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Role of extracellular cysteine residues in dimerization/oligomerization of the human prostacyclin receptor

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Abstract

Prostacyclin activation of prostanoid IP receptors may result in pain sensation, inflammatory responses, inhibition of platelet aggregation, and vasodilation in vascular tissue. The prostanoid IP receptor is a G-protein-coupled receptor. In the present study, we investigated the determinants responsible, at least in part, for the prostacyclin receptor (IP) dimerization/oligomerization. Using co-immunoprecipitation of differentially tagged IP expressed in COS-7 cells, we demonstrate that IP can form dimers and oligomers. Treatment of IP-expressing cells with the stable agonist carbaprostacyclin failed to alter the ratios of oligomeric/dimeric/monomeric forms of the receptor, suggesting that IP dimerization/oligomerization is an agonist-independent process. The reducing agents dithiothreitol and 2-mercaptoethanol were highly efficient in converting the receptor from its oligomeric form to the monomeric state, indicating the involvement of disulfide bonds in IP oligomerization. Immunoblotting of the osteoblastic MG-63 cell line lysates with an anti-IP specific antibody revealed the presence of endogenous IP oligomers which were converted to dimers and monomers upon treatment with dithiothreitol. Individual substitutions of the four extracellular IP Cys residues (Cys^5, Cys^{92}, Cys^{165} and Cys^{170}) for Ser resulted in greatly decreased receptor protein expression in COS-7 cells. The C92–170S double mutant showed receptor protein expression level similar to the individual mutants. However, expression of the C92–165S and C165–170S mutants was drastically reduced, suggesting that there was formation of disulfide bonds between Cys^5 and Cys^{165}, and between Cys^{92} and Cys^{170}. The Cys receptor mutants showed altered oligomer/dimer/monomer ratios. Dimerization/oligomerization likely occurs intracellularly since these Cys receptor mutants could still form dimers/oligomers despite their lack of expression at the cell surface.

Keywords: Prostacyclin; Prostanoid IP receptor; G-protein-coupled receptor; Dimerization; Oligomerization; Cysteine

1. Introduction

G-protein-coupled receptors form the largest class of transmembrane receptors; they are constituted of seven transmembrane α-helices linked by intracellular and extracellular loops and ended at extremities with an extracellular amino-terminal domain and a cytoplasmic carboxy-terminal tail. G-protein-coupled receptors are expressed in a wide variety of cell types and mediate the actions of a vast array of hormones, neurotransmitters and sensory stimuli (Bockaert and Pin, 1999).

For about a decade, there have been many studies elaborated to prove that several G-protein-coupled receptors interact with their associate G proteins in a stoichiometric ratio different of 1:1, suggesting that those G-protein-coupled receptors exist and even function as dimers/oligomers. Evidence of dimerization between two distinct G-protein-coupled receptors was first demonstrated in an indirect manner with two defective chimeric constructs, α_{2c}/M_3 and M_3/α_{2c} receptors, derived from rat α_{2c}-adrenergic and M_3 muscarinic receptors, between which C-terminal receptor portions including transmembrane domains VI and VII were exchanged; co-expression of these two chimeras restored functional and pharmacological properties that were not manifested when α_{2c}/M_3 and M_3/α_{2c} were expressed separately (Maggio et al., 1993). Heterodimerization of other G-protein-coupled receptors was also sug-

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gested to occur since changes in ligand binding affinity and/or potency (Rocheville et al., 2000a,b; Pfeiffer et al., 2001; Maggio et al., 1999; Jordan and Devi, 1999; George et al., 2000; Yoshioka et al., 2001; Gomes et al., 2000; AbdAlla et al., 2000; Le Gouill et al., 1999), signal transduction (George et al., 2000; Gines et al., 2000; Mellado et al., 2001; Ciruela et al., 2001; Lavoie et al., 2002), trafficking (Rocheville et al., 2000a; Pfeiffer et al., 2001, 2002; Lavoie et al., 2002; Jordan et al., 2001) and biological response intensity (Jordan and Devi, 1999; Pfeiffer et al., 2002; Ferre et al., 2002) were observed when two different G-protein-coupled receptors were co-expressed relatively to expression of individual receptors. The first direct evidence of G-protein-coupled receptor dimerization was reported by Hebert et al. (1996) who showed that β_2-adrenoceptor dimerization was dependent on sequences found in transmembrane domain VI of the receptor. Co-immunoprecipitation experiments revealed bands corresponding to multiples of the established molecular weight of many G-protein-coupled receptors (Hebert et al., 1996, 1998; Ng et al., 1996; Romano et al., 1996; Cvejic and Devi, 1997; Bai et al., 1998, 1999; Zeng and Wess, 1999; Schulz et al., 2000; Benkirane et al., 1997; McVey et al., 2001; Babcock et al., 2003; Angers et al., 2000; Vila-Coro et al., 2000; Rodríguez-Frade et al., 1999) suggesting oligomerization of these receptors. Furthermore, it was demonstrated that expression of a functional type B γ-aminobutyric acid ($GABA_B$) receptor at the cell surface depended on an obligatory interaction between the two subtypes of $GABA_B$ receptor: $GABA_B$ receptor 1, which binds the ligand and is retained in the endoplasmic reticulum by a retention signal located in its carboxyl tail, and $GABA_B$ receptor 2, which transduces the signal and dimerizes with $GABA_B$ receptor 1, hiding the endoplasmic reticulum retention signal of the latter and thus allowing its expression at the cell surface (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999; Sullivan et al., 2000; Pagano et al., 2001; Ng et al., 1999; Calver et al., 2001; Margeta-Mitrovic et al., 2001; Robbins et al., 2001).

Similarly, lack of cell surface expression was shown when a wild-type G-protein-coupled receptor was co-expressed with one of its mutant which was not targeted to the cell surface. The fact that the latter could retain the wild-type G-protein-coupled receptor intracellularly suggested that an interaction occurred between the two receptor forms, indicating possible G-protein-coupled receptor dimerization within intracellular compartments (Benkirane et al., 1997; Zhu and Wess, 1998; Karpa et al., 2000). G-protein-coupled receptor homodimerization could also be observed by biophysical techniques like bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET) for the somatostatin subtype 5 (SSTR5) (Rocheville et al., 2000a), β_2-adrenoceptor (Angers et al., 2000), δ-opioid (McVey et al., 2001), thyrotropin-releasing hormone (Kroeger et al., 2001), gonadotropin-releasing hormone (Cornea et al., 2001; Horvat et al., 2001), yeast α-factor (Overton and Blumer, 2002), complement factor 5a (Floyd et al., 2003), thyrotropin (Latif et al., 2002), CCR5 (Issafras et al., 2002), and CXCR4 chemokine (Babcock et al., 2003) receptors.

