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Recycling of the Human Prostacyclin Receptor is Regulated through a Direct Interaction with Rab11a GTPase.

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Key Words: human prostacyclin receptor, internalization, Rab11a, Rab4a, yeast-two-hybrid, GPCR.

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ABSTRACT

The human prostacyclin receptor (hIP) undergoes agonist-induced internalization but the mechanisms regulating its intracellular trafficking and/or recycling to the plasma membrane are poorly understood. Herein, we conducted a yeast-two-hybrid screen to identify proteins interacting with the carboxyl terminal (C)-tail domain of the hIP and discovered a novel interaction with Rab11a. This interaction was confirmed by co-immunoprecipitations in mammalian HEK293 and was augmented by cicaprost - stimulation. The hIP co-localized to Rab11-containing recycling endosomes in both HEK293 and endothelial EA.hy 926 cells in a time-dependent manner following cicaprost-stimulation. Moreover, over-expression of Rab11a significantly increased recycling of the hIP, while the dominant negative Rab11^{S25N} impaired that recycling. Conversely, while the hIP co-localized to Rab4-positive endosomes in response to cicaprost, ectopic expression of Rab4a did not substantially affect overall recycling nor did Rab4a directly interact with the hIP. The specific interaction between the hIP and Rab11a was dependent on a 22 amino acid (Val²⁹⁹ – Gln³²⁰) sequence within its C-tail domain and was independent of isoprenylation of the hIP. This study elucidates a critical role for Rab11a in regulating trafficking of the hIP and has identified a novel Rab11 binding-domain (RBD) within its C-tail domain that is both necessary and sufficient to mediate interaction with Rab11a.

INTRODUCTION

The prostanoid prostacyclin (prostaglandin (PG)_I₂) plays a central role in haemostasis acting as a potent inhibitor of platelet aggregation and as an endothelium-derived vasodilator [1]. It exerts pro-inflammatory and *anti*-proliferative properties *in vitro* [2, 3] and offers cytoprotection during acute myocardial ischemia or hypoxia [4]. The actions of prostacyclin generally counteract those of thromboxane (TX) A₂, and, thus, factors dictating their relative levels and/or the cellular responses are central to haemostasis and vessel tone [5]. Imbalances in prostacyclin or TXA₂ are widely associated with a range of vascular diseases, including thrombosis, stroke, systemic and pulmonary hypertensions, myocardial infarction and atherosclerosis [6-10]. Moreover, mice deficient in prostacyclin receptors (IPs) show impaired responses to thrombotic stimuli [2] and develop more severe pulmonary hypertension and vessel remodelling following chronic hypoxia [11].

The prostacyclin receptor (IP), a member of the G-protein coupled receptor (GPCR) superfamily, is primarily coupled to G_s/adenylyl cyclase activation but may regulate other secondary effectors including G_q/phospholipase (PL)C activation, G_i/adenylyl cyclase activation, amongst others [12-15]. The IP is subject to a number of post-translational modifications that are critical in regulating its function. For example, the human IP (hIP) undergoes both isoprenylation and palmitoylation within its carboxyl-terminal (C)-tail domain and collectively these lipidations modulate its G-protein/effector signaling and internalization post-agonist activation [16-18]. Both the human (h) and mouse (m) IPs are isoprenylated through attachment of a C-15 farnesyl isoprenoid to a Cys within its highly conserved –‘CAAX’ motif [16, 17]. The hIP is dually palmitoylated at Cys³⁰⁸ and Cys³¹¹ and together with the farnesyl-Cys³⁸³-methyl ester, at Cys³⁸³, confers a double loop structure within its C-tail domain to orientate and provide the critical structural domains for interaction with coupling G proteins and, possibly, with components of the protein trafficking machinery to modulate both G protein/effector signaling and receptor internalization post-agonist activation [16-18].

A critical feature of GPCR signaling is desensitization, the process(es) that regulates its ability to respond to repeated challenge to its own ligand or indeed regulates cross-talk between other related or unrelated signaling systems [19, 20]. Desensitization is usually initiated by phosphorylation, such as by the second messenger-protein kinases and/or by the G protein-coupled receptor kinases (GRKs) [19, 20]. GRK phosphorylation of the agonist-occupied GPCR may in turn recruit the non-visual β -arrestin1/2 which binds with high affinity sterically hindering G-protein coupling, desensitizing GPCR signalling [20-22]. Moreover, depending on the receptor, β -arrestin(s) may serve as adapters targetting the GPCR to components of the endocytic machinery, such as to clathrin coated pits through association with clathrin and the β 2-adaptin subunit of the heterotetrameric AP2 adaptor complex [20, 21] to induce sequestration and internalization of the desensitized GPCR from the plasma membrane. Following

internalization, the GPCR may undergo further intracellular trafficking, such as through Rab-dependent vesicular transport, ultimately leading to either GPCR dephosphorylation and recycling (resensitization), GPCR degradation (down-regulation) or indeed GPCR recruitment into scaffolds/subcellular microdomains for participation in other novel/distinct signalling cascades [20-24]. The Rab GTPases control multiple steps of the trafficking [25, 26], either independently of /or in association with the β -arrestin/adaptor mechanisms. Rab5a is present at the plasma membrane and in early endosomes and mediates endocytosis and transport or fusion of endocytic vesicles with early endosomes [27]. Rab4a and Rab11a are associated with early and late recycling endosomes and can mediate GPCR recycling through either the so-called “short pathway” and the “long pathway”, respectively [28] while Rab7 is also associated with late endosomes and can regulate transport to lysosomes [29]. While much is known about the events leading to GPCR desensitization and internalization, significantly less is understood about the intracellular sorting mechanisms dictating GPCR recycling and /or transport to the lysosomes for degradation.

A number of independent studies have established that the prostacyclin receptor (IP) undergoes agonist-induced phosphorylation, desensitization and down-regulation in human platelets and other cell types, providing an important mechanism of fine-tuning the cellular responses to prostacyclin *in vivo* [30-34]. While the hIP undergoes agonist-induced protein kinase (PK)C phosphorylation within its C-tail domain, desensitizing its signaling [33], its internalization is entirely independent of both PKC and the classic GRK/ β -arrestin-mediated mechanism [34]. Through recent investigations, we have established that the hIP undergoes rapid agonist-induced internalization through a mechanism involving its direct interaction with Rab5a [35]. In general, it is thought that the carboxyl-terminal (C)-tail domain of a given GPCR can act as an important determinant of desensitization and internalization, frequently being enriched in Ser/Thr residues for phosphorylation and/or containing structural motifs for interaction with components of various trafficking pathways, such as β -arrestin or Rab protein binding domains [20, 21, 36, 37]. In the case of the hIP, while deletion of its C- tail domain of the hIP did not fully impair agonist-induced internalization or, indeed, association with Rab5 *per se*, trafficking of the truncated hIP (hIP ^{Δ 312} and hIP ^{Δ 307}) was substantially altered suggesting the C-tail domain contains structural determinant(s) for hIP sorting post Rab5-mediated endocytosis [35]. Hence, the aim of the current study was to apply the yeast-two-hybrid (Y2H) screening approach to identify interactants of the C-tail domain of the hIP, in particular with the view to possibly identifying those proteins that influence hIP trafficking in response to agonist stimulation. Herein, we have discovered a novel interaction with Rab11a that is enhanced by agonist-activation of the hIP. The functional implications of this interaction with Rab11a, and the possible role of Rab4a, in regulating agonist-induced trafficking of the hIP are investigated.

EXPERIMENTAL PROCEDURES

Materials.

Cicaprost was obtained from Schering AG (Berlin, Germany). Rat monoclonal *anti*-HA-3F10 horse radish peroxidase (HRP)-conjugated antibody (25µg/ml) was obtained from Roche. Mouse monoclonal *anti*- hemagglutinin (HA)- 101R antibody (1 mg/ml) was obtained from Eurogentec; *anti*-FLAG[®] M2 monoclonal antibody (F3165) was sourced from Sigma; *anti*-Myc (M911) M2 mouse monoclonal antibody was from Cell Signalling Technology; *anti*-GFP (FL), *anti*-Rab4 (D-20), *anti*-Rab11 (H-87), HRP-conjugated goat *anti*-mouse (400 µg/ml) and HRP-conjugated goat *anti*-rabbit (400 µg/ml) antibodies were from Santa Cruz; mouse *anti*-Tfr (A-11130) was from molecular probes; *Anti* HDJ-2 antibody was from Neomarkers. AlexaFluor594 goat *anti*-mouse (2 mg/ml) and AlexaFluor488 goat *anti*-rabbit (2 mg/ml) antibodies were from Molecular Probes.

Yeast-2-Hybrid Screening and Yeast Matings.

A human kidney cDNA library cloned in-frame with the activation domain of the yeast GAL4 transcriptional activator in the yeast prey plasmid pACT2 and pre-transformed into *Saccharomyces cerevisiae* (*S.c*) Y187 was obtained from Clontech (3.5 X 10⁶ independent clones; HY4043AH). cDNA fragments encoding the carboxyl-terminal (C)-tail domain of the human prostacyclin receptor (hIP), corresponding to amino acids 299 – 386 of either the wild type hIP, namely hIP^{299-386WT}, or of its isoprenylation deficient variant, namely hIP^{299-386SSLC} (Figure 1A), were subcloned in-frame with the DNA-binding domain of GAL4 into the vector pGBKT7. The resulting bait pGBKT7:hIP^{299-386WT} and pGBKT7:hIP^{299-386SSLC} plasmids were transformed into *S.c* AH109 strain, according to standard protocols (Matchmaker[™] Two-Hybrid System 3, Clontech, Protocol No. PT3024-1) and grown on SD Leu⁻ plates. Thereafter, the *MATa* bait strain *S.c.* AH109 (pGBKT7:hIP^{299-386,SSLC}) was mated with the *MATα* *S.c.* Y187 (pACT2; human kidney cDNA library), at a density of 2 x 10⁶ cells per ml and a ratio of 30: 1 bait : prey cells, where 1 X 10⁸ individual transformants/diploids were screened. After 24 hr growth in SD/Trp⁻,Leu⁻ (DDO, double dropout) at 30 °C, resulting diploids were plated on SD/Trp⁻, Leu⁻, His⁻, Ade⁻ medium (quadruple drop out (QDO) medium; Clontech, Protocol No. PT3024-1) and plates were maintained for 15 days at 30 °C. Recombinant pACT2 plasmid DNA was extracted from each putative interactant [38] and transformed into supercompetent *Escherichia (E) coli* cells. Following re-transformation of the pACT2- based plasmid from putative interactants into *S.c* Y187 and remating with a range of bait strains including *S.c* AH109 (pGBKT7: hIP^{299-386WT}), *S.c* AH109 (pGBKT7: hIP^{299-386SSLC}) and AH109 (pGBKT7), positive interactants were selected on the basis of their ability to express the HIS3, ADE2 and lacZ genes by growth on the high stringency QDO medium and by their ability to cleave X-β-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), as measured by the filter lift assay from SD/Trp⁻

Leu⁻ medium plates according to standard protocols (Clontech, Protocol No. PT3024-1). Thereafter, plasmids from positive interactants were purified, cDNA inserts identified by DNA sequence analysis and subsequent interrogation of the National Center for Biotechnology Information (NCBI) databases using Basic Local Alignment Search Tool (BLAST).

