 on intracellular vesicles and at the perinuclear ERC (Fig. 7A). However, in PMA-treated cells, there was extensive colocalization between GFP-Rip11 and Rab11 at the plasma membrane (Fig. 7A). Rab11 plasma membrane colocalization was also observed in cells expressing GFP-RCP and GFP-Rab11-FIP2 (data not shown). Therefore, Rab11 can traffic to the plasma membrane upon translocation of the class I Rab11-FIPs, and dissociation is not required before translocation can occur.

Interestingly, when the localization of the transferrin receptor (TfnR) was examined in PMA-stimulated cells, we did not observe a similar dramatic translocation (Fig. 7B). The TfnR was observed in fewer but larger vesicles than in unstimulated cells, but these vesicles did not traffic to the plasma membrane. EGF also did not stimulate the translocation of the TfnR (data not shown). Because the TfnR does not translocate to the plasma membrane upon PMA or EGF treatment, this suggests that the class I Rab11-FIPs also function along a pathway distinct from the TfnR-recycling pathway.

Class I Rab11-FIPs localize with Akt/PKB in EGF-treated cells

Akt/PKB possesses a PH domain that binds PtdIns(3,4,5)P3 and PtdIns(3,4)P2 (Klippel et al., 1997). Activation of PI-3-kinase by growth factors results in its translocation from the cytosol to the plasma membrane (Watton and Downward, 1999). We used Akt/PKB in EGF-stimulated cells as a marker for PtdIns(3,4,5)P3. In cells co-expressing HA-tagged Akt/PKB and each GFP-fused class I Rab11-FIP, we observed colocalization at the plasma membrane in EGF-treated cells (Fig. 8).

Discussion

Rab11 is a member of the Rab family of small GTPases. It is localized primarily to the ERC in eukaryotic cells and plays a role in regulating membrane traffic from this compartment to the plasma membrane, and also to the TGN. Several Rab11 interacting proteins have been identified in recent years. These include Rabphilin-

Fig. 4. Endogenous RCP and Rab11-FIP2 translocate to the plasma membrane upon PtdIns(3,4,5)P3 and PA synthesis in A431 cells. (A) Cells were unstimulated, treated with 100 ng ml⁻¹ EGF, for 20 minutes at 37°C, or pretreated with 100 nM wortmannin before incubation with EGF. The cells were then fixed and processed for immunofluorescence with antibodies to RCP or Rab11-FIP2. (B) Cells were unstimulated, treated with 1 µM PMA for 30 minutes at 37°C or pretreated with 100 nM wortmannin followed by processing for immunofluorescence as above. (C) Cells were either treated with DMSO or 1 µM PMA for 30 minutes at 37°C before fixation. The cells were then stained for RCP (green) and ZO-1 (red). Scale bar, 10 µm.
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11/Rab11BP, myosin Vb, Rip11, RCP, Rab11-FIP1, Rab11-FIP2, Rab11-FIP3, Rab11-FIP4 and Rab6IP1 (Hales et al., 2001; Lapierre et al., 2001; Lindsay et al., 2002; Mammoto et al., 1999; Nizak et al., 2003; Prekeris et al., 2001; Prekeris et al., 2000; Wallace et al., 2002; Zeng et al., 1999). Rip11, RCP, Rab11-FIP2, Rab11-FIP1, Rab11-FIP3 and Rab11-FIP4 have been grouped into a family of proteins called the Rab11-FIPs. The role of the Rab11-FIPs is unclear. Expression of truncation mutants of the class I Rab11-FIPs inhibits the receptor recycling pathway (Lindsay et al., 2002; Lindsay and McCaffrey, 2002; Prekeris et al., 2000), whereas equivalent class II Rab11-FIP truncation mutants do not have such an effect (Hickson et al., 2003; Wallace et al., 2002). RCP interacts with both Rab4 and Rab11 (Lindsay et al., 2002), and Rab11-FIP3 and Rab11-FIP4 interact with several Arf GTPases (Hickson et al., 2003; Shin et al., 2001; Shin et al., 1999). It is possible that these proteins serve as ‘molecular links’ that connect the transport pathways regulated by the different small GTPases. This role has been proposed for the divergent Rab4- and Rab5-interacting proteins Rabaptin-5 and Rabenosyn-5 (de Renzis et al., 2002; Deneka and van der Sluijs, 2002; Vitale et al., 1998).

