

***Structure function studies on prostanoid receptors:
Thromboxane A₂ receptor (TP) and Prostacyclin
receptor (IP)***

by

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Abstract

Cell membrane receptors help to mediate communication between the cell and its environment. The largest group of these membrane receptors belong to the family of G protein-coupled receptors (GPCRs). GPCRs contain seven transmembrane (TM) helices and signal predominantly through heterotrimeric G proteins in response to diverse extracellular stimuli. Previously, three levels of amino acid conservation were proposed to understand the structure and function of a GPCR. This includes “signature” amino acids, “group –conserved” amino acids and amino acids conserved only within a specific subfamily. The group-conserved residues in class A GPCR family involve amino acid conservation of up to 99% when considered as a group of small and weakly polar residues (Ala, Gly, Ser, Cys and Thr). These group-conserved residues have been proposed as key determinants in helix-helix interactions. Therefore, I selected these residues for structure-function analysis in the amine and the prostanoid receptor sub-families of class A GPCRs. Molecular and biochemical assays clearly demonstrate the importance of group-conserved residues in β_2 -adrenergic receptor and thromboxane A₂ receptor (TP) structure and function. These studies led to the identification of a non-synonymous single nucleotide polymorphic variant (nsSNP) A160T in TP to be a constitutively active mutant (CAM). Further, the TP-CAM was used as a pharmacological tool that enabled classification of well-known TP-blockers, into neutral antagonists and inverse agonists. The role of TP-A160T in prostanoid receptors, TP- Prostacyclin receptor (IP) heterodimerization and signaling was investigated. Activation of a GPCR ultimately leads to structural changes in its intracellular loops (ICLs), which in turn activates G-protein. TP activates its cognate G protein (G_{αq}), while IP mediates signaling, through G_{αs}. Using TP-IP chimeric receptors, molecular modelling, and site directed mutagenesis studies I determined the specific ICL regions required for G-

protein coupling in TP and IP. Significant challenges exist in expressing and purifying GPCR-CAMs in amounts required to pursue biophysical studies. Using tetracycline inducible HEK293S system, A160T was expressed at high-levels and CD spectropolarimetry studies were successfully pursued on the purified A160T. The CD spectra showed that the loss of thermal stability of the A160T mutant is due to the subtle changes in the secondary structure of the A160T protein. These studies involving molecular, biochemical and pharmacological approaches provide novel insights into the structure and function of prostanoid receptors TP and IP.

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Chapter 2A. Structural and functional role of small group-conserved amino acids present on TM7 in the β_2 -AR

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List of Abbreviations

AA	Arachidonic acid
AC	Adenylate cyclase
ANOVA	One-way analysis of variance
β_2 -AR	β_2 -adrenergic receptor
BSA	Bovine serum albumin
BRET	Bioluminescence resonance electron transfer
Ca^{2+}	Calcium
CAM	Constitutively active mutants
cAMP	Cyclic AMP
CCR5	Chemokine receptor
CD	Circular dichroism
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CHS	Cholesterol hemisuccinate
COX-2	Cyclooxygenase 2
DAG	Diacylglycerol
DM	Detergent n-dodecyl- β -D-maltoside
DNA	Deoxy ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EP	Prostaglandin E2 receptor
ECL1, 2, 3.	Extracellular loop-1, 2, 3
FACS	Fluorescence activated cell sorter
FRET	Fluorescence resonance electron transfer
FSH	Follicle-stimulating hormone

GnTI ⁻	N-acetylglucosamine transferase 1
GPCRs	G protein-coupled receptors
GPCRDB	G protein-coupled receptor database
GRKs	G protein receptor kinases
GSK3	Glycogen synthase 3 kinase
HEL	Human erythroleukemia cells
hASMC	Human aortic smooth muscle cells
HEK293	Human embryonic kidney cells 293
ICLs 1, 2, 3.	Intracellular loops 1, 2, 3
IP	Prostacyclin receptor
IP ₃	Inositol-1, 4, 5-trisphosphate
KDa	Kilo dalton unit
LH	Luteinizing hormone
Meg 01	Human megakaryocytes cells
MFI	Mean fluorescence intensity
NMR	Nuclear Magnetic Resonance
NSAID	Non-steroidal anti-inflammatory drug
OG	Octyl-β-D-glucoside
PBS	Phosphate-buffered saline
PDE	Phosphodiesterase
PGI ₂	Prostacyclin
PGs	Prostaglandins
PKA/PKC	Protein Kinase A/C
PLPs	Platelet like particles

PLA ₂	Phospho lipase A ₂
PMSF	Phenylmethanesulfonyl fluoride
PFA	Paraformaldehyde
RFU	Relative fluorescence unit
RLU	Relative Luminescence unit
RNA	Ribo nucleic acid
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
<i>TBXA2R</i>	TP α gene
TM	Transmembrane
TP	Thromboxane A ₂ receptor
TxA ₂	Thromboxane
UV	Ultra violet
WT	Wild-type
μ M	Micro molar

Chapter 1

Introduction

1.1 Prostanoids and prostanoid receptors

1.1.1 Prostanoids

Prostanoids are known to be a family of lipid mediators with diverse biological actions. They are derived from arachidonic acid (AA), a C20 unsaturated fatty acid. AA acid is primarily stored in an esterified form in the glycerol backbone of the membrane phospholipids, and cannot be metabolised to prostaglandins (PGs) [1, 2]. Once hydrolyzed by Phospholipase A₂ (PLA₂), AA gets converted to PGs by prostaglandin G/H synthases now more specifically known as cyclooxygenase (COX), lipoxygenases and cytochrome P450s [1]. The COX isoforms which are shown to be constitutively expressed within the human body play important role in maintaining the physiological functions, such as platelet aggregation, and renal function.

The COX protein exists in two isoforms, COX1 and COX2, which on entry into the endoplasmic reticulum, catalyze the conversion of AA to intermediate unstable PGG₂ which then gets converted to PGH₂, the common precursor for a number of PGs [2, 3]. Prostanoid synthesis is completed by cell specific synthases, like TXA synthase in platelets, PGI synthase in endothelial cells and PGE synthase in renal tubular cells. Structural chemistry of PGs consists of cyclopentane ring, and based on the modifications of the cyclopentane ring, PGs can be classified into four naturally occurring subtypes: prostaglandin D (PGD), prostaglandin E (PGE), prostaglandin F (PGF) and prostaglandin I (PGI). Similarly thromboxane contains a cyclohexane ring and is subdivided into Thromboxane A₂ and B₂ (TxA₂ and TxB₂) [4].

The importance of prostanoids and prostanoid signaling in pathophysiological conditions of humans is highlighted by the use of aspirin, a pharmacological inhibitor of COX, which is still used as a palliative medicine. Furthermore, genetic knockout mice

models have also provided insights into the importance of prostanoids on human physiological systems and diseases. The role of prostanoids in development, inflammation and various pathophysiological conditions such as cancer, renal dysfunction, hypertension is well established [2]. This makes it important to understand their downstream signaling effects and the underlying molecular mechanisms.

1.1.2 Prostanoid receptors

G protein-coupled receptors (GPCRs) constitute a diverse family of membrane proteins and play crucial role in transducing extracellular stimuli into intracellular signals by predominantly coupling to heterotrimeric G proteins. [5, 6]. GPCRs contain seven transmembrane (TM) helices, three intracellular loops (ICLs), three extracellular loops (ECLs), an extracellular N-terminus and an intracellular C-terminus. The first step in GPCR signal transduction is ligand binding in the extracellular or TM regions. Once the ligand (agonist) binds, it causes a conformational change in the TM domain of the receptor, which in turn facilitates G protein binding in the intracellular side of the receptor so that a variety of downstream signaling can be activated [7].

GPCRs are broadly divided into five families; Rhodopsin-like (class A), Secretin-like (class B), Adhesion, Metabotropic glutamate (class C), and frizzled (class D) receptor family [5]. According to the study by (Fredriksson et al., 2003) [5] there are more than 800 GPCRs in the humans. Among these ~700 were in the rhodopsin family or class A GPCRs and, of these, more than 300 were olfactory receptors [5]. The presence of high numbers of olfactory receptors thus allows humans to detect a wide variety of olfactory ligands. The rhodopsin family of GPCRs are further subdivided into sub-classes like amine, peptide, prostanoid, nucleotide like, and cannabinoid.

The prostanoid receptors are poorly characterized in terms of structure and molecular mechanisms of activation. Some of the well-known members of PGs include thromboxane, leukotrienes, lipoxins, hydroperoxy and hydroxyeicosatetraenoic acids [8]. The major PGs, PGD₂, PGF_{2α}, PGI₂, TxA₂ and PGE₂ are known to signal via their receptors prostaglandin D receptor (DP), prostaglandin F receptor (FP), prostacyclin receptor (IP), thromboxane A₂ receptor (TP), (**Fig. 1.1**) and prostaglandin E receptor (EP) (**Fig 1.3B**). These receptors referred to as the prostanoid receptors belong to the class A GPCRs [3].

In my thesis the major focus is on the structure function studies on prostanoid receptors thromboxane A₂ receptor (TP) and prostacyclin receptor (IP).

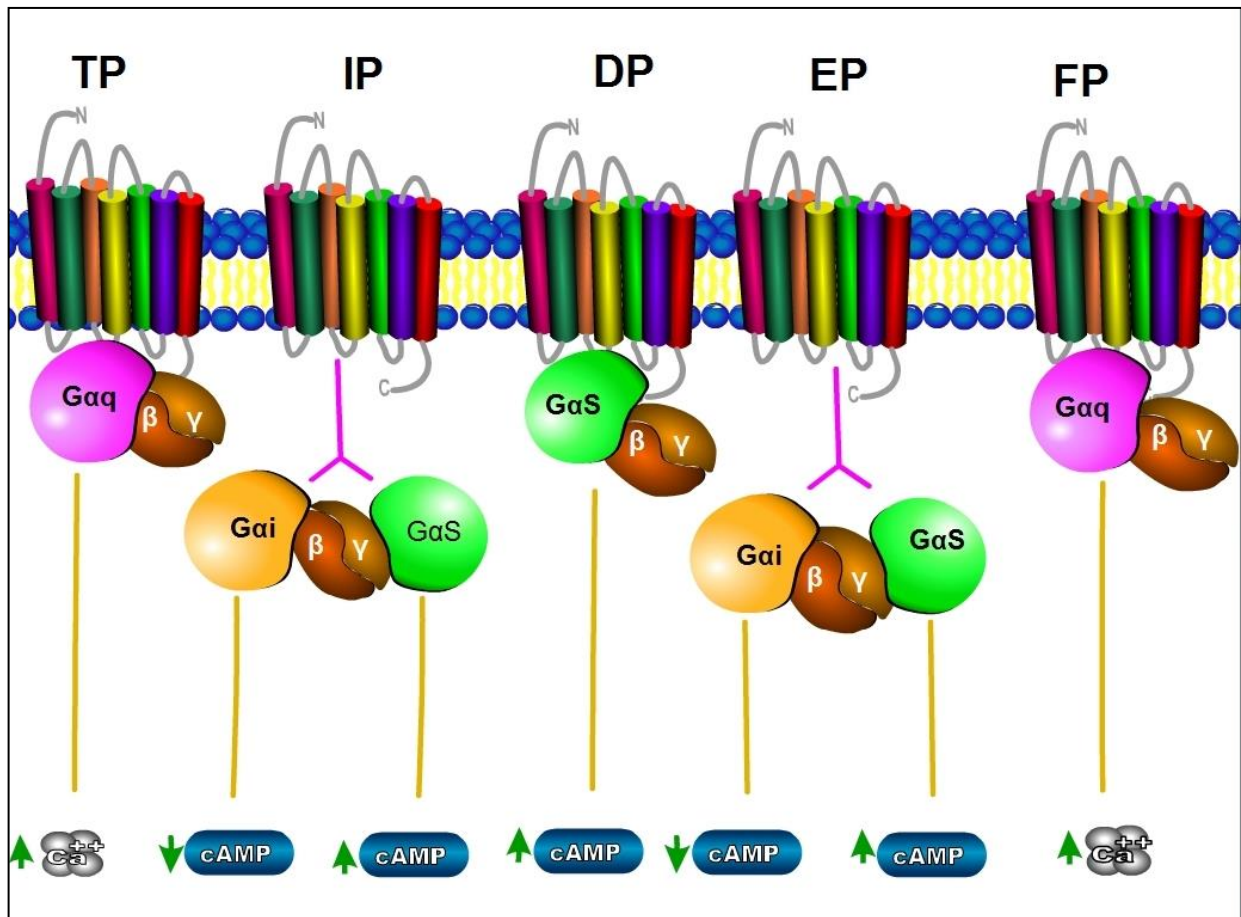


Fig. 1.1. Prostanoid receptors signaling pathways.

The different prostanoid receptors TP, IP, DP, EP and FP along with their cognate G proteins are illustrated. TP and FP receptors predominantly couple with Gαq and the subsequent signaling through PLCβ results in intracellular calcium increase. IP and EP, can couple to two different G proteins, either Gαs or Gαi resulting in increase or decrease in cAMP, respectively. DP is known to couple to only Gαs resulting in increase in cAMP.

1.2 Thromboxane A₂ receptor (TP)

The thromboxane A₂ receptor (TP) belongs to the family of Class A or Rhodopsin like GPCRs. TxA₂ is the endogenous ligand for TP. The receptor is encoded by *TBXA2R* gene located on chromosome 19p.13.3. TP is known to exist in two isoforms, TP α originally cloned from the placenta [9] and TP β cloned from the endothelium [10]. TP β is made up of 423 amino acids, whereas TP α is made up of 343 amino acids. Both the receptors have their initial 328 amino acids similar, and only differ in their C terminus.

1.2.1 Ligand binding pocket of TP

Studies over the last decade using multiple approaches such as chimeric receptors, site directed mutagenesis, and antibody affinity tag, have shown that the TM domains, as well as extracellular loops, ECL2 and ECL3 are important for ligand binding in TP [11-13]. Studies by Funk *et al.*, have shown mutation of the residues in TM7 of TP, L291F, and R295G (known to be conserved across all prostanoid receptors), resulted in complete loss of antagonist SQ 29,548 binding [12]. Another mutation in TM7 W299G resulted in the preferential binding of agonist to the receptor and complete loss of antagonist binding (**Fig. 1.2**). Similarly, mutations of S201A in TM5 as well as S255A in TM6, resulted in loss of agonist I-BOP binding to the TP, whereas no effect was observed in terms of antagonist SQ 29,548 binding [14]. Studies by the same group reported Phe184, Asp193, Thr186, Ser191 in the ECL 2 were important for SQ 29,548 binding to TP, whereas Glu190 did not affect ligand binding in terms of agonist and or antagonist binding compared to WT-TP [15]. Interestingly, the TM5 mutants F196A, F200A, S201T, though located in close proximity of the ECL2 did not affect the ligand binding properties of TP. High resolution nuclear magnetic resonance studies (NMR) of a TP ECL2 peptide segment suggested

ligand contacts with Val176, Leu185, Thr186 and Leu187. Mutation of these residues lead to a decrease in SQ 29,548 binding, whereas of the four residues only Val176 exhibited normal activity compared to WT-TP [13].

