Interaction of the Human Prostacyclin Receptor with Rab11

CHARACTERIZATION OF A NOVEL Rab11 BINDING DOMAIN WITHIN α-HELIX 8 THAT IS REGULATED BY PALMITOYLATION*

Received for publication, January 21, 2010, and in revised form, April 14, 2010. Published, JBC Papers In Press, April 15, 2010, DOI 10.1074/jbc.M110.106476

Helen M. Reid¹, Eamon P. Mulvaney¹, Elizabeth C. Turner, and B. Therese Kinsella²

From the School of Biomolecular and Biomedical Sciences, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland

The human prostacyclin receptor (hIP) undergoes agonist-induced internalization of membrane vesicles containing the receptor (hIP) and Rab11. Through complementary approaches in yeast and mammalian cells along with computational structural studies, the RBD was proposed to be organized into an eighth–helix domain. Because the proposed RBD contains palmitoylation at Cys 311 in addition to agonist-regulated deacylation at Cys 309, Cys308 and Cys311, in addition to Cys309, that are known to undergo palmitoylation, we sought to identify the structure/function domain of hIP involved in Rab11 recruitment. Through complementary approaches in yeast and mammalian cells along with computational structural studies, the RBD was proposed to be organized into an eighth–helix domain. Because the proposed RBD contains palmitoylation at Cys 311 in addition to agonist-regulated deacylation at Cys 309, Cys308 and Cys311, in addition to Cys309, that are known to undergo palmitoylation, we sought to identify the structure/function domain of hIP involved in Rab11 recruitment.

This article has been withdrawn by the authors. After reviewing the data, the corresponding author learned that there were errors in how some of the microscope images of this manuscript were prepared for presentation. The authors wish to withdraw the article in the interests of maintaining their publication standards and those of the journal. After reviewing the data, the corresponding author learned that there was masking of background surrounding the cell/region of interest in 3 subpanels of Fig. 3C (hIP⁰, 0 hr; hIP⁰, 4 hr; hIP⁰, 4 hr), in 1 subpanel of Fig. 4A (hIP⁰, 0 hr, non-permeabilized), and in 2 subpanels of Fig. 5B (hIP⁰, 2 hr, Anti-α-HA; hIP⁰, 2 hr, GFP), which was not specified in the corresponding figures or legends. In addition, one image was displayed twice in both Fig. 3C (hIP⁰, 2 hr) and in Fig. 8C (hIP⁰, 2 hr), which was not specified in the figure legend. The authors state that these errors did not impact the findings or overall conclusions of the published article. The authors stand by the original scientific results as published. The authors apologize for the errors, but wish to voluntarily withdraw the article to respect the highest standards of transparency and reliability of their research and of the JBC. Replica data sets for each of the figures in question that the authors state fully validate the findings and conclusions of the published article are available. A revised version of the manuscript with those replica data sets can be obtained by contacting the corresponding author.

* This work was supported by Science Foundation of Ireland Grant SFI: 05/IN.1/B19.
[¹] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–10.
[²] Both authors contributed equally to this work.
[1] To whom correspondence should be addressed. Tel.: 353-1-7166727; Fax: 353-1-2837211; E-mail: therese.kinsella@ucd.ie.
membrane (TM) topography (15, 22). The IP is primarily coupled to Gs/adenyl cyclase activation but may regulate other effectors in a cell- and/or species-specific manner (23, 24). The IP is somewhat unusual amongGPCRs in that it undergoes both isoprenylation and palmitoylation within its carboxy-terminal tail (C-tail) domain, modifications that are critical for its signaling and function (24–30). More specifically, in the case of the human (hIP), it undergoes farnesylation at Cys308 within an evolutionarily conserved -CAA motif (24, 25) and is palmitoylated at Cys308 and Cys311, whereas an intervening Cys309 was found not to be palmitoylated, at least under the experimental conditions used (27). Although neither lipidation affected its ligand binding properties, it is proposed that farnesylation in addition to palmitoylation of the hIP may confer a double loop structure within its C-tail domain to provide and/or orientate the critical structural domains for its G protein/effector(s) coupling and, possibly, for its interaction with components of the intracellular trafficking machinery to modulate its internalization after agonist activation (24, 25, 27). More specifically, although disruption of farnesylation effectively abolishes agonist-induced Gs/adenyl cyclase activation and cAMP generation by the hIP, palmitoylation at either Cys308 or Cys311 is sufficient to maintain functional Gs coupling, whereas disruption of palmitoylation at both sites abolishes that signaling (24, 25, 27).

Through recent studies, we have established that the IP undergoes agonist-induced internalization that occurs via a Rab5a-dependent mechanism (31) rather than a Rab7-dependent mechanism typical of many other GPCRs. Although its C-tail domain did not impair its subsequent trafficking and/or internalization, the region of interaction with Rab11a was dependent on a 22-α-helical domain, comprising Val299–Val307, which bears limited overall structural similarity to the Rab11/25 binding domain (RBD) first identified within members of the Rab5a/Rab7Rab11/Rab25 family of Rab11-box proteins, referred to as FIPs (5).

The RBD of the hIP contains two nascent α-helices, which bears limited overall structural similarity to the Rab11/25 binding domain (RBD) first identified within members of the “family of Rab11-interacting proteins” (FIPs) (5). It is proposed that the α-helical domain corresponds to a putative α-helix 8 (α-H8) within the overall structure of the hIP, a feature originally identified by x-ray crystallography data within rhodopsin and, more recently, in other GPCRs, including the human β2 adrenergic and the A2A adenosine receptors (33–36). Data are presented to suggest that agonist-regulated palmitoylation and/or depalmitoylation (deacylation), in particular at Cys308 > Cys309, in addition to palmitoylation at Cys311, each of which lie outside of α-H8, may be important in positioning or anchoring the helical region of the RBD in proximity to Rab11a, to regulate the intracellular trafficking of the hIP in response to activation. Because many GPCRs are predicted to contain a similar α-H8 domain, in addition to adjacent palmitoylated residue(s), data generated in this study are likely to have broader functional significance for trafficking of other members of the wider receptor superfamily.

**Experimental Procedures**

**Materials**

Cicaprost was obtained from Schering AG (Berlin, Germany). Ampliﬁ™ was from GE Healthcare. Mouse monoclonal anti-hemagglutinin (HA) 101R antibody was from Cambridge Biosciences, a rabbit monoclonal anti-Rab11 (H-87), rabbit polyclonal anti-Rab5 (S-19), and chicken polyclonal anti-arrestin-depen-
dent activator from Santa Cruz; mouse monoclonal anti-Rab11 mouse monoclonal anti-EEA1 (clone 1) was from Abcam. AlexaFluor594-conjugated and AlexaFluor488-goat anti-rabbit antibodies from Roche Applied Science. Mouse monoclonal anti-EEA1 (clone 2E11); rabbit polyclonal anti-S6K (clone 1A5); and rabbit polyclonal anti-PKB (clone 9252) antibodies from Abcam; goat anti-mouse and anti-rabbit horseradish peroxidase conjugates from Pierce; and rabbit polyclonal anti-Rab11 (H-87) antibody was from Santa Cruz, mouse monoclonal anti-Rab5 (S-19) antibody from Molecular Probes. pCRE-Luc was from Clontech.

**Cloning and Site-directed Mutagenesis**

The plasmids pHM6:hIPWT and pHM6:hIPSSLC, encoding HA epitope-tagged forms of the wild type human prostacyclin receptor (hIP) or isoprenylation-defective hIPSSLC, have been described previously (25), pHM6:hIPC308S, pHM6:hIP C309S, pHM6:hIP C311S, pHM6:hIP C308S,C309S, pHM6:hIP C308S,C311S, pHM6:hIP C309S,C311S, and pHM6:hIP C308S,C309S,C311S have been described (27). The plasmids pGBKT7:hIP299–386, pGBKT7:hIP299–386,C308S, pGBKT7:hIP299–386,C309S, pGBKT7:hIP299–386,C311S, pGBKT7:hIP299–386,C308S,C309S, pGBKT7:hIP299–386,C308S,C311S, pGBKT7:hIP299–386,C309S,C311S, pGBKT7:hIP299–386,S6S, pGBKT7:hIP299–386,S6S,C308S, pGBKT7:hIP299–386,S6S,C309S, pGBKT7:hIP299–386,S6S,C311S, pGBKT7:hIP299–386,S6S,C308S,C309S, pGBKT7:hIP299–386,S6S,C308S,C311S, pGBKT7:hIP299–386,S6S,C309S,C311S, and pGBKT7:hIP299–386,S6S,C308S,C309S,C311S were generated by subcloning amino acids 299–386 of the wild type or the respective mutated hIPs from the corresponding pHM6-based plasmids into the EcoRI-BamHI sites of the yeast bait vector pGBK7 (Clontech), such that fragments were in frame with the DNA-inking domain of the yeast GAL4 transcrip-
tional activator.

The plasmids pHM6:hIPV299A, pHM6:hIPF300A, pHM6: hIPQ301A, pHM6:hIPP302A, pHM6:hIPL303A, pHM6:hIPK304A, pHM6:hIPR305A, pHM6:hIPV306A, pHM6:hIPV307A, pHM6: hIPC308A, pHM6:hIPF309A, pHM6:hIPL310A, pHM6:hIPC311A, and pHM6:hIPL312A were generated by QuickChange™ site-

*JOURNAL OF BIOLOGICAL CHEMISTRY*  VOLUME 285 • NUMBER 24 • JUNE 11, 2010

WITHDRAWN
### Table 1

| Plasmid                  | Oligonucleotide primer† |
|--------------------------|-------------------------|
| pHM6:hiP<sub>WT</sub>    | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |
| pHM6:hiP<sub>299A</sub>  | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |
| pHM6:hiP<sub>300A</sub>  | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |
| pHM6:hiP<sub>301A</sub>  | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |
| pHM6:hiP<sub>302A</sub>  | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |
| pHM6:hiP<sub>303A</sub>  | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |
| pHM6:hiP<sub>304A</sub>  | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |
| pHM6:hiP<sub>305A</sub>  | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |
| pHM6:hiP<sub>306A</sub>  | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |
| pHM6:hiP<sub>307A</sub>  | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |
| pHM6:hiP<sub>308A</sub>  | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |
| pHM6:hiP<sub>309A</sub>  | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |
| pHM6:hiP<sub>310A</sub>  | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |
| pHM6:hiP<sub>309A</sub>  | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |
| pHM6:hiP<sub>310A</sub>  | 5′-CTTTCGGGCAAGCTGCTCAAGCGATCAAG-3′ |

† Sequences presented correspond to those of the sense primer only, where the antisense sequence is inferred, and the identity of the mutator codon is in boldface italic type.

