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Investigation of a functional requirement for isoprenylation by the human prostacyclin receptor

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In the current study, we have established that the human (h) prostacyclin receptor (IP) is isoprenylated in whole cells. Through site directed mutagenesis and generation of the isoprenylation defective hIP^{SSLC}, it was established that while isoprenylation of hIP does not influence ligand binding, it is obligatory for agonist activation of adenylyl cyclase and cAMP generation. Overexpression of $G\alpha_S$ significantly augmented cAMP generation by the hIP but not by the hIP^{SSLC}. Moreover, $G\alpha_S$ co-immunoprecipitated with hIP following agonist activation but did not co-immunoprecipitate with hIP^{SSLC}. Whereas hIP mediated concentration-dependent activation of phospholipase C (PLC); the extent of PLC activation by hIP^{SSLC} was impaired compared to hIP. Co-expression of $G\alpha_q$ significantly augmentated intracellular calcium mobilization by the hIP but not by hIP^{SSLC}. Moreover, whereas $G\alpha_q$ co-immunoprecipitated with hIP, it failed to co-immunoprecipitate with hIP^{SSLC}. While both the hIP and hIP^{SSLC} underwent agonist-induced internalization, the kinetics and extent of hIP^{SSLC} internalization was impaired compared to hIP. Altering the CAAX motif of the hIP from a farnesyl (–CSLC) to a geranylgeranyl (–CSLL) isoprene acceptor, to generate hIP^{CSLL}, did not affect ligand binding and yielded a receptor that exhibited identical signalling through both G_s- and G_q-coupled effectors to that of hIP.

Thus, whereas isoprenylation of hIP does not influence ligand binding, it is functionally imperative in regulating post-receptor events including agonist-activation of adenylyl cyclase, for efficient activation of PLC and for receptor internalization. Though the nature of the isoprenoid attached to hIP does not act as a major determinant, the presence of an isoprenoid group, for example farnesyl or geranylgeranyl, is required for functional receptor–G protein interaction and coupling and for efficient agonist-induced receptor internalization.

Keywords: prostacyclin; receptor; isoprenylation; internalization; signaling.

The prostanoid prostacyclin [prostaglandin (PG)I_2] plays a key role in the local control of vascular hemostasis acting as a potent inhibitor of platelet aggregation and as an endothelium derived vasodilator [1]. It exerts pro-inflammatory and antiproliferative properties *in vitro* [2,3] and may offer a cytoprotective effect against tissue injury during acute myocardial ischemia or in response to hypoxia in vascular endothelial cells [4]. Knockout mice deficient in prostacyclin receptors exhibit an impaired response to thrombotic stimuli [2] and develop more severe pulmonary hypertension and vascular remodelling following chronic hypoxic exposure [5] relative to wild-type mice. Within the vasculature, the actions of prostacyclin generally counteract those of thromboxane A_2 and, thus, the relative levels of these two prostanoids in the circulation are central to the maintenance of vascular hemostasis and tone [6,7].

Prostacyclin signals via interaction with its specific cell surface G protein-coupled receptor (GPCR) termed the prostacyclin receptor or IP [8]. IP is primarily coupled to activation of adenylyl cyclase, with concomitant rises in cAMP levels, but may also couple to activation of phospholipase C (PLC), leading to phosphatidyl inositol turnover and mobilization of intracellular calcium ($[Ca^{2+}]_i$) [7,9–13]. The mouse (m)IP may couple to G_s-, G_i- and G_q-regulated effector systems in a mechanism involving cAMP dependent protein kinase A (PKA)-mediated phosphorylation and switching from G_s- to G_i- and to G_q-coupled effector signalling [14]. A number of independent studies have established that the IP undergoes rapid agonist-mediated receptor internalization and down-regulation in human platelets and other cell types, providing an important mechanism of regulating cellular responses to prostacyclin *in vivo* [15–18].

Isoprenylation is a post-translational modification whereby C15 farnesyl or C20 geranylgeranyl isoprenoids, derived from the mevalonate/cholesterol biosynthetic pathway, are attached via stable thioether linkages to specific C-terminal cysteine residues located in distinct 'isoprenylation motifs' of proteins, most commonly within 'CAAX' motifs where C represents the target isoprene acceptor cysteine [19]. A major determinant of whether a protein is farnesylated or geranylgeranylated depends on the nature of the residue in the X position [20]. Of the three isoprenylation sequence

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Abbreviations: cAMP, adenosine 3′, 5′-cyclic monophosphate; $[Ca^{2+}]_i$, intracellular calcium; ELISA, enzyme-linked immunosorbent assay; FTase, farnesyl protein transferase; GGTase, geranylgeranyl protein transferase; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HMG CoA, hydroxy methyl glutaryl coenzyme A; IP, prostacyclin receptor, PG, prostaglandin; PLC, phospholipase C; PVDF, poly(vinylidene difluoride).

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motifs that have been identified, a putative consensus CAAX motif, with the sequence CSLC, exists at the C-termini of the IP from a number of species [21–23]. We have recently confirmed that the mIP may be somewhat unique among GPCRs in that it is isoprenylated in whole cells (*in vivo*) through attachment of a C15 farnesyl isoprenoid and that recombinant forms of both the mIP and human (h)IP may be isoprenylated *in vitro* [13]. Whereas isoprenylation of mIP was shown not to influence ligand binding, it is absolutely required for receptor activation of adenylyl cyclase and for its efficient coupling to PLC [13].

The statins, widely used in the management of hypercholesterolemia and in the prevention of coronary heart disease, act by blocking the endogenous synthesis of cholesterol through the competitive inhibition of hydroxy methylglutaryl coenzyme A (HMG CoA) reductase, the rate-limiting enzyme of cholesterol biosynthesis [24]. Mevalonate, the product of the HMG CoA reductase reaction, is however, the precursor of many nonsteroidal isoprenoid metabolites including farnesyl and geranylgeranyl pyrophosphate. Thus, statin therapy may not only lower cholesterol but may exhibit pleiotrophic effects not necessarily associated with their cholesterol lowering properties and may, for example, potentially interfere with protein isoprenylation. In a recent study, the statins cerivastatin and lovastatin were shown to impair mIP and hIP coupling to G_s- and G_q-mediated effector signalling [25], most likely by inhibiting the endogenous production of mevalonate and thus potentially interfering with downstream isoprenylation. Other therapeutic agents that can interfere with protein isoprenylation are of course the farnesyl protein transferase (FTase) inhibitors currently under development as novel anti-$p21^{ras}$ chemotherapeutic agents [26]. The fact that the farnesyl moiety of $p21^{ras}$ is absolutely required for its membrane association and oncogenic transformation, coupled to the fact that most isoprenylated proteins contain C20 geranylgeranyl group(s) rather than the C15 farnesyl moiety found on all cellular forms of ras (H-, K- and N-), represents the basis for the development of the FTase inhibitors as novel anti-$p21^{ras}$ chemotherapeutics [26]. However, the lack of specificity of the FTase inhibitors under development in exclusively targeting the ras proteins strongly cautions that these FTase inhibitors may potentially interfere with the isoprenylation of other farnesylated proteins, such as the hIP.

In this study, in view of the central role of the IP in the regulation of vascular hemostasis coupled to the fact that it may be a potential indirect target of a number of important therapeutics in humans, such as the statins and the FTase inhibitors, we thought it imperative to establish whether the hIP is isoprenylated in whole cells (*in vivo*) and to investigate the role of isoprenylation with respect to its G protein coupling characteristics and effector signalling. We also sought to investigate the role of isoprenylation in mediating internalization/desensitization of the hIP in response to agonist activation. Moreover, through site directed mutagenesis, whereby the wild-type receptor hIP^{CSLC} was mutated to hIP^{CSLL}, we investigated the effects of altering the CAAX motif of the hIP from a putative farnesyl acceptor motif to that of a putative geranylgeranyl motif on the functional properties of the hIP.

EXPERIMENTAL PROCEDURES

Materials

Cicaprost was obtained from Schering AG (Berlin, Germany). Fura2/AM was purchased from Calbiochem. [^{3}H]mevalonolactone (15–30 Ci·mmol^{-1}) and [^{3}H]cAMP (15–30 Ci·mmol^{-1}) were purchased from American Radiolabeled Chemicals Inc. [^{3}H]Iloprost (15.3 Ci·mmol^{-1}) was purchased from Amersham Pharmacia Biotech. Poly(vinylidene difluoride) (PVDF) filters, *Taq* DNA Polymerase, Chemiluminescence Western blotting kit, and rat monoclonal 3F10 peroxidase-conjugated anti-hemagglutinin (HA) serum were purchased from Roche Molecular Biochemicals. Oligonucleotides were synthesized by Genosys Biotechnologies. Anti-$G\alpha_s$ serum (K-20), Anti-$G\alpha_{q/11}$ serum (C-19) and horseradish peroxidase-conjugated goat anti-(rabbit IgG) serum were obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-(HA 101R) serum was obtained from BabCO. Secondary fluorescein isothiocyanate (FITC)-conjugated anti-(mouse IgG) serum was purchased from Jackson Immunoresearch laboratories, Inc.