The plasmids pGBKT7:hIP^{299-386WT}, pGBKT7:hIP^{299-386SSLC}, pGBKT7: hIP^{320-386WT}, pGBKT7:hIP^{320-386,SSLC}, pGBKT7:hIP^{IC1}(IC1^{Ser39-Ala50}), pGBKT7:hIP^{IC2}(IC2^{E116-L138}), pGBKT7:hIP^{IC3}(IC3^{Ser209-ILeu239}), pGBKT7:hIP²⁹⁹⁻³²⁰, pGBKT7: hIP^{312-386,WT}, pGBKT7: hIP^{307-386,WT}, pGBKT7: hIP^{312-386SSLC} and pGBKT7:hIP^{307-386SSLC} were generated by subcloning their respective cDNA fragments into the EcoRI-BamHI sites of the bait vector pGBKT7 in-frame with the DNA-binding domain of GAL4. The control plasmids pGBKT7:p53 and pTDI, encoding p53 and the SV40 large T antigen, respectively, were obtained from Clontech. All pGBKT7-based bait plasmids were transformed into *S.c* AH109 (*Mata*) while all prey plasmids (pACT2-based and pTDI) were transformed into *S.c* Y187 (*Mata*) and were mated, according to standard protocols with selection on synthetic media (SD) with double drop-out (DDO) of Leu and Trp (SD/DDO, Leu⁻, Trp⁻; Clontech).

For analysis of protein expressions in the transformed *S.c.* AH109 (pGBKT&) bait or *S.c* Y187 (pACT2) prey strains, protein was extracted according to recommended protocols (Clontech, Protocol No. PT3024-1), resolved by SDS-PAGE, on 12.5% acrylamide gels, and screened by western blot analysis using *anti-Myc* (9B11) or *anti-FLAG* M2 monoclonal antibodies, respectively, followed by chemiluminescence detection [16].

Subcloning and Site-Directed Mutagenesis.

The plasmid pcDNA3:Rab11a was obtained from the Missouri S&T cDNA Resource Center. Conversion of Ser²⁵ to N²⁵ to generate pcDNA3:Rab11a^{S25N} was performed using pcDNA3:Rab11a as template and the sense/antisense primer pair (5'TCT GGT GTT GGA AAG **AAT** AAT CTC CTG TCT CGA). The plasmid pcDNA3:Rab11a^{Q70L} was generated using pcDNA3:Rab11a as template and sense/antisense primer pair (5'TGG GAC ACA GCA GGG **CTA** GAGCGA TAT CGA GCT). The plasmid pCMV5:Rab4 was previously described [39]. Conversion of Ser²² to N²² to generate pCMV5:Rab4^{S22N} was achieved using pCMV5:Rab4a as template and the sense/antisense primer pair (5' GCA GGA ACT GGC AAA **AAT** TGC TTA CTT CAT CAG). The plasmid pCMV5:Rab4a^{Q67L} was generated using pCMV5:Rab4a as template and sense/antisense primer pair (5' TGG GAT ACA GCA GGA **CTA** GAA CGA TTC AGG TCC). For each primer pair, sequences shown correspond to the sense primer and the identity of the mutator codon is in boldface italics. All site directed mutagenesis was performed using QuikChangeTM (Stratagene) site-directed mutagenesis and mutations were validated by DNA sequence analysis. The full length cDNAs encoding Rab11a, Rab11a^{S25N}, Rab11a^{Q70L}, Rab4a, Rab4a^{S22N} and

Rab4a^{Q67L} were subcloned in frame into the Xho1-BamH1 sites of pEGFPC1 to generate pEGFPC1:Rab11a, pEGFPC1:Rab11a^{S25N}, pEGFPC1:Rab11a^{Q70L}, pEGFPC1:Rab4a, pEGFPC1:Rab4a^{S22N} and pEGFPC1:Rab4a^{Q67L}, respectively.

Cell Culture and Transfections.

Human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection and grown in minimal essential medium (MEM) containing 10% foetal bovine serum (FBS).

Routinely, approximately 48 hr prior to transfection, cells were plated at a density of 2×10^6 cells/10 cm culture dish in 8 ml media. Thereafter, cells were transiently transfected with 10 µg of pADVA [40] and 25 µg of pCMV-, pcDNA3- or pEGFPC1-based vectors using the calcium phosphate/DNA co-precipitation procedure, as previously described [41]. Transiently transfected cells were harvested 48 hr after transfection, unless otherwise stated.

HEK.hIP^{WT}, HEK.hIP^{Δ307} and HEK.hIP^{Δ312} cells stably over-expressing HA-tagged forms of hIP^{WT}, hIP^{Δ307} and hIP^{Δ312} respectively, have been previously described [16, 18]. HEK.β-galactosidase (HEK.β-Gal) cells stably over-expressing the HA-tagged β-galactosidase (β-Gal) from *Escherichia coli* were generated essentially as previously described [16, 18].

EA.hy 926 cells were obtained from the Tissue Culture Facility at UNC Lineberger Comprehensive Cancer Center, North Carolina and were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10 % FBS [42].

Co-immunoprecipitations.

HEK.hIP, HEK.hIP^{Δ307}, HEK.hIP^{Δ312} and, as controls HEK 293 or HEK.β-galactosidase (β-Gal) cells were transiently co-transfected with pADVA and either pEGFPC1, pEGFPC1:Rab11a, pEGFPC1:Rab11a^{S25N}, pEGFPC1:Rab11a^{Q70L} or pEGFPC1:Rab4a using the calcium phosphate/DNA co-precipitation procedure [41]. Some 48 hr post-transfection, co-immunoprecipitations were carried out as previously described [35]. Immunoprecipitates and whole cell lysates were resolved by SDS-PAGE on 10% gels and subjected to immunoblotting with *anti*-GFP (1:1000), *anti*-Rab4 (1 :1000), *anti*-Rab11 (1 :1000), *anti*-HA 3F10-HRP (1:500) antibodies followed by chemiluminescence detection [16].

ELISA-based Recycling Assays.

HEK.hIP cells (10-cm dishes, 60-70% confluent) were transiently co-transfected with 10 µg pADVA along with 25 µg pcDNA3:Rab11a, pcDNA3:Rab11a^{S25N}, pcDNA3:Rab11a^{Q70L}, pCMV5:Rab4a, pCMV5:Rab4a^{Q67L} or pCMV5:Rab4a^{S22N} or, as controls, with the empty vector equivalents using the calcium phosphate/DNA co-precipitation procedure, as previously described [41]. Some 24 hr post-

transfection, cells were transferred to poly-L-lysine (0.001%) pre-coated 24-well plates at a density of 1×10^5 cells per well and were cultured for an additional 48 hr. Thereafter, to assess recycling of hIP post-agonist stimulation, the media was changed to serum-free MEM and cells were treated with cicaprost (1 μ M), or as a control, with vehicle (MEM) for 1 hr at 37 °C to allow receptor internalization to commence. Cicaprost-containing media was removed by washing cells twice in MEM and the cells were further incubated at 37 °C in fresh serum-free MEM for varying times between 0 and 4 hr. Cells were washed twice in ice-cold PBS prior to fixation in 3.7 % paraformaldehyde, 1x TBS, pH 7.4, for 5 min at room temperature. After washing the cells three times in TBS (20 mM Tris-HCl, pH 7.4, 0.1 M NaCl), non-specific sites were blocked with Blocking Buffer (1 % bovine serum albumin, BSA, in 1 x TBS) for 1 hr at room temperature. Thereafter, cells were incubated with *anti*-HA 101R antibody (1: 2000 in Blocking Buffer) for 1 hr at room temperature. The antibody solution was removed and the cells were washed 3 times in TBS, prior to incubation with goat *anti*-mouse HRP (1 : 2000) for 1 hr at room temperature. Following this, the cells were washed three times in TBS and net changes in cell surface expression of HA-tagged receptor expression was determined colorimetrically at 650 nm using the K-Blue substrate (Neogen Corp), as previously described [17].

Assessment of Agonist-Induced Trafficking by Confocal Microscopy.

HEK.hIP cells (10-cm dishes, 60-70% confluent) were transiently co-transfected with 10 μ g pADVA along with 25 μ g pEGFPC1:Rab11a, pEGFPC1:Rab11a^{S25N}, pEGFPC1:Rab11a^{Q70L} or pEGFPC1:Rab4a using the calcium phosphate/DNA co-precipitation procedure, as previously described [41]. Seeding of cells onto coverslips, pre-labelling of cell surface hIPs and treatment with Cicaprost was carried out as described previously [35].