Directed mutagenesis (Stratagene) using pHM6:hiP<sub>WT</sub> as template and the sense/antisense primer pairs presented in Table 1. The plasmids pGBK7:hiP<sub>299A</sub>, pGBK7:hiP<sub>300A</sub>, pGBK7:hiP<sub>301A</sub>, pGBK7:hiP<sub>302A</sub>, pGBK7:hiP<sub>303A</sub>, pGBK7:hiP<sub>304A</sub>, pGBK7:hiP<sub>305A</sub>, pGBK7:hiP<sub>306A</sub>, pGBK7:hiP<sub>307A</sub>, pGBK7:hiP<sub>308A</sub>, pGBK7:hiP<sub>309A</sub>, pGBK7:hiP<sub>310A</sub>, and pGBK7:hiP<sub>311A</sub> were generated by subcloning amino acids 299–386 from pHM6-based plasmid into the EcoRI-BamHI sites of pHM6-based plasmid, and the resulting plasmids were transformed into the AH109 strain, present in the latter was based on the ability of three independent colonies selected from respective DDO media to produce blue (+) or white (−) colonies due to expression of β-galactosidase activity, where, as indicated in the figure legends, ++ was used to indicate that cells developed blue color within 30 min of assay, and a minus sign indicates that cells remain white over the period of the assay (4 h).

For analysis of protein expression in S. cerevisiae AH109 (pGBK7) bait or S. cerevisiae Y187 (pACT2) prey transformants, protein was extracted, resolved by SDS-PAGE, and screened by Western blot analysis using anti-Myc (9B11), with chemiluminescence detection.

### Yeast Two-hybrid Screening and Yeast Matings

Y2H screening of a human kidney cDNA library with the bait plasmids pACT2:Rab11a, expressed in the yeast prey plasmid pGBKT7:p53, encoding the GAL4 DNA-binding domain alone or as a fusion with p53, was obtained from Clontech. All yeast protocols were standard procedures as described previously (32).

In brief, all pGBK7-based bait plasmids were transformed into Saccharomyces cerevisiae AH109 (MATα strain) while pACT2-based prey plasmids were transformed into S. cerevisiae Y187 (MATα strain) and were mated with selection of diploids on synthetic double-drop-out (DDO) media (SD/Leu<sup>−</sup>, Trp<sup>−</sup>, His<sup>−</sup>, Ade<sup>−</sup>) and for the ability to cleave X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactoside), as measured by the filter lift assay of β-galactosidase activity. The scoring system used in the latter was based on the ability of three independent colonies selected from respective DDO media to produce blue (+) or white (−) colonies due to expression of β-galactosidase activity, where, as indicated in the figure legends, ++ was used to indicate that cells developed blue color within 30 min of assay, and a minus sign indicates that cells remain white over the period of the assay (4 h).

For analysis of protein expression in S. cerevisiae AH109 (pGBK7) bait or S. cerevisiae Y187 (pACT2) prey transformants, protein was extracted, resolved by SDS-PAGE, and screened by Western blot analysis using anti-Myc (9B11), with chemiluminescence detection.

### Cell Culture and Transfection

Human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were grown in minimal essential medium (MEM), 10% fetal bovine serum and were transiently or stably transfected using the calcium phosphate/DNA co-precipitation procedure, as previously described (31, 32). In this way, HEK.hIP<sub>WT</sub>, HEK.hIP<sub>V299A</sub>, HEK.hIP<sub>F300A</sub>, HEK.hIP<sub>Q301A</sub>, HEK.hIP<sub>R302A</sub>, HEK.hIP<sub>W306A</sub>, HEK.hIP<sub>303A</sub>, HEK.hIP<sub>304A</sub>, HEK.hIP<sub>305A</sub>, HEK.hIP<sub>309A</sub>, HEK.hIP<sub>310A</sub>, HEK.hIP<sub>311A</sub>, and HEK.hIP<sub>312A</sub> stably overexpressing HA-tagged forms of the respective mutated and wild type and mutated hIPs, respectively, have been described (27). Similarly, HEK.hIP cells stably overexpressing the native, non-epitope-tagged wild type hIP were generated using pcDNA3:hIP and characterized effectively as previously described (27). Primary human umbilical vein endothelial cells (HUVECs), obtained from Lonza (IR79–048-0904D), were routinely cultured in M199 medium (Sigma) supplemented with 0.4% (v/v) endothelial cell growth supplement/heparin (PromoCel), 20% (v/v) fetal bovine serum, and 0.2% (v/v) l-glutamine. Primary HUVECs were used between passages 2 and 8.

For preparation of human platelets, blood was drawn from healthy volunteers, who had not taken any medication for at least 10 days, into 0.15% (v/v) citrate/dextrose anticoagulant (38 mM citric acid, 75 mM sodium citrate, 124 mM d-glucose). Blood was centrifuged at 160 × g for 15 min at room temperature, and platelet-rich plasma was removed and centrifuged at 160 × g for 10 min to remove contaminating blood cells. Platelets were pelleted from platelet-rich plasma by centrifugation at 720 × g for 10 min at room temperature and resuspended in a modified Tyrode buffer (130 mM NaCl, 10 mM trisodium citrate, 9 mM NaHCO<sub>3</sub>, 6 mM d-glucose, 0.9 mM MgCl<sub>2</sub>, 0.81 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris, pH 7.4). Platelets were counted using a Sysmex hematology analyzer (TOA Medical Electronics, Kobe, Japan).
Immunoprecipitations

Immunoprecipitations from mammalian HEK 293 cell lines were carried out essentially as described previously (31, 32). To assess the effect of 2-bromopalmitate (2-BP) on the interaction between the hIP and Rab11, HEK.hIPWT cells, transiently transfected with pEGFPC1:Rab11a, were incubated with 2-BP or, as control, with 0.1% DMSO (vehicle) for 16 h at 37 °C prior to cicaprost stimulation.

A polyclonal anti-hIP antibody was generated in rabbits immunized with a peptide corresponding to its intracellular domain 2 (residues 121–134) that had been conjugated to keyhole limpet hemocyanin carrier protein. The antisera was subsequently purified on affinity resin (SulfoLink Coupling gel; Thermo Fisher Scientific) to which the immunizing peptide was coupled. The specificity of the affinity-purified anti-hIP antibody was confirmed by a number of independent approaches, including immunocyto- and immunohistochemistry, immunoprecipitation, and competition of the specific signal in each of those assays using the immunizing peptide. For example, as indicated in supplemental Fig. 1B, the specificity of the anti-hIP antibody was confirmed to specifically immunoprecipitate the hIP from HEK.hIP cells but not from HEK 293 cells; moreover, the preimmune serum did not result in immunoprecipitation of the hIP from HEK.hIP cells (data not shown).

For co-immunoprecipitation of endogenous Rab11a, washed human platelets (2 × 10⁶ platelets/ml) were incubated with 1 μM cicaprost or, as control, with 0.1% DMSO (vehicle) for 16 h at 37 °C prior to cicaprost stimulation. Thereafter, cells were washed twice in ice-cold PBS to stop stimulation of the specific signal in each of those assays using the immunizing peptide.

Assessment of Agonist-induced Internalization by Confocal Microscopy

Agonist-induced Co-localization of the hIP with Rab11a and Rab5a—In order to monitor changes in cell surface expression of the hIP and/or its co-localization with Rab11a or Rab5a as a function of cicaprost stimulation, HEK.hIP cells were transiently transfected with pADVA (10 μg) and pEGFPC1:Rab11a (25 μg) or pEGFPC1:Rab5a (25 μg), encoding green fluorescent protein (GFP)-tagged forms of the Rab11a or Rab5a protein, respectively, by calcium phosphate/DNA co-precipitation (37). Some 24 h later, cells were seeded onto poly-L-lysine-pretreated coverslips in 6-well plates to achieve 60–70% confluence following 48 h of incubation at 37 °C. Thereafter, cells were washed in serum-free MEM and then prelabeled with anti-HA 101R (1:1000 dilution in MEM) at 4 °C for 1 h to label cell surface receptors. Unbound antibody was removed by washing twice with MEM, following which cells were either analyzed immediately (0 h) or were incubated with 1 μM cicaprost in MEM for 0–4 h at 37 °C, as indicated in the respective figures. For treatment of cells with acid wash, cells were incubated in low pH acid wash (0.15 M NaCl, HCl, pH 2.5) for 5 min on ice. Thereafter, all cells were washed twice in ice-cold PBS prior to fixation in 3.7% paraformaldehyde, PBS, pH 7.4, for 15 min at room temperature. After washing three times in PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min on ice, followed by washing three times in TBS. Nonspecific sites were blocked by incubating with Blocking Buffer (1% BSA in TBS), and HA-tagged receptor was immunolabeled with the secondary AlexaFluor594 goat anti-mouse antibody (1:5000, in 1% BSA, TBS). Data shown are representative images from three independent experiments (n ≥ 3), from which quantification is presented at ×63 magnification, following analysis plugin available with WCIF ImageJ software (version 1.37c), where quantification is presented as percentage of co-localized pixels in supplemental Figs. 3, 6, and 10.
in TBS) prior to immunolabeling with anti-hIP antibody (1:500, in 5% nonfat milk, TBS), followed by detection with anti-rabbit AlexaFluor594-conjugated secondary antibody (1:2000, in 5% nonfat milk, TBS). In parallel, nuclei were counterstained with 4',6-diamidino-2-phenylindole (0.5 µg/ml, 1 min). To compare agonist-induced internalization of the HA epitope-tagged and non-tagged hIP, previously characterized HEK 293 cell lines stably overexpressing either the HA.hIP or the hIP (27) were plated onto glass coverslips. Some 48 h later, cells were incubated with 1 µM cicaprost for 0–4 h at 37 ºC and processed for immunofluorescence, as above.