Subcloning and site-directed mutagenesis of human prostacyclin receptor

Site directed mutagenesis of codon 383, to mutate Cys383 to Ser383 within the C-terminal tail of hIP, was performed by PCR mutagenesis using pHM6:hIP as a template [13] and the oligonucleotide primers: 5′-dGAGAAGCTTATGGCG GATTCGTGCAGG-3′ (sense primer; the sequence corresponding to the initiation codon is underlined) and 5′-dT AGGAATTCAGCAGAGGGA**G**GAGGCGACGCT-3′ (antisense primer; the sequence complimentary to the single mutator base is highlighted in boldface). Similarly, site directed mutagenesis of codon 386, to mutate Cys386 to Leu386 within the C-terminal tail of hIP, was performed using pHM6:hIP as a template and the oligonucleotide primers: 5′-dGAGAAGCTTATGGCGGATTCGTG CAGG-3′ (sense primer; the sequence corresponding to the initiation codon is underlined) and 5′-dATATGA ATTCA**CA**AGAGGGAGCAGGCGACGCT-3′ (antisense primer; the sequence complimentary to the mutator bases is highlighted in boldface). The full length cDNAs encoding the mutants $hIP^{S383SLC}$ and $hIP^{CSLL386}$, herein designated hIP^{SSLC} and hIP^{CSLL}, respectively, were subcloned inframe into the *Hin*dIII–*Eco*RI sites of pHM6 to generate the plasmids pHM6:hIP^{SSLC} and pHM6:hIP^{CSLL}. Subcloning of hIP^{SSLC} and hIP^{CSLL} into pHM6 facilitated their expression with an amino-terminal epitope tag corresponding to the 9-amino-acid hemagglutinin (HA) epitope tag [27]. All plasmids were verified by dsDNA sequencing, using SEQUENASE Version 2.0 (United States Biochemical Corp.).

Stable cell lines and transfections

Human embryonic kidney (HEK) 293 cells were cultured in minimal essential medium (MEM) with Earle's salts, 10% fetal bovine serum. HEK.hIP cells stably over-expressing a hemagglutinin (HA)- epitope tagged form of the wild type hIP have been previously described [25]. To create the HEK.hIP^{SSLC} and HEK.hIP^{CSLL} stable cell lines, HEK 293

cells (2×10^6 cells per 10-cm dish) were transfected with 10 μg of *Sca*1-linearized pADVA plus 25 μg of *Pvu*1-linearized pHM6:hIP^{SSLC} or pHM6:hIP^{CSLL}, respectively, using the calcium phosphate/DNA coprecipitation procedure [28]. Forty-eight hours post transfection, G418 (0.8 mg·mL^{-1}) selection was applied and, after ≈ 21 days, G418 resistant colonies were selected. Individual HEK.hIP^{SSLC} and HEK.hIP^{CSLL} stable cell isolates were examined for IP expression by radioligand binding assay. The plasmids pCMV5, pCMV:Gα_q and pCMV:Gα_S, encoding the short form of Gα_S, have been previously described [13,28]. To enhance recombinant protein expression, HEK 293 cells were cotransfected with the previously described plasmid pADVA [29]. Thus, for transient transfections, cells were routinely transfected with pHM, pcDNA-, pCMV- or pMEV based vector (25 μg per 10-cm dish) plus pADVA (10 μg per 10-cm dish) using the calcium phosphate/DNA coprecipitation procedure [28] and cells were harvested 48 h post transfection.

Isoprenylation of prostacyclin receptor in transfected HEK 293 cells

To investigate isoprenylation of the hIP *in vivo*, metabolic labelling of whole cells was performed essentially as previously described [13]. Briefly, HEK.hIP, HEK.hIP^{SSLC} and HEK.hIP^{CSLL} cells were transiently transfected with pMEV, encoding the membrane-bound mevalonolactone transporter [30] plus pADVA. As a positive control, HEK 293 cells were transiently cotransfected with pHM6:HA-ras plus pMEV (25 μg per 10-cm dish) plus pADVA (10 μg per 10-cm dish) or, as a negative control, with pHM6 plus pMEV (25 μg per 10-cm dish) plus pADVA (10 μg per 10-cm dish). At 36 h post transfection, lovastatin (15 μM) was added to deplete the intracellular pool of mevalonate and its metabolites. After 12 h, the medium was replaced with fresh medium (3 mL MEM plus 10% fetal bovine serum per 10-cm dish) containing lovastatin (20 μM) and 150 μCi [^{3}H]mevalonolactone (15–30 μCi·mmol^{-1}) or, as a control, in the presence of an equivalent concentration of nonradiolabelled mevalonolactone. Following incubation at 37 °C for 22 h, the cells were harvested and aliquots (70 μg) of whole cell protein were resolved by SDS/PAGE and electroblotted onto PVDF membrane. The remaining whole cell protein (600 μg) was subjected to immunoprecipitation using the anti-(HA 101r) serum as previously described [31]. Immunoprecipitates were then resolved by SDS/PAGE followed by electroblotting onto PVDF membrane. Blots were soaked in Amplify for 30 min followed by autoradiography using Kodak Xomat XAR film for 60 days at −70 °C. Thereafter, membranes were screened by immunoblot analysis using the peroxidase conjugated anti-(HA 3F10) serum followed by chemiluminescence detection. In parallel experiments, immunoprecipitation of nonmetabolically labelled hIP, hIP^{SSLC}, hIP^{CSLL} and HA-ras followed by immunodetection was performed to confirm equal protein loading and expression.

Radioligand binding studies

Cells were harvested by centrifugation at 500 ***g*** at 4 °C for 5 min followed by washing three times with phosphate-buffered saline (NaCl/P$_i$). Following their resuspension in Homogenization Buffer (25 mM Tris/HCl, pH 7.5, 0.25 M sucrose, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride), cells were homogenized and then centrifuged at 100 000 ***g***, 40 min at 4 °C. The pellet fractions (P$_{100}$), representing crude membranes, were resuspended in resuspension buffer (10 mM Mes/KOH, pH 6.0, 10 mM $MnCl_2$, 1 mM EDTA, 10 mM indomethacin). Protein determinations were carried out using the Bradford assay [32]. Radioligand binding assays were carried out at 30 °C for 1 h in a final assay volume of 100 μL using 35–100 μg of membrane (P$_{100}$ fraction) protein per assay in the presence of 4 nM [^{3}H]iloprost (15.3 Ci·mmol^{-1}), for saturation binding studies, or in the presence of 0.1–200 nM [^{3}H]iloprost for Scatchard analysis. Nonspecific binding was determined in the presence of 0.2 mM iloprost, for saturation binding studies, or in the presence of 500-fold molar excess of nonlabelled iloprost for Scatchard binding isotherm studies. Reactions were terminated by the addition of 4 mL ice-cold resuspension buffer followed by filtration through Whatman GF/C filters; filters were washed three times with resuspension buffer (3 mL per wash) and then subject to liquid scintillation counting in scintillation fluid (5 mL per filter). Radioligand binding data was analysed using the PRISM 2 computer program (GRAPHPAD Software Inc., San Diego, CA, USA) to determine the K_d and B_{max} values. The suitability of both one-site and two-site binding models was examined using the *F*-test. The level of significance of the results of the *F*-test were tested to $p < 0.05$.

Measurement of cAMP

cAMP assays were carried out as described previously [13]. Briefly, cells were harvested by scraping and washed three times in ice-cold NaCl/P$_i$; cells (≈ $1–2 \times 10^6$ cells) were resuspended in 200 μL of Hepes/NaCl/P$_i$ (140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose, 15 mM Hepes/NaOH, pH 7.4) containing 1 mM 3-isobutyl-1-methylxanthine and were preincubated at 37 °C for 10 min. Thereafter, cells were stimulated for 10 min with 10^{-12}–10^{-6} M cicaprost (50 μL). As a control, cells were incubated in the presence of 50 μL Hepes/NaCl/P$_i$ in the absence of cicaprost. To examine the effect of cotransfection of Gα_s on cAMP generation, HEK 293, HEK.hIP, HEK.hIP^{SSLC} and HEK.hIP^{CSLL} cells were transiently cotransfected with pCMV:Gα_s (25 μg per 10-cm dish) plus pADVA (10 μg per 10-cm dish).

In each case, cAMP reactions were terminated by heat inactivation at 100 °C for 5 min and the level of cAMP produced was quantified using the cAMP binding protein assay [13]. Levels of cAMP produced by ligand-treated cells over basal stimulation, determined in the presence of Hepes/NaCl/P$_i$, were determined as pmol cAMP per mg cell protein and are expressed as fold stimulation relative to basal (fold increase ± SEM). Data were analyzed using an unpaired Student's *t*-test. *P*-values of less than or equal to 0.05 were considered to indicate a statistically significant difference.