Yet, little is understood on how G-protein-coupled receptors can homodimerize. Other than the β_2-adrenoceptor (Hebert et al., 1996), transmembrane domain interactions were also involved in the cases of vasopressin V_2 (Schulz et al., 2000) and dopamine D2 receptors dimerization (Ng et al., 1996; Lee et al., 2003), whereas extracellular amino-terminal domain was involved in bradykinin B_2 receptor dimerization (AbdAlla et al., 1999). According to other studies, G-protein-coupled receptor homodimerization occurs primarily through intermolecular disulfide bonds between cysteines located at extracellular regions of the G-protein-coupled receptor like the amino-terminal domain of the calcium-sensing (Bai et al., 1998; Ward et al., 1998; Ray et al., 1999; Zhang et al., 2001) and the metabotropic glutamate receptors (Romano et al., 1996, 2001; Ray and Hauschild, 2000), and the first and second extracellular loops of the M_3 muscarinic receptor (Zeng and Wess, 1999).

We have investigated the possible dimerization of the human prostacyclin receptor (IP). The prostanoid IP receptor mediates the actions of its principal ligand, prostacyclin (PGI_2), generally through G_s protein activation, leading to subsequent adenylate cyclase activation and increase of intracellular cAMP (Boie et al., 1994), though in some studies other G proteins than G_s (G_q, G_{11} and G_i) were reported to be activated (Smyth et al., 1996; Lawler et al., 2001). The major physiological roles of PGI_2 are the inhibition of platelet aggregation and vasodilation (Whittle et al., 1980; Sinzinger et al., 1987; Numaguchi et al., 1998; Zucker et al., 1998; Harada et al., 1999). Other probable biological functions ensued from PGI_2 activation of prostanoid IP receptors include pain sensation and inflammatory responses (Murata et al., 1997; Bley et al., 1998). Understanding the molecular mechanisms implicated in the regulation of this receptor may thus be important to the pathophysiology and pharmacology of vascular and inflammatory diseases.

In the present study we generated prostanoid IP receptor deletion and site-specific mutants which cover different regions throughout the receptor to characterize the nature of the molecular interactions leading to its dimerization. Our results indicate that prostanoid IP receptor dimerization/oligomerization likely occurs in intracellular compartments primarily through disulfide bond formation.

2. Materials and methods

2.1. Materials

Oligonucleotides were acquired from Invitrogen/Life Technologies and Sigma/Genosys. Taq and Tgo DNA

polymerases, T4 ligase and restriction endonucleases were obtained from Roche Applied Science. The human prostanoid IP receptor (IP) cDNA was kindly provided by Dr. Mark Abramovitz (Merck Frosst, Montreal, QC) and the pcDNA3-HA and pcDNA3-FLAG vectors were previously described (Parent et al., 1999). Dulbecco's modified Eagle's medium (DMEM) and fetal bovin serum were bought from Invitrogen/Life Technologies and ICN Biomedicals, while FuGENE-6 transfection reagent was purchased from Roche Applied Science. The PGI_2 analog carbaprostacyclin ($cPGI_2$) was purchased from Cayman Chemical. IGEPAL CA-630, poly-L-lysine hydrobromide, iodoacetamide, bovin serum albumin, *N*-ethylmaleimide, 2-mercaptoethanol, protease inhibitors such as chymostatin, leupeptin, antiparin and pepstatin, and mouse monoclonal anti-FLAG M2 antibodies came from Sigma-Aldrich. The other antibodies were mouse monoclonal (Covance) and rabbit polyclonal anti-HA (Santa Cruz Biotechnology), horseradish peroxidase-conjugated goat anti-mouse IgG (γ-immunoglobulin) and anti-rabbit IgG (Amersham Biosciences) and goat anti-mouse IgG–alkaline phosphatase conjugate (Bio-Rad Laboratories). Protein A-agarose was from Santa Cruz Biotechnology Hybond™-ECL™ nitrocellulose membranes, ECL™ Western blot detection reagents and ECL™ hyperfilms were obtained from Amersham Biosciences whereas Alkaline Phosphatase Substrate kit was from Bio-Rad Laboratories. Finally, DL-dithiothreitol (DTT) was obtained from Promega.

2.2. DNA constructs, site-directed mutagenesis and subcloning of human prostanoid IP receptor (IP) and its mutants

Amino-terminal hemagglutinin (HA) or FLAG tagging of prostanoid IP receptor was achieved basically as we described before (Parent et al., 1999). The cDNA encoding the prostanoid IP receptor was amplified by polymerization chain reaction (PCR) using sense primer F1 (5′-GCTCCG GGCGAATTC**ATGGCGGATTCGTGCAGG**-3′) and antisense primer R1 (5′-GAGCTCGA G**TCAGCAGAGGGAGCAGGCGACGCTGGCTTCTGCTTTGG**-3′) and then was subcloned in-frame into the *Eco*RI–*Xho*I sites of pcDNA3-HA and pcDNA3-FLAG (Parent et al., 1999) to create pcDNA3-HA-IP and pcDNA3-FLAG-IP, respectively.

The prostanoid IP receptor deletion mutants were generated by PCR using pcDNA3-HA-IP as template and the following oligonucleotides: sense primer F1, sense primer F3 (5′-GAGGAATTC***ATG*** **CAGC AGTACTGCCCCGGCAGCTGGTGCTTC**-3′), antisense primer R2 (5′-GAGCTCGA G***TCA***cagatcctcttcagagatgagtttctgttc**CCCCATCTCACTGCTGCT**-3′), antisense primer R3 (5′-GAGCTCGAG***TCA*** cagatcctcttcagagatgagtttctgttc**CGGCTGGGCCCAGCGCAT**-3′) and antisense primer R4 (5′-GAGCTCGAG***TCA***cagatcctcttcagagatgagtttctgttc**GCACAGGGCG GGGCCGCC**-3′). In all primers, nucleotides corresponding to prostanoid IP receptor DNA sequences are in boldface, inserted methionine or stop codons are in underlined boldface italics and a carboxy-terminal c-myc tag sequence is shown in lowercase letters. These primers were used to generate the F1R4, F3R2, F1R2 and F1R3 constructs, as illustrated in Fig. 3.

Prostanoid IP receptor transmembrane domains point mutants were produced by PCR site-directed mutagenesis, replacing Val^{28}, Gly^{32} and Gly^{36} in transmembrane domain I and Val^{247}, Cys^{251} and Leu^{255} in transmembrane domain VI by Ala codons as seen in Fig. 3. pcDNA3-IP was used as the template and the designed primers were sense FTM1 (5′-ATGTTCGTGGCCGGT***GCA*** GTGGGCAAC***GCA***-CTGGCCCTG***GCA***AT CCTGAGCGCACGGCGA-3′) and antisense RTM1 (5′-GCGCGCCGGTCGCCGTGCGCTCA GGAT***TGC***CAGGGCCAG***TGC***GTTGCCCAC***TGC***ACCG-GC-3′) for the transmembrane domain I (TM1) mutant and sense FTM6 (5′-GCCCTCATGACAGTG***GCA***ATGGCCG-TG***GCA***TCCCTGCCT***GCA***AC GATCCGCTGCTTCACC-3′) and antisense RTM6 (5′-GACAGCCTGGGTGAAG-CAGCGGA TCGT***TGC***AGGCAGGGA***TGC***CACGGCC-AT***TGC*** CACTGT-3′) for the transmembrane domain VI mutant (TM6) (Ala codons are in underlined boldface italics). The TM1–TM6 mutant was obtained by site-directed mutagenesis of the TM6 mutant with the FTM1 and RTM1 primers.