To monitor the co-localization of hIP and hIP\textsubscript{C308S,C309S,C311S} with the early endosomal marker EEA1 as a function of cicaprost-stimulation, HA-tagged receptors were prelabeled with anti-HA 101R (1:1000 dilution in MEM) at 4 ºC for 1 h to label cell surface receptors, essentially as described under “Agonist-induced co-localization of the hIP with Rab11a and Rab5a.” After removal of unbound antibody, cells were either analyzed immediately (0 h) or incubated with 1 µM cicaprost in MEM for 0–4 h at 37 ºC, as indicated in the respective figures. Thereafter, cells were fixed and permeabilized prior to immunolabeling with anti-EEA1 antibody (1:500, in 1% BSA, TBS), followed by detection with anti-rabbit AlexaFluor488-conjugated secondary antibody (1:2500, in 1% BSA, TBS). HA-tagged hIPs were detected with anti-mouse AlexaFluor488-conjugated secondary antibody (1:5000, in 1% BSA, TBS), as described under “Agonist-induced co-localization of the hIP with Rab11a and Rab5a.”

All confocal microscope images were captured as single slices using Carl Zeiss laser scanning LSM imaging software or microscope and Axioplan V (Carl Zeiss) system and represented in the figures at ×63 magnification, where the horizontal bar represents 10 µm.

**Palmitoylation of the hIP**

Palmitoylation was carried out essentially as described previously (27, 38). Briefly, cells were plated 48 h in advance such that they would achieve a density of ~3 × 10^5 cells/10-cm dish (~80% confluence) on the day of metabolic labeling. Cells were washed once in PBS and then metabolically labeled in serum-free minimal essential medium (1.5 ml) containing 1.5 mCi of [3H]palmitic acid (60 Ci/mmol; PerkinElmer Life Sciences) for 4 h in the absence or presence of 1 µM cicaprost for 0–4 h at 37 ºC. To assess the effect of 2-BP on palmitoylation, cells were preincubated with 100 µM 2-BP or, as control, with 0.1% DMSO (vehicle) for 16 h at 37 ºC prior to metabolic labeling and agonist stimulation. Labeling was terminated by washing the cells in ice-cold PBS, and HA-tagged hIPs were immunoprecipitated as described (27, 38). Immunoprecipitates were resolved by SDS-PAGE and electrophobbed onto polyvinylidene difluoride membrane. Blots were soaked in Amplify\textsuperscript{TM} for 30 min followed by fluorography using Eastman Kodak Co. X-Omat XAR film for 60 days at ~70 ºC. In addition, the [3H] fluorograms were subjected to PhosphorImage analysis, and intensities of cicaprost-induced palmitoylation of the hIP were determined and expressed, in arbitrary units, as mean percentage palmitoylation ± S.E. of hIP\textsuperscript{WT} relative to basal levels detected at 0 h. Following fluorographic exposure, polyvinylidene difluoride membranes were screened by immunoblot analysis using the anti-HA 3F10 peroxidase-conjugated antibody followed by chemiluminescence detection.

**Measurement of Agonist-induced cAMP Generation**

A reporter gene assay was performed to investigate changes in the intracellular levels of cAMP in response to cicaprost stimulation, essentially as described previously (39). In brief, the plasmids pHM6: hIP\textsuperscript{WT}, pHM6: hIP\textsubscript{C308S}, pHM6: hIP\textsubscript{C309S}, pHM6: hIP\textsubscript{C311S}, pHM6: hIP\textsubscript{C308S,C309S}, pHM6: hIP\textsubscript{C308S,C311S}, pHM6: hIP\textsubscript{C309S,C311S}, pHM6: hIP\textsubscript{C308S,C309S,C311S}, pHM6: hIP\textsubscript{V299A}, pHM6: hIP\textsubscript{F300A}, pHM6: hIP\textsubscript{Q301A}, pHM6: hIP\textsubscript{R302A}, pHM6: hIP\textsubscript{V303A}, pHM6: hIP\textsubscript{L304A}, pHM6: hIP\textsubscript{L305A}, pHM6: hIP\textsubscript{W306A}, pHM6: hIP\textsubscript{Y307A}, pHM6: hIP\textsubscript{C308A}, pHM6: hIP\textsubscript{C309A}, pHM6: hIP\textsubscript{L310A}, pHM6: hIP\textsubscript{L311A}, pHM6: hIP\textsubscript{L312A}, or pHM6 (1.5 µg) as a negative control were each transiently co-transfected into HEK 293 cells with the luciferase reporter pCRE-Luc (1.0 µg) and the Renilla luciferase activity reagent as per the manufacturer's instructions. Firefly and Renilla luciferase activity were measured 18 h after transfection using the Dual Luciferase Assay System. Firefly to Renilla luciferase activities (arbitrary units) were calculated as a ratio and were expressed in relative luciferase units.

**Computational Structure Predictions**

Structure prediction of the hIP and its C-tail domain (hIP\textsuperscript{299–386}) were generated by online submission to the iterative TASSER (I-TASSER) algorithm, three-dimensional protein structure prediction software that builds models based on multiple-threading consensus target-to-template alignments by LOMETS (Local Meta-Threading-Server) and I-TASSER simulations (40, 41). I-TASSER predictions were in agreement those generated from independent predictions using PHYRE (available on the World Wide Web) and were further analyzed for helical content using the AGADIR prediction algorithm (available on the World Wide Web). Jpred 3 (42), a web system of different secondary structure prediction algorithms was used to confirm that mutations generated, such as the Ala-scanning mutagenesis, per se did not affect the formation of the $\alpha$-helical domain within the RBD.

**Data Analyses**

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

Investigation of the Role of Cysteines within the RBD of the hIP in Mediating its Interaction with Rab11a—Rab11a was recently identified as a direct interactor of the hIP (32). Consistent with this, immunoprecipitation of endogenous hIP expressed in human platelets confirmed the constitutive association of endogenous Rab11a with the hIP and that this association was increased in response to stimulation with cicaprost, a selective IP agonist (supplemental Fig. 1, A and B). The previous studies suggested that Val\textsuperscript{299}–Gln\textsuperscript{320} (Fig. 1A), adjacent to TM7 of the hIP, may be critical for its interaction with Rab11a (32). Notably, this 22-residue putative “RBD” contains three Cys residues, where Cys\textsuperscript{308} and Cys\textsuperscript{311}, but not Cys\textsuperscript{309}, have been established to undergo palmitoylation to regulate both hIP-G protein coupling and effector signaling (27). Hence, in the current study, it was first sought to investigate the role of those Cys residues and/or the requirement for their palmitoylation in influencing the interaction between the hIP and Rab11a.

Initially, exploiting the Y2H-based approach to interrogate such protein-protein interactions, the effect of mutation of Cys\textsuperscript{308}, Cys\textsuperscript{309}, and Cys\textsuperscript{311} to corresponding Ser residues, either singly or in combination, on the interaction between the C-tail domain of either the wild type (hIP\textsuperscript{299–386,WT}) or isoprenylation-deficient (hIP\textsuperscript{299–386,SSLC}) hIP was investigated. In agreement with previous studies (32), both the hIP\textsuperscript{299–386,WT} and hIP\textsuperscript{299–386,SSLC} showed a strong interaction with Rab11a (Fig. 1B). Although each of the bait strains (S. cerevisiae AH109 (pGBK7)-based) mated successfully with the prey strain (S. cerevisiae Y187 (pACT2:Rab11a)), control diploids either harboring the empty vector pGBK7 or encoding p53 failed to show any interaction with Rab11a (Fig. 1B). Mutation of Cys\textsuperscript{308} and Cys\textsuperscript{311}, along with Cys\textsuperscript{309}, did not affect the interaction between the hIP\textsuperscript{299–386,WT} or hIP\textsuperscript{299–386,SSLC} and Rab11a (Fig. 1B), but there was no specific interaction between the hIP\textsuperscript{299–386,WT} or hIP\textsuperscript{299–386,SSLC} and Rab11a (Fig. 1B), and Cys\textsuperscript{308} and Cys\textsuperscript{311} were selected on QDO media and by their ability to express reporter genes, respectively, due to positive interaction between either hIP\textsuperscript{299–386,WT} or hIP\textsuperscript{299–386,SSLC} and Rab11a (Fig. 1B). The previous studies suggested that Val\textsuperscript{299}–Gln\textsuperscript{320} (Fig. 1A), adjacent to TM7 of the hIP, may be critical for its interaction with Rab11a (32). Notably, this 22-residue putative “RBD” contains three Cys residues, where Cys\textsuperscript{308} and Cys\textsuperscript{311}, but not Cys\textsuperscript{309}, have been established to undergo palmitoylation to regulate both hIP-G protein coupling and effector signaling (27). Hence, in the current study, it was first sought to investigate the role of those Cys residues and/or the requirement for their palmitoylation in influencing the interaction between the hIP and Rab11a.

Initially, exploiting the Y2H-based approach to interrogate such protein-protein interactions, the effect of mutation of Cys\textsuperscript{308}, Cys\textsuperscript{309}, and Cys\textsuperscript{311} to corresponding Ser residues, either singly or in combination, on the interaction between the C-tail domain of either the wild type (hIP\textsuperscript{299–386,WT}) or isoprenylation-deficient (hIP\textsuperscript{299–386,SSLC}) hIP was investigated. In agreement with previous studies (32), both the hIP\textsuperscript{299–386,WT} and hIP\textsuperscript{299–386,SSLC} showed a strong interaction with Rab11a (Fig. 1B). Although each of the bait strains (S. cerevisiae AH109 (pGBK7)-based) mated successfully with the prey strain (S. cerevisiae Y187 (pACT2:Rab11a)), control diploids either harboring the empty vector pGBK7 or encoding p53 failed to show any interaction with Rab11a (Fig. 1B). Mutation of Cys\textsuperscript{308} and Cys\textsuperscript{311}, along with Cys\textsuperscript{309}, did not affect the interaction between the hIP\textsuperscript{299–386,WT} or hIP\textsuperscript{299–386,SSLC} and Rab11a (Fig. 1B), but there was no specific interaction between the hIP\textsuperscript{299–386,WT} or hIP\textsuperscript{299–386,SSLC} and Rab11a (Fig. 1B), and Cys\textsuperscript{308} and Cys\textsuperscript{311} were selected on QDO media and by their ability to express reporter genes, respectively, due to positive interaction between either hIP\textsuperscript{299–386,WT} or hIP\textsuperscript{299–386,SSLC} and Rab11a (Fig. 1B). The previous studies suggested that Val\textsuperscript{299}–Gln\textsuperscript{320} (Fig. 1A), adjacent to TM7 of the hIP, may be critical for its interaction with Rab11a (32). Notably, this 22-residue putative “RBD” contains three Cys residues, where Cys\textsuperscript{308} and Cys\textsuperscript{311}, but not Cys\textsuperscript{309}, have been established to undergo palmitoylation to regulate both hIP-G protein coupling and effector signaling (27). Hence, in the current study, it was first sought to investigate the role of those Cys residues and/or the requirement for their palmitoylation in influencing the interaction between the hIP and Rab11a.