Measurement of intracellular Ca^{2+} mobilization

Measurements of $[Ca^{2+}]_i$ in FURA2/AM preloaded cells were carried out essentially as previously described [28]. Cicaprost was diluted in HBSSHB (modified Ca^{2+}/Mg^{2+}-

free Hanks' buffered salt solution containing 20 mM Hepes, pH 7.67, 0.1% BSA plus 1 mM $CaCl_2$) to the appropriate concentration such that addition of 20 μL of the diluted cicaprost to 2 mL of cells resulted in the correct working concentrations (10^{-8}–10^{-5} M). In separate experiments, to examine the effect cotransfection of $G\alpha_q$ on $[Ca^{2+}]_i$ mobilization, HEK.hIP, HEK.hIP^{SSLC} and HEK.hIP^{CSLL} cells were transiently cotransfected with pCMV:$G\alpha_q$ (25 μg per 10-cm dish) plus pADVA (10 μg/ per 10-cm dish). For each $[Ca^{2+}]_i$ measurement, calibration of the fluorescence signal was performed in 0.2% Triton X-100 to obtain the maximal fluorescence (*R*max) and 1 mM EGTA to obtain the minimal fluorescence (R_{min}). The ratio of the fluorescence at 340 and 380 nm is a measure of $[Ca^{2+}]_i$ assuming a K_d of 225 nM Ca^{2+} for FURA2/AM. The results presented in the figures are representative data from at least four independent experiments and are plotted as changes (Δ) in $[Ca^{2+}]_i$ mobilized as a function of time(s) upon ligand stimulation or, alternatively, were calculated as mean changes in $[Ca^{2+}]_i$ mobilized ($\Delta[Ca^{2+}]_i \pm$ SEM; $n = 4$).

Co-immunoprecipations of the hIP and its associated G-proteins

HEK.hIP, HEK.hIP^{SSLC}, HEK.hIP^{CSLL} or HEK 293 cells were transfected with pADVA (10 μg per 10-cm dish) and pCMV:$G\alpha_q$ or pCMV:$G\alpha_s$ encoding the short form of $G\alpha_s$, or as a negative control with pCMV5 (25 μg per 10-cm dish) using the calcium phosphate/DNA coprecipitation procedure [28]. After 48 h post-transfection, cells were stimulated with 1 μM cicaprost for 10 min at 37 °C, 5% CO_2 or, as a control, with an equivalent volume of the vehicle Hepes/NaCl/P_i. Reactions were terminated and HA-tagged IP receptors were immunoprecipitated using the anti-(HA 101R) serum (1 : 300) essentially as previously described [31]. Immunoprecipitates were resuspended in 1 × solubilization buffer [10% 2-mercaptoethanol (v/v), 2% SDS (w/v), 30% glycerol (v/v), 0.025% bromophenol blue (w/v), 50 mM Tris/HCl, pH 6.8; 100 μL], were boiled for 5 min, resolved by SDS/PAGE and were electro-blotted onto poly(vinylidene difluoride) (PDVF) membranes. Thereafter, membranes were screened with either the anti-$G\alpha_s$ serum (K-20) (1 : 3000) or anti-$G\alpha_{q/11}$ serum (C-19) (1 : 3000) antisera followed by secondary screening with horseradish peroxidase-conjugated goat anti-(rabbit IgG) serum (1 : 3000). Membranes were then stripped and reprobed using the anti-(HA 3F10) serum horseradish peroxidase conjugate (1 : 500) to check for quantitative recovery of each receptor type. All immunoreactive proteins were visualized using the chemiluminescence detection system essentially as described by the manufacturer (Roche).

Internalization assays

For quantification of receptor internalization, ELISA assays were performed essentially as previously described [33]. Briefly, 96-well plates were coated with poly L-lysine (0.1 g·mL^{-1}; 1 min). Thereafter, HEK.hIP and HEK.hIP^{SSLC} cells were seeded at 1×10^3 cells per well. After 48 h, the medium was replaced with fresh medium (MEM, 10% fetal bovine serum) containing varying concentrations of cicaprost (10^{-12}–10^{-6} M) for 4 h. For time-course studies, cells were exposed to cicaprost (1 μM in MEM, 10% fetal bovine serum) for 0–4 h. The cells were then washed three times with NaCl/P_i followed by fixing with 2% formaldehyde in NaCl/P_i, pH 7.4 for 15 min at room temperature. Following washing, nonspecific binding was blocked with NaCl/Tris (20 mM Tris-Cl, pH 7.2, 0.1 M NaCl) containing 2% dried skimmed milk powder for 1 h at room temperature Thereafter, the cells were incubated with the anti-(HA 101r) serum (1 : 2000 in NaCl/Tris, 2% dried milk powder) for 1 h at room temperature. Following washing of the cells three times with NaCl/P_i, the cells were incubated with horseradish peroxidase-conjugated goat anti-(mouse IgG) serum (1 : 2000 in NaCl/Tris, 2% dried milk powder). After 1 h, the cells were washed three times with NaCl/P_i followed by the addition of a colorimetric alkaline phosphatase substrate (75 μL; K-blue substrate, Neogen Corp). After 10 min, the reactions were terminated by the addition of stop solution (20 μL; Neogen Corp). The A_{650} measurements were determined using a SpectraMax 190 ELISA plate reader (Molecular Devices). Internalization of HA-tagged receptors was quantified as the mean percentage (%) of cell surface receptor in agonist treated cells relative to vehicle (NaCl/P_i) treated cells ± SEM, where control, vehicle treated cells were assigned a value of 100%.

Immunofluorescence analysis

Immunofluorescence analysis was performed essentially as previously described [34]. Briefly, coverslips (2.2 cm^2) were coated with poly L-lysine (0.1 g·mL^{-1}; 1 min). HEK.hIP and HEK.hIP^{SSLC} cells were seeded at 0.5×10^5 cells per 10-cm dish in MEM, 10% fetal bovine serum. After 24 h, the cells were stimulated with cicaprost (1 μM; 4 h) with vehicle (NaCl/P_i) treated cells serving as a control. The cells were washed twice with NaCl/P_i followed by fixing of the cells with 2% formaldehyde in NaCl/P_i, pH 7.4 for 15 min at room temperature followed by washing three times with NaCl/P_i. The cells were then rinsed with Blotto solution (20 mM Tris-Cl, pH 7.2, 0.1 M NaCl, 5% dried skimmed milk powder) followed by incubation with the anti-(HA 101r) serum (1 : 2000) for 1 h at room temperature. Following washing of the cells three times with NaCl/P_i, cells were incubated with secondary FITC-conjugated anti-(mouse IgG) serum (1 : 120 dilution) for 30 min at room temperature. Cells were then washed once with NaCl/P_i, followed by gentle rinsing with distilled H_2O. Slides were then mounted followed by immunofluorescent localization of hIP and hIP^{SSLC} using an Axioplan 2 imagine fluorescence microscope (Zeiss Vision GmbH, Munich, Germany).

Data analyses

Statistical analysis was carried out using the unpaired Student's *t*-test using GRAPHPAD PRISM v2.0 programme (GraphPad Software Inc., San Diego, CA, USA). *P*-Values of less than or equal to 0.05 were considered to indicate a statistically significant difference. Radioligand binding data was analysed using the PRISM 2 computer program (GraphPad Software Inc., San Diego, CA, USA) to determine the K_d and B_{max} values. The suitability of both one-site and two-site binding models was examined using the *F*-test. The level of significance of the results of the *F*-test were tested to $p < 0.05$.