Substitution of Cys^{92}, $Cys^{165}$and Cys^{170} codons for Ser residues in the prostanoid IP receptor was performed by PCR site-directed mutagenesis using pcDNA3-HA-IP template and the following primers: sense primer FC92S (5′-GCCCGAGGCGGCCCCGCCCTG***TCA*** GATGCCTT-CGCCT TCGCCATGACCTTC-3′) and antisense primer RC92S (5′-GGCGAAGGCGAAGGCAT C***TGA***-CAGGGCGGGGCCGCCTCGGGCCAGGCCCAG-3′), sense primer FC165S (5′-CTGGGCCAACACCAGCA-GTAC***TCA***CCCGGC-3′) and antisense primer RC165S (5′-GCC GGG***TGA*** GTACTGCTGGTGTTGGCCCAGGC-3′), sense primer FC170S (5′-GGCAGCTG G***TCA*** TTCCTCCGCATGCGC-3′) and antisense primer RC170S (5′-GCCCGGCTGGGCCCA GCGCATGCGGAGGAA-***TGA*** CCAGCTGCC-3′) (the substituted serine codons are in underlined boldface italics) (Fig. 3). Sense primer FC5S (5′-CGCGAATTC**ATGGCGGATTG C*TCA*AGGAACC-TCACCTACGTG**-3′) was used to mutate Cys^5 to Ser in combination with the R1 primer by single-step PCR site-directed mutagenesis. The double C92–165S, C92–170S and C(165–170)S mutants were generated by the use of the primers described above on the various newly obtained Cys mutants as template.

Subcloning of all the PCR-amplified cDNAs were carried out into the *Eco*RI–XhoI site of pcDNA-3-HA and pcDNA-3-FLAG. Constructs were verified by DNA sequencing (University of Calgary, Calgary; University of Sherbrooke, Sherbrooke) using T7 and Sp6 primers.

2.3. Cell culture and transfection

COS-7 and MG-63 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. Transient transfections of COS-7 cells grown to 75–90% confluence were performed using FuGENE 6™ according to the manufacturer's instructions. Empty pcDNA3-HA vector was added to keep the total DNA amount added per plate constant. Cells grown on 60-mm plates were transfected using 6 μg of total DNA vectors whereas cells grown on six-well plates were transfected using 1 μg/well of total DNA vectors.

2.4. Whole cell lysates and immunoprecipitations

Six well plates of COS-7 cells were transfected with different combinations of prostanoid IP receptor constructs as indicated in the Results section. Transfected cells were maintained as described above for 48 h. The MG-63 or the transfected COS-7 cells were then harvested and washed with ice-cold phosphate-buffered saline before being lysed in lysis buffer (150 mM NaCl, 50 mM Tris, pH 8, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM $Na_4P_2O_7$, 5 mM EDTA) supplemented with protease inhibitors (9 nM pepstatin, 9 nM antipain, 10 nM leupeptin and 10 nM chymostatin). The lysis buffer volumes vary from 100 μl for whole cell lysates to 800 μl for immunoprecipitations. After the cells were incubated in lysis buffer for 60 min at 4 °C with slow rotation, the lysates were clarified by centrifugation for 20 min at 14 000 rpm at 4 °C. Supernatants were used for immunoblotting and immunoprecipitation investigations. For the latter purposes, 100 μl of the supernatants were kept as control lysates and the remainder 700 μl processed for immunoprecipitation. Five micrograms of specific monoclonal antibodies were then added and samples incubated for 60 min at 4 °C, after which 50 μl of 50% protein A-agarose pre-equilibrated in lysis buffer was added, followed by an overnight incubation at 4 °C, always with slow rotations. Samples were then centrifuged at 5000 rpm for 1 min in a microcentrifuge and washed three times with lysis buffer. Immunoprecipitated proteins were eluted by addition of 50 μl of SDS sample buffer followed by a 45–60 min incubation at room temperature. Initial lysates and immunoprecipitated proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting using specific antibodies.

2.5. Receptor cell surface expression assay

Cell surface expression of prostanoid IP receptor and its various mutants described in this study was assessed by enzyme-linked immunosorbent assay (ELISA) experiments using transiently transfected COS-7 cells as we described previously. Briefly, 1.2×10^6 cells were grown overnight in 60-mm plates. The cells were then transfected with empty pcDNA3-HA vector or with the different epitope-tagged prostanoid IP receptor constructs as indicated in Results. Transfected cells were maintained as described above for 24 h. Thereafter, 2×10^5 cells were transferred to 24-well plates precoated with 0.1 mg/ml poly-L-lysine and were maintained for an additional 24 h. The cells were then fixed with 3.7% of formaldehyde in Tris-buffered saline (20 mM Tris, pH 7.5, 150 mM NaCl) for 5 min at room temperature. The cells were then washed three times with Tris-buffered saline and nonspecific binding blocked with Tris-buffered saline containing 1% bovine serum albumin for 45 min at room temperature. The cells were then incubated with either a HA-specific (Covance) or a FLAG M2-specific (Sigma) monoclonal antibody at a dilution of 1:1000 in Tris-buffered saline/bovine serum albumin for 1 h at room temperature. Three washes with Tris-buffered saline followed, and cells were briefly reblocked for 15 min at room temperature. Incubation with goat anti-mouse conjugated alkaline phosphatase diluted 1:1000 was carried out for 1 h at room temperature. The cells were then incubated with an alkaline phosphatase-conjugated goat anti-mouse antibody at a 1:1000 dilution (Parent et al., 1999) for 1 h at room temperature followed by three washes with Tris-buffered saline. The colorimetric Alkaline Phosphatase Substrate (Bio-Rad Laboratories) was then added as specified by the manufacturer. The resulting colorimetric reactions were measured using a Titertek MultisKan MCC/340 spectrophotometer with the 405 nm filter. Cells transfected with pcDNA3 were studied concurrently to determine background. All experiments were done in triplicate.