1.2.2 Role of cysteine residues in TP

Sequence alignment of amino acids, in TP and other eicosanoid receptors have shown that conserved cysteine amino acids are present in the ECLs, and in the intracellular loop 3 (ICL 3) regions. Studies by Angelo *et al.*, have shown the importance of cysteine residues for their role in ligand binding as well as receptor-G protein coupling in TP [16]. In that study seven cysteine residues were targeted, Cys11 (N terminal domain), Cys35 (TM1), Cys68 (TM2), Cys102 and Cys105 (ECL1), Cys183 (ECL 2), Cys223 (ICL3) and Cys257 (TM6) [16]. The results showed that cysteine replacement with serine at positions 11, 68, 223 and 257 did not affect agonist binding by TP. Interestingly, mutations of the three residues Cys102, Cys105 and Cys184 which are highly conserved across Class A GPCRs, are shown to affect agonist binding and subsequent decrease in calcium signaling compared to WT-TP. The two cysteine residues Cys105 and Cys184 readily form a disulphide bond required for proper folding, trafficking as well as ligand binding in TP. Another interesting finding of the study was the role of Cys223 in ICL3. The C223S mutant was devoid of calcium signaling and the Cys223 residue was found to be critical for receptor-G protein coupling [16]. GPCRs are shown to undergo post-translational modifications, with palmitoylation being one of the most important [17]. Studies on TP isoforms TP α and TP β revealed that TP β gets palmitoylated at position Cys347 in the C terminus, and to a lesser extent at positions Cys373 and Cys377, whereas TP α does not undergo palmitoylation [17]. Interestingly, the C347S mutant in TP β , did not affect

receptor expression or ligand binding, but instead showed a reduced calcium signaling compared to WT-TP β . The results indicate the important role of Cys347 for effective G α_q coupling and subsequent PLC β activation. Furthermore, the study showed that palmitoylation at Cys373, and Cys377 is critical for TP β internalization [17].

1.2.3 Role of protein kinase A/C and G protein-coupled receptor kinases (GRKs) in TP regulation

GPCRs are shown to undergo desensitization on repeated agonist challenge [18]. These proteins undergo desensitization principally by second messenger activated serine or threonine kinases, and GRKs, resulting in receptor internalization and endocytosis. TP like other GPCRs has been shown to be regulated by different second messenger kinases as well as GRKs [19, 20]. Evidence suggests that both TP α and TP β undergo U46619 mediated desensitization due to phosphorylation of Ser45 at ICL1 and Ser331 in C-terminus by PKC [18]. Similarly, it is now well established that it is TP β , and not TP α , which undergoes agonist induced internalization mediated by GRK2/3 at positions Ser239 in ICL2 and Ser 357 in the c-terminus [18]. Mutation of these serine residues to alanine resulted in complete loss of desensitization and TP β receptor internalization [18]. In addition, TP β and not TP α undergo tonic internalization [21].

TP can also be regulated by PKA. Studies by Walsh *et al.*, investigated TP and IP crosstalk and showed that PKA inhibitor: H89, and not PKC inhibitor, GF109203X, resulted in IP mediated TP α but not TP β , desensitization [22]. The Ser329 in c-terminus of TP α was found to be responsible for the observed effect [22]. In another group of studies, the effect of hypoxic condition on TP α phosphorylation and desensitization was investigated. Hinton *et al.*, showed that under hypoxic conditions TP phosphorylation is

decreased compared to normoxic control, in neonatal porcine pulmonary artery myocytes [23, 24]. The decrease in phosphorylation in turn resulted in higher $G_{\alpha q}$ binding and the subsequent TP hyper response. A subsequent study by Santosh, *et al.*, showed that sensitization of TP is due to the IP inactivation in hypoxia [25]. Interestingly, PKA activity was also found to be lower under hypoxic condition compared to normoxic condition. A mechanism was proposed using milrinone, a Phosphodiesterase (PDE) inhibitor. Milrinone treatment resulted in less degradation of cAMP, with subsequent activation of PKA. The activated PKA resulted in phosphorylation of TP. This study was further extended to elucidate the residues involved in PKA mediated phosphorylation. Mutation S324A in the c-terminus of TP α remained insensitive to PKA activation or inhibition by H89 compound [26]. Similarly, mutation S331A in c-terminus though showed decreased affinity both in terms of agonist or antagonist binding, compared to WT-TP, but remained insensitive to PKA inhibition or activation. Interestingly, this S331A mutant showed a lower agonist response curve both in hypoxia and normoxia suggesting the importance of phosphorylation at this site [26]. These studies clearly indicate that phosphorylated Ser324 could be the target for PKA activity and plays a crucial role in reducing the hyperactivity of TP. Ser331 was shown to be an important residue for TP structure and function both in hypoxic and normoxic conditions [26].

1.2.4 G protein binding to TP and downstream signaling

The activation of TP leads to its coupling to different heterotrimeric G proteins in order to regulate a wide variety of physiological and pathophysiological processes involving vasoconstriction, thrombosis, heart disease, hypertension, and acute myocardial infarction [27, 28]. Extensive biochemical and biophysical studies have been done to delineate the distinct TP signaling pathways. TP is known to bind to at least four different G proteins $G_{\alpha q}$, $G_{\alpha 13}$, $G_{\alpha 12}$ and $G_{\alpha i}$ in both cell and tissue specific manner to activate a large number of downstream signaling molecules [29]. Probably the most well established and well characterized TP signal transduction pathway is through its cognate G protein $G_{\alpha q}$ [30, 31]. TP activation by its ligand TxA_2 results in the coupling of $G_{\alpha q}$ and subsequent dissociation of α and $\beta\gamma$ subunits of the heterotrimer, the $\beta\gamma$ subunits then activate phospholipases C, $PLC\beta$, which in turn release inositoltriphosphate (IP_3) and diacylglycerol (DAG) [32, 33]. The IP_3 molecules bind with the IP_3 receptors on the surface of endoplasmic reticulum (ER) resulting in the release of intracellular Ca^{2+} which then mediate various downstream effects. In addition, DAG as well as the Ca^{2+} mobilized has been shown to activate PKC which carries out important physiological processes inside the cell [34, 35]. TP has been shown to communicate with $G_{\alpha i}$ [36-39]. $G_{\alpha i}$ is widely known to inhibit adenylate cyclase activity thereby down regulating cAMP production [40, 41]. Stimulation of $G_{\alpha 13}$ has been shown to activate Rho/ Rho kinase signaling pathway and subsequent myosin light chain phosphorylation in platelets, resulting in actin formation and cytoskeleton rearrangement leading to platelet shape change [28]. The TP- $G_{\alpha 13}$ has also been involved in the regulation of cell motility, contraction and cell proliferation [27]. It has been shown that TP activation response in platelets is abolished in absence of $G_{\alpha 13}$ [42]. Interestingly, it is shown that platelet aggregation requires $G_{\alpha q}$, however platelet

shape change which is crucial in blood clotting depends on G α 13 [43]. Similarly, in smooth muscle cells TP activation of G α 13 is shown to cause calcium sensitization [44, 45]. The structural requirement for TP to bind to G α i or G α 13 is poorly characterized and much remains to be understood.

Structural studies to identify possible G protein binding sites in TP are limited. Over the last decade considerable interest has been focussed on the Glu (E)/Asp (D)-Arg (R)-Tyr (Y) motif or the E/DRY motif, located at the intersection of TM3 and ICL2 in all class A GPCRs [46]. In TP, the DRY motif consists of Glutamate at position 129 and Arginine at position 130. Mutation of these residues were done either to conservative amino acids (for example amino acids with similar charge) or non-conservative amino acids (for example, replacing charged amino acid to a bulkier amino acid or a simple amino acid without any charge) [46]. Interestingly it was found that E129V showed a 10- fold decrease in receptor expression in terms of B_{max} (picomole of receptor /mg of protein) but the agonist affinity and efficacy for G protein binding increased 2-6 fold. These results indicated the importance of Glu129 in maintaining the conformation of TP in an inactive state, while its mutation resulted in relaxing the inactive structure towards an active conformation [46]. Similarly, R130V mutation in TP impaired IP₃ signaling, whereas R130K mutation restored the signaling of TP. The results indicated that the charged amino acid Arg130 is important for receptor activation and signaling for TP [46]. Most of the prostanoid receptors, have a highly conserved ERY(C)xxV(I)xx PL sequence in the ICL2, with the last amino acid being a bulky hydrophobic amino acid (Leu, Ile, Val, or Phe) [47]. In TP, the last amino acid is Phenylalanine at position 138. Studies by Zhou *et al.*, have shown that mutation of the phenylalanine resulted in the receptor incapable of binding to G α q and transduce signal [47]. Similarly, in TP it was shown that when Cys223 in ICL3 was mutated to serine, the

receptor showed impaired calcium signaling, and the residue was found to be critical for receptor-G protein coupling [16]. NMR studies using a 15 amino acids small peptide sequence also showed the importance of the residues Arg130, Phe138, Cys223 in Gαq binding [48]. Though a number of studies have tried to identify potential residues involved in G protein binding, the regions in the ICLs as well as precise amino acids of TP required for Gαq binding are not yet known.

1.2.5 Naturally occurring polymorphic variants of TP

Thromboxane has emerged as a key player in the development of cardiovascular disease. The recent discovery of genetic variants or single nucleotide polymorphisms (SNPs) of the human TP provides unprecedented opportunity to investigate the structural and biochemical basis for disease association (**Fig. 1.2**). Recent studies have identified SNP- R60L in the ICL1 of TP to be associated with an autosomal bleeding disorder [49]. Another naturally occurring point mutation in the highly conserved NPxxY motif in TM7 of TP, D304N also showed bleeding disorder in patients. These nsSNP had reduced agonist and antagonist binding [50]. Two studies by a UK study group have recently shown that N42S and W29C naturally occurring variations in TP also caused increased bleeding in patients [51, 52].

Recent studies by Gleim *et al.*, [53] targeted six TP non- synonymous (change in nucleotide sequence resulting in change in amino acid sequence of protein) polymorphic variants or nsSNPs, V68S, V80E, E94V, V176E, A160T and V217I listed in SNP database to determine their association in a cardiovascular patient cohort (**Fig. 1.2**). In a novel megakaryocytic cell (MEG-01) line mimicking the human platelet system their studies highlighted that the five mutants V68S, V80E, E94V, V176E, and A160T showed a

significant decrease in saturation binding, with antagonist SQ 29,548, compared to WT-TP. However no such effect was observed for the mutants A160T and V217I. Similarly, while most of the mutants displayed similar binding characteristics compared to WT-TP, the mutants A160T and V80E, located close to the ligand binding domain of TP showed increased or significantly decreased affinity, respectively. V80E mutant showed dramatically lower response when stimulated with agonist which was similar to D304N or R60L phenotype and might be responsible for the bleeding disorder. In future, it will be really interesting to actually involve larger cohorts of high risk cardiovascular patient pools across Americas, as well as groups from Asian subcontinents, who are prone to cardiovascular diseases to further identify these genetic variants and find out their possible roles in disease.

1.3 Prostacyclin receptor (IP)

Prostacyclin or PGI₂ has been shown to be one of the important eicosanoids, and is well documented for its critical role in maintaining cardiovascular homeostasis [54]. It is known to inhibit platelet aggregation by counteracting TxA₂ [4]. Therefore, there is a general concern that using selective COX2 inhibitors might be responsible for decreasing PGI₂ production from endothelial cells, without actually disturbing the COX1 derived TxA₂ production from platelets predisposing individuals towards an increased risk of thrombosis [55]. PGI₂ acts through its receptor IP. The receptor has a molecular mass of ~40 KDa but is often detected around ~46-66 KDa due to Asn (N) -linked glycosylation.

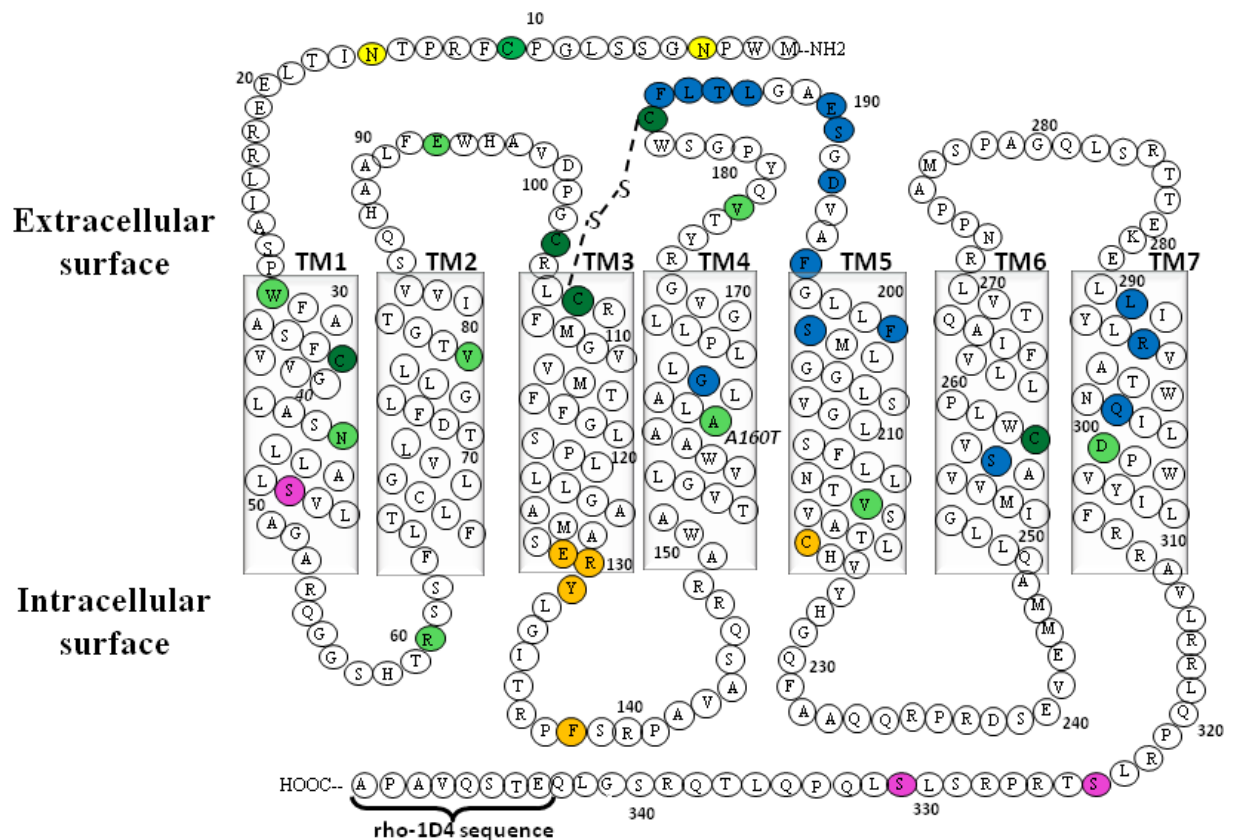


Fig. 1.2. Two dimensional representation of Thromboxane A2 receptor α (TPA).

The receptor has seven transmembrane helices (TM1–TM7) having three extracellular loops (ECLs) and 3 intracellular loops (ICLs). Amino acids are shown in single-letter codes. Yellow circles show N-glycosylation sites. Blue circles show important residues for ligand binding see text (1.1.4). Orange circles represent G protein binding sites. Pink circles indicate important residues for PKA or PKC mediated phosphorylation. Light green circled residues are the genetic variants which have been characterised section (1.1.5). The dark green residues are the cysteine residues which have been characterised in terms of structure function.

1.3.1 Ligand binding pocket of IP

Similar to other GPCRs, the structural topology of IP is made up of 7 TM domains connected by 3 ECLs and 3 ICLs and a long C-terminus (**Fig. 1.3**). Most GPCRs are known to have N-linked glycosylation sites in their N-terminus or in the ECLs [56, 57]. The IP is no exception, and has been shown to have N-glycosylation sites at Asn7 (N-terminus) and Asn78 (ECL1). Mutagenesis studies have shown that Asn78 plays a crucial role in receptor membrane localization, ligand binding and signal transduction of IP [58].

Similarly, four cysteine residues Cys92-Cys170 and Cys5-Cys165 have been shown to form disulphide bonds in IP (**Fig. 1.3**). Mutation of these cysteine residues resulted in loss of ligand binding, as well as receptor trafficking and signaling [59]. Cys118 located in TM3 of IP when mutated to alanine, showed no ligand binding and reduced receptor expression. The observed effect might be caused due to absence or disturbance in the interhelical bonding between TM3 and TM5, known to be crucial for GPCR activation [59]. Another important residue, Cys251 in TM7, is conserved across all prostanoid receptors and mutation to alanine resulted in defective ligand binding and activation of the receptor [59]. Molecular modelling studies have indicated that there might be interaction between Cys251 and Phe278 in the ligand binding pocket, and the mutation might have disturbed this interaction, resulting in defective ligand binding [59].