RESULTS

Isoprenylation of the human prostacyclin receptor in whole cells

We have previously established that a recombinant form of the hIP may be isoprenylated through attachment of a C15 farnesyl isoprenoid *in vitro* [13]. Thus, to investigate whether the hIP is isoprenylated in whole cells, mammalian cell lines stably over-expressing hemagglutinin (HA) epitope tagged forms of the wild type hIP (HEK.hIP cells) and its site directed variant hIP^{SSLC} ($HEK.hIP^{SSLC}$ cells), in which the Cys383 from the putative CAAX motif of hIP was mutated to Ser383, were metabolically labelled in the presence of [^{3}H]mevalonolactone. Initially, saturation radioligand binding studies confirmed comparable levels of hIP and hIP^{SSLC} expression in their respective cell lines (Table 1). To optimize the efficiency of metabolic labelling, cells were transiently cotransfected with pMEV to enhance the cellular uptake of [^{3}H]mevalonolactone and, to deplete the intracellular pool of mevalonate and its metabolites, cells were preincubated with and metabolically labelled in the presence of the HMG-CoA reductase inhibitor lovastatin. As a positive control for the assay conditions, isoprenylation of a HA-epitope tagged form of Harvey (H)-ras, herein designated HA-ras, was also examined. Efficient labelling of HA-ras (Fig. 1A, lane 4) and of endogenous isoprenylated proteins (Fig. 1A, lane 5) were observed. In immunoprecipitation experiments using the monoclonal anti-(HA 101R) Ig, the identity of the 21–23 kDa protein to be that of HA-ras was confirmed (Fig. 1B, lane 4). It was noteworthy that three other isoprenylated proteins between the 46–66 kDa molecular mass markers were evident in the immonoprecipitates and were present in all lanes (Fig. 1B, lanes 1–5) and therefore most likely correspond to nonspecific immunoprecipitated proteins. The presence of an additional isoprenylated protein of 43 kDa in the immunoprecipitates from HEK.hIP, but not from $HEK.hIP^{SSLC}$ or HEK 293 cells, indicated that the hIP is indeed isoprenylated in whole cells (Fig. 1B, compare lanes 1–2 and 5). In parallel experiments, immunoblot analyses confirmed the expression of hIP, hIP^{SSLC} and HA-ras in the immunoprecipitates from HEK.hIP, $HEK.hIP^{SSLC}$ and HEK 293 cells transfected with HA-ras (Fig. 1C, lanes 1, 2 and 4), but not from control HEK 293 cells (Fig. 1C, lane 5). In the case of the hIP and hIP^{SSLC}, two major immunoreactive bands of 43 kDa and 46–66 kDa, corresponding to the nonglycosylated and glycosylated forms of hIP, respectively, were present in the immunoprecipitates from HEK.hIP and $HEK.hIP^{SSLC}$ (Fig. 1C, lanes 1 and 2).

Table 1. Radioligand binding assays. The assays were carried out on membrane fractions in the presence of 4 nM [^{3}H]iloprost. Data are presented as the mean ± SEM (n = 4)

Cell type	[^{3}H]Iloprost bound (pmol·mg protein^{-1})
HEK.hIP	1.58 ± 0.15
$HEK.hIP^{SSLC}$	1.31 ± 0.08
$HEK.hIP^{CSLL}$	0.91 ± 0.03

Effect of isoprenylation on the ligand binding properties of the human prostacyclin receptor

The functional requirement for isoprenylation by hIP was then investigated. Initially, using [^{3}H]iloprost as selective radioligand, we investigated the ligand binding properties of the wild-type hIP comparing it to those of the hIP^{SSLC}. Scatchard analysis demonstrated that both the hIP and hIP^{SSLC} were expressed at comparable levels in their

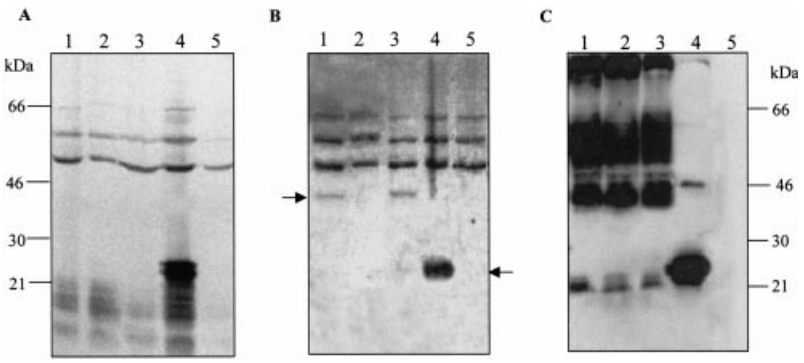

Fig. 1. Analysis of isoprenylation in HEK.hIP, $HEK.hIP^{SSLC}$ and $HEK.hIP^{CSLL}$ cells. (A–C) HEK.hIP (lanes 1), $HEK.hIP^{SSLC}$ (lanes 2) and $HEK.hIP^{CSLL}$ (lanes 3) cells or, as a positive control, HEK 293 cells transiently transfected with pHM6:Ha-ras (lanes 4) or, as a negative control, HEK 293 cells transiently transfected with pHM6 (lanes 5), were co-transfected with pMEV. (A) Following metabolic labelling with [^{3}H]mevalonolactone, cells were harvested and aliquots (70 μg) of whole cell protein were resolved by SDS/PAGE followed by electroblotting onto PVDF membrane. (B) Following metabolic labelling with [^{3}H]mevalonolactone, the HA-tagged hIP, hIP^{SSLC}, hIP^{CSLL} and HA-ras or HEK 293 cells cotransfected with pHM6 plus pMEV, serving as a negative control, were immunoprecipitated using anti-(HA 101r) serum. Immunoprecipitates were resolved by SDS/PAGE followed by electroblotting onto PVDF membrane. Blots in (A) and (B) were then soaked in Amplify for 30 min and exposed to X-Omat AR 5 film for 60 days at −70 °C. (C) HA-tagged proteins immunoprecipitated from nonmetabolically labelled cells were resolved by SDS/PAGE followed by electroblotting onto PVDF membrane; thereafter, membranes were screened using the peroxidase conjugated anti-(HA 3F10) serum followed by chemiluminescent detection. The positions of the molecular weight markers (kDa) are indicated to the left and right of (A) and (C), respectively. The arrows to the left of (B) indicate the positions of the metabolically labelled hIP (lane 1) and hIP^{CSLL} (lane 3). The arrow to the right of (B) indicates the position of HA-ras (lane 4). Data presented are representative of three independent experiments.

Table 2. Scatchard analysis. Scatchard analysis was carried out on membrane fractions of HEK.hIP, HEK.hIP^{SSLC} and HEK.hIP^{CSLL} cells. Data are presented as the mean ± SEM (n = 4).

Cell Type	High Affinity state K_d [a]	Low affinity state K_d [a]
HEK.hIP	0.65 ± 0.07	66.6 ± 12.7
HEK.hIP^{SSLC}	0.75 ± 0.14	72.2 ± 17.8
HEK.hIP^{CSLL}	0.68 ± 0.13	71.4 ± 14.4

[a] Iloprost (nM).

respective stable cell lines and that each exhibited two affinity binding sites, a high affinity and a low affinity site, for iloprost (Table 2). Moreover, values obtained for the high ($P \geq 0.64$) and low ($P \geq 0.80$) affinity iloprost binding sites were not significantly different between hIP and hIP^{SSLC} (Table 2) suggesting that isoprenylation of hIP does not affect its ligand binding properties.

Role of isoprenylation with respect to functional coupling of the prostacyclin receptor to adenylyl cyclase

To investigate the possible functional role of isoprenylation of hIP with respect to its intracellular signalling, we then investigated agonist-mediated cAMP generation in HEK.hIP and in HEK.hIP^{SSLC} cells and, as controls, in nontransfected HEK 293 cells in response to stimulation with 10^{-12}–10^{-6} M cicaprost (Fig. 2A). Whereas the hIP exhibited significant, concentration dependent increases in cAMP, the levels of cAMP generation by hIP^{SSLC} were not significantly different from those generated by the control HEK 293 cells throughout the range of concentrations examined (Fig. 2A). However, at the higher concentrations of cicaprost (10^{-7}–10^{-6} M), a small, though significant, increase in cAMP generation was observed in HEK.hIP^{SSLC} cells relative to HEK 293 ($P \leq 0.05$).

To investigate whether failure of hIP^{SSLC} to induce cAMP generation may be due to its altered ability to interact with G_s, HEK.hIP or HEK.hIP^{SSLC} cells were cotransfected with the cDNA encoding the Gαs and the ability of the coexpressed $G\alpha_s$ to augment cAMP generation in response to cicaprost (1 μM) stimulation was investigated (Fig. 2B). Whereas cotransfection of $G\alpha_s$ resulted in a significant augmentation (1.81-fold) of cAMP generation in HEK.hIP cells, $G\alpha_s$ did not significantly alter cAMP generation in HEK.hIP^{SSLC} cells (Fig. 2B). Western blot analysis demonstrated efficient over-expression of $G\alpha_s$ in both HEK.hIP and HEK.hIP^{SSLC} cells (Fig. 2C).