3. Results

3.1. Detection of prostanoid IP receptor dimers and oligomers by co-immunoprecipitation and Western blotting

COS-7 cells transiently co-transfected with the indicated constructs (Fig. 1) were subjected to immunoprecipitation with a mouse monoclonal anti-FLAG antibody, SDS-PAGE, and immunoblotting using a rabbit polyclonal anti-HA antibody as described under Materials and methods. Immunoprecipitation of FLAG-IP resulted in the co-immunoprecipitation of HA-IP when both receptor forms were co-expressed (Fig. 1, lane 3). Protein bands of molecular weights corresponding to the monomeric (~ 40 kDa), dimeric (~ 80 kDa) and oligomeric (~ 120 kDa) prostanoid IP receptors were detected. No such species were revealed when only one of the two epitope-tagged receptors was expressed or when empty pcDNA3-HA was transected as a negative control (Fig. 1, lanes 1, 2 and 4). Similar results were obtained when HA-tagged receptors were immunoprecipitated and FLAG-tagged IP detected (not shown). On the other hand, no prostanoid IP receptor co-immunoprecipitation could be observed when separately grown COS-7 cells expressing either HA-IP or FLAG-IP

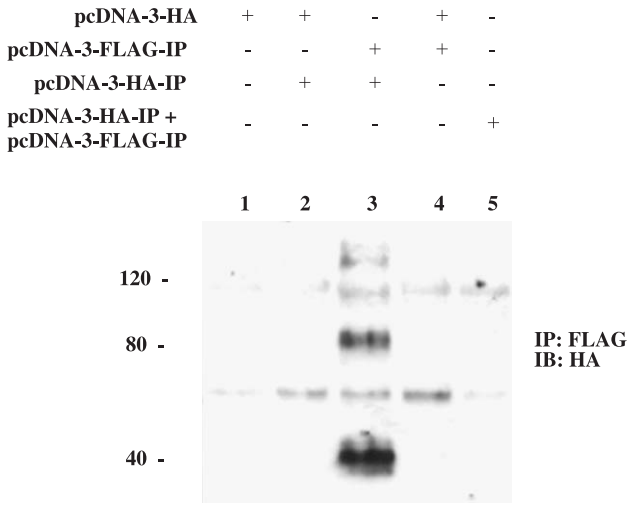

Fig. 1. Detection of prostanoid IP receptor dimers/oligomers through co-immunoprecipitation and Western blot analysis. COS-7 cells transiently transfected with the indicated constructs were lysed and immunoprecipitated (IP) with a FLAG-specific monoclonal antibody as described under Materials and methods. The immunoprecipitated receptors were subjected to SDS-PAGE and detected through immunoblotting (IB) with a HA-specific polyclonal antibody. Molecular weights of the monomeric, dimeric and oligomeric species of the receptor are shown in kDa. Lane 5 shows that mixing two populations of cells individually expressing either the HA- or FLAG-tagged IP receptors prior to immunoprecipitation failed to produce the same receptor species. The blot shown is representative of three independent experiments.

were mixed together prior to cell lysis (Fig. 1, lane 5). These observations suggest that prostanoid IP receptor dimerization and oligomerization occurred in living cells and not during cell lysis and protein solubilization. Thus, co-immunoprecipitation of the differentially tagged receptors and their detection in molecular weights that are multiple of the monomeric form indicate that the prostanoid IP receptor can dimerize and oligomerize.

3.2. Agonist-independent prostanoid IP receptor dimerization

Dimerization of some G-protein-coupled receptors was shown to be increased or decreased following agonist stimulation (Hebert et al., 1996; Angers et al., 2000; Kroeger et al., 2001; Horvat et al., 2001; Cornea et al., 2001; Rocheville et al., 2000a,b; Latif et al., 2002). To examine whether prostanoid IP receptor dimerization was influenced by agonist treatment, we incubated COS-7 cells transfected with pcDNA3-HA-IP in DMEM containing 0.5% bovine serum albumin, 20 mM HEPES and 1 μM of carbaprostacyclin ($cPGI_2$) (a stable PGI_2 analog) for time periods varying from 0 to 3 h at 37 °C. After cell harvest and lysis, cell extracts were subjected to SDS-PAGE and Western blot analysis with the use of a mouse monoclonal anti-HA antibody. Fig. 2A shows that presence of $cPGI_2$ over the time periods tested does not alter the ratio of dimeric to monomeric forms of the receptor. This is further confirmed by the densitometry analysis shown in Fig. 2B, where no statistical differences were observed in bands intensity when stimulated or not by $cPGI_2$. Similarly, amounts of the higher oligomeric forms of the receptor were not affected by agonist exposure (data not shown).

3.3. Identification of residues/domains of the prostanoid IP receptor involved in its dimerization/oligomerization

As discussed in Introduction, residues or domains involved in G-protein-coupled receptor dimerization differ depending on the receptor. Thus, we next attempted to determine the prostanoid IP receptor domains responsible for its dimerization/oligomerization. To this end, several receptor mutants were generated. Fig. 3 schematically describes the deletion and site-specific mutant constructs that were used in this study. Using the corresponding primers (Fig. 3), the HA-tagged F1R2, F1R3, F1R4 and F3R2 deletion mutants were initially made. The capacity of each deletion mutant to co-immunoprecipitate with full-length FLAG-tagged IP was then evaluated. Cell lysates from COS-7 cells co-transfected with FLAG-IP and either of the HA-tagged deletion constructs were immunoprecipi-

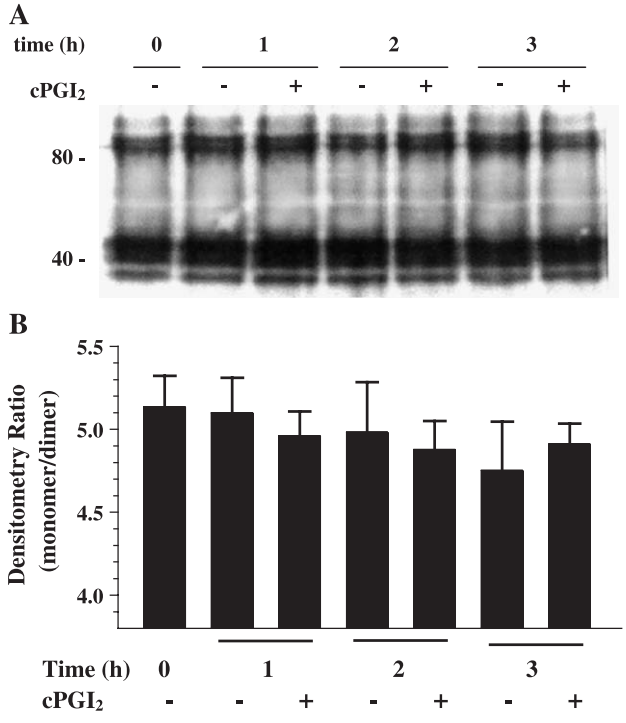

Fig. 2. (A) Effect of $cPGI_2$ stimulation on prostanoid IP receptor dimerization. COS-7 cells transiently expressing HA-IP were stimulated with 1 μM $cPGI_2$ for the indicated times. Cell lysates were then subjected to SDS-PAGE and Western blot analysis carried out with a monoclonal specific anti-HA antibody as described under Materials and methods. Molecular weights are indicated in kDa. The blot shown is representative of three independent experiments. (B) Densitometry analysis of the prostanoid IP receptor monomer/dimer ratio. Densitometry was done using the Scion Image Software. Statistical analysis showed no significant differences ($P>0.05$) between the conditions shown ($n=3$).