Proline residues in GPCRs are known to act as molecular hinges along which TM domains might swivel when activated, and may also contribute towards ligand binding. To determine the role of proline(s) Stitham, J *et al.*, mutated the 9 proline residues in IP [60]. Among the proline mutations, P154A in TM4, P179A in TM5, and P254A in TM6 have been linked to defective ligand binding and receptor activation. However, P17A in TM1, P69A in TM2, and P89A in TM3 are present towards the extracellular side of the receptor

and closer to the ligand binding pocket, did not affect the ligand binding to the receptor. The results clearly indicated the importance of the proline residues in IP activation [60].

A chimeric receptor study using mouse DP and IP has shown that it is TM6 and TM7 which could be important for prostacyclin binding [61]. Further studies using site directed mutagenesis, identified amino acids Tyr75 and Phe95 in TM3, Phe278 and Arg279 in TM7, to be important for PGI₂ binding and receptor activation [62]. It will be very interesting to see the high resolution crystal structure of IP bound to its agonist or antagonist validating the above mentioned data.

1.3.2 G protein binding to IP and downstream signaling

IP binds predominantly to G α s and leads to subsequent activation of adenylate cyclase (AC), however, IP can also activate G α q and PLC β in a cell and tissue specific manner [37, 63]. In addition, IP has been shown to bind to G α i and inhibit adenylate cyclase activity, though controversy exists on the IP coupling to G α i. For example, no evidence has been found for IP coupling to G α i in Chinese hamster ovary or CHO cells as well as human embryonic kidney 293 cells (HEK293) [38]. However, IP has been shown to bind to G α i in human erythroleukemia cells or HeLa cells resulting in STAT and STAT6 phosphorylation [64]. To better understand the IP /G α s-protein interface, studies by Zhang *et al.*, have shown that over expressing minigenes corresponding to the ICL1 and ICL3 of IP inhibited cAMP production in HEK 293 cells expressing the IP [65].

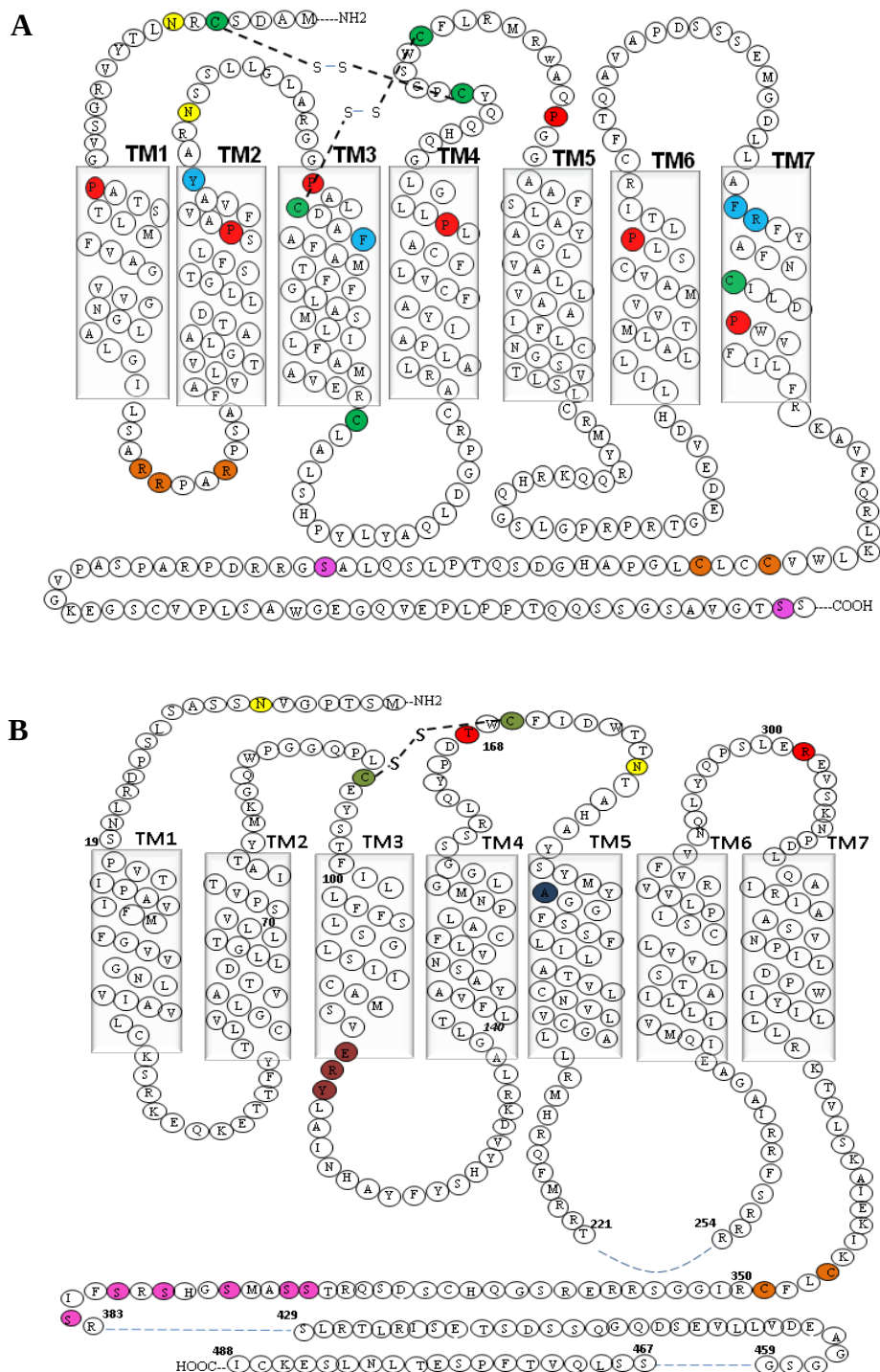


Fig. 1.3. Two dimensional representation of prostacyclin and prostaglandin E receptor 4 (IP and EP4).

Amino acids are shown in single-letter codes. **Panel A (IP)** and **Panel B (EP4)** Red circle residues are proline residues which have been characterised. Yellow circles show N-glycosylation sites. Blue circles indicate important residues for ligand binding see text (section 1.2.1). Orange circles represent G protein binding residues/palmitoylation sites. Pink circles indicate putative residues for PKA/PKC or GRK binding. The dark greens are the cysteine residues which are or could be important for disulphide bonds. For clarity purpose few amino acids in EP4 receptor are removed and shown as dotted lines.

This was similar to data observed in human coronary arterial cells endogenously expressing IP [65]. NMR studies with synthetic peptides of ICL1 of IP and C-terminus (11 residues) of G α s have shown that a triad of arginine residues Arg42, Arg43, Arg45 and Ala44 interact with the G α s peptide sequence. Further experiments using site directed mutagenesis and transient expression in COS7 cells and solution NMR spectroscopy have shown that Arg45 at the centre of ICL1 and residues Arg42 -Ala48 surrounding it, might be critical for receptor activation [66]. The interaction of the c-terminus of G α s with Arg45 of IP was found to be dependent upon the chemical nature of the side-chain of the amino acid, and not the charge of the amino acid. Therefore, when Arg was mutated to Lys the resulting mutation did not rescue receptor activation [66].

The crystal structures of rhodopsin (PDB-ID 3DQB), metarhodopsin II (PDB ID: 3PQR) and the recent β_2 -AR-G α s complex have shown that the G α s-C terminus is away from ICL1 and closer to TM5 and TM6, resulting in speculation over the non-specificity of the interactions observed using such small peptide sequences. In IP, studies by Miggin *et al.*, have shown that Cys308 and Cys311 in the C-terminus get palmitoylated [67]. Amino acid replacements C308S and C311S and C308S, C309S, and C311S, resulted in complete abolishment of G α s mediated signaling, indicating the importance of palmitoylation of either Cys308 or Cys311 in IP signaling [67]. Similarly, they also showed that mutations of C309S and C311S did not affect IP coupling to G α q. However, mutation of C308S resulted in abolishing IP mediated G α q signaling [67].

1.3.3 Role of protein kinase A/C and GRKs in IP regulation

It is now well established that, most of the GPCRs once activated get desensitized i.e. the receptors will not be able to signal even in the presence of agonist. Generally in a two-step process the serine or threonine residues in the c-terminus get phosphorylated by either GRKs or kinases. Once phosphorylated these receptors are recognised by proteins such as β -arrestins, which compete with or sterically hinder the binding of the G-proteins resulting in desensitization [68]. In mouse IP, Ser357 has been shown to be the site for PKA mediated phosphorylation, however, the site analogous to position Ser357 in human is Ser328 and has been shown to be phosphorylated by PKC [37]. Similarly, Ser328 and Ser374 are also important sites for GRK mediated phosphorylation. In one of the studies using the yeast two-hybrid approach, with the C-terminus as bait and human cDNA library from human aortic smooth muscle cells (hASMC) as prey, it was found that the delta subunit of phosphodiesterase-6 (PDE6 δ) interacted with the C-terminus of IP [69]. PDE6 δ protein interaction was studied in hepatocellular carcinoma cells without any endogenous IP or PDE6. When both IP and PDE6 δ were expressed together in the hepatocellular carcinoma cell system, they could be easily co-immunoprecipitated and was dependent on prenylation of the IP C terminus. Treatment with an IP agonist caused internalization of the receptor in 15 mins much faster than the 2 hours' time-frame observed in other cell system, and can be restored back to the surface in 2 hour. Interestingly, when PDE6 was blocked or knocked down, the IP internalization process was slower and suggest that PDE6 might be a crucial player in IP internalization in vascular cells [70]. Few studies indicated that, platelet IP once incubated with iloprost resulted in internalization within 2 hours and can be resensitized after 3 hours indicating the role of agonist dependent internalization and not

receptor desensitization per se [71]. However, little is known about the process of IP internalization in platelets or the specific pathways involved in the process.

1.3.4 Naturally occurring polymorphic variants of IP

Studies on polymorphic variants have shown that V25M mutation in IP had no significant effect on receptor activation in terms of ligand binding or G α s binding. However, a nsSNP mutation R212C in ICL3, was found in patient samples [54]. Pharmacological analysis of the mutant showed decreased signal transduction in terms of G α s coupling and signaling. Even the receptor expression level was lower compared to WT-IP and was predominantly located in the ER. With the known importance of IP in maintaining the cardiovascular homeostasis, the above data provided critical insights on how naturally occurring mutations and dysfunctional IP might predispose patients towards hyperthrombotic state and other diseases [54].

1.4 Homodimerization and heterodimerization in prostanoid receptors

Evidence suggests that GPCRs are able to form dimers and higher oligomeric complexes *in vitro* as well as *in vivo* [72, 73]. Such receptor homodimers or heterodimers display altered functional properties. For example, similar to other GPCRs, IP and TP can physically attach to form homo- and heterodimers [36, 74]. Heterodimerization of TP with IP resulted in coupling of TP α to cAMP generation and rendered TP sensitive to regulation by IP agonists. This implies that when TP and IP are expressed in the same cells, such as vascular smooth muscle cells, cAMP can be generated via the PGI $_2$ -IPIP and TxA $_2$ -IPTP pathways [75].

A previous study showed few individuals to be heterozygous for an IP polymorphic variant [54]; When Arg212 was mutated to Cys patients displayed a significant loss of platelet IP function and were found to accelerate cardiovascular disease [54]. The authors indicated this was due to IP R212C exerting a dominant negative action on the wild type IP and TP α through dimerization [75]. Thus far, most of the studies on TP-IP crosstalk focused only from the IP perspective and none have examined the crosstalk in the context of TP genetic variants. GPCR dimerization studies using Fluorescence resonance electron transfer (FRET) and Bioluminescence resonance electron transfer (BRET) techniques have shown the structural requirements for receptor dimerization but very few have pursued on prostanoid receptors.

In conclusion the variety of prostanoid receptors, their ligands, and their importance in various physiological and pathophysiological conditions makes it important to understand their molecular mechanisms of activation. In addition, growing importance of SNPs in GPCRs and their disease association and absence of high resolution crystal structures of any of the prostanoid receptors, structure-function studies in the prostanoid receptor family will provide new insights towards prostanoid receptor activation and signaling mechanisms.

1.5 Study objectives and rationale

Hypothesis. *Prostanoid receptors exist in multiple signaling states and each of these states has unique structural and functional properties.*

To test this hypothesis, a series of structure-function studies were pursued on the two well-characterized prostanoid receptors, TP and IP. In certain cases, comparison with other Class A GPCRs was made. This study had five objectives listed below.

Specific objective 1.

Role of group-conserved amino acids in the activation mechanism(s) of Class A GPCRs, including the prostanoid receptors.

Rationale and approach.

Recently, in class A GPCRs it has been shown that, in TM helices weakly polar amino acids with small side chains are highly conserved (>90%) when considered as a group for example Gly, Ala, Ser, Thr, and Cys [76, 77]. These group-conserved amino acids could be involved in proper folding of the receptor structure as well as in receptor function. Using site-directed mutagenesis, molecular modelling and biochemical approaches the role of group-conserved residues in two prototypical members of the amine and prostanoid subfamily β_2 -AR and TP were pursued. This study led to characterization of group-conserved amino acid Ala160 in TM4 of TP, and a naturally occurring nsSNP variant at the position A160T (discussed in Chapter 2A and 2B).

Specific objective 2.

Characterize the molecular basis for prostanoid receptor-G protein interaction(s).

Rationale and approach.

Conformational changes in the TM helices of a GPCR through rigid body movement of the receptor are believed to contribute in achieving the active state (s) [78]. These conformational changes in the helices would transmit to the ICLs that bind to specific G proteins, and sustain GTP/GDP exchange, leading to activation of intracellular signaling cascades. Prostanoid receptors couple to a variety of heterotrimeric G proteins to regulate physiological and pathophysiological processes involved in vasoconstriction, thrombosis and heart disease. Some prostanoid receptors, such as TP transduce predominantly through heterotrimeric G proteins that signal through PLC- β causing changes in intracellular calcium. However, others such as IP bind predominantly to $G_{\alpha S}$ and signal through adenylate cyclase (AC) and cause changes in intracellular cAMP. However among the prostanoid receptors, the structural features on the receptors that determine binding of different G_{α} -proteins have not been determined. Using TP-IP chimeric receptors, site-directed mutagenesis, molecular modelling and cell based assays the molecular basis of prostanoid receptor-G protein interactions was pursued (discussed in chapter 3).

Specific objective 3.

High-level expression, purification and characterization of a constitutively active thromboxane A_2 receptor polymorphic variant.

Rationale and approach.

Over the last few years 18 GPCR crystal structures were solved. However, the crystal structure of a member of the prostanoid receptor subfamily is not yet reported. Surprisingly, the structure of only one constitutively active GPCR has been reported. Over-expression of GPCRs in both quantity and quality for structural studies is still a formidable challenge. In this thesis, work on the first objective lead to the characterization of the first constitutively active genetic variant in prostanoid receptors, A160T in TP. To understand the structural basis for the constitutive activity, biophysical studies including high resolution structural studies need to be done. To pursue biophysical studies, expression and purification of WT-TP and A160T in high-levels is required. To achieve this HEK293S tetracycline inducible cell system was utilised (discussed in chapter 4)

Specific objective 4.

Classify the efficacy of TP blockers using the constitutively active A160T genetic variant.

Rationale and approach.

Over the last decade a considerable number of GPCRs was shown to have constitutive activity [79, 80]. This phenomenon of GPCRs became the most important tool in pharmacological classification of ligands into inverse agonists and neutral antagonists [81, 82]. Inverse agonists are compounds or drugs known to reduce the constitutive GPCR activity and are often defined to have a (-1) efficacy whereas neutral antagonists do not affect the basal GPCR activity and have (0) efficacy [83]. In this thesis, using the constitutively active mutant, A160T, pharmacological classification of some of the well-known TP blockers as neutral and inverse agonist was pursued (discussed in chapter 5).

Specific objective 5.