Initially, exploiting the Y2H-based approach to interrogate such protein-protein interactions, the effect of mutation of Cys\textsuperscript{308}, Cys\textsuperscript{309}, and Cys\textsuperscript{311} to corresponding Ser residues, either singly or in combination, on the interaction between the C-tail domain of either the wild type (hIP\textsuperscript{299–386,WT}) or isoprenylation-deficient (hIP\textsuperscript{299–386,SSLC}) hIP was investigated. In agreement with previous studies (32), both the hIP\textsuperscript{299–386,WT} and hIP\textsuperscript{299–386,SSLC} showed a strong interaction with Rab11a (Fig. 1B). Although each of the bait strains (S. cerevisiae AH109 (pGBK7)-based) mated successfully with the prey strain (S. cerevisiae Y187 (pACT2:Rab11a)), control diploids either harboring the empty vector pGBK7 or encoding p53 failed to show any interaction with Rab11a (Fig. 1B). Mutation of Cys\textsuperscript{308} and Cys\textsuperscript{311}, along with Cys\textsuperscript{309}, did not affect the interaction between the hIP\textsuperscript{299–386,WT} or hIP\textsuperscript{299–386,SSLC} and Rab11a (Fig. 1B), but there was no specific interaction between the hIP\textsuperscript{299–386,WT} or hIP\textsuperscript{299–386,SSLC} and Rab11a (Fig. 1B), and Cys\textsuperscript{308} and Cys\textsuperscript{311} were selected on QDO media and by their ability to express reporter genes, respectively, due to positive interaction between either hIP\textsuperscript{299–386,WT} or hIP\textsuperscript{299–386,SSLC} and Rab11a (Fig. 1B).
indicated to the /H11011 (50 IB)
were resolved by SDS-PAGE and were either immunoblotted (IB) versus that of the hIPWT, and there was no cicaprost-in-
ing stimulation with the TXA2 mimetic U46619 (Fig. 2)

To further investigate the influence of palmitoylation on the
interaction between TPβ and Rab11a, co-immunoprecipitation of the hIP with Rab11a—To further examine the influence of palmitoylation, co-localization between either wild type or palmitoylation-defective hIPs with Rab11a in the respective HEK 293 clonal cell lines was investigated by confocal microscopy. First, only HA-tagged hIPs actually located at the cell surface, as opposed to on intracellular membranes, were pre incubated with anti-HA 101R antibody at 4 °C, and thereafter, HA-hIP localization was either
analyzed directly (0 h) or following stimulation with cicaprost
for 2 or 4 h at 37 °C. In the absence of agonist, the prelabeled
hIPWT was detected exclusively at the cell surface as expected
(Fig. 3A, Anti-HA, 0 h), whereas GFP-Rab11a was predomin
antly located in the ERC with no co-localization between the
hIPWT and Rab11a (Fig. 3A, Overlay, 0 h). Following 2-h cicaprost
treatment, there was substantial relocalization of the hIPWT away from the cell surface to punctate intracellular vesicles concomitantly with relocalization of Rab11a to more discrete endocytic vesicles (Fig. 3A, 2 h). Furthermore, there was co-localization between the hIPWT and Rab11a (Fig. 3A, Overlay, 2 h). At 4 h after agonist stimulation, much of the hIPWT had recycled to the cell surface (Fig. 3A, Anti-HA, 4 h).

The prelabeled hIPWT showed altered maturation or expres-
sion in any of the respective HEK 293 cell lines (Fig. 2B, lower panels), whereas efficient immunoprecipitation and expression of the hIP Cys → Ser variants was confirmed in all cases (supplemental Fig. 2, B and C, respectively) (27).

It was notable that although each hIP variant carrying the
Ser mutation (e.g. hIPC311S, hIPC309S, hIPC309S,C311S, and
hIPC308S,C309S,C311S) showed altered patterns of glycosylation (supple-
mental Fig. 2), this did not lead to altered maturation or expres-
sion in any of the respective HEK 293 cell lines, as determined by radioligand
binding (27) or image analyses (e.g. Figs. 3 and 4), or account for the impaired ability of the palmitoylation-defective hIPs and hIPWT mutants to signal per se, as previously reported by us (27).

Influence of Palmitoylation on Co-localization of the hIP with
Rab11a—To further examine the influence of palmitoylation,”
co-localization between either wild type or palmitoylation-defec-
tive hIPs with Rab11a in the respective HEK 293 clonal cell
lines was investigated by confocal microscopy. First, only HA-
tagged hIPs actually located at the cell surface, as opposed to on intracellular membranes, were pre incubated with anti-HA 101R antibody at 4 °C, and thereafter, HA-hIP localization was either
analyzed directly (0 h) or following stimulation with cicaprost
for 2 or 4 h at 37 °C. In the absence of agonist, the prelabeled
hIPWT was detected exclusively at the cell surface as expected
(Fig. 3A, Anti-HA, 0 h), whereas GFP-Rab11a was predomin
antly located in the ERC with no co-localization between the
hIPWT and Rab11a (Fig. 3A, Overlay, 0 h). Following 2-h cicaprost
treatment, there was substantial relocalization of the hIPWT away from the cell surface to punctate intracellular vesicles concomitantly with relocalization of Rab11a to more discrete endocytic vesicles (Fig. 3A, 2 h). Furthermore, there was co-localization between the hIPWT and Rab11a (Fig. 3A, Overlay, 2 h). At 4 h after agonist stimulation, much of the hIPWT had recycled to the cell surface (Fig. 3A, Anti-HA, 4 h).

The prelabeled hIPWT showed altered maturation or expres-
sion in any of the respective HEK 293 cell lines (Fig. 2B, lower panels), whereas efficient immunoprecipitation and expression of the hIP Cys → Ser variants was confirmed in all cases (supplemental Fig. 2, B and C, respectively) (27).

It was notable that although each hIP variant carrying the
Ser mutation (e.g. hIPC311S, hIPC309S, hIPC309S,C311S, and
hIPC308S,C309S,C311S) showed altered patterns of glycosylation (supple-
mental Fig. 2), this did not lead to altered maturation or expres-
sion in any of the respective HEK 293 cell lines, as determined by radioligand
binding (27) or image analyses (e.g. Figs. 3 and 4), or account for the impaired ability of the palmitoylation-defective hIPs and hIPWT mutants to signal per se, as previously reported by us (27).

Influence of Palmitoylation on Co-localization of the hIP with
Rab11a—To further examine the influence of palmitoylation,”
co-localization between either wild type or palmitoylation-defec-
tive hIPs with Rab11a in the respective HEK 293 clonal cell
lines was investigated by confocal microscopy. First, only HA-
tagged hIPs actually located at the cell surface, as opposed to on intracellular membranes, were pre incubated with anti-HA 101R antibody at 4 °C, and thereafter, HA-hIP localization was either
analyzed directly (0 h) or following stimulation with cicaprost
for 2 or 4 h at 37 °C. In the absence of agonist, the prelabeled
hIPWT was detected exclusively at the cell surface as expected
(Fig. 3A, Anti-HA, 0 h), whereas GFP-Rab11a was predomin
antly located in the ERC with no co-localization between the
hIPWT and Rab11a (Fig. 3A, Overlay, 0 h). Following 2-h cicaprost
treatment, there was substantial relocalization of the hIPWT away from the cell surface to punctate intracellular vesicles concomitantly with relocalization of Rab11a to more discrete endocytic vesicles (Fig. 3A, 2 h). Furthermore, there was co-localization between the hIPWT and Rab11a (Fig. 3A, Overlay, 2 h). At 4 h after agonist stimulation, much of the hIPWT had recycled to the cell surface (Fig. 3A, Anti-HA, 4 h).

The prelabeled hIPWT showed altered maturation or expres-
sion in any of the respective HEK 293 cell lines (Fig. 2B, lower panels), whereas efficient immunoprecipitation and expression of the hIP Cys → Ser variants was confirmed in all cases (supplemental Fig. 2, B and C, respectively) (27).

It was notable that although each hIP variant carrying the
Ser mutation (e.g. hIPC311S, hIPC309S, hIPC309S,C311S, and
hIPC308S,C309S,C311S) showed altered patterns of glycosylation (supple-
mental Fig. 2), this did not lead to altered maturation or expres-
sion in any of the respective HEK 293 cell lines, as determined by radioligand
binding (27) or image analyses (e.g. Figs. 3 and 4), or account for the impaired ability of the palmitoylation-defective hIPs and hIPWT mutants to signal per se, as previously reported by us (27).
continued to exhibit ERC staining (Fig. 3A, GFP, 2 hr), and there was no co-localization evident (Fig. 3A, Overlay, 2 hr). At 4 h after cica-
prost stimulation, most of the hIP \( C^{308S,309S,311S} \) remained in intracellular vesicles with substan-
tially reduced recycling to the plasma membrane relative to the hIP \( WT \) (Fig. 3A, Anti-HA, 4 hr).

Moreover, the Rab11a continued to exhibit ERC and diffuse cytosolic staining, and there was no co-local-
ization of the hIP \( C^{308S,309S,311S} \) with Rab11a (Fig. 3A, Overlay, 4 hr).