To further understand the role of the Rab11-FIPs in Rab11-regulated membrane trafficking, we examined the properties of the conserved C2 domain present in Rip11, RCP and Rab11-FIP2. We determined that truncation mutants of the class I Rab11-FIPs that lack their C2 domains display a noticeably altered subcellular distribution, indicating that these domains play an important role in Rab11-FIP localization. We developed two hypotheses for the function of the class I Rab11-FIP C2 domains. First, the C2 domains might target each class I Rab11-FIP to a unique microdomain of the ERC. This would provide a means by which each of these Rab11 binding proteins can localize to a specific region of the ERC, such as the site of transport vesicle delivery from the sorting endosome or the location of vesicle exit to the plasma membrane. Second,
the C2 domain targets the Rab11-FIP vesicle to a separate (acceptor) membrane compartment within the cell, such as the plasma membrane or the TGN. This could be a means by which the class I Rab11-FIPs can regulate the traffic of transport vesicles to their destination.

Phospholipid-binding properties of the class I Rab11-FIPs

Phospholipid-binding modules are common features of many proteins involved in membrane trafficking. As well as C2 domains, such modules include FYVE, Phox homology (PX), PH and ENTH domains. They recruit proteins to specific membrane surfaces within the cell (Lemmon, 2003). These domains are structurally and functionally diverse. The FYVE domain, named after the first four proteins in which it was identified (Fab1p, YOTB, Vac1p and EEA1), binds specifically to PtdIns(3)P and targets several proteins involved in endocytosis to the early sorting endosome. PX domains are found mainly in proteins involved in vesicular trafficking, protein sorting, or lipid modification. Most PX domains, such as the PX domains of the sorting nexins, bind to PtdIns(3)P (Wishart et al., 2001). PH domains are common phosphoinositide-binding modules (approximately 250 PH domains have been identified in the human proteome). The PH domain of phospholipase Cβ1 binds PtdIns(4,5)P2, and recruits phospholipase Cβ1 to the plasma membrane.

To establish the phospholipid-binding properties of the C2 domains of the class I Rab11-FIPs, we employed a protein-lipid overlay technique (Dowler et al., 2002). Recently, this technique has been successfully used to identify the phospholipid binding partners of many proteins (Catz et al., 2002; Chiang et al., 2003; Dowler et al., 1999; Gozani et al., 2003). Using this technique, we determined that the three C2 domains of the class I Rab11-FIPs preferentially bind the phospholipids PtdIns(3,4,5)P3 and PA. Although no consensus binding motifs have been reported for either of these phospholipids, analysis of the primary sequence of the NHD of Rip11, RCP and Rab11-FIP2 identified a region rich in basic amino acids that is a candidate for the PA-binding site (Fig. 3, Table 1). Of the proteins identified so far that interact directly with PA, each of their putative PA-binding domains possess at least one polybasic motif.

PtdIns(3,4,5)P3 and PA

Phosphoinositides are phosphorylated forms of phosphatidylinositol (PtdIns) and are unique among phospholipids in that their levels can be quickly modified by the rapid phosphorylation/dephosphorylation of the head group (Lemmon, 2003). This provides a means by which the cell can transiently create membrane-targeting signals at specific locations within the cell. PtdIns(4,5)P2 and PtdIns(3,4,5)P3 are concentrated in the plasma membrane and generally excluded from endosomes, whereas PtdIns(3)P is an endosomal phospholipid and is not present on the plasma membrane. Among several functions, PtdIns(3,4,5)P3 has a role in directing exocytosis, whereas PtdIns(4,5)P2 directs endocytosis by anchoring several coat proteins to the plasma membrane (Czech, 2003). Even though it only accounts for ~1% of plasma-membrane lipid PtdIns(4,5)P2 is the most abundant of the phosphoinositides. It is present constitutively within the cell. By contrast, PtdIns(3,4,5)P3 is present at very low basal levels, but its concentration increases rapidly upon activation of phosphoinositide-3-kinases (Lemmon, 2003). Phosphoinositide-3-kinases synthesize PtdIns(3,4,5)P3 by phosphorylating the D3 position of PtdIns(4,5)P2, and its levels can be reduced by phosphatases such as SHIP and the tumour suppressor PTEN. Growth factors, hormones and
cytokines can stimulate phosphoinositide-3-kinases to rapidly produce PtdIns(3,4,5)P$_3$. PtdIns(3,4,5)P$_3$ is asymmetrically localized to the cytoplasmic surface of the plasma membrane. It is highly concentrated in membrane ruffles. These ruffles form by the insertion into the plasma membrane of endosome-derived membrane from the recycling pathway (Bretscher and Aguado-Velasco, 1998). Such ruffles are enriched in recycling receptors such as the transferrin and low-density lipoprotein receptors, but receptors that do not recycle are absent. Thus, it is likely that PtdIns(3,4,5)P$_3$ provide a plasma membrane targeting signal for transport vesicles containing the class I Rab11-FIPs.