To monitor agonist-induced changes in the intracellular localization of Rab4 or Rab11 in the human endothelial EA.hy 926 cell line, cells were treated with 1 μ M cicaprost prior to fixation, permeabilization and immunolabelling with *anti*-Rab4 and *anti*-Rab11 antibody and secondary AlexaFluor488 goat *anti*-rabbit antibodies. All slides were imaged, at x 63 magnification, using Carl Zeiss Laser Scanning System LSM 510 and Zeiss LSM Imaging Software for acquiring multichannel images with filters appropriate for enhanced GFP, AlexaFluor488 or AlexaFluor594 fluorescence.

Data analyses.

Statistical analyses were carried out using the unpaired Student's *t* test throughout or, where relevant and specifically indicated in text, using two-way ANOVA employing the GraphPad Prism version 4.00 package. *P*-values of less than or equal to 0.05 were considered to indicate a statistically significant difference.

RESULTS

Identification of Rab11a as an Interactant of the hIP in Yeast and Mammalian Cells.

It has been recently established that the carboxyl-terminal (C) -tail domain of the human prostacyclin receptor (hIP) may contain critical structural determinants regulating its intracellular vesicular trafficking post -agonist stimulation [35]. Hence, herein, a yeast-two-hybrid (Y2H) screen of a human kidney cDNA library was carried out to identify proteins that specifically interact with the C-tail domain of the hIP (Figure 1A). As stated, the hIP is isoprenylated at Cys³⁸³ within its conserved “-CAAX motif”(Figure 1A; - C³⁸³SLC³⁸⁶), a modification that is likely to affect the efficient transport of ‘bait:fusion proteins’ based on the GAL4 DNA binding domain into the nucleus during the Y2H screen itself [43]. Therefore, at the outset, the human kidney cDNA library was screened with an isoprenylation deficient variant of the C-tail domain, namely hIP^{299-386SSLC} (Figure 1A). To begin with, it was confirmed that the GAL4-fusion proteins based on the hIP^{299-386SSLC} and, indeed, the hIP^{299-386WT} were efficiently expressed in the *Saccharomyces cerevisiae* (*S.c*) AH109 host (Figure 1B). Moreover, whilst the hIP^{299-386WT} is expressed as both the isoprenylated and non-isoprenylated forms, the hIP^{299-386SSLC} was expressed exclusively as the non-isoprenylated precursor, consistent with disruption of the “-CAAX motif” (Figure 1B).

Following primary high stringency- and secondary- screening of the human kidney cDNA library (complexity, 3.5 X 10⁶ individual clones; 1 X 10⁸ individual diploids screened), a number of independent clones were identified that encoded Rab11a. This interaction was further tested by direct mating of the *MATα S.c* Y187 (pACT:Rab11a) strain with the *MATα S.c* AH109 bait strain transformed with recombinant pGBKT7 encoding either the original hIP^{299-386SSLC} or the hIP^{299-386WT} and, as controls, with the unrelated bait p53 or the empty pGBKT7. While each of the bait strains mated successfully with the prey strain to generate diploids, strains harbouring either the empty vector pGBKT7 alone or encoding p53 failed to show any interaction with Rab11a (Figure 1C). Conversely, strains encoding both the original bait hIP^{299-386SSLC} and indeed its wild type equivalent hIP^{299-386WT} showed a strong interaction Rab11a (Figure 1C). Based on these data, it appears that the C-tail domain of the hIP can specifically interact with Rab11a and that interaction does not appear to be dependent on the isoprenylation status of the hIP.

Thereafter, the ability of Rab11a to interact with the hIP expressed in mammalian cells was investigated. To this end, a previously characterized HEK.hIP cell line, that stably over-expresses a hemagglutinin (HA)-tagged form of the hIP [18], was co-transfected with plasmids encoding green fluorescent protein (GFP)-tagged forms of Rab11a, Rab11a^{S22N} and Rab11a^{Q70L} and their presence in the *anti*-HA-hIP immunoprecipitates investigated as a function of agonist-stimulation. In the absence of agonist, Rab11a was detected in the *anti*-HA immunoprecipitates from HEK.hIP cells, but was not present in corresponding immunoprecipitates from either the control HEK.β-Gal cells or the parental

HEK 293 cell line (Figure 2A; upper panel). Stimulation of cells with the selective IP agonist cicaprost (1 μ M) led to a significant increase in the amount of Rab11a associated with the hIP immunoprecipitates (Figure 2A; upper panel). Furthermore, this agonist-dependent interaction was time-dependent, reaching a maximum at 2 hr (Figure 2A; upper panel). In addition, hIP immunoprecipitates from HEK.hIP cells were analysed for endogenous Rab11 protein by immunoblotting with an *anti*-Rab11a-antibody. As with the overexpressed Rab11 protein, we found some interaction in unstimulated cells, but the association between hIP and endogenously expressed Rab11 was greatly enhanced by stimulation with cicaprost for 2 hr (Figure 2B; upper panel). The GTP-binding defective variant of Rab11a^{S22N} also co-precipitated with the hIP even in the absence of agonist and, similar to the wild type Rab11a, this interaction was significantly increased in response to cicaprost (Figure 2C; upper panel). Likewise, the constitutively active, GTPase deficient Rab11a^{Q70L} also co-precipitated with the hIP in the absence and presence of cicaprost to levels that were comparable to that of Rab11a and Rab11a^{S22N} even in the absence of agonist (Figure 2C, upper panel). The increase in co-precipitation of the Rab11a proteins in response to agonist was not due to differences in hIP (data not shown) or Rab11a (Figure 2A-2C; lower panels) expression levels or in the efficiency of the immunoprecipitations *per se* (Figure 2A-2C; middle panels). Hence, these data confirm a physical interaction between the hIP and Rab11a in mammalian HEK 293 cells that is constitutive but enhanced in response to cicaprost stimulation and which is independent of the guanine nucleotide (GDP/GTP) binding status of Rab11a itself.

Effect of Rab11a on Recycling of the hIP Post-Cicaprost Stimulation.

To further examine the interaction of the hIP with Rab11a in mammalian cells, its co-localization with GFP-tagged Rab11a, Rab11a^{S22N} and Rab11a^{Q70L} in HEK.hIP cells was investigated by confocal microscopy. Initially, only HA-hIPs located at the cell surface were pre-labelled with *anti*-HA 101R at 4 °C; thereafter, HA- hIP expression was either analysed directly (0 hr) or following incubation of cells with 1 μ M cicaprost at 37 °C for 2 hr or 4 hr. In the absence of agonist stimulation, the pre-immunolabelled HA-hIPs were detected exclusively at the cell surface as expected, whereas GFP-Rab11a exhibited a small amount of preformed vesicular staining, but was mainly present in a diffuse cytosolic pattern with no co-localization between the hIP and Rab11a evident (Figure 3A, Rab11a^{WT}, 0 hr). Following 2 hr cicaprost-treatment, there was significant re-localization of the hIP away from the cell surface to small punctate intracellular vesicles (Figure 3A, 2 hr). Coincident with this, there was also a significant re-localization of Rab11a to more discrete vesicular structures and there was significant co-localization between the hIP and Rab11a in small punctate vesicles, reminiscent of late endosomes as evident in the over-laid images (Figure 3A, Rab11a^{WT}, 2 hr). At 4 hr post-agonist stimulation, a significant amount of the hIP population had recycled to the cell surface (Figure 3A, *anti*-HA panel), but

the portion that remained intracellular was largely associated with late endosomal vesicles and co-localized with GFP-Rab11a (Figure 3A, overlay panel). In cells over-expressing GFP-Rab11a^{S25N}, the pre-labelled hIP was exclusively detected at the plasma membrane while Rab11a^{S25N} itself showed diffuse cytosolic staining with no co-localization evident in resting cells (Figure 3B, 0 hr). At 2 hr post-cicaprost stimulation, much of the hIP re-localized from the plasma membrane to small punctate, intracellular vesicular structures (Figure 3B, 2 hr, *anti*-HA) while Rab11a^{S25N} continued to exhibit diffuse cytosolic staining (Figure 3B, 2 hr, GFP), and co-localization between the hIP and Rab11a^{S25N} was substantially reduced relative to that of wild type Rab11a (Figure 3B, 2 hr, overlay). At 4 hr post-cicaprost stimulation, most of the hIP remained associated with punctate, intracellular vesicular structures with reduced evidence of recycling (Figure 3B, 4 hr, *anti*-HA). Moreover, the Rab11a^{S25N} continued to exhibit diffuse cytosolic staining and there was little co-localization of the hIP with Rab11a^{S25N} (Figure 3B, 4 hr). In contrast, even in the absence of cicaprost, the constitutively active GFP-Rab11a^{Q70L} showed punctate-type staining reminiscent of preformed endosomes while the pre-labelled hIP was exclusively detected at the plasma membrane, as expected (Figure 3C). At 2 hr post-cicaprost stimulation, most of the hIP re-localized from the plasma membrane to punctate endosomal vesicles with substantially enhanced co-localization to Rab11a^{Q70L}-endosomes, relative to those associated with the wild type Rab11a (Figure 3A & 3C, 2 hr). At 4 hr post-agonist stimulation, some of the hIP had recycled to the cell surface (Figure 3C, *anti*-HA panel), but largely remained intracellular, associating with endosomal vesicles and co-localized with GFP-Rab11a^{Q70L} (Figure 3C, 4hr). Taken together, these data confirm that the hIP co-localizes to Rab11a containing endosomes in response to cicaprost-stimulation with maximal association evident at 2 hr while at 4 hr, a significant proportion of the hIP recycled to the plasma membrane. Moreover, while the dominant negative Rab11a^{S25N} did not impair the internalization *per se*, it significantly reduced the ability of the hIP to recycle to the plasma membrane, such as at 4 hr post -agonist activation. Consistent with a role for Rab11a in mediating recycling, the hIP also showed enhanced co-localization with GFP-Rab11a^{Q70L} endosomes at 2 hr and 4 hr post-agonist stimulation.