Decipher the role of polymorphic variant A160T in mediating signaling cross-talk between the prostanoid receptors TP and IP.

Rationale and approach.

Previous work showed that heterodimerization of TP with IP resulted in coupling of TP α to cAMP generation and rendered the TP sensitive to regulation by IP agonists. This implies that when TP and IP are expressed in same cells, cAMP can be generated via the PGI₂-IP and TxA₂-IPTP pathways. However, dimerization of IP in the presence of a TP genetic variant A160T and under stress conditions such as hypoxia is yet to be elucidated. In this thesis, an attempt was made to decipher the crosstalk between TP and IP in the presence of TP genetic variants and hypoxia using cell based signaling assays and biophysical approaches (discussed in chapter 6).

Chapter 2

**Role of group-conserved amino acids in two Class A GPCRs,
Beta₂-adrenergic receptor (β_2 -AR) and Thromboxane A₂ receptor (TP α)**

2A. Structural and functional role of small group-conserved amino acids present on TM7 in the β_2 -AR.

Arakawa M, Chakraborty R, et al., *Biochimica et biophysica acta* 1808(4): 1170-8, 2011

2A.1 Introduction

G protein-coupled receptors (GPCRs) share a seven transmembrane (TM) helix architecture and activate cognate G-proteins in response to agonist binding. The most extensively studied ligand-activated GPCR is the β_2 -adrenoreceptor (β_2 -AR), which mediates physiological responses to epinephrine and norepinephrine. The β_2 -AR was the first ligand-activated GPCR to be cloned [84], and the first whose crystal structure was determined at high resolution [85]. The crystal structure of β_2 -AR was solved with the inverse-agonist carazolol bound, and provided a direct comparison with the crystal structure of the dark, inactive state of rhodopsin, the dim-light photoreceptor [86, 87]. The two structures revealed the positions of amino acids that are conserved across the family of class A GPCRs, but have left largely unanswered questions concerning their contribution to stabilizing the structure of the inactive receptor or guiding the transition to an active receptor conformation.

Fig. 2a.1 presents a two-dimensional representation of the β_2 -AR sequence with the conserved amino acids highlighted by different colors. There are at least three levels of conservation that can be considered in understanding the structure and function of a given receptor or subfamily of receptors within the GPCR superfamily. The most important set of conserved residues in the class A GPCR family are the “signature” amino acids with sequence identities of >70%. There are 15 signature residues in the transmembranes

(TM1–TM7) including the highly conserved (E/D) RY motif on TM3 and NPxxY motif on TM7. The second level of conservation involves the “group-conserved” residues in the class A GPCR family with conservation of up to 99% when considered as a group of small and weakly polar residues (Ala, Gly, Ser, Cys and Thr). These amino acids have been identified in membrane proteins as key determinants in helix-helix interactions [88, 89]. We have previously suggested that TM helices TM1–TM4 form a tightly packed core on the basis of the location of the group-conserved positions [77]. The group-conserved residues present on TM2, TM3 and TM4 in β_2 -AR have both structural and functional roles [90]. The most significant changes in receptor expression and activity were observed upon replacement of the amino acids Ser161^{4.53} and Ser165^{4.57} on TM4. (The amino acid numbering used in this manuscript incorporates the residue number from the receptor sequence (e.g. Ser165) and a residue number (e.g. 4.57) from a generic numbering system developed by Ballesteros and Weinstein) [91]. Substitution at Ser161^{4.53} and Ser165^{4.57} by larger residues lowered the expression and activity of the receptor, but did not affect specific binding to the antagonist ligand dihydroalprenolol. Substitution of other group-conserved residues on TMs, TM2–TM4 by larger amino acids lowered receptor activity in the order Ala128^{3.47}, Ala76^{2.47}, Ser120^{3.39}, and Ala78^{2.49} [90]. The third level of conservation includes those residues that have sequence identities of >90% in the amine subfamily. Each class A GPCR subfamily contains a set of residues that makes it uniquely able to respond to its own ligand. For example, Asp113^{3.32} on TM3 is 92% conserved in the amine subfamily. This residue is the counter ion to the positively charged amine [92]. The next highest conservation (92%) is Asn318^{7.45} on TM7. Asn318^{7.45} corresponds to Ser298^{7.45} in rhodopsin, a residue that only has 50% conservation in the opsin subfamily.

Other residues that are highly conserved within the amine subfamily are Asn69^{2.40} (90%), Trp313^{7.40} (88%) and Tyr316^{7.43} (82%).

To fully understand the structure and activation mechanism of any class A GPCR one needs to define the roles of key residues at each level of amino acid conservation. Here, we target two group-conserved residues, Gly315^{7.42} and Ser319^{7.46}, present on TM 7. These residues are of particular interest for several reasons. First, they are connected to two different functional microdomains [93-95], the conserved aromatic cluster on TM6 and the NPxxY motif on TM7. Second, they are flanked by residues that have very high sequence conservation in the amine subfamily of receptors: Trp313^{7.40}, Tyr316^{7.43} and Asn318^{7.45}. Third, while being group conserved throughout the class A GPCR family, they have very high sequence identity within the amine subfamily (69.2% glycine at position 7.42 and 98% serine at position 7.46). To determine the structural and functional roles of these two group-conserved residues on TM7 in the β_2 -AR, mutants were generated by site-directed mutagenesis and transiently expressed in COS-1 or HEK293S cells, and then ligand binding assays were performed using membrane preparations. To elucidate the effect of these mutations on G protein signaling, cAMP levels were measured following stimulation by isoproterenol. We discuss the influence of mutation at Gly315^{7.42} and Ser 319^{7.46} on receptor structure and stability.

2A.2 Materials and Methods

2A.2.1 Materials

The β_2 -AR ligands, alprenolol, isoproterenol, epinephrine and salbutamol were purchased from Sigma (St. Louis, MO, USA). Protease inhibitors and common chemicals were purchased either from Fisher or Sigma (Ottawa, ON, Canada). The detergent n-

dodecyl- β -Dmaltoside (DDM) was purchased from Anatrace (Maumee, OH, USA). The monoclonal antibody, rho-1D4, was prepared by the Cell Culture Center (Minneapolis, MN) from a cell line provided by R.S. Molday (University of British Columbia, Vancouver, Canada).

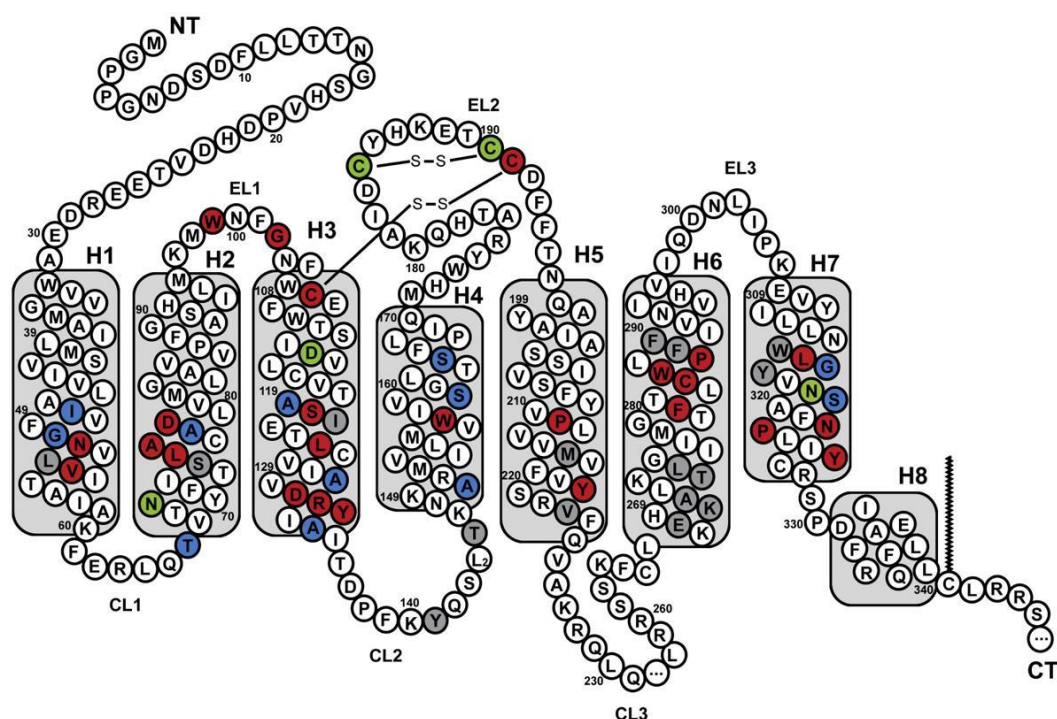


Fig. 2a.1. Two-dimensional representation of the β_2 -AR sequence showing amino acid conservation at the three different levels.

The receptor has seven transmembrane (1–7) and a short amphipathic helix (TM 8) that lies on the cytoplasmic surface of the membrane. Amino acids are shown in single-letter codes. Red circles denote signature-conserved residues that include the (E/D) RY and NPxxY motifs, an asparagine (Asn51^{1.50}) on TM1, aspartic acid on TM2 (Asp79^{2.50}), three prolines on helices TM5 (Pro211^{5.50}), TM6 (Pro288^{6.50}) and TM7 (Pro323^{7.50}) and several hydrophobic residues (Leu75^{2.46}, Trp158^{4.50}). Blue circles represent small and weakly polar group-conserved residues in the Class A GPCRs excluding the olfactory subfamily, and green circles subfamily-specific residues. The residues in gray are between 70% and 90% conserved in the amine receptor subfamily. CL, cytoplasmic loop; CT, C terminus; EL, extracellular loop; NT, N terminus.

Fetal bovine serum was purchased from Sigma and DME High Glucose was from Invitrogen (Burlington, ON, Canada). The β_2 -AR antagonist [3^H] Di hydro alperenolol (DHA) was purchased from GE Healthcare (GE Healthcare Biosciences, Little Chalfont, UK). Synthetic oligonucleotides were purchased from Invitrogen (Carlsbad, CA, USA). Buffers used were as follows: Phosphate-buffered saline (PBS) buffer: 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4 , 10 mM Na_2HPO_4 (pH 7.4); Buffer A (Lysis buffer), 10 mM Tris-HCl, pH 7.4, containing protease inhibitors (1 mM EDTA, 10 μ g/ml benzamidine, 10 μ g/ml leupeptin, 20 μ g/ml soybean trypsin inhibitor, 5 μ g/ml aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride); Buffer B (Storage buffer) 50 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, containing protease inhibitors as in Buffer A; Buffer C (Binding buffer), 75 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, containing protease inhibitors as in Buffer A. Buffer E (solubilization buffer), 20 mM Tris-HCl (pH 7.4), containing 500 mM NaCl, 10% glycerol, 1% DM and the protease inhibitors as in Buffer A.

2A.2.2 Molecular biology and cell culture

Mutant hamster β_2 -AR genes in the plasmid expression vector, pMT4, were constructed using the Quick-Change mutagenesis kit (Stratagene, La Jolla, CA, USA) [90]. The DNA sequences of all the mutated genes were verified by automated DNA sequencing (MICB DNA Sequencing Facility, Winnipeg).

2A.2.3 Cell culture and immunoblot analysis

The WT- β_2 -AR and mutant genes were expressed in COS-1 cells using a DEAE dextran based transient transfection method [96, 97]. For transient transfection of HEK293T cells using the plasmid pMT4, lipofectamine 2000 (Invitrogen) mediated

transfection was used as described by the manufacturer. Membranes were prepared using Buffers A and B and as described previously [98]. The protein concentration in the resuspended membrane pellet was determined using a modified DC protein assay kit from Bio-Rad Laboratories (Hercules, CA). Approximately 2.5 – 5 µg of the total solubilized membrane protein were resolved by 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane. β_2 -AR was visualized by immunodetection with the monoclonal antibody, rho-1D4 [98]. The band at approximately 65 KDa corresponds to the completely glycosylated receptor [96].

2A.2.4 Radioligand binding assays

β_2 -AR receptor radioligand binding assays were carried out in Buffer C, using 2–20 µg of membrane protein and as described earlier [98, 99]. Saturation binding assays were carried out using 0.1 – 20 nM [3 H] DHA. Binding of [3 H] DHA in the presence of 10 µM alprenolol was used as a measure of nonspecific binding. Specific binding was derived by subtracting nonspecific binding from the total binding and is shown in Figure 2a-3. Competition binding assays were performed using 3 nM [3 H] DHA and different concentrations of unlabeled agonists (10^{-2} – 10^{-9} M) and the reactions kept for 2 hr at room temperature. Radioligand binding data obtained from competition curves were analyzed by non-linear regression analysis to determine the K_i and EC_{50} values using PRISM software version 4.03 (GraphPad Software Inc, San Diego, CA).

2A.2.5 Immunofluorescence microscopy

HEK293T cells were seeded into six-well tissue culture plates containing sterilized poly-L-lysine (Sigma)-treated glass cover-slips and transiently transfected with wild-type

β_2 -AR or mutant DNA or according to the aforementioned transfection protocols. Unless specified, all steps were carried out room temperature. Cells were fixed in 3.7% formaldehyde/1x PBS buffer for 15 min, and then permeabilized with 0.05% triton X-100/1x PBS buffer for 30 min. The cells were washed and blocked with 1x PBS buffer containing 2% bovine serum albumin (IgG and Protease free) for 1 hr. β_2 -AR was labeled for 90 min using a 1:1000 dilution of the mouse-anti-rho-1D4 monoclonal antibody (C-terminal tagged β_2 -AR) and a 1:250 dilution of rabbit-anti-calnexin polyclonal antibody (Abcam, MA, USA; endoplasmic reticulum marker). The labeled receptor was washed and incubated with a fluorescent coupled secondary antibody using a 1:500 dilution of anti-mouse-FITC (Jackson ImmunoResearch labs, PA, USA) and anti-rabbit-Texas Red (Jackson ImmunoResearch Laboratories, PA, USA) for 60 min in the dark. The coverslip was washed twice, mounted with Prolong-antifade-gold (Invitrogen, Molecular probes, CA, USA) for 15 min and its edges sealed with nail-polish. Representative cells were selected and visualized using an Olympus BX61 microscope for cytoplasmic or plasma membrane localization.

2A.2.6 Determination of receptor activation by cAMP assays for β_2 -AR and mutants

Functional characterization of the β_2 -AR was carried out with HEK293S cells using a commercially available cAMP assay system (DiscoverX HitHunter TMcAMP XS+, Freemont, CA). HEK293S cells were used as these cells show very small changes in endogenous cAMP upon stimulation with isoproterenol [90, 99]. Measurements of cAMP levels were made according to the directions supplied by the manufacturer. Briefly, 44 hrs after transfection HEK293S cells (100,000 cells/well) were seeded in a 96 well plate and stimulated for 30 minutes with concentrations of agonist (–) isoproterenol ranging from

10^{-6} M to 10^{-12} M. Luminescence was read using a Flex Station 3 plate reader (Molecular Devices, CA, USA). The assays were carried out a minimum of three times, each in duplicate and the data were analyzed using PRISM software version 4.03 (GraphPad Software Inc, San Diego, CA). The cAMP values of the mutants expressed as relative luminescence units (RLUs) were normalized to that of wild type β_2 -AR.

2A.2.7 Occluded surface calculations

The occluded surface (OS) method for the analysis of packing interactions in proteins has been previously described [88, 100]. The method yields packing values of individual amino acids within a protein. Briefly, a packing value is composed of two parameters, the OS area and the distribution of distances to occluded atoms. Using coordinates from a high resolution structure, a molecular dot surface is calculated for each residue using a 1.4-Å probe. The dot density is chosen such that each dot represents 0.215 Å² of the surface area. A normal is extended radially from each dot until it either intersects the van der Waals surface of a neighboring atom or reaches a length of 2.8 Å (the diameter of a water molecule). The OS is defined as that molecular surface area on the originating atom associated with normals that intersect with another atom surface as opposed to reaching the 2.8-Å limit. All other molecular surface area is considered non-occluded or exposed. The packing value (PV) relates the occluded surface to the total surface of the residue (sum of occluded and non-occluded areas). Division by the total molecular surface area normalizes the packing value to account for various sizes of amino acid residues. Structural waters are not included in the calculations, resulting in surface areas that are non-occluded.