PA is an anionic phospholipid that serves as an important second messenger. It is found at various locations within the cell including the plasma membrane, Golgi, endosomes and lysosomes. Most PA is synthesized by PLD, which hydrolyses PC into PA and choline. PLD activity is stimulated by many cell-surface receptors such as cytokines, receptor tyrosine kinases and G protein coupled receptors (Andresen et al., 2002). Phorbol esters can be used to activate PLD, which in turn synthesizes PA. PA has been implicated in protein phosphorylation, activation of oxidative processes and membrane trafficking. It has been shown to act as a plasma-membrane targeting molecule for several proteins – for example, PA recruits cRaf-1 from the cytosol to the plasma membrane, where it is then activated by Ras (Rizzo et al., 1999; Rizzo et al., 2000). Several membrane trafficking regulatory proteins bind directly to PA such as the coatamer complex, Arf6, NSF and kinesin (Manifava et al., 2001).

To determine the effect of PtdIns(3,4,5)P$_3$ and PA synthesis on the localization of the class I Rab11-FIPs, A431 cells were treated with the growth factor EGF or the phorbol ester PMA, and the localization of endogenous RCP and Rab11-FIP2 was compared with their localization in control cells. We observed a significant shift in labelling from cytosolic vesicles and the ERC in control cells to the plasma membrane in EGF-treated cells. EGF stimulates phosphoinositide-3-kinase to synthesize PtdIns(3,4,5)P$_3$ but, when the cells were pretreated with the phosphoinositide-3-kinase inhibitor wortmannin, this plasma membrane translocation was abolished. A similar but more dramatic plasma membrane translocation was observed in PMA-treated cells.

From the data presented here, we propose that the class I Rab11-FIPs mediate transport pathways from the ERC to the plasma membrane. Their N-terminal domains target these proteins to the plasma membrane via binding to PtdIns(3,4,5)P$_3$ and PA. Synthesis of these phospholipids results in the translocation of Rab11-FIP vesicles from their intracellular locations at the ERC and on intracellular transport vesicles to the plasma membrane. Their site of delivery is likely to be membrane ruffles that are rich in PtdIns(3,4,5)P$_3$. In unstimulated cells, there is very little class I Rab11-FIP labelling at the plasma membrane. TfnR-positive vesicles do not translocate to the plasma membrane upon stimulation, suggesting that the class I Rab11-FIPs might function in a

| Table 1. The percentage of basic amino acids in full-length, and subdomains, of the class I Rab11-FIPs |
|---------------------------------------------------------------|
| **Full-length** | **NHD** | **C2 domain** | **NHDAC2** |
|-----------------|---------|---------------|-------------|
| RCP             | 15.56%  | 16.59%        | 11.23%      | 23.91%      |
| (1-649)         | (1-199) | (20-108)      | (109-199)   |
| Rip11           | 13.32%  | 14.22%        | 9.82%       | 19.78%      |
| (1-653)         | (1-218) | (17-128)      | (129-218)   |
| Rab11-FIP2      | 15.82%  | 16.93%        | 12.5%       | 22.34%      |
| (1-512)         | (1-195) | (15-102)      | (103-195)   |

NHD is N-terminal homology domain. NHDAC2 is the NHD minus the C2 domain. Amino acid numbers corresponding to each region are indicated in brackets. There are approximately twice the number of basic amino acids in the NHDAC2 domains, of each of the Rab11-FIPs, compared to their C2 domains, indicating a possible phosphatidic acid binding region.
recycling pathway distinct from the TfnR pathway. It is likely that signals from the plasma membrane trigger the release of Rab11-FIP vesicles from the ERC and their subsequent delivery to plasma membrane docking sites.

Catz and co-workers recently demonstrated that the C2A domain of the tandem C2 domain-containing ATPase JFC1 binds preferentially to PtdIns(3,4,5)_P³ (Catz et al., 2002). JFC1 is a Rab27a effector protein and these authors propose that it positions Rab27a-containing vesicles to a specific docking point on the plasma membrane. This proposed function for the C2 domain of JFC1 is similar to the role we suggest for the C2 domains of the class I Rab11-FIPs.

The dual phospholipid specificity of RCP, Rip11 and Rab11-FIP2 might have several purposes. It may be a means to increase the plasma membrane affinity of the Rab11-FIPs, provide an extra level of regulation for the Rab11-FIP transport pathway or it may function to orientate the protein correctly at its site of entry into the plasma membrane. Several phosphoinositide-binding modules have been shown to bind more than one phospholipid, such as the PH domain of Akt/PKB, which binds both PtdIns(3,4,5)_P³ and its breakdown product PtdIns(3,4)_P² and recruits Akt/PKB to the plasma membrane (Klippel et al., 1997; Watton and Downward, 1999). Interestingly, the PX domain of p47phox binds both PtdIns(3,4)_P² and PA. These two phospholipids can bind synergistically to distinct non-overlapping sites within the PX domain (Karathanassis et al., 2002). This synergistic binding significantly increases the membrane affinity of p47phox.

Further investigations need to be carried out to identify the cargo transported by the class I Rab11-FIP mediated pathways, and the signal-mediated events that control these pathways.

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