Thereafter, the effect of over-expression of Rab11a on recycling of the hIP was examined by monitoring net changes in its cell surface expression by an ELISA-based assay in response to agonist stimulation. To this end, HEK.hIP cells were pre-incubated with cicaprost (1 μ M, 1 hr at 37 °C) to initiate the internalization process; thereafter, following agonist removal, cells were incubated at 37 °C for 0 – 4 hr and the overall net level of recycling of the HA-hIP to the plasma membrane assessed by ELISA. To begin with, it was determined that at 1 hr post-cicaprost stimulation, the level of cell surface hIP expression was 63.2 ± 2.79 % of that pre-agonist stimulation (ie approximately 40% of the hIP internalized) and that over-expression of Rab11a, Rab11a^{S25N} or Rab11a^{Q70L} did not significantly affect the level of internalization *per se* ($p < 0.1$). The presence of significant levels of endogenous Rab11 and

over-expressed Rab11a, Rab11a^{S22N} or Rab11a^{Q70L} in HEK.hIP cells was confirmed by western blot analysis (Figure 4A). Over-expression of Rab11a significantly increased recycling of the hIP to the plasma membrane relative to that of mock (pcDNA)-transfected cells (Figure 4B; $p = 0.0027$, ANOVA), with most significance observed between 1.5 – 2 hours post-agonist stimulation ($p = 0.038$ and $p = 0.009$, respectively). At 3 hr and 4 hr post-cicaprost stimulation, the level of recycling stabilized at approximately 80% cell surface expression, with no significant difference between mock-transfected or Rab11a-transfected cells evident (Figure 4B). Hence, it appears that some 50% of the internalized hIP does not recycle to the plasma membrane and that this is not influenced by the level of Rab11a. Consistent with this, over-expression of the GTPase-deficient Rab11a^{Q70L} did not alter the overall profile or extent of recycling of the hIP relative to the wild type Rab11 at any of the time points examined (Figure 4C; $p = 0.6005$, ANOVA). On the other hand, over-expression of Rab11a^{S22N} significantly impaired recycling of the hIP relative to that of the wild type Rab11a (Figure 4D; $p = 0.0001$, ANOVA) and relative to mock-transfected cells ($p = 0.0001$; ANOVA).

Cicaprost-induced movement of Endogenous Rab11 in EA.hy 926 cells.

In order to further investigate the involvement of Rab11 in trafficking of the hIP, the ability of cicaprost to induce changes in the localization of Rab11 endogenously expressed in the human endothelial EA.hy 926 cell line [42] through stimulation of endogenous hIPs was also examined. In the absence of agonist, Rab11 exhibited dispersed cytosolic staining with little evidence of its expression at the cell surface (Figure 5A, 0 hr). In response to cicaprost-stimulation, Rab11 staining became more pronounced, showing redistribution to small discrete punctate vesicles in a time-dependent manner such that at 2-3 hr post-stimulation they coalesced into fewer but significantly larger vesicles, while at 4 hr Rab11 staining returned to a more dispersed pattern (Figure 5A, 1-4 hr). The enlarged Rab11-positive vesicles observed in the presence of cicaprost also showed positive staining for the transferrin receptor (Tfr), an alternative endosomal marker (Figure 5C). Collectively, data in endothelial EA.hy 926 cells provide further independent evidence of the direct role of Rab11 in agonist-dependent trafficking of the hIP where both Rab11 and the hIP are expressed endogenously at physiologic levels. Moreover, they confirm that agonist-stimulation of the hIP leads to the active engagement of Rab11 in its trafficking and that such effects in EA.hy 926, or indeed in HEK293, cells are not an artifact of a cell line or expression system.

Effect of Rab4 on Cicaprost-Induced Trafficking of the hIP.

Another member of the Rab family shown to be important in GPCR recycling is Rab4, which exhibits overlapping distribution with both Rab5 and Rab11 in early and recycling endosomes [44]. In view of the findings of a direct role for Rab5a in agonist-induced internalization of the hIP [35], coupled to the data

herein involving Rab11a, it was also sought to establish whether Rab4 may also play a role in trafficking/recycling of the hIP in response to cicaprost. Initially, the effect of over-expression of Rab4a, Rab4a^{S22N} and Rab4a^{Q67L} on recycling of the hIP post-cicaprost stimulation (1 μ M, 1 hr at 37 °C) was investigated by monitoring their ability to affect overall or nett changes in cell-surface expression of the HA-hIP, as monitored through the previously described ELISA-based approach. The presence of significant levels of endogenous Rab4 and over-expressed of Rab4a, Rab4a^{S22N} and Rab4a^{Q67L} in HEK.hIP cells was confirmed by western blot analysis (Supplemental Figure 1A). At the outset, it was established that over-expression of either of the Rab4a proteins did not significantly affect internalization *per se* and that, at 1 hr post-cicaprost stimulation, the level of cell surface hIP expression was 61.2 ± 2.15 % of that pre-agonist stimulation (ie approximately 40% of the hIP internalized). Following agonist-removal and incubation of cells at 37 °C over a period of 0 – 4 hr, the overall level of the hIP that recycled to the cell-surface in mock (pCMV5)-transfected cells stabilized at approximately 80% cell surface expression (Supplemental Figure 1A). In addition, over-expression of either Rab4a, the dominant negative Rab4a^{S22N} or the constitutively active Rab4a^{Q67L} variant had no measurable effect on the overall recycling of the hIP relative to that observed in either mock-transfected cells ($p = 0.8784$; $p = 0.7444$ and $p = 0.5842$, respectively; ANOVA) or non-transfected cells (data not shown).

However, as the ELISA-based assay can only measure nett changes in cell surface expression, we also investigated whether the hIP may undergo agonist-induced localization to Rab4 endosomes through confocal imaging. In resting cells, the pre-immunolabelled hIP was detected at the cell surface while GFP-Rab4a displayed diffuse cytosolic staining with no co-localization between the pre-labelled hIP and Rab4a evident (Figure 6A; 0 hr). Consistent with its ability to undergo cicaprost-induced internalization, the hIP moved from the cell surface into discrete intracellular endosomal vesicles in a time-dependent manner, such that at 2 hr post-agonist stimulation most of it was found intracellular while at 4 hr there was evidence of hIP recycling to plasma membrane (Figure 6A; *anti*-HA). Coincident with this, in response to cicaprost-stimulation there was a time-dependent relocalization of GFP-Rab4a into more discrete, enlarged endosomal vesicles, while at 4 hr post-stimulation it regained the diffuse cytosolic-staining pattern associated with resting cells (Figure 6A; GFP-Rab4a). In the overlay images, there was significant time-dependent co-localization of the hIP with many of the enlarged Rab4-associated endosomal vesicles post-cicaprost stimulation (e.g at 0.5 – 2hr), while at 4 hr their co-localization in endosomal structures was substantially reduced (Figure 6A; Overlay). While over-expression of GFP-Rab4a^{S22N} did not impair the ability of the hIP to undergo similar time-dependent internalization and recycling in response to cicaprost-stimulation, GFP-Rab4a^{S22N} showed diffuse cytoplasmic staining and did not relocalize to discrete enlarged endosomal structures and there was no significant co-localization between the hIP and GFP-Rab4a^{S22N} either in the absence or presence of agonist (Supplemental Figure

1B). Conversely, expression of the constitutively active, GTPase deficient GFP-Rab4a^{Q67L} did not affect agonist-induced internalization and recycling of the hIP, it promoted the formation and recruitment of the hIP into enlarged Rab4a^{Q67L} positive endocytic vesicles, with substantial co-localization evident at 0.5 – 2 hr post agonist stimulation, while at 4 hr much of the hIP was located at the cell surface (Supplemental Figure 1C).

Collectively these latter data suggest that while over-expression of Rab4a did not appear to significantly enhance the overall levels/nett recycling of the hIP to the cell surface in response to cicaprost stimulation as measured by the ELISA assay, the imaging data show that there is indeed a transient re-localization of the hIP to Rab4-containing endosomes. Consistent with this, stimulation of endogenous hIPs expressed in the endothelial EA.hy 926 cell line with cicaprost also led to a substantial time-dependent re-localization of endogenous Rab4 (Figure 5B), with evidence of the Rab4-positive endocytic vesicles coalescing into more punctate, some of which were significantly larger vesicles from 1 hr onward and which showed positive co-staining for the transferrin receptor (Figure 5D; 3 hr). These data in EA.hy 926 cells confirm that agonist-activation of the hIP leads to active participation of Rab4 and, similar to that of Rab11, corroborate our findings in HEK293 cells indicating that they are not an artifact of a cell line or expression system.

Thereafter, through co-immunoprecipitation studies, it was also sought to investigate whether Rab4a might physically associate with the hIP expressed in HEK.hIP cells. In the absence of agonist, Rab4a was not detected in the *anti*-HA immunoprecipitates unlike that of Rab11a, which served as a control (Figure 6B). Moreover, consistent with our previous findings (Figure 2A), while stimulation of HEK.hIP cells with cicaprost (1 μ M; 2 hr) yielded a significant increase in the level of Rab11a present in the immunoprecipitates (Figure 6B & 8), Rab4a was not found in the *anti*-HA immunoprecipitates following short term (0 – 120 min; Figure 6D, upper panel) or long term (4 hr; data not shown) exposure to cicaprost. Failure to detect Rab4a in the *anti*-HA immunoprecipitates was not due to differences in hIP (data not shown) or in Rab4a (Figure 6B, upper and lower panels) expression levels or in the efficiency of the immunoprecipitations *per se* (Figure 6B; middle panel). Collectively these data confirm that the hIP co-localizes with Rab4a, albeit transiently, in response to cicaprost stimulation. However, unlike that of Rab11a, these cicaprost-induced responses do not involve a direct association of Rab4a with the hIP in mammalian HEK 293 cells. Furthermore, these latter data confirm the specificity of the direct interaction of Rab11a with the hIP.

Identification of the Domain(s) within the hIP Mediating Interaction with Rab11a.

Hence, thereafter, it was sought to identify the structural domain(s) within the hIP that mediates its interaction with Rab11a initially through the use of the Y2H approach for the identification of interactions

between Rab11a, as prey protein, and various intracellular sub-domains of the hIP acting as specific baits (Figure 7). In all, some 12 bait proteins, corresponding to either the intracellular loops 1–3 (IC₁ – IC₃) of the hIP or various sub-fragments of its C-tail domain were tested (Figure 7).