To further explore the receptor–G protein interaction, HEK.hIP, HEK.hIP^{SSLC} and HEK 293 cells were transiently transfected with $G\alpha_s$ or, as controls with pCMV5; thereafter, cells were stimulated with cicaprost where vehicle (Hepes/NaCl/P_i) treated cells served as a reference (Fig. 3B–D). Receptor–G protein interactions were examined by immunoprecipitating the HA-tagged hIP or hIP^{SSLC} receptors using the anti-(HA 101R) serum, followed by screening the immunoprecipitates for coprecipitation of $G\alpha_s$. Whereas pCMV5 transfected cells or vehicle-treated HEK.hIP cells showed no receptor–G protein interaction (Fig. 3B, lanes 1–3), as shown by an absence of coprecipitation of $G\alpha_s$, stimulation of HEK.hIP cells with cicaprost permitted coprecipitation of $G\alpha_s$ (Fig. 3B, lane 4). In contrast, stimulation of HEK.hIP^{SSLC} cells with cicaprost did not lead to coprecipitation of $G\alpha_s$ with hIP^{SSLC} (Fig. 3C, lane 4). $G\alpha_S$ was not detected in the immunoprecipitates from HEK 293 cells (Fig. 3D, lanes 3 and 4), further confirming the specificity of the hIP:$G\alpha_S$ interaction. The presence of HA-tagged hIP and hIP^{SSLC} in the immunoprecipitates from their respective HEK.hIP, HEK.hIP^{SSLC} cell lines, but not from HEK 293 cells, was confirmed by screening the blots with the peroxidase conjugated antibody anti-(HA 3F10) serum (Fig. 3A). These data demonstrate that whereas hIP can associate with $G\alpha_s$, the hIP^{SSLC} cannot interact with $G\alpha_s$.

Role of isoprenylation with respect to functional coupling of the prostacyclin receptor to phospholipase C

To extend these studies, we also investigate the potential role of isoprenylation of hIP in influencing its $G\alpha_q$:PLCβ coupling by examining cicaprost mediated intracellular calcium ($[Ca^{2+}]_i$) mobilization in HEK.hIP and HEK.hIP^{SSLC} cells. Whereas the hIP and hIP^{SSLC} yielded significant increases in $[Ca^{2+}]_i$ mobilization in response to cicaprost stimulation, the level of $[Ca^{2+}]_i$ mobilization in HEK.hIP cells (Fig. 4A; $\Delta[Ca^{2+}]_i$ = 133 ± 3.71 nM) was significantly greater than that mobilized in HEK.hIP^{SSLC} cells (Fig. 4A; $\Delta[Ca^{2+}]_i$ = 60.0 ± 8.16 nM, $p < 0.0008$).

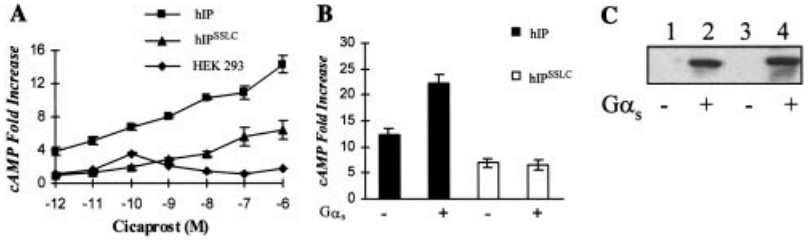

Fig. 2. Cicaprost-mediated cAMP generation in HEK.hIP and HEK.hIP^{SSLC} cells. HEK.hIP, HEK.hIP^{SSLC}, or nontransfected HEK 293 cells were stimulated with 10^{-12}–10^{-6} M cicaprost at 37 °C for 10 min (A). Alternatively, HEK.hIP and HEK.hIP^{SSLC} cells transiently cotransfected with either pCMV5 (–) or with pCMV:$G\alpha_s$ (+) were stimulated with 1 μM cicaprost (B). In each case, basal cAMP levels were determined by exposing the cells to the vehicle Hepes/NaCl/P_i under identical assay conditions. Levels of cAMP produced in ligand-stimulated cells relative to basal cAMP levels were expressed as fold stimulation of basal (fold increase in cAMP ± SEM, n = 4). (C) A typical Western blot confirming over-expression of $G\alpha_s$ in HEK.hIP (lane 2) and HEK.hIP^{SSLC} (lane 4) cells with nontransfected cells serving as controls (HEK.hIP, lane 1; HEK.hIP^{SSLC}, lane 3); 75 μg total cell protein was analysed per lane in each case.

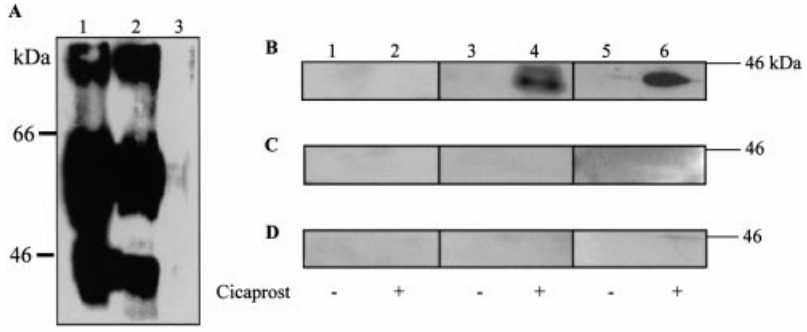

Fig. 3. Co-immunoprecipitation of the hIP and hIP^{SSLC} with G$\alpha_{s\ and}$ Gα_q. (A) HEK.hIP, HEK.hIP^{SSLC}, and HEK 293 cells (lanes 1–3, respectively) were immunoprecipitated using the anti-(HA 101R) serum. Alternatively, HEK.hIP (B), HEK.hIP^{SSLC} (C) and HEK 293 (D) cells were transiently cotransfected with pCMV5 (B–D; lanes 1 and 2), pCMV: Gα_s (B–D; lanes 3 and 4) or with pCMV:Gα_q (B–D, lanes 5 and 6). Thereafter, cells were stimulated for 10 min with 1 μM cicaprost (+) with vehicle-treated cells serving as a control (–). Following immunoprecipitation with anti-(HA 101R) serum, immunoprecipitates were resolved by SDS/PAGE and electroblotted onto PVDF membranes. Immunoblots were screened with peroxidase conjugated anti-(HA 3F10) serum (A), with anti-Gα_s serum (B–D; lanes 1–4) or with anti-Gα_q serum (B–D; lanes 5 and 6) and immunoreactive bands were visualized by chemiluminescence detection. Data presented are representative of three independent experiments. The positions of the molecular mass markers (kDa) are indicated to the left of (A) and to the right of Panels B–D.

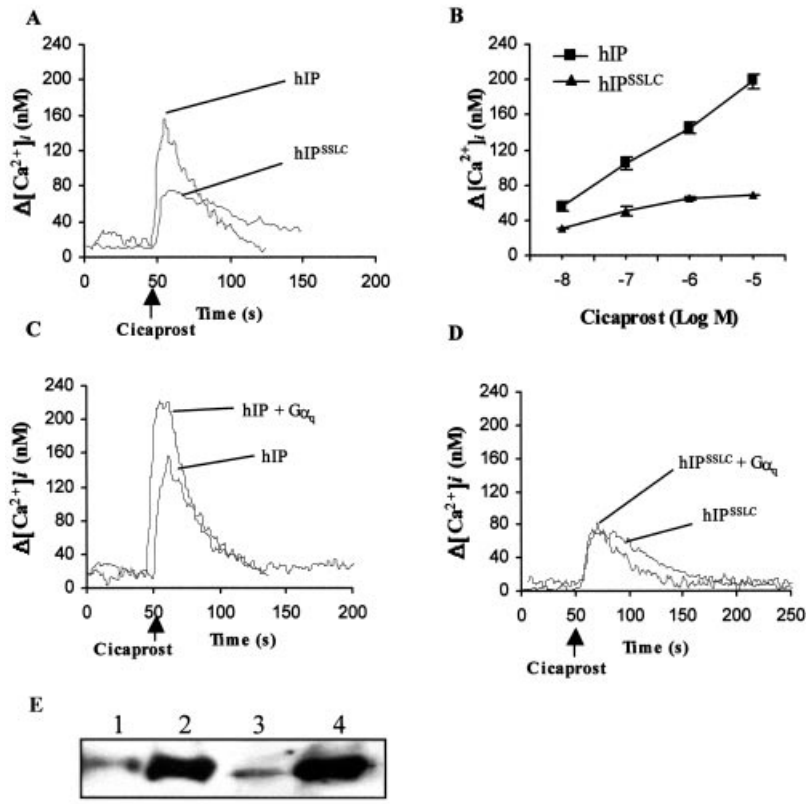

Fig. 4. Cicaprost-mediated $[Ca^{2+}]_i$ mobilization in HEK.hIP and HEK.hIP^{SSLC} cells. HEK.hIP (hIP) or HEK.hIP^{SSLC} (hIP^{SSLC}) cells, preloaded with Fura2/AM, were stimulated with 1 μM cicaprost (A) or 10^{-8}–10^{-5} M cicaprost (B). Data presented in (A) are representative of at least four independent experiments and are plotted as changes in intracellular calcium mobilization ($\Delta[Ca^{2+}]_i$; nM) as a function of time (s) following stimulation, where cicaprost was added at the times indicated by the arrow. In Panel B, mean data are plotted as changes in intracellular calcium mobilization ($\Delta[Ca^{2+}]_i \pm$ SEM; nM; $n = 4$). HEK.hIP (hIP; C) or HEK.hIP^{SSLC} (hIP^{SSLC}; D) cells, transiently cotransfected without or with pCMV: Gα_q (+ Gα_q) were stimulated with 1 μM cicaprost at the times indicated by the arrows. Data presented in panels (C) and (D) are representative of at least four independent experiments and are plotted as changes in intracellular calcium mobilization ($\Delta[Ca^{2+}]_i$; nM) as a function of time (s) following ligand stimulation. Panel E represents a typical Western blot confirming over-expression of Gα_q in HEK.hIP (lane 2) and HEK.hIP^{SSLC} (lane 4) cells with nontransfected cells serving as controls (HEK.hIP, lane 1; HEK.hIP^{SSLC}, lane 3); 75 μg total cell protein was analysed per lane in each case.