The agonist-induced traffic-
king of the hIP and of the hIP \( C^{308S,309S,311S} \) was also exam-
ined following acid stripping in order to further distinguish cell sur-
face from internalized receptor. Both the prelabeled hIP and the hIP \( C^{308S,309S,311S} \) were confirmed to be expressed at the cell surface prior to agonist stimulation, as evi-
denced by the sensitivity of the fluorescent signal to acid stripping (Fig. 3B, 0 hr). At 2 h after cicaprost
stimulation, the prelabeled hIP and hIP \( C^{308S,309S,311S} \) internalized, being insensitive to acid treat-
ment. However, at 4 h, although much of the internalized wild type hIP had recycled to the plasma
membrane, little if any of the hIP \( C^{308S,309S,311S} \) recycled, as indicated by its sensitivity and
resistance to acid stripping, respect-
ively (Fig. 3B).

Co-localization of GFP-Rab11a was also investigated in HEK.hIP \( C^{308S,309S,311S} \) cell lines (Fig.
3C). As expected, the prelabeled hIP Cys → Ser variants were exclusively detected at the plasma membrane
without co-localization with Rab11a in resting cells (Fig. 3C, 0 hr). Although each of the individual hIP Cys → Ser variants underwent cica-

**FIGURE 3.** Effect of palmitoylation on co-localization of Rab11a with the hIP. HEK.hIP \( WT \) and HEK.hIP \( C^{308S,309S,311S} \) cells (A and B) or HEK.hIP \( C^{308S} \), HEK.hIP \( C^{309S} \), HEK.hIP \( C^{311S} \), HEK.hIP \( C^{308S,309S} \), HEK.hIP \( C^{308S,311S} \), and HEK.hIP \( C^{309S,311S} \) cells (C), each transiently transfected with pEGFPCI:Rab11a, were prelabeled with anti-HA 101R antibody for 1 h at 4 °C; thereafter, cells were either analyzed directly (0 h) or were incubated with \( 1 \mu M \) cicaprost at 37 °C for 2 or 4 h. Alternatively, cells were either subject to acid-washing (B, Acid Wash) or analyzed directly following fixation and permeabilization prior to detection of HA-tagged hIPs, with anti-
mouse AlexaFluor594-conjugated secondary antibody (Anti-HA) and enhanced GFP-Rab11a (GFP) expression or both (Overlay), using Carl Zeiss laser-scanning system LSM 510 and Zeiss LSM imaging software. B and C, overlay images only. Data presented are representative images from at least three independent experiments (\( n \geq 3 \)), from which at least 10 fields were viewed at \( \times 63 \) magnification, where the horizontal bar represents 10 µm.
prost- induced internalization, albeit to varying extents (Fig. 3C, 2 hr), they displayed distinct differences in terms of their subcellular relocalizations. For example, hIPC308S localized to punctate vesicles to the perinuclear region of the cell reminiscent of the ERC, with co-localization with Rab11 evident (Fig. 3C, hIPC308S, 2 hr). Similarly, hIPC308S,C309S exhibited some relocalization to the perinuclear region (Fig. 3C, hIPC308S,C309S, 2 hr). After a 4-h cicaprost stimulation, most of the internalized hIPC308S recycled back to the cell surface, whereas a significant amount of hIPC308S,C309S did not recycle but rather remained localized to the ERC (Fig. 3C, hIPC308S,C309S, 4 hr). The pattern of internalization of the hIPC308S was similar to that of hIPWT, in that it co-localized with Rab11a in punctate vesicles after a 2-h cicaprost stimulation, and after 4 hr, it mostly recycled to the cell surface with no evidence of co-localization with Rab11a or of its many variants used in this study to undergo internalization or recycling (supplemental Fig. 4A). Cicaprost-induced internalization of the HA-hIP and of the hIP was examined by indirect immunofluorescence microscopy and confocal imaging (single slices) under permeabilizing conditions using a previously characterized antibody directed to the intracellular loop (IC)2 domain of the hIP (39). Both the HA-hIP and the non-tagged hIP underwent similar cicaprost-induced trafficking, with maximal internalization at 2 hr, whereas at 4 hr, they each recycled to the plasma membrane (supplemental Fig. 4A). Moreover, agonist-induced trafficking of the hIP endogenously expressed in primary HUVECs was also examined and was confirmed to display a pattern somewhat similar to that of the hIP ectopically expressed in the HEK 293 cell lines. More specifically, in primary HUVECs, in the absence of agonist, the hIP

![WITHDRAWN](July 9, 2018)
**Rab11 and the Prostacyclin Receptor**

was predominantly expressed at the plasma membrane. However, in response to cicaprost simulation, it underwent a time-dependent internalization with loss of cell surface expression coinciding with increased intracellular accumulation, with maximal internalization at 1–2 h after agonist stimulation, whereas at 4 h, much of it recycled to the plasma membrane (supplemental Fig. 4B). In keeping with the fact that the anti-hIP antibody was directed to an intracellular epitope (IC3), no immunostaining was observed in non-permeabilized cells (supplemental Fig. 4B).

**Influence of Palmitoylation on the Internalization and Association of the hIP with Rab5a**—To further confirm that the lack of association and co-localization of the palmitoylation-deficient hIP variants with Rab11a is not due to impaired agonist-induced internalization per se or indeed an artifact of overexpression of GFP-Rab11a, their cellular localization/trafficking was also examined by confocal imaging under both non-permeabilizing and permeabilizing conditions in the presence of endogenous Rab proteins only. Initially, the hIPWT and hIPC\(^{308S,309S,311S}\) were predominantly located at the cell surface (Fig. 4A, 0 hr, Non-Permeabilized) with some evidence of intracellular localization, albeit at much lower levels (Fig. 4, 0 hr, Permeabilized). Upon agonist stimulation, cell surface expression of both the hIPWT and hIPC\(^{308S,309S,311S}\) was lost (Fig. 4A, 2 hr, Non-Permeabilized), concomitant with increased intracellular localization (Fig. 4A, 2 hr, Permeabilized) and co-localization with the endosomal marker EEA1 (Fig. 4B). Moreover, in the presence of 2-BP, the hIPWT internalized to punctate vesicles in response to cicaprost (Fig. 4), whereas at 4 h, much of it recycled to the plasma membrane (Fig. 4B, supplemental Fig. 5). In the absence of agonist, the prelabeled hIPWT was detected exclusively at the cell surface, whereas most of the hIPC\(^{308S,309S,311S}\) remained internalized with no cell surface following prolonged (4-h) cicaprost stimulation, coinciding with increased intracellular accumulation, with hIPC\(^{308S,309S,311S}\) predominantly located at the cell surface (Fig. 4B, supplemental Fig. 6). In the absence of agonist, the prelabeled hIPWT was detected exclusively at the cell surface, whereas most of the hIPC\(^{308S,309S,311S}\) remained internalized with no cell surface following prolonged (4-h) cicaprost stimulation, coinciding with increased intracellular accumulation, with hIPC\(^{308S,309S,311S}\) predominantly located at the cell surface (Fig. 4B, supplemental Fig. 6).

As stated, previous studies have established that the hIP undergoes agonist-induced internalization through a Rab5a-dependent mechanism (31). Therefore, in view of findings herein with Rab11a, we also sought to examine whether palmitoylation may affect the actual association, be it direct or indirect, between the hIP and Rab5. Consistent with previous data (31), Rab5a was detected in the anti-HA immunoprecipitates from HEK hIPWT cells, and levels were increased in response to cicaprost (Fig. 5A). In contrast to that observed with Rab11a, Rab5a co-immunoprecipitated with the palmitoylation-deficient hIP\(^{308S,309S,311S}\) and in an agonist-augmented manner (Fig. 5A).

Thereafter, the possible influence of palmitoylation on the actual agonist-induced co-localization of the hIP with Rab5a was investigated by confocal imaging (Fig. 5, B and C, and supplemental Fig. 6A). In the absence of agonist, the prelabeled hIPWT was detected exclusively at the cell surface, whereas the hIP\(^{308S,309S,311S}\) was also examined by confocal imaging under both non-permeabilized and permeabilized conditions. Initially, the hIPWT and hIPC\(^{308S,309S,311S}\) were predominantly located at the cell surface (Fig. 4A, 0 hr, Non-Permeabilized) with some evidence of intracellular localization, albeit at much lower levels (Fig. 4, 0 hr, Permeabilized). Upon agonist stimulation, cell surface expression of both the hIPWT and hIPC\(^{308S,309S,311S}\) was lost (Fig. 4A, 2 hr, Non-Permeabilized), concomitant with increased intracellular localization (Fig. 4A, 2 hr, Permeabilized) and co-localization with the endosomal marker EEA1 (Fig. 4B). Moreover, in the presence of 2-BP, the hIPWT internalized to punctate vesicles in response to cicaprost (Fig. 4), whereas at 4 h, much of it recycled to the plasma membrane (Fig. 4B, supplemental Fig. 5). In the absence of agonist, the prelabeled hIPWT was detected exclusively at the cell surface, whereas most of the hIPC\(^{308S,309S,311S}\) remained internalized with no cell surface following prolonged (4-h) cicaprost stimulation, coinciding with increased intracellular accumulation, with hIPC\(^{308S,309S,311S}\) predominantly located at the cell surface (Fig. 4B, supplemental Fig. 6). In the absence of agonist, the prelabeled hIPWT was detected exclusively at the cell surface, whereas most of the hIPC\(^{308S,309S,311S}\) remained internalized with no cell surface following prolonged (4-h) cicaprost stimulation, coinciding with increased intracellular accumulation, with hIPC\(^{308S,309S,311S}\) predominantly located at the cell surface (Fig. 4B, supplemental Fig. 6).