In agreement with the initial screen, both the hIP^{299-386,WT} and hIP^{299-386,SSLC} showed evidence of interaction with Rab11a as indicated by growth of diploids on QDO selection plates and LacZ reporter gene expression (Figure 7B & Supplemental Figure 2). Conversely, there was no specific interaction between the hIP^{320-386WT} and Rab11a suggesting that residues Val²⁹⁹ – Gln³²⁰, at least, are specifically required for such interaction. Moreover, consistent with this, the equivalent non-isoprenylated hIP^{320-386SSLC} did not interact with Rab11a and further suggested that the interaction with Rab11a is not largely affected by the isoprenylation status of the hIP. To further investigate the requirement of residues Val²⁹⁹ – Gln³²⁰, two additional sub-deletions of the hIP^{299-386WT} / hIP^{299-386SSLC} bait proteins that either retain (hIP^{307-386WT} / hIP^{307-386SSLC}) or lose Cys³⁰⁸ – Cys³¹¹ (hIP^{312-386WT} / hIP^{312-386SSLC}) were tested. Neither the hIP³⁰⁷⁻³⁸⁶ nor the hIP³¹²⁻³⁸⁶ based on either the wild-type -CAAX motif (Figure 7B) or on the -SSLC variant (data not shown) showed any interaction with Rab11a. On the other hand, the hIP²⁹⁹⁻³²⁰ bait protein showed strong interaction with Rab11a, as assessed by growth on QDO plates and LacZ reporter gene expression (Figure 7B & Supplemental Figure 2). These data suggest that residues Val²⁹⁹ – Gln³²⁰ mediate the interaction between the C-tail domain of the hIP and Rab11a.

Thereafter, using a similar approach, we also investigated whether any of the other intracellular domains, namely IC₁ – IC₃, may also interact with Rab11 and in so doing sought to ascertain whether they also contribute to the over-all interaction between the hIP and Rab11. Neither the IC₁, IC₂ nor IC₃ domains showed any interaction with Rab11a through growth on QDO plates or β-Gal assays (Figure 7B). Throughout these studies, the specificity of the interactions between Rab11a and the various bait proteins based on the hIP was confirmed whereby Rab11a failed to show any interaction with either the GAL4 DNA binding domain itself, as encoded by the empty pGBKT7 bait vector, or with p53 protein, acting as an unrelated control protein. Taken together, these data suggest that the Rab11a specifically and directly interacts with the C-tail domain but not with the intracellular loops (IC₁, IC₂ or IC₃) of the hIP and, more specifically, that residues Val²⁹⁹ – Gln³²⁰ are required and sufficient to mediate that interaction, in the *S.cerevisiae*-based expression system at least.

Hence, bearing this in mind, it was sought to investigate the role of the C-tail domain of the hIP in mediating its interaction with Rab11a in mammalian HEK 293 cells and as a function of cicaprost stimulation. To this end, we used two previously characterized cell lines, namely HEK.hIP^{Δ312} and HEK.hIP^{Δ307} cells that stably over-express the truncated variants hIP^{Δ312} (devoid of the terminal residues 312-386, including the ‘-CAAX’ motif) and hIP^{Δ307} (devoid of the terminal residues 307-386, including the palmitoylated residues at Cys³⁰⁸ and Cys³¹¹ and the ‘-CAAX’ motif), respectively [18]. Both the

HEK.hIP^{Δ312} and HEK.hIP^{Δ307} cell lines express near equivalent levels of the hIP^{Δ312} and hIP^{Δ307}, respectively [17, 18]. From co-immunoprecipitation studies, consistent with our previous data (Figure 2), it was confirmed that in the absence of agonist-stimulation, GFP-Rab11a was readily detected in the *anti*-HA -immunoprecipitates from the control HEK.hIP cells and, in response to cicaprost-treatment, there was a time-dependent increase in association of Rab11a with the wild type hIP with maximal response at 2 hr (Figure 8A, upper panel). Conversely, Rab11a was not found in the *anti*-HA immunoprecipitates from HEK.hIP^{Δ312} or HEK.hIP^{Δ307} in the absence of agonist and only showed a modest association with the hIP^{Δ312}, but not with the hIP^{Δ307}, following cicaprost stimulation (Figure 8A, upper panel). Moreover, the specificity of the interaction between the hIP and Rab11a was further confirmed by the fact that Rab11 was not detected in the *anti*-HA immunoprecipitates from the control HEK. β-Gal cells (Figure 8A, upper panel; HEK:β-Gal). The absence of Rab11 in the immunoprecipitates from HEK.hIP^{Δ312} or HEK.hIP^{Δ307} cells was not due to differences in Rab11a expression levels (Figure 8A; lower panel) or in the level of the hIP present in the *anti*-HA immunoprecipitates *per se* (Figure 8A; middle panel). Moreover, through Elisa-based assays it was established that in contrast to that which occurred for the wild type hIP, over-expression of Rab11a did not increase the recycling of hIP^{Δ312} or hIP^{Δ307} over that level observed in the control pcDNA3-transfected cells (Figure 8B & Figure 4B).

Finally, we investigated whether the hIP^{Δ312} and hIP^{Δ307} expressed at the cell surface co-localize with Rab11 endosomes in response to cicaprost, as observed with the wild type hIP (Figure 3A). In the absence of agonist, both the pre-immunolabelled hIP^{Δ312} and hIP^{Δ307} were predominantly detected at the cell surface while GFP-Rab11a exhibited diffuse intracellular staining (Figure 9, 0 hr). Following 2 hr cicaprost-treatment, there was a significant re-localization of Rab11a to more dispersed vesicular endosomes and significant internalization of the wild-type hIP away from the cell surface into punctate intracellular vesicles with significant co-localization of the hIP to the Rab11-containing endosomes evident (Figure 3A & Figure 9, hIP^{WT}, 2 hr). In contrast to this, the recruitment of both hIP^{Δ312} and hIP^{Δ307} into intracellular vesicular structures following 2 hr cicaprost-stimulation was substantially impaired (Figure 9, hIP^{Δ312} or hIP^{Δ307}, 2 hr). Moreover, unlike that which occurred in the HEK.hIP cells, Rab11a did not undergo a significant relocalization to vesicular endosomal structures in either HEK.hIP^{Δ312} or HEK.hIP^{Δ307} cells in response to cicaprost-treatment and the extent of co-localization of the hIP^{Δ312} or hIP^{Δ307} with Rab11a was substantially reduced.

Taken together, these data confirm that the C-tail domain is critical in mediating direct association of the hIP with Rab11a in mammalian cells and that, in response to cicaprost stimulation, the hIP:Rab11 complex co-localize in Rab11-containing late recycling endosomes, an effect not observed in the case of either the hIP^{Δ312} or hIP^{Δ307}. Moreover, from studies in yeast, we have identified a 22 amino

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acid segment, corresponding to Val²⁹⁹ – Gln³²⁰, located proximal to transmembrane 7 as a critical subdomain in mediating direct association between the hIP and Rab11a.

DISCUSSION

Prostacyclin is a critical endogenous autocoid that not only plays a central role in regulating haemostasis and vascular tone, but is also a potent pro-inflammatory and pro-nociceptive mediator and contributes to a variety of (patho)physiologic processes [1, 45]. Hence, understanding the mechanisms whereby the cellular responses to prostacyclin are dynamically regulated is critical to the understanding of its role in such processes at the detailed molecular level. While much is known about the modes of intracellular signalling by the prostacyclin receptor (IP) and the events leading to its desensitization and internalization, significantly less is understood about the intracellular sorting mechanisms dictating its recyclicalization or other trafficking following agonist stimulation [14, 32-35, 46]. Herein, we have uncovered a novel direct association between the hIP and the GTPase Rab11a that regulates its recycling to the plasma membrane following agonist stimulation.

The hIP is somewhat unique among members of the GPCR superfamily in that it undergoes both isoprenylation (farnesylation at Cys³⁸³) and palmitoylation (at Cys³⁰⁸ and Cys³¹¹) within its C-tail domain, and collectively, these modifications are critical in mediating its G-protein: effector coupling /signalling and in regulating its internalization post-agonist stimulation [16-18]. Moreover, consistent with the latter findings, it was recently established that the hIP undergoes rapid agonist-induced internalization through a mechanism involving its direct interaction with Rab5a [35]. While deletion of the C-tail domain of the hIP did not fully impair its internalization or abolish its interaction with Rab5a *per se*, agonist-induced trafficking of its variants hIP^{Δ312} and hIP^{Δ307} was substantially altered suggesting that the C-tail domain may contain key structural determinant(s) regulating its intracellular trafficking post-agonist stimulation [35]. Herein, we employed the Y2H system to screen a human kidney cDNA library for proteins that interact with the C-tail (residues 299-386) domain of the hIP. Amongst those protein interactants, we identified several independent clones encoding Rab11a that specifically interacted with both the non-isoprenylated (hIP^{299-386SSLC}) and wild type, isoprenylated (hIP^{299-386WT}) forms of the hIP. It is noteworthy that from our Y2H screen we also identified cGMP phosphodiesterase (PDE) 6δ as an interactant of the C-tail domain of the hIP but which, consistent with a previous study [47], specifically interacted with the wild type isoprenylated (-CSLC) but not with the non-isoprenylated (-SSLC) form (data not shown).