2A.2.8 Homology modeling

The homology model of G315L β_2 -AR was created in Swiss PDB viewer [101] (<http://www.expasy.org/spdbv/>) by threading the G315L β_2 -AR sequence onto the opsin structure (3CAP). The resulting structure was energy minimized *in vacuo* with the GROMOS96 43B1 parameters set without a reaction field. The energy computations were one with the GROMOS96 implementation of Swiss-PdbViewer.

2A.3 Results

2A.3.1 Influence of mutations on receptor expression and ligand binding

Table 2a-1 presents the saturation binding data of the antagonist DHA for the wild-type β_2 -AR and the Gly315^{7.42} and Ser319^{7.46} mutants. Two types of mutations were made. First, mutations were made to other group-conserved amino acids, for example, glycine to alanine or serine to glycine. The hypothesis is that these conservative substitutions will have a minimal effect on receptor folding and ligand binding. Second, mutations were made to a non-group conserved amino acid with a bulky side chain (i.e. leucine). Total binding, specific binding and non-specific binding of DHA were measured [98]. Specific binding is the difference between the observed total binding and non-specific binding. Binding of [³H] DHA in the presence of 10 μ M alprenolol was used as a measure of non-specific binding. When compared to the wild-type β_2 -AR, the G315A mutant showed a similar affinity for DHA. The G315S mutant exhibited a higher K_D and an increased amount of non-specific binding. High non-specific binding can be due to the improperly folded protein interfering with the radioligand assay, or else it can also be due to the mutation perturbing the ligand binding site of the receptor. In contrast to the receptors with these conservative substitutions, the G315L mutant totally failed to bind to DHA in a

specific manner. The binding affinity of DHA to the β_2 -AR with mutations at the group-conserved site Ser319^{7,46} was similar to the wild-type receptor for the conservative mutations S319A and S319G, but the K_D increased for the non-group conserved leucine substitution, S319L. The level of receptor expression was quantified by immunoblots and saturation binding assays using DHA (**Fig. 2a.2 and 2a.3**). The B_{max} is higher than wild-type for the S319A and S319G mutants, and immunoblot analysis showed heterogeneous expression as indicated by the presence of three predominant bands in the molecular mass range of 45–kDa, with none of the low expressing mutants producing the ~65 kDa band (**Fig. 2a.2**). Previously, photocrosslinking of β_2 -AR expressed in COS-1 cells showed that the band at ~65 kDa corresponds to the completely glycosylated receptor [96]. To elucidate the subcellular localization of the mutant receptors, immunofluorescence microscopy studies on the mutants was carried out in the HEK293T cells (**Fig. 2a.4**).

Immunofluorescence microscopy showed that the G315A, S319A and S319G mutants appear to be predominantly localized on the cell surface with only a small amount of receptor present intracellularly, presumably due to receptor that is in-transit (**Fig. 2a.4**). By contrast, a significant amount of the G315S and S319L mutants appear to be retained in the intracellular compartments, while the G315L mutant is predominantly found localized internally in the cytoplasm (**Fig. 2a.4**). Cell surface localization is a strong indicator of correct folding of the G315A, S319A and S319G mutants because misfolded membrane proteins are typically retained either in the Golgi and/or in the endoplasmic reticulum by the endoplasmic reticulum quality control system and targeted for degradation [102].

Table 2a.1. Summary of DHA binding to the wild type β_2 -AR and Gly315 or Ser319 mutant receptors. The values are expressed as the mean $\pm$ S.E (n=3 to 5 experiments)

Receptor	K_D (nM)	Bmax (pmol/mg)
Wild type	3.1 $\pm$ 0.3	18 $\pm$ 0.6
G315A	4.9 $\pm$ 0.7	7.8 $\pm$ 0.7
G315S ^a	25.3 $\pm$ 7.4	5.3 $\pm$ 0.1
G315L	ND ^b	
S319A	2.8 $\pm$ 0.3	48 $\pm$ 3.0
S319G	3.0 $\pm$ 0.2	42 $\pm$ 2.0
S319L ^a	20.6 $\pm$ 5.1	9.5 $\pm$ 1.5

^a High non-specific binding (>20% of total binding).

^b Not detected (no significant specific binding detected under the assay conditions).

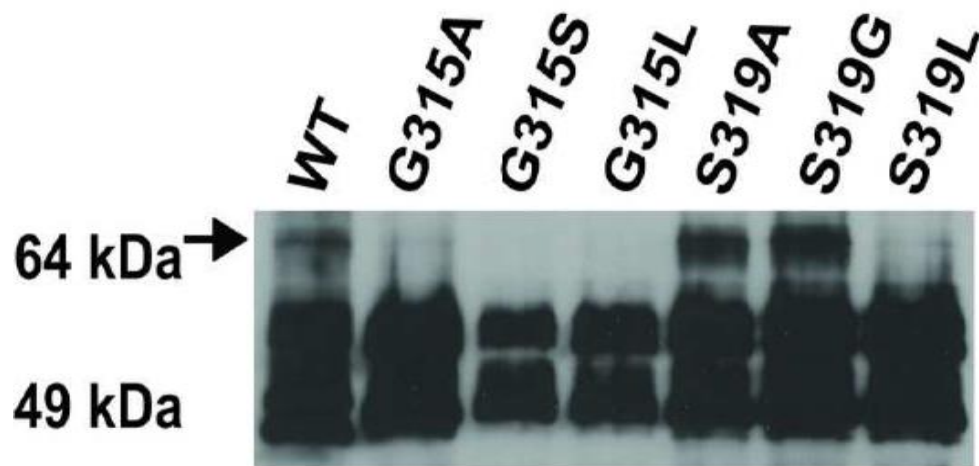


Fig. 2a.2. Immunoblot analysis of β_2 -AR and mutants using the monoclonal antibody rho-1D4.

Immunoblotting was carried out as described in the materials and methods. The arrow indicates the position of the fully glycosylated receptor.

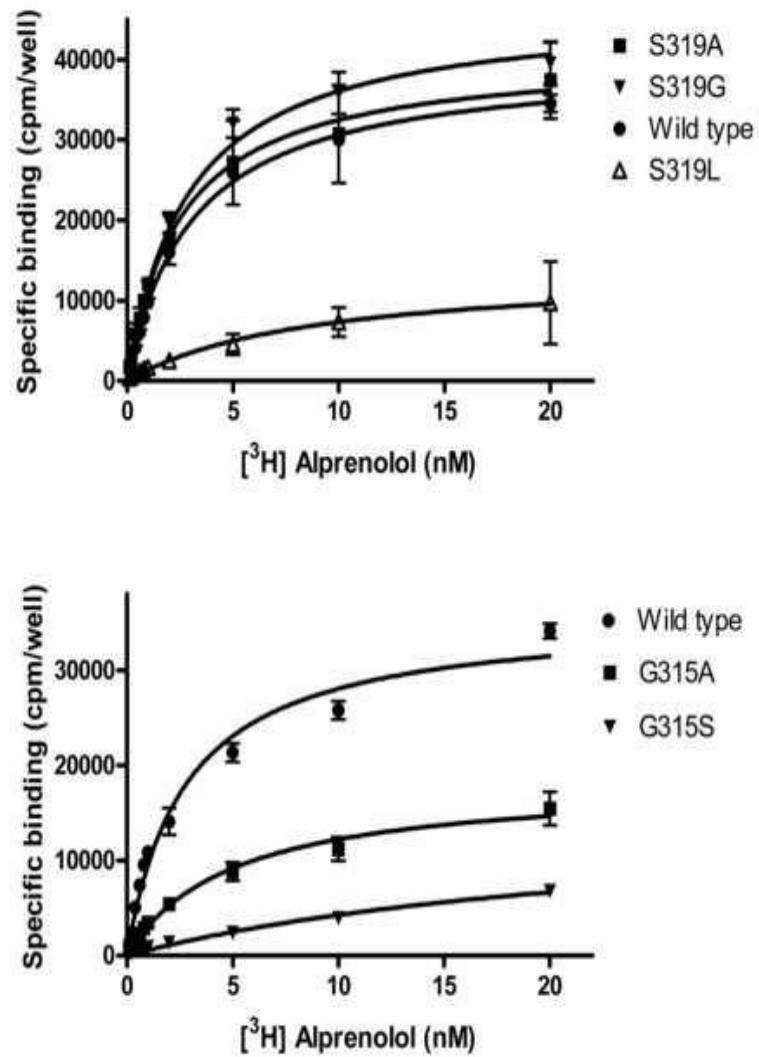


Fig. 2a.3. Saturation binding assays for membrane bound β_2 -AR and mutants from COS-1 cells using the radioligand $[^3\text{H}]$ DHA.

Saturation binding assays were performed as described in the materials and methods.

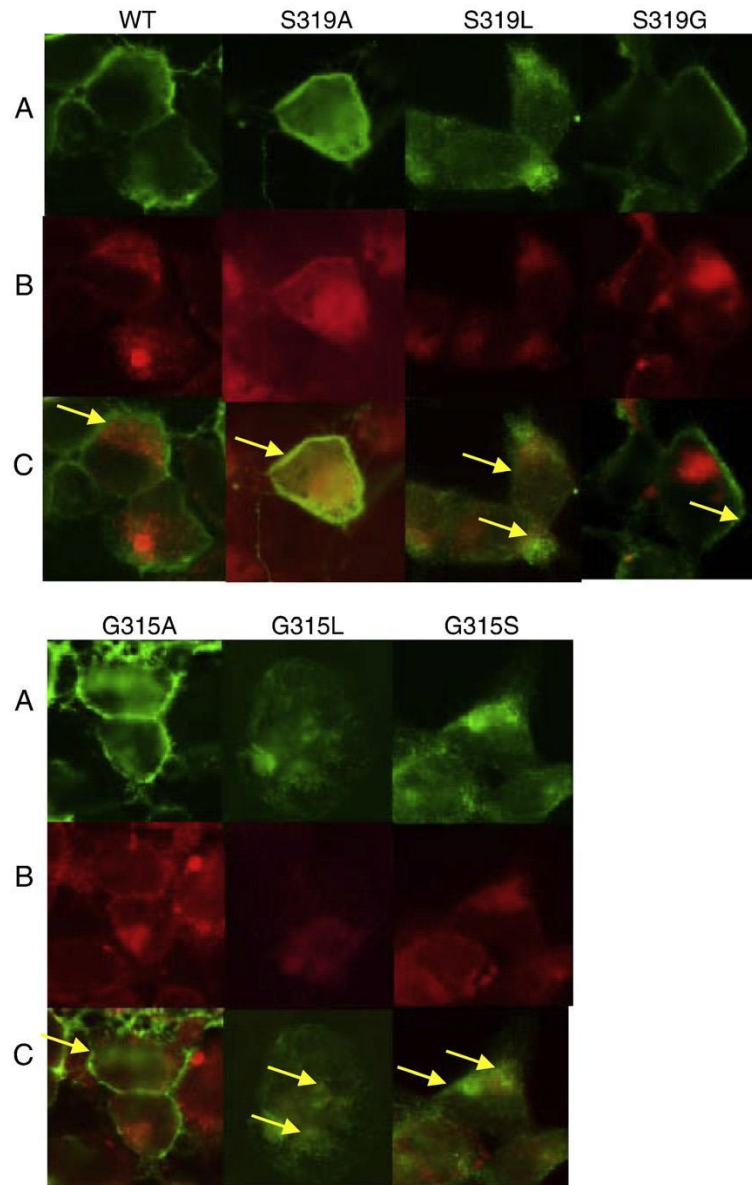


Fig. 2a.4. Immunofluorescence microscopy showing localization of wild-type β_2 -AR and the group-conserved mutants expressed in HEK293T cells.

Immunofluorescence was performed with the mouse rho-1D4 antibody **(A)** and the endoplasmic reticulum marker, rabbit anti-calnexin antibody **(B)**. The mouse rho-1D4 antibody was visualized with anti-mouse-FITC secondary antibody (green), and the rabbit anti-calnexin antibody was visualized with anti-rabbit-Texas Red secondary antibody (red). Overlays of (A) and (B) are shown in (C). Yellow arrows show the locations of receptors. In the wild-type, S319A, S319G and G315A mutants, the receptors were predominantly localized at the plasma membrane, whereas in G315S, G315L and S319L the receptors appear to be located in intracellular compartments.

2A.3.2 Agonist competition assays and G α s mediated signaling

Table 2a.2 presents the binding of unlabelled agonists to the wild type β_2 -AR and mutant receptors as determined by competition with 3 nM [3 H] DHA. We characterized the ligand binding properties of the wild-type β_2 -AR and the G315A, S319A and S319G β_2 -AR mutants with the following β_2 -AR agonists: (–) isoproterenol (full agonist), epinephrine (natural, full agonist) and salbutamol (partial agonist). We could not determine the binding properties of the G315S, G315L and S319L mutants as these mutants showed high nonspecific binding under our assay conditions. Since the affinity of DHA to the wild-type receptor is similar for the G315A, S319A and S319G mutants (**Table 2a.1**), we can estimate the binding affinities of the various agonists from the competition data in **Table 2a.2**. Salbutamol has a lower affinity to the wild-type receptor than either epinephrine or isoproterenol; i.e. the concentration needed to displace DHA is 5–6 times greater for salbutamol. The S319A and S319G mutants showed slight decreases in affinity for all three agonists. The G315A substitution had the most marked effect in the competition assay, requiring a 200-fold increase in agonist concentration to displace the DHA antagonist (**Table 2a.2**). The agonist competition data for S319A and S319G are in agreement with the agonist activation of the wild-type and mutant receptors measured by cAMP assays (**Fig 2a.5**). For the measurements on the Gly315 mutants treated with isoproterenol in a dose-dependent manner, the relative activity decreased in the order G315A > G315S > G315L. For the Ser319 mutants, the relative activity decreased in the order S319A > S319G > S319L. In general, the basal activity of the mutant receptors was higher than that of the wild-type β_2 -AR (**Fig. 2a.5**). The mutants showed agonist independent or constitutive activity to various degrees, for the S319A and S319G it was 15–20% of wild type, while for the G315A and G315S it was 10–15% of wild type. An unexpected finding was the

G315A mutant exhibited high agonist dependent cAMP production even though it had the lowest affinity for (–) isoproterenol of the mutants assayed in **Table 2a-2**.

2A.3.3 Molecular packing of Gly315^{7.42}

The crystal structure of β_2 -AR show Gly315^{7.42} is tightly packed against Cys285^{6.47} and Trp286^{6.48} on TM6 [85] (**Fig. 2a.6A**). The packing interaction can be assessed by the method of occluded surfaces [88, 100]. Occluded surface calculations show that the packing value for Gly315^{7.42} is high (0.65) indicating that the small glycine side chain does not form a water binding site in β_2 -AR. For comparison, the average packing value for amino acids in helices of membrane proteins is 0.44 [88]. In helical membrane proteins, glycine has the highest amino acid packing value (0.52), followed by proline (0.51) and alanine (0.49) [88]. The replacement of Gly315^{7.42} with amino acids having larger side chains results in a steric clash with the side chain of Trp286^{6.48} (packing value 0.56) [88]. Since the glycine side chain is part of the helix backbone, larger amino acids at position 7.42 can be accommodated by rotation of the Trp286^{6.48} side chain, which may be coupled to the rotation of TM6. **Fig 2a-6B** shows a model of the G315L β_2 -AR structure based on homology with the structure of active opsin. The leucine side chain at position 315 is well packed against Trp286^{6.48}. For comparison, **Fig. 2a.6C and 6D** show the structures of inactive rhodopsin and active opsin. In the transition from rhodopsin to opsin, Trp265 moves away from Ala295, whose packing value decreases from 0.55 to 0.44. Displacement of the highly conserved Trp286^{6.48} and rotation of TM6 would explain the constitutive activity observed for the Gly315^{7.42} mutants.