Moreover, in concentration response experiments, hIP yielded significantly more $[Ca^{2+}]_i$ mobilization when compared to hIP^{SSLC} throughout the range of cicaprost concentrations employed (Fig. 4B).

To further investigate whether failure of hIP^{SSLC} to efficiently mobilize $[Ca^{2+}]_i$ may be due to its altered ability to interact with its coupling G protein, the ability of cotransfected $G\alpha_q$ to augment cicaprost-induced $[Ca^{2+}]_i$ mobilization in HEK.hIP and $HEK.hIP^{SSLC}$ cells was investigated. Whereas a significant augmentation of $[Ca^{2+}]_i$ mobilization was observed in HEK.hIP cells cotransfected with $G\alpha_q$ (Fig. 4C; $\Delta[Ca^{2+}]_i = 207 \pm 2.18$ nM by hIP + $G\alpha_q$, $p < 0.0001$), $G\alpha_q$ did not significantly affect $[Ca^{2+}]_i$ mobilization in $HEK.hIP^{SSLC}$ cells (Fig. 4D; $\Delta[Ca^{2+}]_i = 69.5 \pm 5.96$ nM by hIP^{SSLC} + $G\alpha_q$). Transient overexpression of $G\alpha_q$ in HEK.hIP and $HEK.hIP^{SSLC}$ cells was confirmed by Western blot analysis (Fig. 4E).

To examine the receptor–$G\alpha_q$ protein interaction further, HEK.hIP, $HEK.hIP^{SSLC}$ and control HEK 293 cells were transiently transfected with the cDNA encoding $G\alpha_q$ (Fig. 3B–D; lanes 5 and 6) or, as a control, with the vector pCMV5 (data not shown) and cells were either stimulated with cicaprost or with the vehicle, serving as a control. Whereas pCMV5 transfected or vehicle-treated HEK.hIP cells showed no receptor–G protein interaction, as assessed by the absence of coprecipitation of $G\alpha_q$ (data not shown), stimulation of HEK.hIP cells with cicaprost permitted coprecipitation of $G\alpha_q$ (Fig. 3B, lane 6). In contrast, stimulation of $HEK.hIP^{SSLC}$ cells with cicaprost did not result in coprecipitation of $G\alpha_q$ with hIP^{SSLC} (Fig. 3C, lane 6). $G\alpha_q$ was not detected in the immunoprecipitates from HEK 293 cells (Fig. 3D, lanes 5 and 6), further confirming the specificity of the hIP–$G\alpha_q$ interaction. The presence of HA-tagged hIP and hIP^{SSLC} in the immunoprecipitates from their respective HEK.hIP, $HEK.hIP^{SSLC}$ cell lines, but not from HEK 293 cells, was confirmed by screening the blots with the peroxidase conjugated anti-(HA 3F10) serum (Fig. 3A). These data demonstrate that whereas hIP associates with $G\alpha_q$, the hIP^{SSLC} does not interact with $G\alpha_q$.

Effect of alteration of the CAAX motif of the prostacyclin receptor to a putative GGTase I substrate

Taken together, our data has confirmed that the hIP is isoprenylated in whole cells and our previous *in vitro* studies indicate that this occurs through attachment of a C15 farnesyl, rather than a C20 geranylgeranyl, isoprenoid moiety. Moreover, our studies have indeed confirmed a functional requirement for isoprenylation by hIP for its efficient signalling. We next sought to investigate the effect of conversion of the CAAX motif of hIP from a farnesyl acceptor site (CSLC; where the terminal Cys predicts that it is a substrate for FTase) to a geranylgeranyl acceptor site (CSLL; where the terminal Leu predicts that it is a substrate for GGTase I) on hIP signalling [19]. Thus, a stable $HEK.hIP^{CSLL}$ cell line over-expressing a HA-epitope tagged hIP^{CSLL}, in which Cys386 of hIP was converted by site directed mutagenesis to Leu386, was established. Saturation radioligand binding studies confirmed that hIP^{CSLL} exhibited identical radioligand binding properties to those of the hIP (Table 1) and Scatchard analysis confirmed that hIP^{CSLL} exhibited similar high and low affinity binding sites for iloprost (Table 2). Moreover, in whole cell metabolic labelling studies, hIP^{CSLL} was confirmed to be isoprenylated as shown by the presence of a faint isoprenylated protein of 43 kDa in the immunoprecipitates from $HEK.hIP^{CSLL}$ (Fig. 1B, lane 3) which was absent in the control HEK 293 cells (Fig. 1C, lane 5). The identity of the 43 kDa isoprenylated protein in $HEK.hIP^{CSLL}$ cells (Fig. 1B, lane 3) to be the hIP^{CSLL} was confirmed by Western blot analysis (Fig. 1C, lane 3).

hIP^{CSLL} exhibited concentration-dependent increases in cAMP generation in response to cicaprost stimulation (10^{-12}–10^{-6} M) that were not significantly different from those levels of cAMP generated by hIP (Fig. 5A). Coexpression of $G\alpha_s$ significantly augmented (1.78-fold) cicaprost-induced cAMP generation in $HEK.hIP^{CSLL}$ cells (Fig. 5B). Western blot analysis demonstrated the overexpression of $G\alpha_s$ in $HEK.hIP^{CSLL}$ cells (data not shown). hIP^{CSLL} also exhibited efficient agonist-induced, concentration-dependent increases in $[Ca^{2+}]_i$ mobilization (data not shown) and co-expression of $G\alpha_q$ in $HEK.hIP^{CSLL}$ cells significantly augmented cicaprost-mediated $[Ca^{2+}]_i$ mobili-

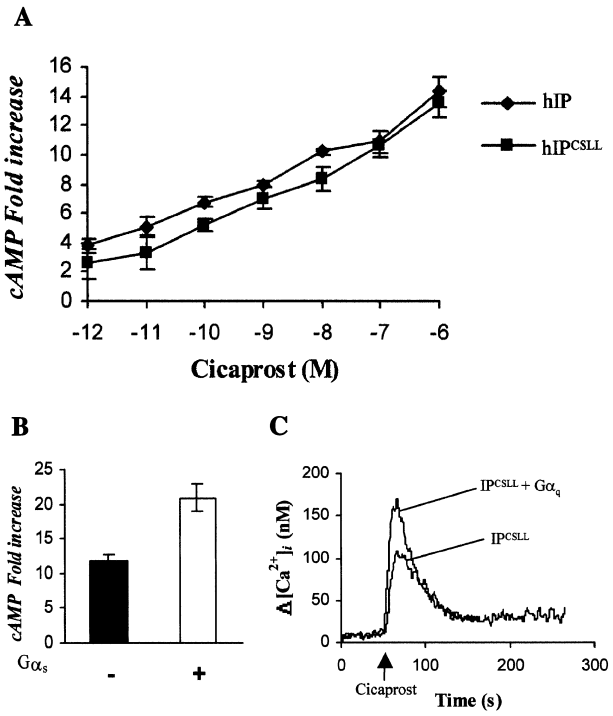

Fig. 5. Cicaprost-mediated signalling by the hIP^{CSLL}. (A,B) HEK.hIP (hIP) and $HEK.hIP^{CSLL}$ (hIP^{CSLL}) were stimulated with 10^{-12}–10^{-6} M cicaprost at 37 °C for 10 min (A). Alternatively, $HEK.hIP^{CSLL}$ cells were transiently cotransfected with either pCMV5 (–) or with pCMV:$G\alpha_s$ (+) were stimulated with 1 μM cicaprost (Panel B). In each case, basal cAMP levels were determined by exposing the cells to the vehicle Hepes/NaCl/P_i under identical assay conditions. Levels of cAMP produced in ligand-stimulated cells relative to basal cAMP levels were expressed as fold stimulation of basal (fold increase in cAMP ± SEM, $n = 4$). (C) $HEK.hIP^{CSLL}$ (hIP^{CSLL}), transiently cotransfected with pCMV:$G\alpha_q$ (+ $G\alpha_q$) or without were stimulated with 1 μM cicaprost at the times indicated by the arrow. Data presented are representative of at least four independent experiments and are plotted as changes in intracellular calcium mobilization ($\Delta[Ca^{2+}]_i$; nM) as a function of time (s) following ligand stimulation.

zation (Fig. 5C; compare $\Delta[Ca^{2+}]_i = 101 \pm 2.33$ nM without $G\alpha_q$ to $\Delta[Ca^{2+}]_i = 149 \pm 2.98$ nM with $G\alpha_q$; $p < 0.0001$).