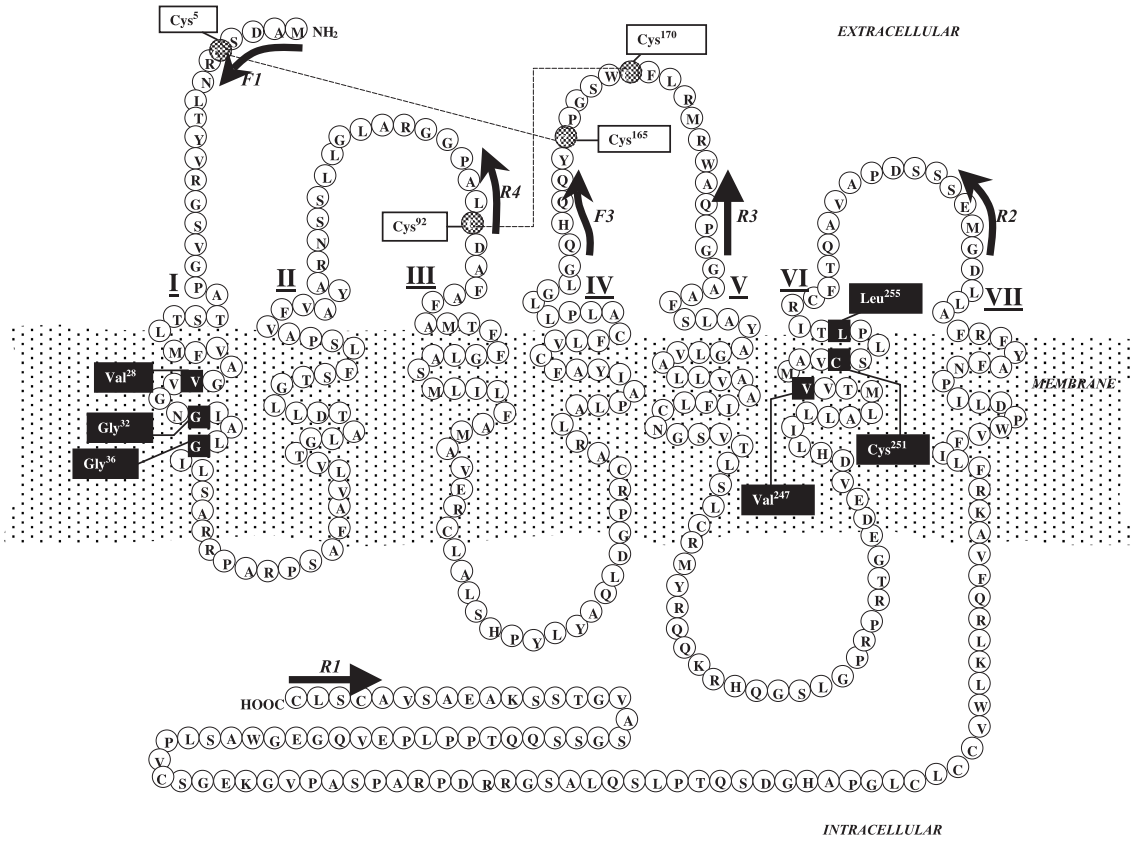

Fig. 3. Schematic representation of the prostanoid IP receptor and of the various mutants that were generated. The circled letters represent the amino acid composition of the prostanoid IP receptor with the seven transmembrane domains numbered from I to VII. F1, F3, R1, R2, R3 and R4 are primers used to construct wild-type IP and the F3R2, F1R2, F1R3 and F1R4 deletion mutants. Residues in transmembrane domains I and VI that were mutated to Ala to generate the TM1, TM6, and TM1–TM6 mutants are illustrated by black squares. The extracellular Cys residues that were substituted by Ser amino acids are indicated by grey circles. The wild-type prostanoid IP receptor and its mutants were subsequently subcloned in pcDNA3-HA and/or pcDNA3-FLAG plasmids to generate amino-terminus epitope-tagged constructs.

tated with a mouse monoclonal anti-FLAG antibody. The samples were then processed for Western blot analysis with a rabbit polyclonal anti-HA antibody (Fig. 4). Our results show that the HA-tagged F1R2 and F1R3 mutants were expressed (Fig. 4, middle panel) and co-immunoprecipitated with FLAG-IP as well as the full-length HA-IP (Fig. 4, upper panel). However, the F1R4 and F3R2 mutants were poorly expressed (Fig. 4, middle panel) but still co-immunoprecipitated with FLAG-IP (Fig. 4, upper panel). It is also seen that F3R2 appeared as a single band whereas the wild-type and the F1R2 and F1R3 mutants were all detected as triplets. This was probably due to the absence of potential *N*-glycosylation sites (Asn^{7} and Asn^{78}) in F3R2 (Fig. 3). Similar results were obtained when each of the FLAG-tagged deletion mutants was co-immunoprecipitated with its own HA-tagged version (data not shown). These data suggest that the deleted domains do not appear to participate in receptor dimerization.

We then looked if dimerization motifs were present in the prostanoid IP receptor amino acid sequence. Two amino acid regions of the prostanoid IP receptor correspond to two different dimerization motifs found in other transmembrane proteins. The first one, located in transmembrane domain I, is comprised of the amino acids ^{27}GVVGNGLALGILS^{39} and similar to the LKTLGIIMGTFTL peptide motif of the β_2-adrenoceptor (Hebert et al., 1996). This motif in the β_2-adrenoceptor was shown to be responsible for its dimerization and is analogous to the LIxxGxxxGxxxT dimerization motif of the glycophorin A protein (Lemmon et al., 1992). A second putative dimerization motif was found in the transmembrane domain VI of the prostanoid IP receptor and corresponds to the amino acid sequence ^{247}VMAVCSLPLTIR^{258}. This region displays similarity to the ILGVICAIILIIIIV and the IMIIICCVILGIIIASTI dimerization motifs of synaptobrevin and syntaxin, respectively (Laage et al., 2000). Amino acids which are underlined in the β_2-adrenoceptor, synaptobrevin and syntaxin motifs were shown to be critical for dimerization (Hebert et al., 1996; Laage et al., 2000). The underlined residues of the putative prostanoid IP receptor motifs (illustrated as black squares in Fig. 3) were mutated. Residues Val^{28}, Gly^{32} and Gly^{36} of transmembrane domain I were mutated to Ala to generate a triple mutant referred to as TM1. Likewise, amino acids Val^{247}, Cys^{251} and Leu^{255} in transmembrane domain VI were changed to Ala to yield mutant TM6. The double-mutant TM1–TM6 was also generated, eliminating both putative dimerization