Table 2a.2. Summary of competition ligand binding of wild type β_2 -AR and mutant receptors

Receptor	Ligands (K_i) μ M (95% confidence intervals)		
	Epinephrine	(-) Isoproterenol	Salbutamol
Wild type	4.9(3.5–7.0)	4.2(2.4–7.5)	25(21–30)
G315A	1014(372–2760)	658(304–1237)	1616(398–5680)
S319A	127(65–246)	31(17–56)	204(85–488)
S319G	141(68–293)	64(30–135)	154(60–394)

Data obtained from determinations of two or more independent transfections and analyzed by non-linear regression as described under methods. The 95% confidence intervals for each measurement are indicated in parenthesis. The binding of unlabeled agonists to β_2 -AR was determined by competition with the antagonist 3 nM [3 H] DHA as the radioligand.

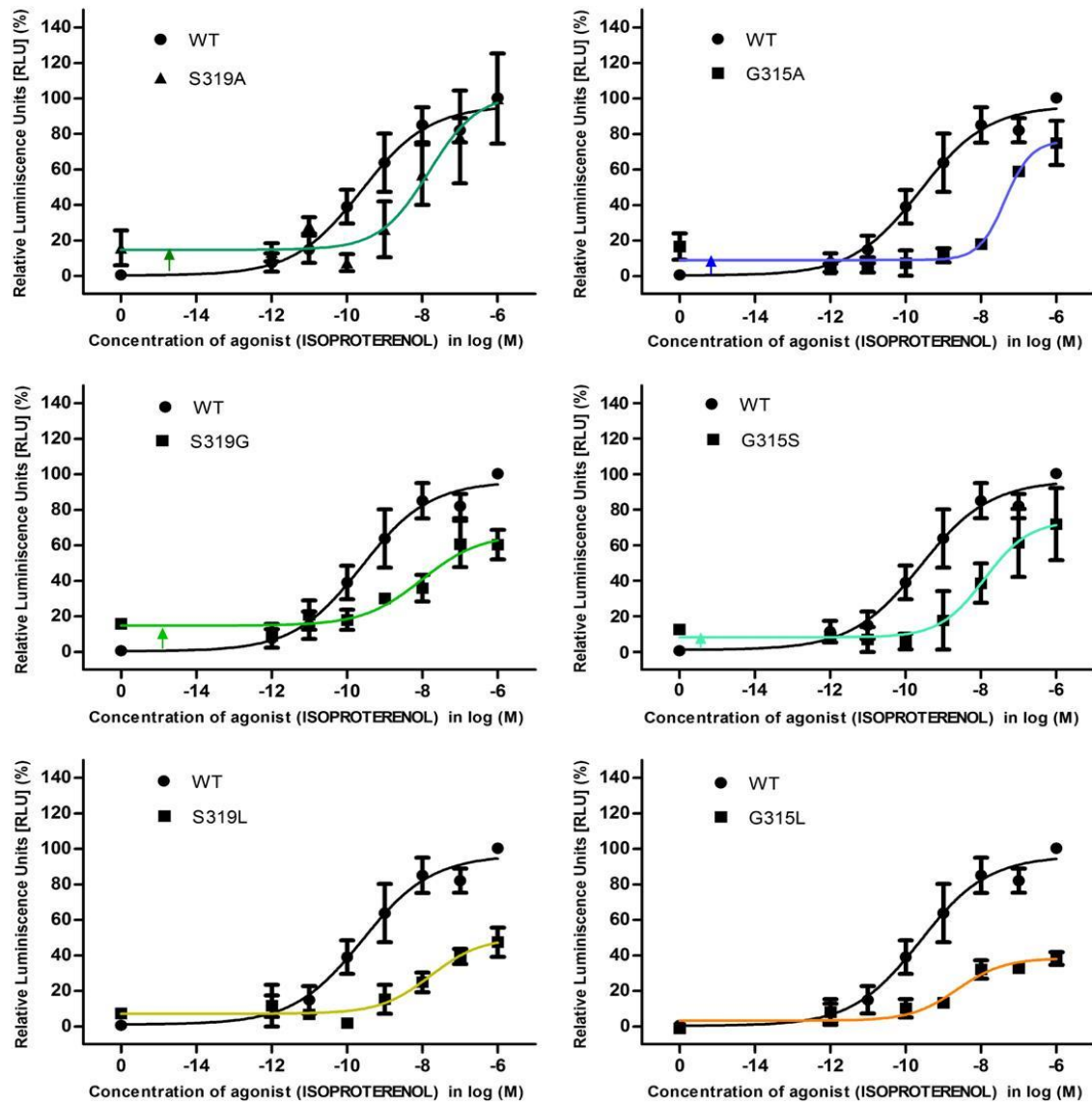


Fig.2a.5. Characterization of *Gas*-mediated signaling of the wild-type and group-conserved TM7 mutants of β_2 -AR.

Receptor activity was determined by measuring cAMP production using transiently transfected HEK293S cells. Shown are the basal (zero concentration) and agonist (isoproterenol) induced cAMP production of the wild-type receptor and the Gly315 and Ser319 mutants. Arrows indicate an increase in the basal activity of the mutants over the wild type receptor. The results are expressed as a percentage of the wild type β_2 -AR activity, and are from at least three independent experiments performed in duplicate.

2A.3.4 Molecular packing of Ser319^{7.46}

Occluded surface calculations yield a smaller packing value (0.51) for Ser319^{7.46} than for Gly315^{7.42}. Nevertheless, the packing value is higher than the average packing value for serine in membrane proteins (0.47) [88] and reflects the tightly hydrogen bonded network surrounding the Ser319^{7.46} side chain (**Fig. 2a.7**). The side chain of Ser319^{7.46} forms hydrogen bonds with Asp79^{2.50} (3.3 Å) and Tyr316^{7.43} (3.1 Å). In addition, Ser319^{7.46} hydrogen bonds with water 534 (3.1 Å), a structural water that also coordinates Asp79^{2.50}, Trp286^{6.48}, and Gly315^{7.42} (**Fig. 2a.7B**). Replacement of Ser319^{7.46} with non-polar residues (S319A, S319G, S319L) results in loss of its hydrogen bonding interactions.

2A.4 Discussion

We targeted Gly315^{7.42} and Ser319^{7.46} because of their position between two key functional microdomains in β_2 -AR. Gly315^{7.42} is in contact with Cys285^{6.47} and Trp286^{6.48} of the conserved CWxP motif on TM6. Ser319^{7.46} hydrogen bonds directly with the conserved Asn51^{1.50} on TM1 and Asp79^{2.50} on TM2, and bridges Trp286^{6.48} and Asn322^{7.49} of the conserved NPxxY motif on TM7 through water mediated hydrogen bonds. Gly315^{7.42} and Ser319^{7.46} are also flanked by amino acids that have high sequence conservation within the amine subfamily of receptors (Trp313^{7.40}, Tyr316^{7.43} and Asn318^{7.45}). As a result, these two group conserved amino acids are strategically positioned between the ligand-binding site on the extracellular side of the receptor and the ionic lock on the cytoplasmic side of the receptor.

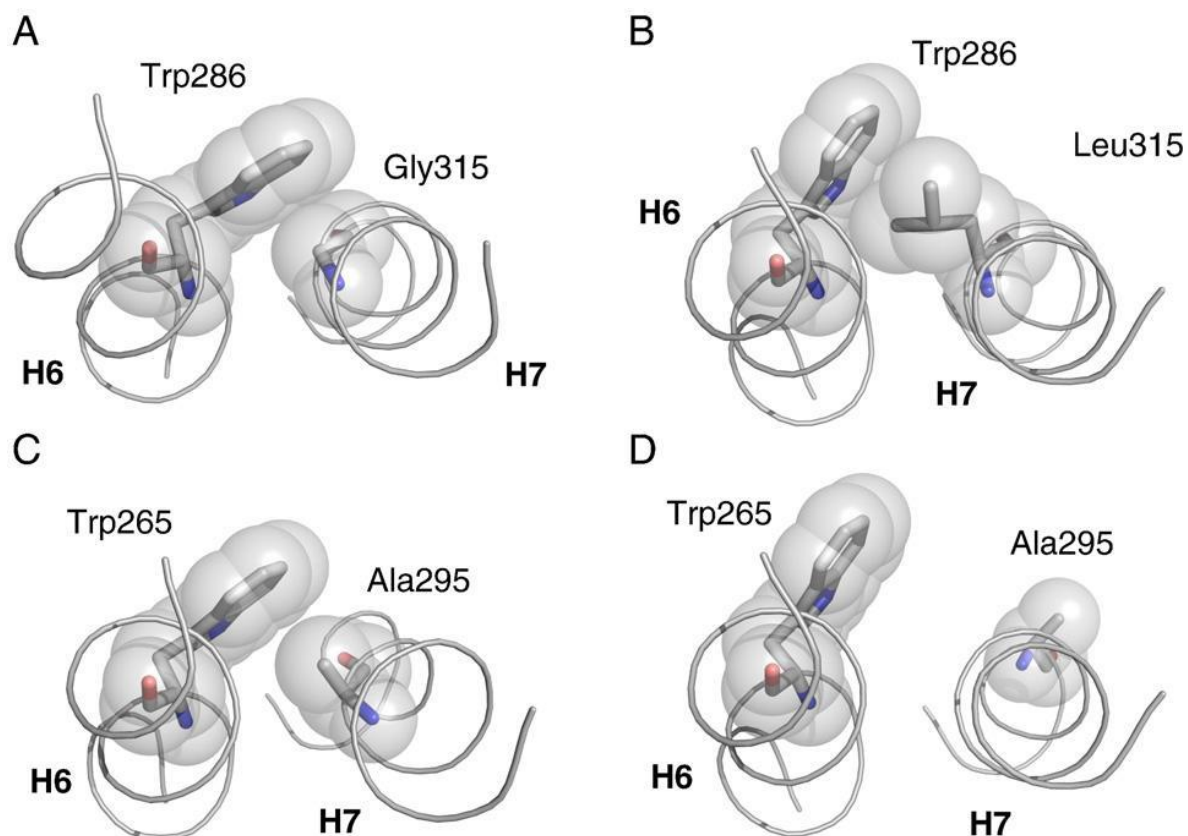


Fig.2a.6. Molecular packing of Gly315^{7.42} and Leu315^{7.42} in β_2 -AR.

(A) Crystal structure of β_2 -AR (2RH1) showing van der Waals interaction of Trp286^{6.48} and Gly315^{7.42}. (B) Homology model of G315L β_2 -AR where the G315L β_2 -AR sequence was threaded onto the structure of active opsin (3CAP) and the resulting structure was energy minimized with 1000 steps steepest descent. The inactive structure of rhodopsin (C) and the active structure of opsin (D) are shown for comparison

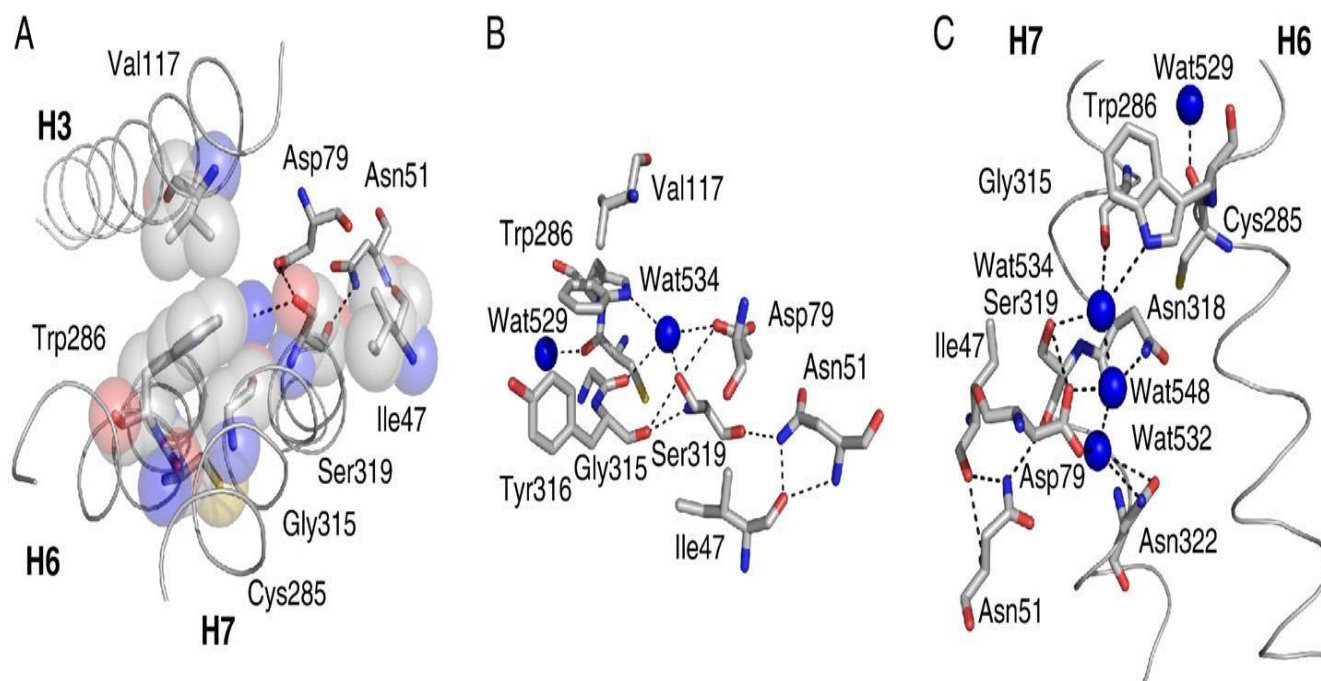


Fig. 2a.7. Crystal structure of the β_2 -AR in the region of Gly315^{7.42} and Ser319^{7.46}.

(A) Packing of Gly315^{7.42} with Trp286^{6.48}. An increase in size of the amino acid at position 7.42 causes a steric clash with Trp286^{6.48} on H6 or TM6 and is predicted to displace the Trp286^{6.48} side chain toward the extracellular surface. (B) Hydrogen bonding interactions of Ser319^{7.46}. The side chain hydroxyl group of Ser319^{7.46} side chain is hydrogen bonded to the carboxyl C(O)OH of Asp79^{2.50} (3.3 Å), to the backbone carbonyl of Tyr316^{7.43} (3.1 Å) and to water 534 (3.1 Å), while the backbone carbonyl of Ser319^{7.46} makes contact with the side chain NH of Asn51^{1.50} (3.1 Å). Water 534 is shown hydrogen bonded to the backbone of Gly315^{7.42}. (C) Hydrogen bonding network connecting the indole NH of Trp286^{6.48} to the amide side chain of Asn322^{7.49}. Water 534 in β_2 -AR mediates hydrogen bonding between the Trp286^{6.48} indole NH (3.48 Å), Ser319^{7.46} C β OH (2.82 Å), Asn318^{7.45} NH₂ (3.11 Å) and Gly315^{7.42} (C=O) (3.38 Å). Water 534 is also hydrogen bonded to Wat548, which in turn is hydrogen bonded to Asn322 through Wat532. The same overall set of interactions appears to exist in the recent crystal structure of the CXCR4 receptor where cysteine occurs at position 7.46 and histidine occurs at position 7.45. The polar imidazole of histidine is located between the conserved indole ring of Trp6.48 and Cys7.46.

Gly315^{7.42} functions as a molecular notch to stabilize Trp286^{6.48} in an inactive position

Fig. 2a.7 shows the close packing interaction of Gly315^{7.42} with the indole ring of Trp286^{6.48}. These residues are 69% and 96% conserved in the amine receptor subfamily, respectively. We found that the conservative mutation of Gly315^{7.42} to alanine exhibits agonist-independent cAMP production. The constitutive activity suggests that Gly315^{7.42} is stabilizing Trp286^{6.48} in an inactive conformation. This packing arrangement is similar to the packing of Ala295^{7.42} against Trp265^{6.48} in rhodopsin [103, 104] where substitution of Ala295^{7.42} with valine results in constitutive activity and leads to autosomal dominant congenital stationary night blindness [105]. A similarly close packing interaction between Ala291^{7.42} and Trp252^{6.48} is observed in the recent structure of the CXCR4 receptor, a member of the large subfamily of GPCRs that bind peptide ligands [106].