The direct interaction between Rab11a and the hIP was confirmed herein in mammalian HEK 293 cells and was found to be constitutive but was significantly enhanced in response to cicaprost-stimulation, with maximal interaction observed at 2 hr post-agonist stimulation. Moreover, the dominant negative Rab11^{S25N} and GTPase defective Rab11^{Q70L} showed similar constitutive and cicaprost-enhanced association with the hIP suggesting that GDP/GTP-exchange on Rab11 is not a rate-limiting determinant for the specific interaction *per se*. Through confocal-imaging, it was found that the cell-surface hIP re-

localized to Rab11a-endosomes with maximal co-localization observed at 2 hr post-cicaprost stimulation and thereafter, a proportion of the internalized hIP (~ 50%) recycled to the plasma membrane at 4 hr. Consistent with the latter, in the presence of Rab11a^{Q70L}, the hIP showed increased cicaprost-induced co-localization while it exhibited substantially less co-localization with- and underwent impaired recycling in the presence of Rab11a^{S25N}. These data were corroborated using an ELISA-based internalization assay to track net changes in cell surface expression of the hIP. In addition, stimulation of endogenous hIPs expressed in the human endothelial EA.hy 926 cells [42] also led to time-dependent redistribution of Rab11 from a dispersed cytosolic localization to more discrete vesicular structures suggesting that observed effect of Rab11 on hIP internalization are not a simple artifact of their over-expression, and can occur in more physiologically relevant cell types and expression levels. It should be noted that in the absence of proprietary antibodies directed to the hIP itself, we sourced and tested several commercial samples, but found that they were not of sufficient quality or specificity to allow for co-immunolocalization of the endogenous hIPs in the EA.hy 926 cells along with Rab11 (data not shown). The time-dependent redistribution of Rab11 in the EA.hy 926 cells paralleled the time-dependent increased interaction of the hIP with Rab11a in HEK293 cells, as monitored by the co-immunoprecipitations and confocal imaging data. Collectively, these data indicate that the hIP directly and constitutively interacts with Rab11a but that in response to cicaprost-stimulation, such interactions are significantly augmented. Hence, as has been suggested for other GPCRs [44], it appears that the hIP does not simply act as passive cargo in the endocytic and recycling process but may directly influence the Rab11a GTPase and, in so doing, directly control its own targeting and transport between intracellular compartments.

Rab proteins are increasingly implicated in the internalization and trafficking of members of the GPCR superfamily, offering alternative or complementary mechanisms to the more classic GRK/ β -arrestin-dependent pathways to fine-tune the signalling responses following agonist activation [20, 44]. Rab4, Rab5 and Rab11 show over-lapping distribution in early and recycling endosomes [44] while Rab11 is also present on the pericentriolar recycling endosome and is associated with the 'long or slow recycling pathway' [48], distinct from the more direct, shorter pathway involving Rab4-positive vesicles [49, 50]. For example, Rab4a mediates the rapid recycling of the transferrin receptor from Rab5a-positive sorting endosomes while Rab11a is required for its slow recycling from perinuclear endosomes [28]. Rab11 also participates in the slow recycling of other GPCRs, including the angiotensin II type 1A receptor, the CXCR2 receptor, the neurokinin 1 receptor, the M₄ muscarinic acetylcholine receptor and β_2 -adrenergic receptors [51-55]. In the context of prostanoid signalling, it is indeed notable that Rab11 regulates the intracellular trafficking of the β -isoform of the human thromboxane (TX) A₂ receptor (TP β) [56]. The direct association of the hIP with Rab11a is consistent with its slow-recycling to the plasma

membrane. However, data herein did not exclude that possibility that the hIP may also undergo more rapid recycling such as through Rab4-containing endosomes. In view of the findings for a direct role of Rab5 in agonist-induced internalization of the hIP [35], coupled to the data herein involving Rab11a, we also investigated whether Rab4 may also play a role in recycling of the hIP in response to cicaprost.

Initially, using the ELISA-based approach, we found no evidence that over-expression of Rab4a, or indeed its constitutively active Rab4a^{Q67L} or dominant negative Rab4a^{S22N}, conferred a measurable change in the recycling of the hIP in response to cicaprost stimulation. In contrast to this, through confocal imaging, there was indeed substantial evidence of co-localization of the hIP to Rab4a-positive endosomes as early as 30 min following cicaprost stimulation. However, that co-localization was transient such that at 4 hr post-agonist stimulation co-localization in the endosomal structures almost disappeared. Moreover, stimulation of endogenous hIPs expressed in the endothelial EA.hy 926 cells with cicaprost led to time-dependent redistribution of endogenous Rab4 to discrete and enlarged intracellular vesicles, confirming that its involvement can occur at more physiologic levels and that it is not an artifact of the mammalian cell line or expression system. Thereafter, through co-immunoprecipitations from mammalian HEK293 cells, unlike that which occurred herein for Rab11a or previously reported for Rab5a ([35]), Rab4a was not found to physically interact with the hIP either constitutively or in response to cicaprost stimulation, even following short- or long-term exposure. Hence, these data confirm that the hIP co-localizes and leads to activation of Rab4a, albeit transiently, in response to cicaprost stimulation. However, unlike that of Rab11, these cicaprost-induced responses do not involve a direct association of Rab4a with the hIP. It can be reasoned that failure to see any role for Rab4a in the recycling of the hIP as measured by ELISA assessment of cell-surface receptors was most likely due to the fact that Rab4a is involved at an early point in the recycling process, as corroborated by the confocal imaging data herein, at a time where there is unlikely to be a net balance between internalization and recycling.

As stated, there are numerous examples in the literature whereby Rab4a and Rab11a play exclusive roles in directing the recycling of various GPCRs, through either the fast and slow recycling mechanisms, respectively [51-53, 55, 56]. Even GPCRs that respond to the same ligand, such as the prostanoid D₂ receptor and the CRTH2 (chemoattractant homologous receptor expressed on TH2 cells) undergo distinct routes of recycling via Rab4 and Rab11, respectively, following stimulation with prostaglandin D₂ [57]. Conversely, substance P (SP) induces the internalization of the neurokinin 1 receptor (NK1R) in epithelial cells through a Rab5a-dependent mechanism but it appears that the route of recycling is dependent on the agonist concentration [54]. Specifically, at low concentrations of SP (1 nM), recycling of the NK1R is from early endosomes through a Rab4a-dependent route while at higher concentrations (10 nM), recycling occurs through a Rab11a-dependent mechanism from perinuclear

endosomes [54]. While we have not, as yet, investigated the possibility of an exclusive agonist concentration-dependent involvement of Rab4a versus Rab11a in the recycling of the hIP, our data herein present a compelling case for a specific and unique involvement of Rab11a in its recycling that involves a direct interaction with the hIP.

Through the Y2H approach, it was established that both the non-isoprenylated hIP^{299-386,SSLC} and the wild type isoprenylated hIP^{299-386,WT} showed strong and comparable interaction with Rab11a, suggesting that the specific interaction is not influenced by the isoprenylation status of the hIP. Conversely, there was no specific interaction between any of the other intracellular domains suggesting that neither IC₁, IC₂ or IC₃ contribute to the overall interaction between the hIP and Rab11a but that, more specifically, the interaction is exclusively dependent on sequences within the C-tail domain, as assessed by the Y2H approach at least. Furthermore, there was no specific interaction between the hIP^{320-386,WT}, or indeed its non-isoprenylated hIP^{320-386,SSLC} equivalent, and Rab11a suggesting that residues Val²⁹⁹ – Gln³²⁰, at least, are specifically required for such interaction. As stated, the hIP is both farnesylated, at Cys³⁸³, and dually palmitoylated, at Cys^{308,311}, and collectively these modifications play a key role in regulating its G protein coupling and signalling and its agonist-induced internalization [16-18, 58]. Hence, we also examined two additional sub-deletions of the hIP^{299-386,WT}, that either retained, namely hIP^{307-386,WT}, or lost, namely hIP^{312-386,WT}, the palmitoylated Cys³⁰⁸ and Cys³¹¹ residues and found that neither the hIP³⁰⁷⁻³⁸⁶ nor the hIP³¹²⁻³⁸⁶ showed any interaction with Rab11a. On the other hand, the bait protein hIP²⁹⁹⁻³²⁰ showed strong interaction with Rab11a. These data clearly demonstrated that residues Val²⁹⁹ – Gln³²⁰ are required and sufficient to mediate the interaction between the C-tail domain of the hIP and Rab11a and that even minimal deletion of residues 299 -307, as in the hIP^{307-386,WT}, or residues 299-312, as in hIP^{312-386,WT}, prevented the interaction. The specific requirement for the C-tail domain of the hIP in mediating its interaction with Rab11a was further confirmed in mammalian HEK 293 cells whereby Rab11a did not substantially co-precipitate with the truncated variants hIP^{Δ312} and hIP^{Δ307} either in the absence of or following cicaprost stimulation. Moreover, unlike that which occurred for the hIP, Rab11a did not re-localize to vesicular endosomal structures in either HEK.hIP^{Δ312} or HEK.hIP^{Δ307} cells in response to cicaprost-treatment and the extent of co-localization of the hIP^{Δ312} or hIP^{Δ307} with Rab11a was significantly reduced. Taken together, these data confirm that the C-tail domain is critical in mediating direct association of the hIP with Rab11a in mammalian cells and, from studies in yeast, we have identified a 22 amino acid segment, corresponding to Val²⁹⁹ – Gln³²⁰, located proximal to transmembrane 7 as a critical subdomain in mediating direct association between the hIP and Rab11. Failure to detect interaction between Rab11a and other subdomains of the hIP as assessed through the Y2H system did not exclude the possibility that other regions or subdomains of the receptor may also contribute to or influence the interaction, such as in mammalian systems. In fact, the co-immunoprecipitation studies did

reveal a weak interaction between Rab11a and hIP^{A312} that increased in response to agonist-stimulation suggesting that other regions of the hIP other than Val²⁹⁹ – Gln³²⁰ may participate in the interaction.

As stated, the hIP is dually palmitoylated at Cys³⁰⁸ and Cys³¹¹ and together with the farnesyl-Cys³⁸³-methyl ester, at Cys³⁸³, it has been proposed to confer a double loop structure within its C-tail domain to orientate and provide the critical structural domains for interaction with coupling G proteins and, possibly, with components of the protein trafficking machinery to modulate both G protein/effector signaling and receptor internalization post-agonist activation [16-18]. We have not, as yet, investigated the specific requirement of Cys³⁰⁸ or Cys³¹¹ for the interaction with Rab11a or indeed the involvement of palmitoylation, such as proposed in the latter model. However, owing to our findings demonstrating the specific involvement Val²⁹⁹ – Gln³²⁰ coupled to requirement of residues N-terminal of Cys³⁰⁸/Cys³¹¹ for interaction with Rab11a, it is indeed tempting to speculate that the proposed double loop structure may indeed provide the platform for interaction with Rab11a. Moreover, agonist-regulated palmitoylation/de-palmitoylation may provide the dynamic platform to modulate such interactions through the provision of increased hydrophobic protein:membrane and/or protein:protein interactions [18, 59]. Through on-going studies, we propose to investigate these possibilities.