![FIGURE 5. Effect of palmitoylation on the agonist-induced interaction of hIP with Rab5a. A, HEK.hIPWT, HEK.hIPC\(^{308S,309S,311S}\), or, as controls, HEK.β-Gal cells, each transiently transfected with pEGFPCI:Rab5a, were either incubated with vehicle (−) or 1 μM cicaprost (+) at 37 °C for 2 h, prior to immunoprecipitation with anti-HA 101R antibody. Immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted (IB) versus anti-Rab5 antibody (upper panels). Aliquots of whole cell lysates (−50 μg/lane) were immunoblotted with anti-HA antibody (lower panels). The relative positions of the molecular size markers are indicated to the left of the panels. n ≥ 3. B and C, HEK.hIPWT and HEK.hIPC\(^{308S,309S,311S}\) cells, each transiently transfected with pEGFPCI:Rab5a, were prelabeled with anti-HA 101R antibody for 1 h at 4 °C; thereafter, cells were either analyzed directly (0 h) or incubated with 1 μM cicaprost at 37 °C for 2 or 4 h. Cells were fixed and permeabilized prior to detection of HA-tagged hIPs, with anti-mouse AlexaFluor594-conjugated secondary antibody (Anti-HA) and enhanced GFP-Rab5a (GFP) expression or both (Overlay). n ≥ 3.](attachment:figure5.png)
GFP-Rab5a exhibited diffuse staining within the cytosol with some evidence of association with preformed endosomes (Fig. 5B, 0 hr). Following 2 h of cicaprost-treatment, there was substantial relocation of the hIPWT to intracellular vesicles reminiscent of early endosomes (Fig. 5B, Anti-HA, 2 hr). Concomitant with this, Rab5a associated with more discrete endosomes (Fig. 5B, GFP, 2 hr), and there was significant co-localization between the hIPWT and Rab5a (Fig. 5B, Overlay, 2 hr). At 4 h, much of the hIPWT recycled to the cell surface, with the remainder co-localizing with GFP-Rab5a in endosomes at or near the cell periphery (Fig. 5B, 4 hr). Similar to the hIPWT, the prelabeled hIPC308S,309S,311S was exclusively detected at the plasma membrane with no co-localization between Rab5a in resting cells (Fig. 5C, 0 hr). At 2 h after cicaprost stimulation, much of the hIPC308S,309S,311S underwent internalization to Rab5a-positive endosomes, but at 4 h, most of it failed to recycle to the plasma membrane, remaining in those endosomes (Fig. 5C, 2 hr and 4 hr). Consistent with this, the prelabeled cell surface hIPC308S,309S,311S, hIPC308S,309S,311S, hIPC308S,311S, and hIPC309S,311S also co-localized with Rab5 in response to cicaprost (supplemental Fig. 6, B and C, 2 hr), which, in cells expressing the Cys311→Ser mutation either alone or in combination (hIPC311S, hIPC308S,311S, and hIPC309S,311S) failed to recycle, remaining in Rab5-positive endosomes (supplemental Fig. 6, B and C, 4 hr). Taken together, these data indicate that although palmitoylation of the hIPWT plays a role in regulating its interaction with Rab11, it does not prohibit its agonist-induced internalization or association with Rab11 and the Prostacyclin Receptor.

Effect of Agonist Stimulation on hIP Palmitoylation

Although the hIP was established to undergo palmitoylation at Cys308 and Cys311 (27), that study did not investigate possible agonist-induced changes in hIP palmitoylation. To further examine the role of palmitoylation of the hIP in regulating its interaction with Rab11, [3H]palmitate labeling of the hIPC308S,309S,311S and hIPC308S,311S, hIPC308S,309S,311S, hIPC309S,311S, and hIPC308S,309S,311S was also investigated (Fig. 6, C and D, 0 hr and 2 hr). In the absence of agonist, palmitoylation of the hIPC308S,309S,311S and hIPC308S,311S, and hIPC309S,311S cells was readily detected, albeit at reduced levels compared with the hIPWT (Fig. 6, C and D, 0 hr), whereas levels of palmitoylation of the hIPC309S,311S, hIPC308S,309S,311S, and hIPC308S,309S,311S cells were even lower (Fig. 6, C and D, 0 hr). The fact that the hIPC308S,311S, but not hIPC308S,309S,311S, underwent palmitoylation suggests that Cys309 is subject to palmitoylation, albeit at low levels, under resting conditions. Cicaprost did not mediate a significant change in palmitoylation of the hIPC308S,311S but led to slight reductions in palmitoylation of hIPC309S,309S,311S (Fig. 6, C and D, 2 h). The net stimulation resulted in an 80% reduction in palmitoylation of the hIPC311S (Fig. 6, C and D, 2 h), whereas reductions in palmitoylation of hIPC308S,309S,311S were observed. These data suggest that, to a much lesser extent, Cys311 may be palmitoylated as a consequence of agonist stimulation, previously, confirmation of equivalent protein expression and efficient immunoprecipitation of the hIPWT and hIPC308S,309S,311S was obtained by subsequent screening of the 3H fluorogram by immunoblot analysis (anti-HA-hIP; supplemental Fig. 7), which also indicated that the lack of detection of palmitoylation of the hIPC308S,309S,311S was not due to failure of its immunoprecipitation.

To further examine the role of palmitoylation of the hIP in regulating its interaction with Rab11, [3H]palmitate labeling of the hIPC308S,309S,311S, hIPC308S,311S, hIPC308S,309S,311S, and hIPC308S,309S,311S was also investigated (Fig. 6, C and D, 0 hr and 2 hr). In the absence of agonist, palmitoylation of the hIPC308S,309S,311S and hIPC308S,311S, and hIPC309S,311S was readily detected, albeit at reduced levels compared with the hIPWT (Fig. 6, C and D, 0 hr), whereas levels of palmitoylation of the hIPC309S,311S, hIPC308S,309S,311S, and hIPC308S,309S,311S cells were even lower (Fig. 6, C and D, 0 hr). The fact that the hIPC308S,311S, but not hIPC308S,309S,311S, underwent palmitoylation suggests that Cys309 is subject to palmitoylation, albeit at low levels, under resting conditions. Cicaprost did not mediate a significant change in palmitoylation of the hIPC308S,311S but led to slight reductions in palmitoylation of hIPC309S,309S,311S (Fig. 6, C and D, 2 h). The net stimulation resulted in an 80% reduction in palmitoylation of the hIPC311S (Fig. 6, C and D, 2 h), whereas reductions in palmitoylation of hIPC308S,309S,311S were observed. These data suggest that, to a much lesser extent, Cys311 may be palmitoylated as a consequence of agonist stimulation, previously, confirmation of equivalent protein expression and efficient immunoprecipitation of the hIPWT and hIPC308S,309S,311S was obtained by subsequent screening of the 3H fluorogram by immunoblot analysis (anti-HA-hIP; supplemental Fig. 7).
Additionally, although not reaching statistical significance over four individual metabolic labeling experiments, the level of palmitoylation of the hIPC309S was consistently lower than in the wild type hIP, further suggesting that Cys309 is palmitoylated independently of Cys308 or Cys311.

Identification of the Domain(s) within the hIP Mediating the Interaction with Rab11a—As stated, the putative RBD region within the hIP required for its interaction with Rab11a was localized to a 22-amino acid segment, corresponding to Val299–Gln320, adjacent to TM7 (Fig. 1A) (32). I-TASSER (40, 41) was used to generate a three-dimensional model of the hIP (Fig. 7A) and was found to have greatest structural similarity with that recently determined for the human A2A adenosine receptor (Protein Data Bank entry 3EML (34)). In light of this, we sought to further define the minimal structural domain(s) within the hIP299–320 region that mediates the interaction with Rab11a through additional deletional analysis and Y2H-based protein interactions. Consistent with the previous study (32), the hIP299–386 and hIP299–320 subfragments supported the interaction (Fig. 7B). Conversely, removal of the 299–320 region or successive amino-terminal deletion of residues within the α-H8 subdomain, as in the cases of the hIP299–386, hIP303–386, hIP307–386, or hIP312–386, respectively, failed to support the interaction with Rab11a (Fig. 7B). Amino-terminal removal of any residues within the α-H8 domain, as in the cases of hIP303–316 and hIP303–320, impaired the hIP-Rab11 interaction, whereas carboxyl-terminal deletions, as exemplified by hIP299–316 and hIP299–312, established that residues between Val299 and Leu312 are sufficient for the interaction, whereas residues Gly313–Gln320 are not actually required. Hence, the minimum region required for interaction with Rab11a is the 14-residue hIP299–312.
predicted to be engaged in the α-H8 domain adjacent to the palmitoylated residues at Cys^{308}–Cys^{311} (Fig. 7A).

**Disruption of hIP and Rab11a Interaction through Ala-scanning Mutagenesis—**Ala-scanning mutagenesis, in combination with the Y2H screening approach, was used to further define the critical residue(s) within the minimum hIP^{299–312} RBD required for interaction with Rab11a, where the mutations introduced were confirmed not to affect the formation of the α-H8 structure *per se* (42). Consistent with previous data involving the Cys → Ser mutations (Fig. 1B), both the hIP^{299–386,C308A} and hIP^{299–386,C311A}, but not hIP^{299–386,C309A}, showed positive interaction with Rab11a, further confirming a specific requirement for Cys^{309} for that interaction (Fig. 8A). Moreover, Ala-scanning mutagenesis of residues Phe^{300}, Arg^{302}, Leu^{303}, Lys^{304}, Lys^{305}, and Val^{307} disrupted the interaction between the hIP^{299–386} with Rab11a, whereas mutation of Val^{299}, Gln^{301}, Trp^{306}, Leu^{310}, and Leu^{312} had no measurable affect (Fig. 8A). Hence, it appears that there is a structural requirement for numerous hydrophobic in addition to positively charged Lys and Arg residues between Val^{299} and Leu^{312} rather than one or more residue(s) being responsible for the interaction between the hIP and Rab11a. This is consistent with the specific requirement for the predicted α-H8 domain adjacent to the palmitoylated residues at Cys^{308}–Cys^{311} within the hIP RBD as necessary for Rab11a binding.