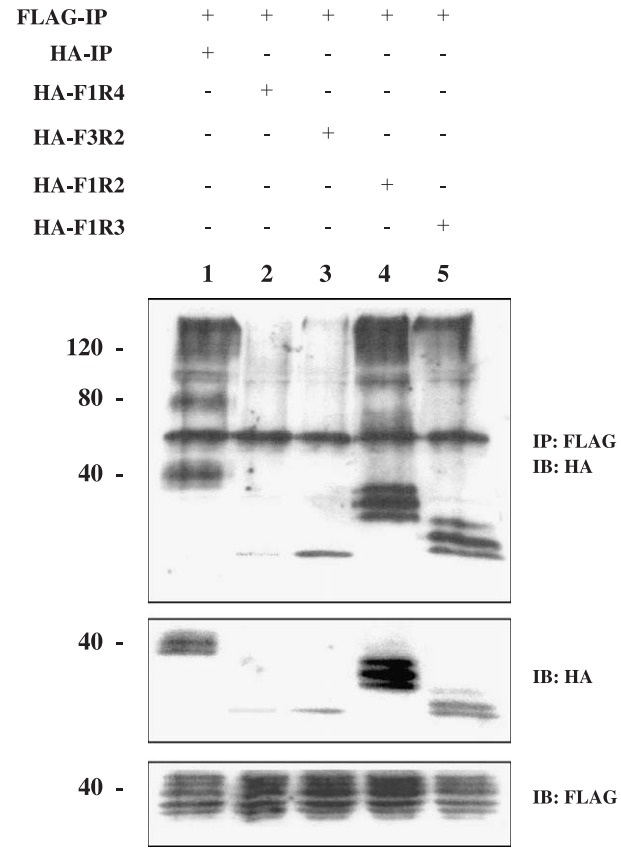

Fig. 4. Co-immunoprecipitation of the prostanoid IP receptor deletion mutants with the wild-type receptor. COS-7 cells were transiently transfected with pcDNA3-FLAG-IP and either pcDNA3-HA-IP (lane 1), pcDNA3-HA-F1R4 (lane 2), pcDNA3-HA-F3R2 (lane 3), pcDNA3-HA-F1R2 (lane 4) or pcDNA3-HA-F1R3 (lane 5). Cell lysates were immunoprecipitated with a mouse monoclonal anti-FLAG antibody and processed for Western blot analysis as described under Materials and methods. Expression levels in cell lysates of each HA-tagged constructs is shown in the middle panel. Co-immunoprecipitation of the HA-tagged deletion mutants with the wild-type FLAG-tagged receptor can be seen in the upper panel. The lower panel indicates that the FLAG-tagged wild-type prostanoid IP receptor was expressed at comparable levels in all samples. These Western blots are representative of three independent experiments.

motifs. The TM1, TM6, and TM1–TM6 mutants were all tested for their ability to dimerize with the wild-type receptor and with themselves. All HA-tagged mutants co-immunoprecipitated as well as the wild-type HA-tagged receptor with either the FLAG-tagged wild-type receptor or their respective FLAG-tagged versions (data not shown), suggesting that these two putative motifs are not involved in prostanoid IP receptor dimerization.

The fact that we were unable to identify a receptor region involved in its dimerization with our previous approaches prompted us to look at the possible role of disulfide bonds in this process. Indeed, when we look at the different elements present in the various deletion mutants that we used, extracellular Cys residues are always present. Examples of G-protein-coupled receptor dimerization through disulfide bond formation between conserved extracellular Cys residues sensitive to reducing agents have been reviewed in Introduction. Thus, we tested if prostanoid IP receptor/dimerization was sensitive to the reducing agents 2-mercaptoethanol and dithiothreitol (DTT). COS-7 cells transiently expressing HA-IP were lysed and samples prepared with loading buffers with either no reducing agents, or with increasing concentration of 2-mercaptoethanol or DTT. Proteins were then processed for SDS-PAGE and Western blot analysis using a mouse monoclonal anti-HA antibody. Fig. 5A shows that the HA-IP receptor can be detected mostly in oligomeric and dimeric forms in absence of 2-mercaptoethanol. Incubation of samples with increasing concentrations of 2-mercaptoethanol resulted in the total disappearance of the oligomeric form at low concentrations, whereas the amount of dimeric HA-IP also decreased at higher concentrations of the reducing agent. Accordingly, the monomeric form of the receptor increased dramatically in samples treated with 2-mercaptoethanol. Similar results were obtained when samples were incubated with DTT but with a more striking effect (Fig. 5B). Addition of DTT resulted in a complete conversion of the oligomeric form of the receptor to its monomeric form, even at very low concentrations of the reducing agent. These data strongly suggest that disulfide bonds are involved in the prostanoid IP receptor dimerization/oligomerization. To confirm our findings, we used MG-63 cells, which endogenously express the prostanoid IP receptor (manuscript in preparation). To detect endogenous IP receptors, we used an anti-IP receptor polyclonal antibody, as previously described (Fortier et al., 2001). Fig. 5C shows that oligomerization of endogenously expressed prostanoid IP receptors is detected in MG-63 cells and that the addition of DTT also disrupt the oligomer complexes to give rise to multimeric, dimeric and monomeric forms of the receptor.

To ascertain if the disulfide linkages implicated in the generation of the prostanoid IP receptor dimers/oligomers were not formed artefactually during cell lysis, COS-7 cells transiently expressing HA-IP were lysed in presence or absence of the sulfhydryl alkylating agent iodoacetamide. Fig. 6 demonstrates that, although the amount of receptor protein is slightly different, the overall repartition or proportion of oligomeric/dimeric/monomeric forms of the receptor was not altered by 100 mM iodoacetamide treatment. These results strongly suggest that specific disulfide bonds formed prior to cell lysis are determinant in dimerization/oligomerization of the prostanoid IP receptor.