To further explore the hIP^{CSLL}–G protein interaction, HEK.hIP^{CSLL} cells were transiently cotransfected with the cDNA encoding $G\alpha_s$ and $G\alpha_q$ or, as a control with pCMV5 and cells were stimulated with cicaprost (Fig. 6B,C) or with the vehicle (data not shown). Whereas vehicle-treated HEK.hIP^{CSLL} cells showed no receptor–G protein interaction as shown by an absence of coprecipitation of $G\alpha_s$ or $G\alpha_q$ (data not shown), stimulation of HEK.hIP^{CSLL} with cicaprost permitted coprecipitation of $G\alpha_s$ (Fig. 6B, lane 2) and $G\alpha_q$ (Fig. 6C, lane 2). The presence of hIP^{CSLL} in the immunoprecipitates was confirmed by screening the blots with the peroxidase conjugated anti-(HA 3F10) serum (Fig. 6A).

Role of isoprenylation of the prostacyclin receptor with respect to agonist-mediated internalization

The IP has been widely reported to undergo rapid agonist-induced internalization and desensitization following receptor activation. To examine the potential influence of isoprenylation on hIP internalization, HEK.hIP and HEK.hIP^{SSLC} cells were stimulated with cicaprost and loss of cell surface receptor expression was then examined by immunofluorescence microspcopy and by ELISA assay. Nonstimulated HEK.hIP and HEK.hIP^{SSLC} cells expressed similar levels of hIP and hIP^{SSLC} on the cell surface (Fig. 7A,C). Both the hIP and hIP^{SSLC} underwent cicaprost-mediated internalization, as shown by a decrease in their surface expression (Fig. 7B,D); however, the extent of

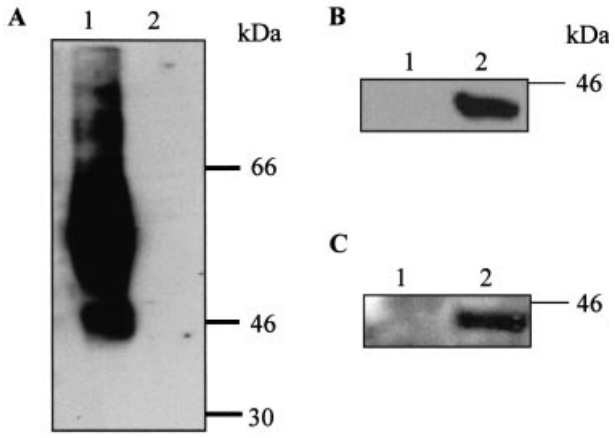

Fig. 6. Co-immunoprecipitation of the hIP^{CSLL} with $G\alpha_s$ and $G\alpha_q$. (A) HEK.hIP^{CSLL} cells (lane 1) or HEK 293 (lane 2) cells were immunoprecipitated using the anti-(HA 101R) serum. Alternatively, HEK.hIP^{CSLL} cells (B,C) were transiently cotransfected with pCMV5 (B,C; lane 1) or with pCMV:$G\alpha_s$ (B; lane 2) or with pCMV:$G\alpha_q$ (C; lane 2); cells were stimulated with 1 μM cicaprost for 10 min (B,C; lanes 1 and 2) and vehicle treated cells served as a control (data not shown). Cells were immunoprecipitated using the anti-(HA 101R) serum and immunoprecipitates were resolved by SDS/PAGE and electroblotted onto PVDF membranes. Immunoblots were screened with peroxidase conjugated anti-(HA 3F10) serum (A) or were screened with anti-$G\alpha_s$ serum (B) or anti-$G\alpha_q$ serum (C) and immunoreactive bands were visualized by chemiluminescence detection. Data presented are representative of three independent experiments. The positions of the molecular mass markers are indicated to the right of (A–C).

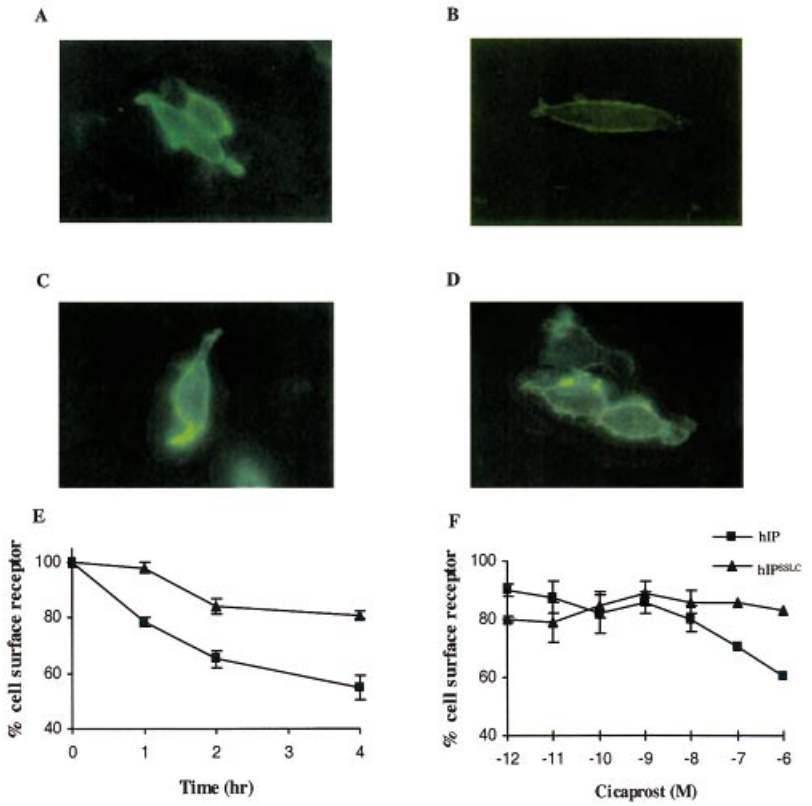

Fig. 7. Role of isoprenylation with respect to cicaprost-mediated prostacyclin receptor internalization. HEK.hIP (A,B) and HEK.hIP^{SSLC} (C,D) cells were stimulated with cicaprost (1 μM, 4 h; B,D) with vehicle treated cells serving as a control (A,C). Thereafter, immunofluorescence analysis was performed using an anti-(HA 101r) serum, as described in Experimental procedures. Results are representative of three independent experiments. Images in (A–D) represent X 630-fold magnification. (E,F) HEK.hIP (hIP) and HEK.hIP^{SSLC} (hIP^{SSLC}) cells were stimulated with cicaprost (1 μM) for 0–4 h (E) or with 10^{-12}–10^{-6} M cicaprost for 4 h (F) and internalization of HA-tagged receptors was quantified by Elisa assay as described in Experimental procedures. Results are presented as the mean percentage (%) of cell surface receptor relative to vehicle (MEM) treated cells ± SEM, where vehicle treated control cells were assigned a value of 100%.

agonist-induced internalization of hIP^{SSLC} was appreciably less than that of the hIP suggesting that isoprenylation of hIP may be required for its efficient agonist-mediated internalization.

To quantify the levels of receptor internalization, ELISA analysis of HEK.hIP and HEK.hIP^{SSLC} cells was performed following cicaprost treatment (1 μM; 0–4 h). Whereas a significant loss of cell surface hIP occurred (58.2 ± 4.51% cell surface hIP receptor remaining relative to vehicle treated cells following 4 h), internalization of hIP^{SSLC} was significantly impaired ($P < 0.009$; 81.0 ± 1.53% cell surface hIP^{SSLC} receptor remaining relative to vehicle treated cells at 4 h; Fig. 7E) when compared to hIP. Moreover, following concentration response studies (10^{-12}–10^{-6} M, 4 h), whereas significant internalization of hIP^{SSLC} occurred relative to nonstimulated cells, the extent of internalization of hIP^{SSLC} was significantly impaired relative to that of the hIP, particularly at the higher agonist concentrations (Fig. 7F).

DISCUSSION

In the present study, we provide the first direct evidence that, like the mIP [13], the hIP is isoprenylated in mammalian cells. Specifically, metabolic labelling of cells with [^{3}H]mevalonolactone identified a 43-kDa isoprenylated protein in HEK.hIP cells whose identity was confirmed to be that of hIP. Failure to observe isoprenylation of the larger glycosylated form of the hIP (46–66 kDa) may indicate that this form of the receptor is not actually isoprenylated or, alternatively, may be due to the fact that generally glycosylated proteins/GPCRs are heterogeneous in size and thus, should they be labelled in the presence of [^{3}H]mevalonolactone, the relatively weak radioactive ^{3}H signal may be too diffuse to generate a fluorographic image. In view of the strong functional requirement for isoprenylation by hIP, as is shown by our data presented herein, we would argue that the latter situation is most likely the case and that both the glycosylated and nonglycosylated forms of the hIP are isoprenylated.