To confirm these data in mammalian cells, Ala-scanning mutagenesis of Val^{299}–Leu^{312} was also performed on HA-tagged forms of the hIP to allow for assessment of their interaction with Rab11a through co-immunoprecipitation (Fig. 8B) and immunolocalization (Fig. 8C) studies. Initially, it was necessary to compare expression and signaling of the wild type hIP with that of each of its Ala-scanning variants (Val^{299}–Leu^{312}) and indeed

---

**FIGURE 7.** Structure and function analysis of the RBD of the hIP. A, I-TASSER three-dimensional structural analysis of the hIP predicts that it contains seven TM domains, typical of its GPCR structure, and that the Val^{299–320} region (inset) contains an eighth α-helical domain between residues Val^{299} and Val^{307} adjacent to the palmitoylation residues Cys^{308}–Cys^{311}. B, the S. cerevisiae Y187 (pACT2:Rab11a) prey strain was mated with the respective S. cerevisiae AH109 (pGBKT7:hIP^{299–386,WT}) bait strains, as listed, or as controls with S. cerevisiae AH109 (pGBKT7:p53) or S. cerevisiae AH109 (pGBKT7). Diploids were selected on DDO media, whereas interactants were selected on QDO media and by their ability to express β-galactosidase due to positive interaction between the bait and prey proteins. n ≥ 3.
### Rab11 and the Prostacyclin Receptor

![Image](https://via.placeholder.com/150)

**FIGURE 8.** Alanine-scanning mutagenesis of hIP_{299–312}, A, the *S. cerevisiae* Y187 (pACT2:Rab11a) prey strain was mated with the respective *S. cerevisiae* AH109 (pGBKT7) bait strains harboring the various Ala-scanning mutations of residues Val_{299}–Leu_{312}, as listed, or as controls with *S. cerevisiae* AH109 (pGBKT7:p53) or *S. cerevisiae* AH109 (pGBK7). Diploids were selected on DDO medium, whereas interactants were selected on QDO medium and by their ability to express β-galactosidase due to positive interaction between the bait and prey proteins (n ≥ 3). B. HEK 293 cells, co-transfected with plasmids encoding GFP-tagged Rab11a and HA-tagged hIP_{WT}, hIP_{V299A}, hIP_{Q301A}, hIP_{R302A}, hIP_{L303A}, hIP_{C304A}, hIP_{L305A}, hIP_{V306A}, hIP_{V307A}, hIP_{C308A}, hIP_{C309A}, hIP_{L310A}, hIP_{K311A}, or hIP_{L312A} were incubated with either vehicle (−) or with 1 μM cicaprost for 2 h (+), as indicated, prior to immunoprecipitation with anti-HA 101R antibody. Immunoprecipitates were resolved by SDS-PAGE and were immunoblotted (IB) with anti-Rab11 antibody. Immunoprecipitates were resolved by SDS-PAGE and were immunoblotted (IB) with anti-Rab11 antibody. Immunoprecipitates were resolved by SDS-PAGE and were immunoblotted (IB) with anti-Rab11 antibody.

| (A) | WT | V299A | F300A | Q301A | R302A | L303A | C304A | L305A | W306A | V307A | C308A | C309A | L310A | K311A | L312A |
|-----|----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| WT | 299 | 300A | 301A | 302A | 303A | 304A | 305A | 306A | 307A | 308A | 309A | 310A | 311A | 312A |
| 386 | 308A | 309A | 310A | 311A | 312A | 308A | 309A | 310A | 311A | 312A | 308A | 309A | 310A | 311A | 312A |

| (B) | WT | V299A | F300A | Q301A | R302A | L303A | C304A | L305A | W306A | V307A | C308A | C309A | L310A | K311A | L312A |
|-----|----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Cicaprost | 62 kDa | 47.5 kDa | 62 kDa | 47.5 kDa | 62 kDa | 47.5 kDa | 62 kDa | 47.5 kDa | 62 kDa | 47.5 kDa | 62 kDa | 47.5 kDa | 62 kDa | 47.5 kDa | 62 kDa |

| Insetting | WT | V299A | F300A | Q301A | R302A | L303A | C304A | L305A | W306A | V307A | C308A | C309A | L310A | K311A | L312A |
|-----------|----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Anti-Rab11 | 0 hr | 2 hr | 4 hr | 0 hr | 2 hr | 4 hr | 0 hr | 2 hr | 4 hr | 0 hr | 2 hr | 4 hr | 0 hr | 2 hr | 4 hr |

**Withdrawn**

July 9, 2018
immunoprecipitations carried out with the Cys → Ser mutations (hIP<sup>C308S</sup>, hIP<sup>C309S</sup>, and hIP<sup>C311S</sup>; Fig. 2B), the hIP<sup>C308A</sup>, hIP<sup>C309A</sup>, and hIP<sup>F311A</sup> also showed impaired ability to co-immunoprecipitate Rab11a in the absence of agonist (Fig. 8B, top), but similar to the hIP<sup>C311A</sup>, the hIP<sup>C311A</sup> retained the agonist-induced responsiveness, whereas the Cys<sup>308</sup> (hIP<sup>C308S</sup> and hIP<sup>C308A</sup>) and Cys<sup>309</sup> (hIP<sup>C309S</sup> and hIP<sup>C309A</sup>) equivalents did not.

In agreement with the co-immunoprecipitation data, confocal imaging established that in general, mutation of many of the residues within the Val<sup>299</sup>–Leu<sup>312</sup> RBD domain resulted in impaired co-localization of the internalized receptor to Rab11a-positive vesicles and altered intracellular trafficking relative to the wild type hIP (Fig. 8C and supplemental Fig. 10). For example, although the hIP<sup>F300A</sup> internalized normally to discrete intracellular vesicles, there was reduced co-localization to Rab11a-positive vesicles (Fig. 8C, F300A, 0 hr and 2 hr). Moreover, following 4 hr of agonist stimulation, the hIP<sup>F300A</sup> did not efficiently recycle to the cell surface compared with the hIP<sup>WT</sup> (Fig. 8C, F300A, 4 hr). On the other hand, both the hIP<sup>V299A</sup> and the hIP<sup>Q301A</sup> behaved similarly to the hIP<sup>WT</sup>, whereby they internalized to discrete vesicles co-localizing with Rab11a after 2 h of cicaprost stimulation. Furthermore, they each efficiently recycled to the cell surface 4 hr poststimulation (Fig. 8C, V299A and Q301A, 0, 2, and 4 hr). In contrast, although the prelabeled hIP<sup>L303A</sup> internalized in response to agonist (2 h), it remained in distinct vesicles, failing to follow prolonged exposure to agonist (Fig. 8C, L303A, 4 hr). Hence, although there is evidence of association between the hIP<sup>L303A</sup> with Rab11a in the absence of agonist and in regulating its intracellular traffic after agonist stimulation. Computational structural modeling along with complementary biochemical approaches in yeast and mammalian cells support the proposal that the 14–amino acid RBD domain is organized into an α-H8 domain, comprising residues Val<sup>299</sup>–Val<sup>303</sup>, adjacent to the palmitoylated residues at Cys<sup>308</sup>–Cys<sup>311</sup>. Palmitoylation at Cys<sup>311</sup> in addition to agonist-regulated deacylation at Cys<sup>309</sup> > Cys<sup>308</sup>, outside of the α-helical domain, are likely to be important in positioning or anchoring the helical RBD of the hIP in proximity to Rab11a, to regulate its intracellular traffic after agonist stimulation.

**DISCUSSION**

Agonist-induced trafficking plays a critical role in regulating the signaling responses by members of the GPCR superfamily (2, 4). A number of independent studies have established that the IP undergoes desensitization and internalization in human platelets and other cell types, fine tuning the responses to prostacyclin in vivo, but that it does not involve the classic G protein-coupled receptor kinase/β-arrestin-mediated mechanism (31, 44). We have recently established that agonist-induced internalization of the hIP occurs through a Rab5α-dependent mechanism (31). Through a subsequent Y2H screen, Rab11a was identified as a direct and specific interactor of the hIP and was found to play a critical role in its agonist-induced trafficking, mainly through the slow or late endosome recycling pathway (32). Furthermore, the interaction with Rab11a was dependent on a 22-amino acid hydrophobic sequence within the C-tail domain of the hIP, adjacent to TM7 and bearing certain structural similarity to the RBD first identified within members of the FIPs/Rab11 effector proteins (5). The current study confirmed that the interaction between the hIP and Rab11a occurs in human platelets and is regulated in response to agonist.

Palmitoylation or, more correctly, thio(lys)-acylation, is a post-translational modification that occurs through attachment of C16 palmitate to Cys residue(s) via labile thioester bonds and is thought to regulate the association of proteins or domains thereof with membranes in a dynamic or transient manner (27, 45, 46). A large number of GPCRs contain at least one palmitoylable Cys typically located some 10–14 amino acids downstream of the palmitoylation site. In the case of the hIP, in addition to its established interaction between the hIP and Rab11a and to identify the key structural determinants of the RBD.