Trp286^{6.48} has been proposed to be part of the rotamer toggle switch involved in receptor activation [107], although the recent crystal structure of active opsin shows only a displacement of Trp265^{6.48} rather than a change in the side chain rotamer angle (**Fig. 2a.6D**). Evidence for displacement of the Trp265^{6.48} side chain in activated rhodopsin comes from NMR measurements showing the gain of a direct packing interaction between the retinal C19 methyl group and the Trp265^{6.48} side chain, in concert with a loss of packing interactions with the retinal C18 and C20 methyl groups [103, 108]. Substitution of larger residues at position 7.42 in the β_2 -AR would be expected to displace the conserved tryptophan as suggested by computational studies of activated β_2 -AR [109]. Gly315^{7.42} is also in close contact with Cys285^{6.47}. Cysteine is highly conserved at position 6.47; mutation to serine in the β_2 -AR shows normal ligand binding, but reduced activity [107, 110], while mutation to threonine results in constitutive activity [107]. The mechanism of

receptor activation in the C285T mutant may involve a steric clash of the β -branched threonine methyl group with Gly315^{7.42} rather than a change in rotamer angle since the predominant conformer for threonine in α -helices is *gauche+* [107], which is the observed conformer for Cys285^{6.47} in the carazolol-bound structure of β_2 -AR. Moreover, the Cys285^{6.47} side chain is more exposed to cysteine reactive reagents in a constitutively active mutant of β_2 -AR [111] and upon agonist binding [112]. Together with our mutational results on Gly315^{7.42}, these cysteine accessibility studies are consistent with a more open structure in the active receptor being due to displacement of Trp286^{6.48} from a molecular notch formed by Gly315^{7.42}.

Ser319^{7.46} mediates hydrogen bonding interactions between Trp286^{6.48} and Asn322^{7.49}

Ser319^{7.46} is highly conserved (98%) in the amine subfamily and is in a key position between Asn51^{1.50}, Asp79^{2.50}, Trp286^{6.48}, and Asn322^{7.49}. These residues are all highly conserved across the class A GPCRs. Both the backbone carbonyl and side chain hydroxyl groups of Ser319^{7.46} form hydrogen bonds that have structural and functional importance. The Ser319^{7.46} carbonyl does not form an *intra*-helical hydrogen bond due to its position one helix turn above Pro323^{7.50} of the conserved NPxxY sequence. The backbone carbonyl of Ser319^{7.46} forms an *inter*-helical hydrogen bond with the amide NH₂ functional group of Asn51^{1.50}, the most highly conserved residue in the class A GPCRs. Along with the backbone carbonyl at position 1.46, another group-conserved residue, this hydrogen bond functions to orient the conserved Asn51^{1.50} side chain (**Fig. 2a.7B**) [113].

The Ser319^{7.46} side chain is in the center of a hydrogen-bonding network connecting Trp286^{6.48} to Asn322^{7.49}. Asn322^{7.49} is part of the conserved NPxxY motif on TM7 that is

involved in receptor activation. We found that the conservative mutation of Ser319^{7.46} to alanine results in constitutive activity. Unlike the Gly315^{7.42} to alanine mutation in β_2 -AR and the Ala295^{7.42} to valine mutation in rhodopsin that lead to constitutive activity, the S319A mutation does not increase the molecular volume of the group-conserved side chain, but rather removes its ability to hydrogen bond. Ser319^{7.46} is hydrogen bonded to water 534, conserved structural water within the transmembrane core of the receptor. In rhodopsin, water 2015 mediates interaction of Trp265^{6.48}, Ser298^{7.45} and Tyr301^{7.48}. In both β_2 -AR and rhodopsin, this conserved water is hydrogen bonded to several additional conserved water molecules that form a shell surrounding the side chain of Asn322^{7.49}. While the details of the structural changes between Trp 265^{6.48} and Asn 322^{7.49} have not been determined by crystallography or NMR spectroscopy in either the β_2 -AR or rhodopsin, NMR measurements of rhodopsin show that displacement of Trp265^{6.48} upon activation weakens the water-mediated hydrogen bond between the Trp265^{6.48} indole NH and Asn302^{7.49} amide side chain [103]. As a result, motion of Trp286^{6.48} away from Gly315^{7.42} in the β_2 -AR upon agonist binding would be expected to alter the hydrogen bonding network illustrated in **Fig. 2a.6C**.

The idea that conserved structural waters can act as allosteric regulators of GPCR function was introduced by Palczewski and colleagues [114]. These authors showed that the interior water molecules do not exchange with bulk solvent suggesting that structural waters represent an intrinsic part of the interfaces between TMs, TM1, TM2, TM6 and TM7. We propose that the structural waters in combination with the group-conserved residues on these helices (**Fig. 2a.7A**) create an interface that facilitates the rotation of TM 6 relative to TM7 during activation.

Interplay between the three levels of amino acid conservation in class A GPCRs

Fig. 2a.6 presents the core conserved transmembrane region of the β_2 -AR to illustrate the interplay between the signature, group-conserved and subfamily specific amino acids. Trp286^{6.48} is sandwiched between Gly315^{7.42} and Val117^{3.36}. The residue at position 3.36 on TM3 appears to play a role that parallels that of group-conserved residue 7.42 on TM 7. In rhodopsin, residue 3.36 is a glycine, which has very high conservation (95%) in the rhodopsin subfamily of receptors. Replacement of this glycine with larger residues results in dark activity of rhodopsin, where the activity is linearly correlated with the residue volume [115]. In a similar fashion, mutation of the residue at position 3.36 in receptors of the amine subfamily has been shown to modulate the receptor activity. The specific residue at this position in the 5HT⁴ and histamine H₁ receptors has been associated with constitutive (basal) activity, and mutation can either increase or decrease this activity. For example, in the histamine H₁ receptor substitution of the wild-type serine with cysteine or threonine increases activity, while substitution with alanine decreases activity [116]. Pardo and coworkers proposed that when Trp 6.48 rotates upon ligand binding, Ser3.36 forms an interhelical hydrogen bond with Asn7.45 on TM7 [116]. Asn7.45 has the second highest level of subfamily conservation in the amine receptors (92%). Threonine at position 3.36 has the highest constitutive activity and may be correlated with a preference for the *g*⁺ rotamer. In the 5HT₄ receptor, the wild-type residue is threonine and substitution with alanine or serine decreases activity [117]. The role of the residue at position 3.36 extends beyond the amine and rhodopsin subfamilies to other class A GPCRs. Schwartz and colleagues concluded residue 3.36 often influences the function of the Trp6.48 rotamer toggle switch, but that it acts in a highly context-dependent manner [109]. In the ghrelin peptide hormone receptor, mutation of the wild-type threonine residue at 3.36 to alanine

was found to increase, rather than decrease, receptor activity [118]. Together, these results illustrate the interplay between the three levels of class A receptor conservation: signature conserved (Trp6.48, Asp2.50, Asn7.49), group-conserved (Cys 6.47, Gly/Ala7.42, Ser/Ala7.46), subfamily conserved (Gly/ Ser/Thr/Val3.36 and Asn7.45).

2B. Site-directed mutations and the polymorphic variant Ala160Thr in the human thromboxane receptor uncover a structural role for transmembrane helix 4.

Chakraborty R, et al., *PLoS One* 7(1): e29996, 2012

2B.1 Introduction

Membrane receptors present on the cell surface mediate the communication between the cell and its environment. The largest group of these membrane receptors belong to the family of G-protein coupled receptors (GPCRs) [119, 120]. GPCRs contain seven transmembrane helices (TM) and are activated by diverse extracellular stimuli including hormones, tastants, light, peptides and neurotransmitters. The majority of GPCRs belong to the Class A family and are important pharmacological targets, with 40-50% of prescription drugs targeting these receptors. The human thromboxane A₂ receptor (TP) belongs to the prostanoid subfamily of Class A GPCRs, and is primarily activated by the prostanoid, thromboxane A₂ (TxA₂). TP mediates thrombosis and vasoconstriction, thereby playing an important pathophysiological role in heart disease and stroke [2, 121].

The receptor knockout studies have implicated TxA₂ as a key regulator of atherosclerosis. Surprisingly little is known about the influence of TP gene (*TBXA2R*) variations on cardiovascular disease. With TxA₂ being a powerful airway constrictor, the major research has focused on the association of variants with severity and susceptibility to asthma [122],[123] [124],[125] and atopic dermatitis [126, 127]. Structural studies on TP are very limited and few have addressed the functional significance of the polymorphic variants in this receptor [12, 13, 50, 128]. A point mutation (R60L) in the first cytoplasmic loop of the TP, however, has been identified in an autosomal dominant bleeding disorder characterized by defective platelet response [49] and another mutation D304N was also

associated with bleeding [50]. None of these studies have provided the details for structural perturbations or evidence for constitutive activity as outlined in our study.

Recent crystal structures and mutational studies of rhodopsin and the β_2 -AR show that TM1-TM4 form a helical bundle core, with other helices moving around this core upon activation [90, 129, 130]. Homo- and heterodimerization studies on GPCRs have shown that TM4 is an important part of the dimer interface [131, 132]. TM4 is one of the shortest helices in GPCRs, yet it performs important structural and functional roles and is a hot spot for naturally occurring GPCR variants. More than 700 GPCRs are identified in the human genome, with a substantial number of these harbouring genetic variants [133], including nucleotide insertion or deletion as well as single nucleotide changes referred to as single nucleotide polymorphisms (SNP). SNPs are sequence changes that can result in either synonymous (i.e. change in DNA sequence but no change in amino acid sequence) or non-synonymous (change in DNA and amino acid sequence) mutations.

Mutations that affect protein structure and function tend to occur at evolutionarily conserved sites and are usually buried in protein structure [134]. One such region in Class A GPCRs, is the TM4 from residues 4.53 to 4.57 (numbering according to Ballesteros and Weinstein nomenclature [135]). For example, in rhodopsin the non-synonymous (ns) SNP A164V^{4.53} destabilizes helix packing resulting in protein misfolding and causes retinitis pigmentosa [136], similarly the T164I^{4.56} nsSNP in β_2 -AR is hypofunctional and is associated with coronary and peripheral artery disease [137]. In the TP receptor, currently there are 7 ns SNPs listed in the GPCR natural variant database [133], a G/A change at position 478 in the nucleotide sequence causes a codon change from GCG > ACG, resulting in A160T in TM4.

In this manuscript, we target two amino acids present in TM4 of TP receptor, Ala160^{4.53} and Gly164^{4.57} for structure-function analysis (**Fig. 2b.1**). These residues are of particular interest for multiple reasons, the region is highly conserved in Class A GPCRs (categorized as group-conserved residues) [77, 90], with conservation of up to 99% when considered as a group of small and weakly polar residues (Gly, Ala or Ser). These amino acids have been identified in membrane proteins as key determinants in helix-helix interactions [89]. Furthermore, A160T is a novel nsSNP in TP that is yet to be characterized and Gly164 is present in close proximity, just one helical turn away and towards the extracellular surface. In this study, mutants were generated by site-directed mutagenesis and transiently expressed in COS-1 or HEK293T cells, and ligand binding assays were performed using membrane preparations. To elucidate the effect of these mutations on G-protein signaling, changes in intracellular calcium levels were measured following stimulation by agonist U46619. Agonist-independent signaling was also measured to assess constitutive activity. Guided by molecular modeling, a series of compensatory mutations were made on TM3, in order to accommodate the bulkier replacements (example, replacement with valine) at positions 4.53 and 4.57 on TM4. Our results show that Ala160^{4.53} and Gly164^{4.57} in TP receptor form a packing motif and have a structural role in the tight packing of helices TM4 and TM3. The nsSNP variant A160T decreased receptor stability and demonstrated increased affinity towards the agonist U46619. Both the A160T and A160V mutants displayed agonist-independent signaling. Furthermore, molecular modeling analysis suggested that the G164V mutation at the extracellular end of TM4 causes loss of hydrogen bonding contact of Ser191 in the extracellular loop-2 (ECL2) with antagonist SQ 29,548. Previous studies have shown that the residues on ECL2 in TP receptor are important for SQ 29,548 binding [13, 15].

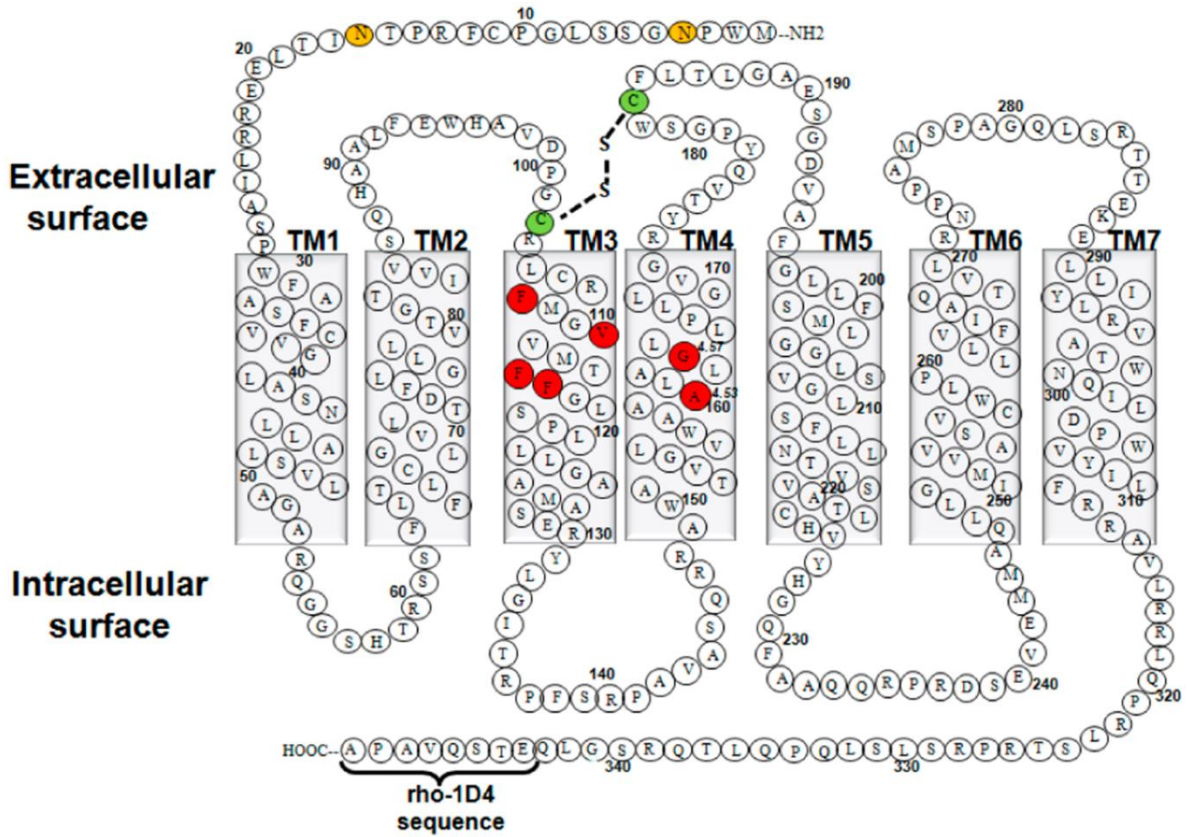


Fig. 2b.1. Two-dimensional representation of the TP α amino acid sequence.

Amino acids are shown in single-letter codes. Shown are the seven transmembrane helices, the disulphide bond between the Cys102 and Cys183 (green colored residues), the N-glycosylated residues Asn4 and Asn16 (orange colored residues), and the rho-1D4 octapeptide epitope tag at the C-terminus. The two conserved residues Ala160^{4.53} and Gly164^{4.57} on TM4 along with the residues on TM3 mutated in this study are shown in red.