Of course the hIP is not the only GPCR shown to have a direct physical interaction with Rab11a. Recently Takahashi et al., demonstrated that Rab11a can co-immunoprecipitate with the CXCR2 in neutrophils in response to the *anti*-inflammatory agent curcumin [60]. Moreover and more specifically in the context of prostanoid receptor signalling, Hamelin et al., [56] provided the first demonstration of a direct interaction between Rab11 and a GPCR, namely the TPβ isoform of the human TXA₂ receptor. Moreover, they established that amino acids 335-344 within the C-tail, proximal to TM7, of TPβ were specifically required for that interaction with a minor role for the IC₁ also identified [56]. Whilst they too did not discuss the possible involvement of palmitoylation in regulating TPβ interaction with Rab11a, it is also notable that, coincident with that of the hIP, the critical residues within the C-tail domain contain a Cys (namely Cys³⁴³) that has been confirmed to undergo palmitoylation [61] and the interacting residues are also located proximal to TM7 within both TPβ and the hIP.

The structural requirement for a hydrophobic binding domain is, in fact, a common feature of other groups of proteins capable of specifically interacting with Rab11 in addition to GPCRs, as exemplified by TPβ [56] and the hIP herein. For example, members of the recently described 'family of Rab11-interacting proteins (FIPs)' contain a highly conserved 20 amino acid motif at their C termini, known as the Rab11/25 binding domain (RBD). Moreover, RBD is found predominantly in a coil-coiled or helical conformation allowing for highly conserved hydrophobic residues therein to form a hydrophobic Rab11 binding patch and substitution of these hydrophobic residues inhibits Rab11 binding to FIPs [62]. The Rab11a binding/interacting domain identified herein within the hIP, corresponding to

the 22 amino acid Val²⁹⁹ – Gln³²⁰ region, is of a similar size to the RBD of the FIPS and, like that of TPβ, is both enriched in hydrophobic residues and is also capable of forming a helical conformation [63]. Through on-going investigations, we propose to more precisely define the Rab11a binding domain (RBD) within the hIP and, additionally, to identify the region(s) within Rab11a involved in that interaction.

In summary, we have identified a novel interaction between the hIP and Rab11a that functionally plays a central role in regulating its recycling following agonist-activation. These studies greatly add to the knowledge and understanding of the molecular events regulating the intracellular trafficking of the hIP and of the structural domains required. Moreover, bearing in mind the similar biochemical properties of the Rab11 binding domain identified herein within the hIP to that previously identified within TPβ [56], both in terms of the presence of palmitoylable -Cys residues and capacity to adopt a helical coil-conformation coupled to their location within the C-tail domains of their respective receptors proximal to transmembrane (TM) 7, these studies may have uncovered a more general mechanism for regulating Rab11-mediated intracellular trafficking of other members of the GPCR superfamily.

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Abbreviations:

C-tail, carboxyl-terminal tail; FBS, foetal bovine serum; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK, GPCR receptor kinase; HA, hemagglutinin; HEK, human embryonic kidney; hIP, human IP; IC, intracellular; IP, prostacyclin receptor; TM, transmembrane; Y2H, yeast-two-hybrid.

FIGURES

Figure 1. Interaction of Rab11a with the C-tail domain of the human prostacyclin receptor (hIP).

Panel A: Schematic of the amino acid sequence of the carboxyl-terminal (C)-tail domain of the human prostacyclin receptor (hIP), corresponding to amino acids 299 – 386 of either the wild type hIP (hIP^{299-386WT}) or of its isoprenylation deficient variant hIP^{SSLC} (hIP^{299-386SSLC}). Panel B: Protein extracted from the bait strains *S.c* AH109 (pGBKT7:hIP^{299-386WT}), *S.c* AH109 (pGBKT7:hIP^{299-386SSLC}) or, as a control, *S.c* AH109 (pGBKT7) were resolved by SDS-PAGE and were immunoblotted versus *anti-Myc* (9B11) antibody, followed by chemiluminescence detection. The arrows to the left of the panel indicate that the hIP^{299-386WT} is expressed both as a non-isoprenylated and isoprenylated form, corresponding to the upper and lower bands, respectively, while the hIP^{299-386SSLC} is expressed as a non-isoprenylated form only. Panel C: *S.c* Y187 (pACT:Rab11) was mated with either the bait strains *S.c* AH109 (pGBKT7:hIP^{299-386WT}), *S.c* AH109 (pGBKT7:hIP^{299-386SSLC}) or, as controls, with *S.c* AH109 (pGBKT7:p53) and *S.c* AH109 (pGBKT7). Resulting diploid strains were either grown on double dropout (DDO) media (Leu⁻, Trp⁻; selection for diploid formation) or on quadruple dropout (QDO) media (Leu⁻, Trp⁻, Ade⁻, His⁻; selection for positive GAL4-dependent transcriptional regulation owing to interaction between bait and prey proteins).

Figure 2. Agonist-dependent Association of Rab11a with the hIP in Mammalian Cells.

HEK.hIP, or as controls, HEK 293 and HEK.β-Gal cells, transiently transfected with either pEGFPCI:Rab11a (Panel A), pEGFPCI:Rab11a^{S25N} or pEGFPCI:Rab11a^{Q70L} (Panel C), or non-transfected (Panel B), were incubated with either vehicle (0 hr) or with 1 μM cicaprost for 1, 2 or 3 hr, as indicated, prior to immunoprecipitation with *anti*-HA 101R antibody. Immunoprecipitates (IP) were resolved by SDS-PAGE and were either immunoblotted (IB) versus *anti*-GFP antibody (A & C, upper panels), or *anti*-Rab11 antibody (B, upper panel), or *anti*-HA 3F10-HRP antibody (middle panels), as indicated to the right of the panels. To verify expression of the Rab11 proteins, aliquots of whole cell lysates (approx. 50 μg /lane) were also resolved by SDS-PAGE and immunoblotted (IB) with *anti*-GFP-antibody (A & C; lower panels) or *anti*-Rab11-antibody (B; lower panel). The relative positions of the molecular size markers are indicated to the left of the panels. Results presented are representative of at least 3 independent experiments.

Figure 3. Co-localization of the hIP with Rab11a.

HEK.hIP^{WT} cells, transiently transfected with pEGFPC1:Rab11a (Panel A), pEGFPC1:Rab11a^{S25N} (Panel B) or pEGFPC1:Rab11a^{Q70L} (Panel C) were pre-labelled with *anti*-HA 101R antibody for 1 hr at 4 °C; thereafter, cells were either analyzed directly (0 hr) or were incubated with 1 μM cicaprost at 37 °C for 2 hr or 4 hr. Cells were fixed and permeabilized prior to detection of HA-tagged hIPs, with *anti*-mouse AlexaFluor594 conjugated secondary antibody, and enhanced GFP:Rab11 expression using a Zeiss fluorescence microscope and AxioVision Software. Data presented are representative images from 3 independent experiments from which at least 10 fields were viewed at x 63 magnification, where the horizontal bar represents 10 μm.

Figure 4. Effect of Rab11a on Recycling of the hIP post-Cicaprost Stimulation.

Panel A. HEK.hIP cells transiently transfected with either pcDNA3, pcDNA3:Rab11a, pcDNA3:Rab11a^{S22N} and pcDNA3:Rab11a^{Q70L}, or control non-transfected cells (-), were analyzed by SDS-PAGE (50 μ g whole cell protein per lane) followed by immunoblotting with *anti-Rab11* antibody and chemiluminescence detection. Panels B-D: HEK.hIP cells, transiently transfected with either the control vector pcDNA3 (Panel B), pcDNA3:Rab11a (Panels B-D), pcDNA3:Rab11a^{Q70L} (Panel C), or pcDNA3:Rab11a^{S22N} (Panel D), were stimulated with 1 μ M cicaprost for 1 hr at 37 °C. Cells were washed and then incubated

at 37 °C in pre-warmed serum-free MEM for 0 - 4 hr. Thereafter, any net changes in cell surface expression of the HA-tagged hIPs was detected by the ELISA-based assay using *anti-HA* 101R antibody. Results are expressed as mean cell surface expression as a percentage of that at 0 hr post-agonist stimulation (% Cell Surface Expression $\pm$ S.E.M.) as a function of time (hr) and are representative of at least three independent experiments, each carried out in triplicate.

Figure 5. Cicaprost-induced re-localization of Rab11 and Rab4 in EA.hy 926 cells.

EA.hy 926 cells were stimulated with vehicle or 1 μ M cicaprost at 37 $^{\circ}$ C for 0 - 4 hr (Panels A & B) or for 0 & 3 hr (Panels C & D). Cells were fixed and permeabilized prior to detection of endogenous Rab11

(Panels A & C), Rab4 (Panels B & D) or transferrin receptor (Tfr, Panels C & D) with rabbit *anti*-Rab11, *anti*-Rab4 and mouse *anti*-Tfr, respectively, followed by *anti*-rabbit AlexaFluor488 or *anti*-mouse AlexaFluor594 conjugated secondary antibodies using a Zeiss fluorescence microscope and software. Data presented are representative images from 3 independent experiments from which at least 10 fields were viewed at x 63 magnification, where the horizontal bar represents 10 μ m. The inset represents a further 3-fold (x 3) magnification of the boxed area.

Figure 6. Effect of Rab4 on Recycling of the hIP.