The C-terminal four amino acids are conserved in the human, mouse, rat and bovine IPs and represent a typical CAAX motif, where X is cysteine [12,21–23]. Site directed mutagenesis of Cys^{383}SLC to Ser^{383}SLC abolished isoprenylation of hIP^{SSLC}, confirming that the four terminal amino acids of hIP behave as a typical CAAX motif and that Cys383 is the acceptor amino acid. The hIP has two affinity binding sites for iloprost and K_d values obtained for the high and low affinity binding sites compare well with those of the mIP [13] and with those previously reported for the hIP [35]. Moreover, the mutant hIP^{SSLC} exhibited K_d values for the high and low affinity binding sites that were not significantly different from those of the hIP confirming that isoprenylation of the hIP is not required for ligand binding.

The primary intracellular signalling pathway utilized by the IP is its $G\alpha_s$ dependent activation of adenylyl cyclase [7,10,14,25]. In the present study, while the hIP mediated significant increases in cAMP generation, levels of cAMP generation by hIP^{SSLC} were not significantly different from those generated by control HEK 293 cells. Whereas cotransfection of HEK.hIP cells with $G\alpha_s$ resulted in a significant increase in cAMP generation, no observable augmentation was seen in HEK.hIP^{SSLC} cells cotransfected with $G\alpha_s$. Moreover, while $G\alpha_s$ co-immunoprecipitated along with the hIP, in an agonist-dependent manner, $G\alpha_s$ did not coprecipitate with hIP^{SSLC}. In addition to its Gs coupling, IP may also mediate increases in intracellular Ca^{2+} via PLCβ activation [7,9–14]. In the present study, while hIP mediated concentration dependent increases in $[Ca^{2+}]_i$ mobilization, the level of $[Ca^{2+}]_i$ mobilization by hIP^{SSLC} was significantly impaired relative to that of the wild-type hIP and cotransfection with $G\alpha_q$ failed to augment mobilization of $[Ca^{2+}]_i$ in HEK.hIP^{SSLC} cells. Moreover, whereas $G\alpha_q$ coprecipitated with the hIP in an agonist dependent manner, $G\alpha_q$ did not associate with hIP^{SSLC}. Taken together, these data demonstrate that isoprenylation of hIP is required for its efficient association with and coupling to both $G\alpha_s$ and $G\alpha_q$ and, hence, for concomitant adenylyl cyclase and PLC activation. In a recent study, we have demonstrated that the statins lovastatin and cerivastatin impair IP-mediated cAMP generation and $[Ca^{2+}]_i$ mobilization [25]. we have defined the mechanism by which the statins inhibit IP signalling, namely inhibition of isoprenylation of hIP and thus abolition of the physical association of hIP with both $G\alpha_s$ and $G\alpha_q$ and concomitant inhibiton of associated effector signalling cascades.

Recently, Hayes *et al.* [13] demonstrated that recombinant forms of the mIP and hIP are isoprenylated by C15 farnesyl groups *in vitro*. To determine the effect of altering the CAAX motif of hIP from a putative farnesyl transferase (FTase) to a geranylgeranyl transferase type I (GGTase I) substrate, site directed mutagenesis of hIP was performed whereby $CSLC^{386}$ was mutated to $CSLL^{386}$. Herein, we demonstrate that hIP^{CSLL} displayed similar radioligand binding properties to that of hIP (Tables 1 and 2) and is isoprenylated in mammalian cells, suggesting that its mutated CAAX motif $CSLL^{386}$, is modified, not by a C15 farnesyl group, but by a C20 geranylgeranyl group. Thereafter, the ability of hIP^{CSLL} to regulate both G_s- and G_q-coupled effector systems was investigated. The hIP^{CSLL} mediated significant concentration dependent increases in cAMP generation at levels that were not significantly different from those of the hIP and cotransfection with $G\alpha_s$ significantly augmented hIP^{CSLL}–mediated cAMP generation. hIP^{CSLL} also mediated concentration dependent increases in $[Ca^{2+}]_i$ mobilization that was significantly augmented by cotransfection with $G\alpha_q$. Taken together, these data demonstrate that although the CAAX motif of hIP^{CSLL} is most likely a GGTase I substrate, hIP^{CSLL} is capable of interacting with the main effector signalling cascades activated by hIP, namely G_s-mediated increases in cAMP generation and G_q-mediated increases in intracellular $[Ca^{2+}]_i$ mobilization. Moreover, co-immunoprecipitation experiments demonstrated that hIP^{CSLL} is capable of functionally associating with both $G\alpha_s$ and $G\alpha_q$. Thus, the nature of the isoprenoid group post-translationally attached to the hIP, be it a C15 farnesyl group or a C20 geranylgeranyl group, does not interfere with the specificity of hIP–G protein coupling and effector activation.

Of the diverse array of proteins that are farnesylated, the ras (proto)oncoproteins are of particular interest whereby farnesylation of ras serves to increase protein hydrophobicity and may explain its affinity for and association with the plasma membrane [20]. Recently, it has been demonstrated

that although H-, K-, and N-ras are normally farnesylated, both K-ras and N-ras are capable of being geranylgeranylated in the presence of FTase inhibitors [36]. Our studies have demonstrated that alteration of the CAAX motif of the hIP from a FTase substrate to a proposed GGTase I substrate does not affect the signalling properties of hIP. Thus, we propose that should the hIP be geranylgeranylated through a compensatory mechanism in the presence of FTase inhibitors, such as the FTase inhibitors under development as novel anti-ras serum chemotherapeutics, signalling by hIP would remain unaffected. Whether the hIP undergoes alternative geranylgeranylation, similar to that of K-ras and N-ras in the presence of the FTase inhibitors, remains to be established experimentally.

Classically, agonist induced phosphorylation of GPCRs by the G protein coupled receptor kinases (GRKs) stimulates the binding of the arrestins, leading to uncoupling of the receptor from its cognate G protein(s), ultimately resulting in receptor desensitization and/or internalization [37,38]. Internalization and sequestration of the hIP has been reported in platelets, fibroblasts and HEK 293 cells and may represent an important mechanism whereby the cellular responses to prostacyclin are regulated in a dynamic manner [17,18,39]. However, sequestration of the hIP occurs subsequent to, but independent of, desensitization and involves trafficking of the receptor to clathrin-coated pits following agonist stimulation [18]. To further dissect the mechanism of hIP internalization, in the present study, we sought to investigate whether the isoprenylation status of the hIP influences its internalization in response to agonist stimulation. Herein, we have demonstrated that the hIP is internalized in a concentration and time dependent manner with a net loss of $\approx$ 50% of surface receptors following 4 h. Whereas the isoprenylation defective mutant hIP^{SSLC} does internalize, the kinetics and extent of internalization of the hIP^{SSLC} is significantly impaired relative to that of the hIP. Thus, isoprenylation mediates, at least in part, internalization of hIP, possibly by presenting the hIP in the correct tertiary structure to facilitate its cellular trafficking. Interestingly, Smyth *et al.* [18] reported that truncation of the C-terminal region of the hIP (C-del) completely abolished receptor trafficking in response to agonist and proposed that specific determinants of the hIP internalization and sequestration are located within its C-terminal tail region. In this study, we have identified isoprenylation as one in the key elements involved either directly or, more likely, indirectly in the mediation of hIP internalization. Definition of the precise role of isoprenylation with respect to the mechanism of agonist-induced IP internalization will require detailed molecular investigation.

These studies investigating the isoprenylation of the hIP are in close agreement with our previous findings with the mIP but provide extensive new data highlighting the functional requirement for isoprenylation by the hIP. Though the nature of the isoprenoid group attached to hIP does not dictate the receptor–G protein coupling specificity, the absence of an isoprenoid group, be it a farnesyl or a geranylgeranyl moiety, impairs functional agonist-induced receptor–G protein coupling and internalization. Herein, we propose that the isoprenoid group increases the hydrophobicity of the C-terminal tail and, thus, the isoprenoid moiety may become integrated into the plasma membrane resulting in the formation of a fourth intracellular loop and, through possible alterations in the tertiary structure, appears to be essential for hIP–G protein (i.e protein–protein) interaction. Moreover, isoprenylation of the hIP appears to play a role in regulating the events post-receptor activation, including agonist mediated G protein interaction and coupling, effector activation and receptor internalization, emphasizing the essential, functional requirement for isoprenylation by the hIP. Thus, it is indeed likely that, depending on their clinical efficacy and potency, therapeutic agents that interfere with the isoprenylation of the hIP, be they the statins [25] or the FTase inhibitors [26], may adversely affect essential IP signalling and function in humans.

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