3.4. Extracellular Cys substitutions in the prostanoid IP receptor altered its dimerization/oligomerization and expression

G-protein-coupled receptors comprise a conserved Cys pair located in the first and second extracellular loops which are thought to be linked by a disulfide bridge. Zeng and Wess (1999) showed that replacement of this M_3 muscarinic receptor Cys pair (Cys^{140} and Cys^{220}) by Ala residues almost completely inhibited receptor dimerization, indicat-

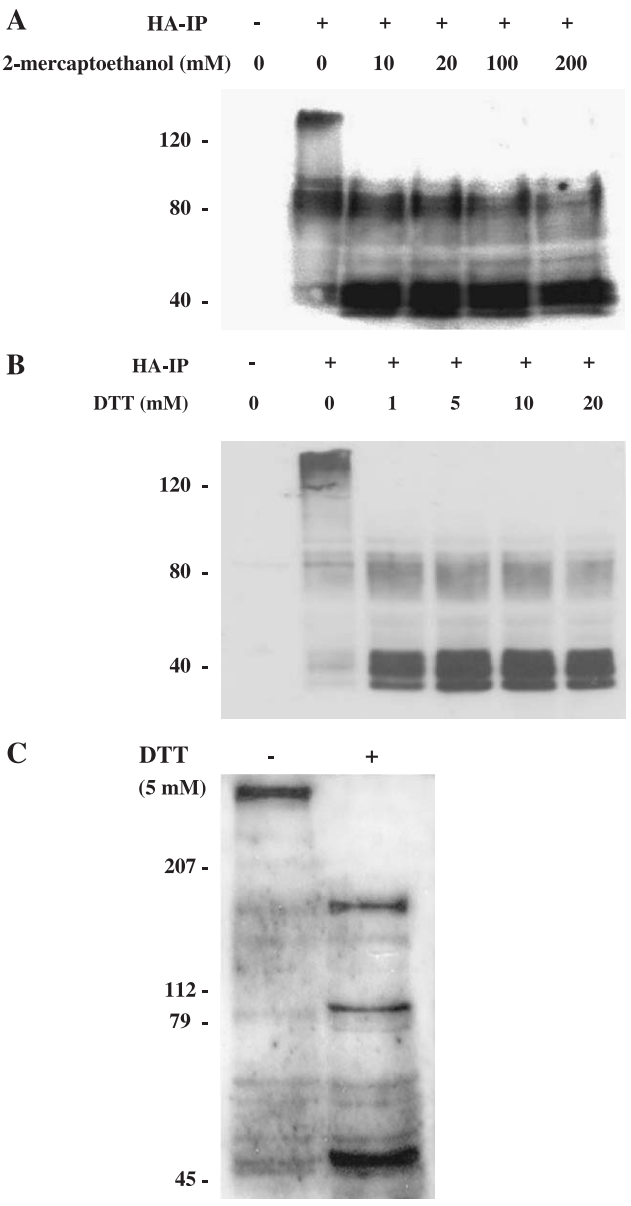

Fig. 5. Effects of reducing agents on prostanoid IP receptor dimerization/oligomerization. COS-7 cells were transiently transfected with pcDNA3-HA or with pcDNA3-HA-IP and then harvested and lysed as described under Materials and methods. Samples were prepared in loading buffer containing either no reducing agents or increasing concentrations of the reducing agents 2-mercaptoethanol (A) or DL-dithiothreitol (DTT) (B). Samples were then subjected to SDS-PAGE and immunoblotted with a mouse monoclonal anti-HA antibody. (C) MG-63 cells were cultured in DMEM with 10% fetal bovine serum and lysed as described in Materials and methods. The samples were subjected to SDS-PAGE with or without DTT in loading buffer, and immunoblotted with a rabbit polyclonal anti-IP antibody (diluted 1:1000) (Fortier et al., 2001). Approximate molecular weights in kDa of monomeric, dimeric and oligomeric species are shown. Western blots are representative of three independent experiments.

ing that these residues are crucial in the formation of disulfide-linked M_3 muscarinic receptor dimers. Four extracellular Cys are found in the prostanoid IP receptor: Cys^{5} within the amino-terminus, Cys^{92} within the first extracellular loop, and Cys^{165} and Cys^{170} located within the second

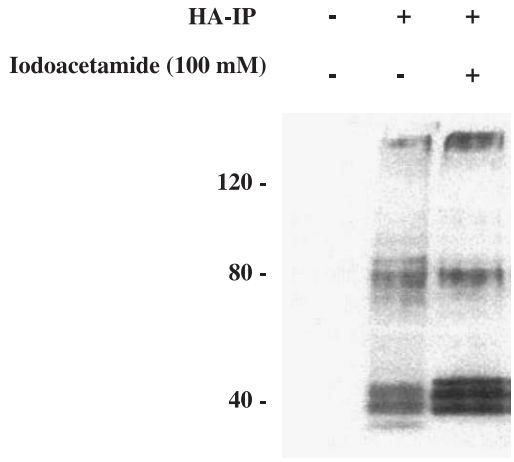

Fig. 6. Effect of iodoacetamide on detection of prostanoid IP receptor dimers/oligomers. COS-7 cells were transiently transfected with pcDNA3-HA alone (as negative control) or with pcDNA3-HA-IP, and then harvested and lysed in presence or absence of 100 mM of iodoacetamide. The prostanoid IP receptors were subjected to SDS-PAGE and detected by Western blot analysis with a mouse monoclonal anti-HA antibody. Approximate molecular weights in kDa of monomeric, dimeric and oligomeric species are indicated. These Western blots are representative of three independent experiments.

extracellular loop (Fig. 3, grey circles). Each of these Cys were mutated to Ser individually and in various combinations to generate the C5S, C92S, C165S, C170S, C92–165S, C92–170S and C165–170S mutants, all HA-tagged for their detection. COS-7 cells were transfected with pcDNA3-HA (negative control), pcDNA3-HA-IP, or either of the Cys mutant constructs. Cells were harvested 48 h post-transfection and processed for Western blot analysis with a mouse monoclonal anti-HA antibody. Fig. 7 reveals interesting results. First, all of the Cys mutants displayed reduced receptor protein expression. More particularly, the C92–165S and C165–170S mutants showed dramatically decreased receptor signals. This was intriguing since the

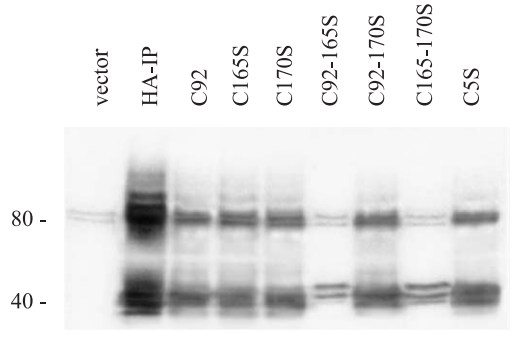

Fig. 7. Implication of extracellular cysteines in prostanoid IP receptor expression and dimerization. COS-7 cells were transiently transfected with pcDNA-3-HA alone, pcDNA3-HA-IP (WT), pcDNA3-HA-C5S, pcDNA3-HA-C92S, pcDNA3-HA-C165S, pcDNA3-HA-C170S, pcDNA3-HA-C92-165S, pcDNA3-HA-C92-170S, or pcDNA3-HA-C165-170S. Cells were then harvested, and processed for Western blot analysis with a mouse monoclonal anti-HA antibody. Approximate molecular weights are shown in kDa. Western blots are representative of three independent experiments.

individual mutations reduced receptor expression to the same extent, while the double C92–165S and C165–170S mutants drastically decreased detection of the receptor. Secondly, the Cys mutants all showed a significantly lower dimer/monomer ratio when compared to the wild-type HA-IP receptor.