Panel A: HEK.hIP^{WT} cells, transiently transfected with pEGFPCI:Rab4a were pre-labelled with *anti*-HA 101R antibody for 1 hr at 4 °C; thereafter, cells were either analyzed directly (0 hr) or were incubated with 1 µM cicaprost at 37 °C for 0.5 - 4 hr. Cells were fixed and permeabilized prior to detection of HA-tagged hIPs, with *anti*-mouse AlexaFluor594 conjugated secondary antibody, and enhanced GFP expression using a Zeiss fluorescence microscope and AxioVision Software. Data presented are representative images from 3 independent experiments from which at least 10 fields were viewed at x 63 magnification, where the horizontal bar represents 10 µm. Panel B: HEK.hIP cells, transiently transfected with either pEGFPCI:Rab4a (left panel) or, as a positive control, with pEGFPCI:Rab11a (right panel), were incubated with either vehicle (0) or with 1 µM cicaprost for 10, 30, 60, 120 min prior to immunoprecipitation with *anti*-HA 101R antibody. Immunoprecipitates (IP) were resolved by SDS-PAGE and were either immunoblotted (IB) with *anti*-GFP antibody (upper panels) or *anti*-HA 3F10-HRP antibody (middle panels), as indicated to the right of the panels. To verify expression of HA-hIP, Rab4 and Rab11, aliquots of whole cell lysates (approx. 50 µg /lane) were also resolved by SDS-PAGE and immunoblotted with *anti*-HA 3F10-HRP antibody (middle panels) or with *anti*-GFP antibody (upper and lower panels). The relative positions of the molecular size markers are indicated to the left of the panels. Results presented are representative of at least 3 independent experiments.

Figure 7: Mapping of Domains within the hIP that Interact with Rab11a.

Panel A: Schematic of the hIP including identification of the seven transmembrane domains (I – VII), intracellular (IC) loops 1 – 3 and the carboxyl-terminal (C)-tail domain. The hIP is palmitoylated at Cys³⁰⁸ & Cys³¹¹ and is farnesylated at Cys³⁸³ within its –CSLC motif and together these two types of lipid modifications have been predicted to introduce a double-loop structure within its C-tail domain. Panel B: Identification of the sub-domains of the hIP that interact with Rab11a through analysis of yeast mating. The *MATα* prey strain *S.c* Y187 (pACT:Rab11a) was mated with transformants of the *MATα* bait strain *S.c* AH109 harbouring recombinant pGBK:T7 plasmids encoding either hIP^{IC1,39-50}, hIP^{IC2,116-138}, hIP^{IC3,209-239}, hIP^{299-386WT}, hIP^{299-386,SSLC}, hIP^{320-386WT}, hIP^{320-386,SSLC}, hIP^{312-386WT}, hIP^{307-386WT} & hIP²⁹⁹⁻³²⁰. Diploids were selected on DDO (SD, Leu⁻, Trp⁻) media while GAL4-dependent transcriptional regulation of the *HIS3*, *ADE2* and *LacZ* reporter genes, due to positive interactions between bait and prey proteins, in resulting diploids was either selected on QDO (SD, Leu⁻, Trp⁻, Ade⁻, His⁻) media or by their ability to cleave the chromogenic substrate X-β-gal, as measured by the filter lift assay of β-Galactosidase (β-Gal) activity. Data presented are representative of at least 3 independent experiments. The scoring system above represents the ability of 3 independent colonies selected from respective DDO media to exhibit equivalent growth (+) or not (-) on QDO (SD, Leu⁻, Trp⁻, Ade⁻, His⁻) media or to produce blue (+) or white (-) colonies owing to expression of β-Gal activity. -SSLC signifies the isoprenylation defective forms of the hIP, where the –CAAX (–C³⁸³SLC³⁸⁶) motif present on the wild type (WT) hIP was mutated.

Figure 8. Co-immunoprecipitation of Rab11a with the hIP^{Δ312} and hIP^{Δ307}

Panel A: HEK.hIP, HEK.hIP^{Δ312}, HEK.hIP^{Δ307} or, as controls, HEK.β-Gal cells, transiently transfected with pEGFPC1:Rab11a, were incubated with either vehicle (0) or with 1 μM cicaprost for 1 - 4 hr, prior to immunoprecipitation with *anti*-HA 101R antibody. Immunoprecipitates (IP) were resolved by SDS-PAGE and were either immunoblotted (IB) with *anti*-GFP antibody (upper panels) or *anti*-HA 3F10-HRP antibody (middle panels), as indicated to the right of the panels. To verify expression of the HA-tagged hIP or Rab11 proteins, samples of whole cell lysates (approx. 50 μg /lane) were also resolved by SDS-PAGE and immunoblotted (IB) with *anti*-HA 3F10 (middle panel) or *anti*-GFP-antibody (upper & lower panels). The relative positions of the molecular size markers are indicated to the left of the panels. Results presented are representative of at least 3 independent experiments. Panel B: HEK.hIP, HEK.hIP^{Δ312}, and HEK.hIP^{Δ307} cells, transiently transfected with either the control vector pcDNA3 (□; white bars) or pcDNA3:Rab11a (■; black bars) were stimulated with 1 μM cicaprost for 1 hr at 37 °C. Cells were washed and then incubated at 37 °C in pre-warmed serum-free MEM for 0 or 2 hr. Thereafter, any net changes in cell surface expression of the HA-tagged hIPs was detected by the ELISA-based assay using *anti*-HA 101R antibody. Results are expressed as mean cell surface expression as a percentage of that at 0 hr post-agonist stimulation (% Cell Surface Expression ± S.E.M.) and are representative of at least three independent experiments, each carried out in triplicate.

Figure 9. Co-localization of Rab11a with the hIP^{Δ312} and hIP^{Δ307}

HEK.hIP, HEK.hIP^{Δ312} and HEK.hIP^{Δ307} cells, transiently transfected with pEGFP::Rab11a, were pre-

labelled with *anti*-HA 101R antibody for 1 hr at 4 °C; thereafter, cells were either analyzed directly (0 hr) or were incubated with 1 μM cicaprost for 2 hr at 37 °C. Cells were fixed and permeabilized prior to detection of HA-tagged hIPs, with *anti*-mouse AlexaFluor594 conjugated secondary antibody, and enhanced GFP expression using a Zeiss fluorescence microscope and AxioVision Software. Data presented are representative images from 3 independent experiments from which at least 10 fields were viewed at x 63 magnification, where the horizontal bar represents 10 μm.

SUPPLEMENTAL FIGURES

SUPPLEMENTAL FIGURE 1.

Supplemental Figure 1: Effect of Rab4 on Recycling of the hIP.

Panel A: HEK.hIP cells, transiently co-transfected with the control vector pCMV5 were pre-stimulated with 1 μ M cicaprost for 1 hr at 37 °C. Cells were washed and then incubated at 37 °C in pre-warmed serum-free MEM for 0- 4 h. Thereafter, net changes in cell surface expression of HA-tagged hIPs were detected by ELISA assay using *anti*-HA 101R antibody. Results are expressed as mean cell surface expression as a percentage of that at 0 hr post-agonist stimulation (% Cell Surface Expression $\pm$ S.E.M.) as a function of time (hr) and are representative of at least three independent experiments, each carried out in triplicate. Inset to Panel A: HEK.hIP cells transiently transfected with either pCMV5, pCMV5:Rab4a, pCMV5:Rab4a^{S22N}, pCMV5:Rab4a^{Q67L} or control non-transfected cells (-) were analyzed by SDS-PAGE (50 μ g whole cell protein per lane) followed by immunoblotting with *anti*-Rab4 antibody and chemiluminescence detection. HEK.hIP^{WT} cells, transiently transfected with pEGFPCI:Rab4a^{S22N} (Panel B) or pEGFPCI:Rab4a^{Q67L} (Panel C) were pre-labelled with *anti*-HA 101R antibody for 1 hr at 4 °C; thereafter, cells were either analyzed directly (0 hr) or were incubated with 1 μ M cicaprost at 37 °C for 0.5 - 4 hr. Cells were fixed and permeabilized prior to detection of HA-tagged hIPs, with *anti*-mouse AlexaFluor594 conjugated secondary antibody, and enhanced GFP expression using a Zeiss fluorescence microscope and AxioVision Software. Data presented are representative images from 3 independent experiments from which at least 10 fields were viewed at x 63 magnification, where the horizontal bar represents 10 μ m.

Supplemental Figure 2

hIP Domain	Rab11a		pTDI	
	DDO	QDO	DDO	QDO
hIP ^{IC1,39-50}				
hIP ^{IC2,116-138}				
hIP ^{IC3,209-239}				
hIP ^{C-tail,299-386WT}				
hIP ^{C-tail,299-386SSLC}				
hIP ^{C-tail,320-386WT}				
hIP ^{C-tail,320-386SSLC}				
hIP ^{C-tail,307-386WT}				
hIP ^{C-tail,312-386WT}				
hIP ^{C-tail,299-320}				

Supplemental Figure 2: Identification of the Domains within the hIP that Interact with Rab11a.

The MAT α prey strain *S.c* Y187 (pACT:Rab11a) or, as controls, *S.c* Y187 (pTDI), encoding the SV40 large T antigen (Clontech), were mated with transformants of the MAT α bait strain *S.c* AH109 harbouring recombinant pGBK:T7 plasmids encoding either hIP^{IC1,39-50}, hIP^{IC2,209-239}, hIP^{299-386WT}, hIP^{299-386,SSLC}, hIP^{320-386WT}, hIP^{320-386,SSLC}, hIP^{312-386WT}, hIP^{307-386WT} & hIP²⁹⁹⁻³²⁰. Diploids were selected on double drop-out (DDO; SD, Leu⁻, Trp⁻) media while GAL4-dependent transcriptional regulation of the *HIS3*, *ADE2* and *LacZ* reporter genes, due to positive interactions between bait and prey proteins, in resulting diploids was selected on quadruple drop-out (QDO; SD, Leu⁻, Trp⁻, Ade⁻, His⁻) media or by their ability to cleave the chromogenic substrate X- β -Gal, as measured by the filter lift assay of β -Galactosidase (β -Gal) activity (data not shown). Data presented are representative of at least 3 independent experiments. Each independent experiment was scored by the ability of 3 individual colonies selected from respective DDO plates to exhibit equivalent growth (+) or not (-) on QDO plates, as shown above, or to produce blue (+) or white (-) colonies owing to expression of β -Gal activity (data not shown). -SSLC signifies the isoprenylation defective forms of the hIP, where the -CAAX (-C³⁸³SLC³⁸⁶) motif present on the wild type (WT) hIP was mutated.