Based on the experimental and computational data presented herein, an overall model is presented in Fig. 9 to explain the direct and specific interaction of the hIP with Rab11a in regulating its intracellular trafficking following agonist stimulation. It is proposed that a major part of the RBD (Val<sup>299</sup>–Leu<sup>307</sup>) overlaps with the putative α-H8 domain located within the cytoplasmic C-tail region, in proximity to TM7, and adjacent to the palmitoylated residues at Cys<sup>308</sup>–Cys<sup>311</sup>. It is noteworthy that the α-H8 equivalent of several other GPCRs, including that of rhodopsin and the A<sub>2A</sub> adenosine receptor, have been proposed to lie perpendicular to the TM bundle in the presence of an interface, such as when associated in a lipid or micelle environment, but may adopt a looplike or disordered structure in an aqueous environment or in the conformationally active receptor (35, 48, 49). Moreover, the putative α-H8 domains in many of those receptors, as exemplified by rhodopsin or the β<sub>2</sub> adrenergic receptor, contain adjacent palmitoylated Cys residues (35, 36). As outlined in Fig. 9C, it is proposed that Cys<sup>311</sup> is the major site of palmitoylation either in the absence or presence of agonist and is required to anchor or orientate the α-H8 domain of the hIP for interaction with Rab11a. Moreover, cicaprost-regulated palmitoylation, particularly deacylation at Cys<sup>309</sup> > Cys<sup>308</sup>, may allow for agonist-
induced conformational flexibility within α-H8, as proposed for other GPCR equivalents, to allow for optimal agonist-dependent interaction with Rab11a. Consistent with the proposed requirement and role for palmitoylation of Cys311, it is the most abundantly palmitoylated residue, and all variants carrying that hIPC311S mutation, either alone or in combination, internalize but remain in endocytic vesicles and do not recycle or co-localize with Rab11a. Consistent with the proposed requirement and role for palmitoylation of Cys308 and Cys309 to regulate the agonist-induced association with Rab11a, these are the residues that are most sensitive to agonist-induced deacylation and, in the case of the hIPC308S and hIPC309S variants, still show a limited agonist-induced association/immunoprecipitation with Rab11a. Notably also, both the single hIPC308S and hIPC309S undergo recycling, somewhat similar to that of the hIPWT, whereas the double hIPC308S,C309S does not, suggesting that palmitoylation at either residue at any given time may be required. Moreover, studies in yeast involving the hIPC309S and hIPC309A highlight a critical role for Cys309 for the interaction with Rab11a. It is proposed that failure of the Y2H studies to identify the critical role for Cys308, as in the case of the single hIPC308S or hIPC309A, for interaction with Rab11a is likely to be due to compensatory palmitoylation at the adjacent Cys309 residue and/or due to the preferred palmitoylation of
Cys$^{309}$, as opposed to Cys$^{308}$, by the yeast palmitoyl transferase. Moreover, failure of the Y2H studies to identify the critical role for Cys$^{311}$, as in the case of the single hIP$^{C311S}$ or hIP$^{C311A}$, is likely to be due to fact that the proposed membrane association role of the palmitoyl-Cys$^{311}$ would not be required, or indeed be permissible, for the Y2H “bait versus prey” interaction and resulting GAL4-dependent transcription to occur (supplemental Fig. 1C). Despite showing normal maturation/expression at the plasma membrane, as evidenced by both radioligand binding data (27) and extensive immunolocalization data herein, each of the hIP variants harboring the C311S mutation stably expressed in HEK 293 cells showed altered or delayed patterns of glycosylation. Hence, because the hIP$^{C308S,C311S}$ and hIP$^{C308S,C309S,C311S}$, either stably or transiently expressed (27) (this work), did not display normal agonist-induced signaling, despite normal radioligand binding, expression at the plasma membrane, and agonist-induced internalization, the possibility that their altered recyclyization/co-localization with the plasma membrane, and agonist-induced internalization, the possibility that their altered recyclyization/co-localization with Rab11a may be due to non-functional receptors, as opposed to altered palmitoylation, cannot be fully excluded. However, the fact that the hIP$^{C311S}$ and hIP$^{C309S,C311S}$ variants actually displayed normal signaling but, similar to hIP$^{C308S,C311S}$ and hIP$^{C308S,C309S,C311S}$, did not show co-localization with Rab11a or recyclyization in response to agonist stimulation makes this less likely. Hence, these data strongly suggest that the impairment of Rab11a-mediated recyclyization of all of the C311S variants is due to impaired palmitoylation as opposed to altered glycosylation. It will be of interest to determine whether the hIP$^{C308S,C309S,C311S}$ undergoes agonist-induced translocation and association with Rab5a (Fig. 9A) and subsequent recyclyization or association with Rab11a (Fig. 9B) and mediate intracellular trafficking (this work).

Thus, although residues within the RBD are critical for the agonist-induced trafficking of the hIP, such as by orienting and/or providing possible flexibility to the RBD, it is proposed that hydrophobic residues mainly orientated on one face of the RBD domain provide the binding surface to which Rab11a may or may actually bind (Fig. 9, A and B). Although studies in yeast and co-immunoprecipitations in HEK 293 cells suggest that positively charged Arg$^{302}$ and Lys$^{304}$ residues may also be important in influencing the interaction between the α-H8 with Rab 11a, this is inconclusive due to the fact that the G304A did not display normal maturation/expression at the plasma membrane (supplemental Fig. 8B). The reason for the failure of the latter hIP$^{R302A}$ and hIP$^{K304A}$ variants to mature and traffic to the plasma membrane is unclear and will be the subject of a separate investigation, being beyond the scope of the current study.

The putative α-H8 has been proposed to act as a conformational switch between the active and inactive states of certain other GPCRs (35, 36). Moreover, peptides based on that domain from several GPCRs have been proposed both to be conformationally flexible, depending on the solvent environment (35, 36), and to inhibit signaling by rhodopsin (50), the β$_2$ adrenergic receptor (51), and the cannabinoid 1 receptor (52), and to activate G$_{i/o}$ proteins (53). To our knowledge, data presented in this study represent the first demonstration of a direct interaction between the α-H8 of any GPCR with Rab11a. Given the conservation of the α-H8 along with the presence of adjacent palmitoylatable Cys residue(s), it is indeed formally possible that such an interaction may also occur between other GPCRs and/or other members of the Rab11 or wider Rab superfamily. Indeed, the first GPCR proposed to have a direct interaction with Rab11 is the TPβ isofrom of human TXA$_2$ receptor, where amino acids 335–344 within its C-tail were proposed to be the critical RBD therein (7). Due to the presence of Cys$^{347}$, a residue established to be palmitoylated (38), in close proximity to the RBD within TPβ, somewhat similar to that found within the RBD of the hIP, the role of palmitoylation in influencing the interaction between TPβ and Rab11 was investigated herein but was found not to be significant in modulating that interaction. However, a notable difference between the RBD within the hIP and that of the TPβ is that the region of interaction in the latter receptor, namely residues 335–344 (7), does not actually map to the α-H8 but rather to an additional putative α-helical domain within its C-terminal tail region.

The identification of Rab11a筷adders to a limited list of proteins that directly interact with Rab11 members, which also includes certain other members of the Rab superfamily and the α5β1 integrin (54), or Rip11 in regulating insulin-dependent GLUT 4 transport in adipocytes (61), the identification of the Rab11 effector(s) involved in coordinating the agonist-dependent recycling of the α5β1 integrin (54), or Rip11 in regulating insulin-dependent GLUT 4 transport in adipocytes (61), the identification of the Rab11 effector(s) involved in the trafficking of the hIP after agonist stimulation remains to be investigated. Given the exquisitely specific relationship that exists between certain Rab11 members and their effectors, as exemplified by Rab25 and Rab-coupling protein in coordinating the agonist-dependent recycling of the α5β1 integrin (54), that direct the trafficking of the hIP after agonist stimulation remains to be investigated. Given the exquisite specific relationship that exists between certain Rab11 members and their effectors, as exemplified by Rab25 and Rab-coupling protein in coordinating the agonist-dependent recycling of the α5β1 integrin (54), Rip11 in regulating insulin-dependent GLUT 4 transport in adipocytes (61), the identification of the Rab11 effector(s) involved in the trafficking of the hIP is likely to add significantly to the understanding of the true physiologic significance of the direct interaction between the hIP and Rab11.

REFERENCES

1. Pfeffer, S. R. (2005) J. Biol. Chem. 280, 15485–15488
2. Schwartz, S. L., Cao, C., Plypenko, O., Rak, A., and Wandinger-Ness, A. (2007) J. Cell. Sci. 120, 3905–3910
3. Gould, G. W., and Lippincott-Schwartz, J. (2009) Nat. Rev. Mol. Cell Biol. 10, 287–292
4. Seachrist, J. L., and Ferguson, S. S. (2003) Life Sci. 74, 225–235
5. Horgan, C. P., Oleksy, A., Zhdanov, A. V., Lall, P. Y., White, I. J., Khan, A. R., Futter, C. E., McCaffrey, J. G., and McCaffrey, M. W. (2007) Traffic 8, 414–430
6. Prekeris, R. (2003) Science World J. 3, 870–880
7. Hamelin, E., Thériault, C., Laroche, G., and Parent, J. L. (2005) J. Biol. Chem. 280, 36195–36205
8. Parent, A., Hamelin, E., Germain, P., and Parent, J. L. (2009) Biochem. J. 418, 163–172
9. Takahashi, M., Ishiko, T., Kamohara, H., Hidaka, H., Ikeda, O., Ogawa, M., and Baba, H. (2007) Mediators Inflammm. 2007, 10767
10. Cheng, K. W., Ladah, J. P., Gray, J. W., and Mills, G. B. (2005) Cancer Res. 65, 2516–2519
11. Cheng, K. W., Ladah, J. P., Kuo, W. L., Lapuk, A., Yamada, K., Auersperg,
Interaction of the Human Prostacyclin Receptor with Rab11: CHARACTERIZATION OF A NOVEL Rab11 BINDING DOMAIN WITHIN α-HELIX 8 THAT IS REGULATED BY PALMITOYLATION

Helen M. Reid, Eamon P. Mulvaney, Elizebeth C. Turner and B. Therese Kinsella

J. Biol. Chem. 2010, 285:18709-18726.
doi: 10.1074/jbc.M110.106476 originally published online April 15, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.106476

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2010/04/15/M110.106476.DC1

This article cites 61 references, 26 of which can be accessed free at
http://www.jbc.org/content/285/24/18709.full.html#ref-list-1

WITHDRAWN
July 9, 2018
Interaction of the human prostacyclin receptor with Rab11. CHARACTERIZATION OF A NOVEL Rab11 BINDING DOMAIN WITHIN α-HELIX 8 THAT IS REGULATED BY PALMITOYLATION.

Helen M. Reid, Eamon P. Mulvaney, Elizebeth C. Turner, and B. Therese Kinsella

In Fig. 2A, the images used to represent anti-Rab11 immunoblots (IB) from HEK 293 cells overexpressing WT and C308S,C309S,C311S mutant human prostacyclin receptor (hIP) were mistakenly used to also represent immunoblots from control TPβC347S,C573S,C377S and β-Gal cells, respectively. The correct anti-Rab11 immunoblot images for the control TPβC347S,C573S,C377S and β-Gal cells have been inserted into Fig. 2A. This correction does not affect the interpretation of the results or the conclusions of this work.

In addition, the same images of yeast colonies used to represent deletion mutagenesis results in Fig. 7B were mistakenly used to also represent the results of alanine-scanning mutagenesis experiments in Fig. 8A. The duplicated images have been replaced with the correct images in the revisedig. 8A. This correction does not affect the interpretation of the results or the conclusions of this work.

Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.