Cell surface expression of the various Cys mutants was then evaluated. COS-7 cells were transfected with pcDNA3-HA (background), pcDNA3-HA-IP, or either of the HA-tagged Cys mutant constructs, and cell surface expression determined by ELISA 48 h post-transfection as described under Materials and methods. All of the Cys mutants failed to be expressed at the cell surface (data not shown) which precluded pharmacological characterization of the receptor mutants on the plasma membrane. Altogether, our data show that disulfide bonds and extracellular Cys residues are involved in dimerization/oligomerization, protein folding/stability, and cell surface expression of the prostanoid IP receptor.

4. Discussion

In the present study, we demonstrated in COS-7 cells that the prostanoid IP receptor can dimerize/oligomerize. Using the "classic" co-immunoprecipitation of differentially tagged receptors, we showed that the prostanoid IP receptor can exist in dimeric and oligomeric forms. Detection of dimers/oligomers was not an artefact occurring during preparation of the samples since mixing cells expressing HA-IP with FLAG-IP-expressing cells prior to cell lysis failed to result in co-immunoprecipitation of the receptors. Ratios between monomeric, dimeric and oligomeric forms were left unaltered by agonist treatment. This is in contrast to the β_2-adrenoceptor (Hebert et al., 1996; Angers et al., 2000), thyrotropin-releasing hormone (Kroeger et al., 2001), gonadotropin-releasing hormone (Horvat et al., 2001; Cornea et al., 2001; Kroeger et al., 2001) and SSTR5 receptors (Rocheville et al., 2000a), and with SSTR5–dopamine D2 heterodimer (Rocheville et al., 2000b) for which agonist stimulation resulted in increased dimer formation, whereas agonist treatment of the thyrotrophin receptor promoted the conversion of the dimeric to the monomeric form of this receptor (Latif et al., 2002). However, dimerization of the M_3 muscarinic receptor was shown to be unaffected by application of the agonist (Zeng and Wess, 1999).

We then attempted to identify a receptor domain responsible for its dimerization/oligomerization. Our results revealed that all of the TM1, TM6, TM1–TM6, and receptor deletion mutants could still dimerize/oligomerize, indicating that the corresponding regions or amino acids were not the determinants we were looking for. A common denominator to all the receptor mutants that were constructed was the presence of Cys residues which could potentially be involved in the formation of disulfide bonds governing receptor dimerization/oligomerization. Supporting this hypothesis, addition of the reducing agent DTT potently converted the dimeric/oligomeric receptors to the monomeric form (Fig. 5B). The use of the osteoblastic MG-63 cell line indicated that the oligomerization also occurs with endogenously expressed prostanoid IP receptors, that this oligomerization is sensitive to DTT, and that it is not the result of overexpression of the receptors or only restricted to COS-7 cells (Fig. 5C). Treatment with the cell permeable SH group alkylating reagent iodoacetamide did not affect the oligomer/dimer/monomer ratio (Fig. 6), indicating that disulfide bonds were not formed artefactually during cell lysis.

Involvement of disulfide bonds between extracellular domain Cys residues was reported for the $mGlu_1$ (Ray and Hauschild, 2000), $mGlu_5$ (Romano et al., 1996, 2001), CaR (Zhang et al., 2001; Ray et al., 1999; Ward et al., 1998; Bai et al., 1998) and M_3 muscarinic receptors (Zeng and Wess, 1999). To further confirm that the same phenomenon might happen with the prostanoid IP receptor, we substituted Cys^5, Cys^{92}, Cys^{165} and Cys^{170} by Ser residues. Our data show that all of these residues are important for proper receptor protein expression, as every Cys mutants displayed reduced expression levels. The C92–165S and C165–170S mutants were particularly poorly expressed. This strongly suggests that Cys^{92} is forming a disulfide bond with Cys^{170}, whereas Cys^5 would be involved in the same way with Cys^{165}. This is indicated by a few observations if we start with the premise that two disulfide bonds can be formed between these four Cys residues. First, all the individual Cys mutants showed greatly reduced but significant receptor protein expression. This could be explained by the loss of one disulfide link but not the other, still procuring some degree of receptor conformation or stability enough to ensure protein expression. The fact that the C92–170S mutant displayed the same level of expression as the individual C92S and C170S mutants would support the concept of the formation of a disulfide bond between Cys^{92} and Cys^{170}, since eliminating both of them had the same consequence as the individual mutation. On the other hand, the C92–165S and C165–170S mutations resulted in substitution of Cys residues involved in two distinct disulfide bonds (illustrated in Fig. 3), probably leading to misfolding of the protein and its elimination. Formation of a disulfide bond between Cys^{92} and Cys^{170} is also supported by the fact that they correspond to the extremely well-conserved G-protein-coupled receptor extracellular Cys residues engaged in such linkages (Narumiya et al., 1999; Smyth and Fitzgerald, 2002). It is also interesting to note that the poorly expressed F1R4 and F3R2 constructs (Fig. 4) have one set of Cys residues but lack their respective disulfide bond partners. In contrast, the F1R2 and F1R3 deletion mutants possessed all of the extracellular domain Cys residues and were expressed as well as the wild-type receptor. Moreover, it seemed like the formation of the proposed disulfide links are involved in

receptor dimerization since all the Cys mutants that were generated showed a significantly altered dimer/monomer ratio when compared to the wild-type receptor, which was concurred by co-immunoprecipitation experiments (data not shown). Worthy of note, none of these mutants was expressed at the cell surface, indicating that dimerization occurs within intracellular compartments. Interestingly, it was reported that $GABA_B$ receptor 1 needed co-expression of, and dimerization with, the $GABA_B$ receptor 2 to be expressed at the cell surface (Jones et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999; White et al., 1998; Calver et al., 2001; Pagano et al., 2001). It is tempting to speculate that the prostanoid IP receptor Cys mutants were not expressed at the cell surface because of their faulty dimerization. However, we cannot distinguish between the requirement of IP dimerization for cell surface expression or if misfolded receptor proteins were targeted for degradation instead of the cell surface.

In summary, we showed in this study that the prostanoid IP receptor can dimerize/oligomerize in a process which is highly dependent on disulfide bond formation. Cys residues of the extracellular domains are determinant in receptor expression and dimerization possibly by forming disulfide links between Cys^5 and Cys^{165}, and between Cys^{92} and Cys^{170}. Dimerization/oligomerization was unaffected by agonist treatment and likely occurred intracellularly since receptor mutants not expressed at the cell surface could still form dimers/oligomers.

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