2B.2 Materials and Methods

2B.2.1 Materials

The TP antagonist [³H] SQ 29,548 was purchased from PerkinElmer (NET936250UC, PerkinElmer, MA, USA). Unlabelled TP antagonist SQ 29,548 and agonist U46619 was purchased from Cayman Chemicals Company (Michigan, USA).

2B.2.2 Molecular biology and cell culture

TP α receptor amino acid substitutions were introduced into the synthetic TP α gene carried by the expression vector pMT4 as described previously [99, 138]. DNA sequences of all the mutated genes were verified by automated DNA sequencing. To minimize variations in transfection efficiency, the total amount transfected DNA was kept constant in all cases at 1 μ g of DNA per 7×10^5 cells. The wild type *TP* or mutant genes were expressed in COS-1 cells using a DEAE-dextran based transient transfection method [99, 138], and in HEK293T cells using lipofectamine 2000 (Invitrogen). Except unless specified, the membranes prepared from COS-1 cells were used for radioligand binding assays and immunoblots. For intracellular calcium determination assays and immunofluorescence imaging, HEK293T cells were used. For transient transfections of HEK293T cells using the plasmid pMT4, lipofectamine 2000 (Invitrogen) mediated transfection was used as described by the manufacturer. Membranes were prepared using Buffers A and B and as described previously [99, 138]. The protein concentration in the resuspended membrane pellet was determined using a modified DC protein assay kit from Bio-Rad Laboratories (Hercules, CA).

2B.2.3 Radioligand binding assays

For TP α receptor also the Buffer C was used for 60 min at room temperature, using 2 to 20 μ g of membrane protein and different concentrations of [3 H] SQ 29,548 (0.5 nM to 20 nM). Binding of [3 H] SQ 29,548 in the presence of 10 μ M SQ 29,548 was used as a measure of nonspecific binding. Competition binding assays were performed using 4 nM [3 H] SQ 29,548 and different concentrations of unlabeled agonists (10^{-3} – 10^{-9} M) and the reactions kept for 2 hr at room temperature. Binding was terminated by filtering under vacuum on GF/A filters (Millipore). Filter-bound radioactivity was measured using a liquid scintillation counter. Equilibrium dissociation constants (K_D) were determined from saturation isotherms using PRISM software version 5.0 (GraphPad Software Inc, San Diego, CA, USA). The K_i values were calculated from the IC_{50} , using the equation of Cheng and Prusoff by PRISM software version 5.0. Where applicable, statistical significance of the data was evaluated using analysis of variance (ANOVA) and/or unpaired t test.

2B.2.4 Immunofluorescence microscopy

HEK293T cells were seeded into six-well tissue culture plates containing sterilized poly-L-lysine (Sigma)-treated glass cover-slips and transiently transfected with wild-type TP or mutant DNA or according to the aforementioned transfection protocols. Briefly, the cells were washed and blocked with 1x PBS buffer containing 2% bovine serum albumin (IgG and Protease free) for 60 min. Briefly, TP and the mutants were labelled for 90 min using a 1:500 dilution of the mouse-anti-rho-1D4 monoclonal antibody (C-terminal tagged TP) and a 1:100 dilution of rabbit anti-calnexin polyclonal antibody (Abcam, MA, USA; endoplasmic reticulum marker). The transfected cells were washed and incubated with

fluorophore-conjugated secondary antibodies using a 1:2000 dilution of goat anti-mouse Alexafluor 488 (Invitrogen) and 1:300 dilution of goat anti-rabbit Alexafluor 594 (Invitrogen) for 60 min. Prolong-antifade gold (Invitrogen, Molecular probes, CA, USA) was used to mount the coverslips on slides, and the edges sealed with nail-polish. Representative cells were selected and visualized using an Olympus BX81 microscope for cytoplasmic or plasma membrane localization.

2B.2.5 Determination of intracellular calcium for TP α receptor and mutants

Changes in intracellular calcium were measured by using the fluorescent calcium sensitive dye Fluo-4NW (Invitrogen). After 6-8 hours of transient transfection of HEK293T cells using lipofectamine 2000, 100,000 viable cells were plated into each well of a 96-well tissue culture treated BD-falcon optilux plates. Cells mock transfected with vector pMT4 alone were used as a negative control. Following 24 hours of incubation at 37°C, the media was removed and cells were incubated with the dye Fluo-4NW (Invitrogen) containing 77 μ g/ml of probenecid for 1 hour, as recommended by the manufacturer. Receptor activation was determined by measuring changes in intracellular calcium after application of different concentrations of agonist U46619 for TP and mutants, using Flexstation-3 fluorescence plate reader (Molecular Devices, CA, USA) at 525 nm following excitation at 494 nm. Dose–response curves were generated and EC₅₀ values calculated by nonlinear regression analysis using PRISM software version 5.0 (GraphPad Software Inc, San Diego, CA) after subtracting the responses of mock-transfected cells stimulated with same concentrations of agonists.

For estimation of calcium mobilized using the non-ratiometric calcium indicator Fluo-4NW, the $\Delta F/F$ ratio which approximately indicates calcium is calculated using the

equation [139], $\Delta F/F = (F - F_{\text{base}}) / (F_{\text{base}} - B)$. Where F is the measured fluorescence intensity of Fluo-4NW, F_{base} is the fluorescence intensity of Fluo-4NW in the cell before stimulation, and B is the background signal determined from areas adjacent to the cell. For determination of basal Ca^{2+} levels for agonist-independent signaling, the Ca^{2+} mobilized ($\Delta F/F$) was corrected for receptor expression levels (B_{max} in picomoles).

2B.2.6 Thermal sensitivity assays

Aliquots containing membranes of TP or mutant receptors were incubated at 25°C, 37°C and at 42°C for 1 to 5 hrs in buffer C. At the specified time point, aliquots containing the membranes were removed from the water bath and the receptors were incubated with a single saturating concentration (20nM) of [^3H] SQ 29,548 for 60 minutes at room temperature and binding was terminated by filtering under vacuum on GF/A filters (Millipore). Filter bound radioactivity was measured using a scintillation counter. Binding of [^3H] SQ 29,548 in the presence of 10 μM SQ 29,548 was used as a measure of nonspecific binding. The activity of the receptor at starting time point (zero) is taken as 100% for the wild type and respective mutants, and the activity remaining at different time points is expressed as a percentage of the starting time point.

2B.2.7 Homology modelling

The basal model of the TP α was built by homology modeling using the crystal structure of $\beta_1\text{AR}$ (PDB ID, 2VT4) as template. The transmembrane regions of TP were modelled using MODELLER 9V7 [140]. Loops of the receptors were modelled using loop database of

SPDBV4.0.1 [101] based on the available 2D NMR structures of loop regions [141]. Side chains of the molecules were refined with SCWRL4 database [142]. The whole molecule was energy minimized by 1000 steps of steepest descent (SD) and 1000 steps of conjugate gradients by using SPDBV 4.0.1[101]. Molecular dynamics (MD) simulations were performed for the basal model with OpenMM Zephyr [143]. The quality of model was verified using PROCHECK [144]. This model was used for further mutational and docking studies. The mutants were built using PyMol, and these models were further simulated with OpenMM Zephyr.

The model was then docked with either antagonist SQ 29,548 or agonist U46619 using AUTODOCK VINA[145]. The binding site of ligand on the receptor was defined by forming a cube with dimensions 60 x 80 x 70 around the protein with a grid point spacing 0.375Å and center grid boxes -51.807, -12.467 and 38.921 in X, Y and Z dimensions respectively. We performed 50 runs for each ligand. In each run the best pose or energy minimized conformation was saved. Finally, all poses were superimposed and the most frequent orientation of the ligand was taken as final pose. Receptor ligand complex was further simulated using Desmond 2.4.2.1 molecular dynamics simulation software [146]

2B.3 Results

The role of TM4 in TP and prostanoid receptors in general is unclear. We used a combination of naturally occurring and site-directed mutations, and molecular modelling studies to determine the critical role for this highly conserved region in TP. In humans, TP exists as two isoforms TP α and TP β which are splice variants of a single gene [9]. These variants differ only in their intracellular carboxyl terminal regions. As the current study is focused on residues involved in helix packing and that are conserved in both the isoforms,

the shorter isoform of TP α was used. The conserved residues Ala160^{4.53} and Gly164^{4.57} are present on the inward-facing side of TM4 in TP. Elucidating the role of Ala160^{4.53}, may also decipher the mechanism of the nsSNP variant A160T. Our initial strategy was to replace Ala160^{4.53} and Gly164^{4.57} by amino acids containing both small and large molecular volumes and to study the effect of these replacements upon receptor expression, activity, and binding of the antagonist SQ 29,548.

2B.3.1 Expression and ligand binding properties of TP and mutants

The ligand binding properties of the TP mutants were measured using the antagonist SQ 29,548 (**Table 2b.1**). Conservative substitutions of Ala160^{4.53} and Gly164^{4.57} with small amino acids were better tolerated with the A160S and G164A mutants showing an increase in expression of functional receptor as quantified by Bmax values (**Table 2b.1**), and saturation isotherms in (**Fig. 2b.3**). A one way ANOVA analysis without any post hoc test showed that except for G164V in TM4 there were no significant difference between TP and the mutants at significance level of $p < 0.05$. Replacement with larger amino acids at position Ala160^{4.53} caused significant reduction in receptor expression (**Fig. 2b.3**). Gly164^{4.57} was more sensitive with the G164V mutant losing the ability to bind to the antagonist SQ 29,548 (**Fig. 2b.3**). To verify whether this loss is due to protein misfolding, G164V immunofluorescence microscopy showed that the receptor was properly expressed on the cell surface (**Fig. 2b.2**). The A160T and A160V mutants with bulky β -branched amino acids were expressed at half of the levels of wild type as reflected by the lower Bmax values (**Table 2b.1**). This is in agreement with previous studies on TM4 residues, Ser161^{4.53} and Ser165^{4.57} of β_2 -AR, where it was observed that conservative substitutions

with small amino acids such as alanine to serine had a minimal effect on receptor folding and ligand binding, whereas mutations made to a non-group conserved amino acid with bulky side chains, such as alanine or serine to valine and leucine, reduced receptor expression [90, 147].

Substitution of the two residues Ala160^{4.53} and Gly164^{4.57}, on TM4 with large amino acids may affect the proper packing of the helices due to steric interactions. If this is the case, introduction of a second mutation at an appropriate site on an opposing helix may compensate for the steric clash and restore correct packing. Using the ligand-free TP molecular model as a template, possible compensatory mutants were designed by selecting residues within 5 Å of Ala160^{4.53} and Gly164^{4.57}, for mutagenesis. In the ligand-free TP model, Ala160^{4.53} is close to Phe114^{3.34} and Phe115^{3.35}, whereas Phe107^{3.27} and Val110^{3.30} are in proximity of Gly164^{4.57}. Based upon molecular modeling, a series of compensatory mutations F107A, V110A, F114A and F115A were made on TM3, in order to try to accommodate the bulkier residues (**Table 2b.1**). The single mutants on TM3 bound to antagonist with affinities similar to TP (**Table 2b.1**). All the compensatory double mutations except for A160V/F115A lacked the ability to bind to the antagonist. The A160V/F115A double mutant showed a moderate increase in expression level compared to either A160V or F115A single mutants. To elucidate whether the double mutants were misfolded and/or unable to bind to the antagonist, immunofluorescence microscopy was performed and A160V/F115A, G164V/V110A mutants were found localized on the cell surface. A160V/F114A and G164V/F107A double mutants were predominantly retained in

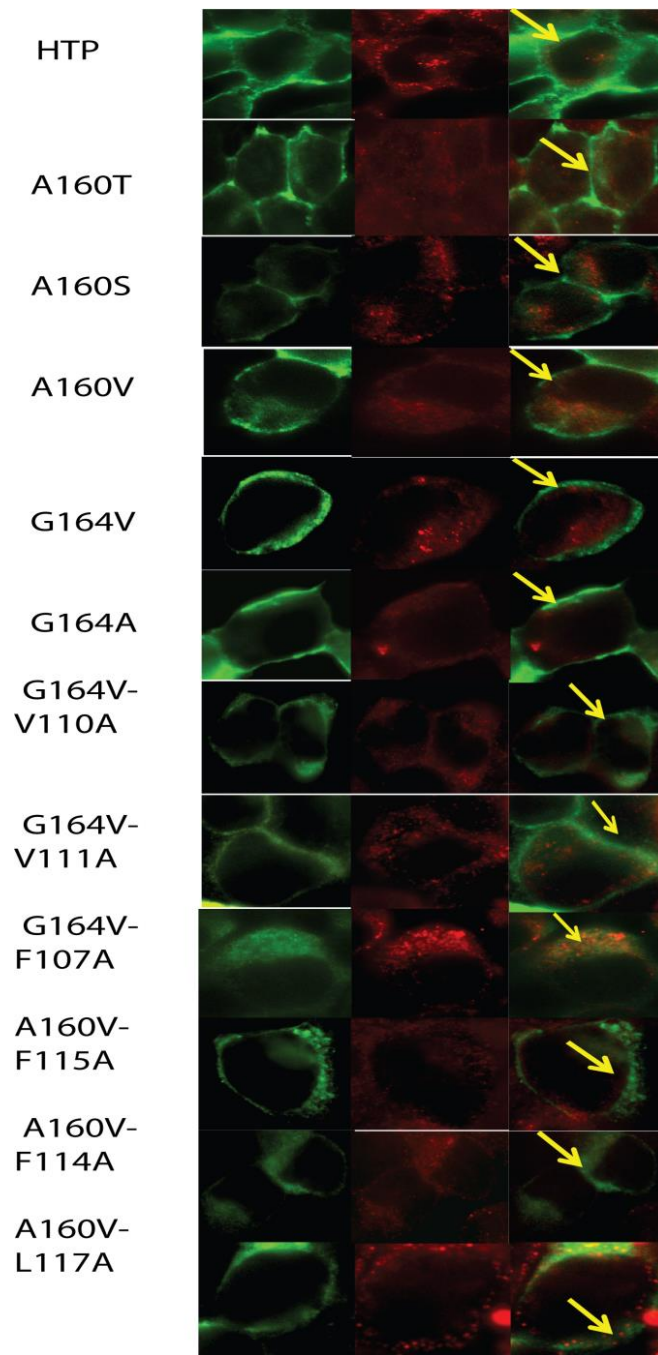


Fig. 2b.2. Cellular localization of the WT-TP and mutants in HEK293T cells.

Double-label immunofluorescence was performed using mouse monoclonal anti-rho-1D4 antibody which recognizes the C-terminal octapeptide tag on the expressed receptors, and rabbit polyclonal anti-calnexin antibody which localizes to the endoplasmic reticulum (ER). The wild type and mutant receptors were visualized using goat anti-mouse Alexafluor 488 secondary antibody (**panel A**) and the ER was visualized with goat anti-rabbit Alexafluor 594 secondary antibody (**panel B**). The overlay of the receptor and ER is shown in panel C (location of the expressed receptor is indicated by an arrow).

Table 2b.1. Functional characterization of WT-TP and mutants

Receptor	Transmembrane helix	K_d ¹ (nM)	95% confidence intervals	B_{max} ² (pmol/mg)	EC_{50} ³ (nM)
WT-TP		3.7	3.02 to 4.44	3.8±0.3	13.1±0.8
A160S ^{4,53}	IV	6.3	3.64 to 8.90	5.8±0.5	9.3±0.3
A160T	IV	4.8	3.17 to 6.42	1.8±0.4	5.5±0.3
A160V	IV	3.8	1.08 to 6.47	1.9±0.3	9.5±0.2
G164A ^{4,57}	IV	5.9	3.88 to 7.99	6.3±0.4	2.5±0.2
G164V [†]	IV	-			38.0±2.1
F107A ^{3,27}	III	2.6	1.33 to 3.92	1.8±0.2	2.5±0.7
V110A ^{3,30}	III	4.6	3.72 to 5.44	1.8±0.3	2.7±0.2
F114A ^{3,34}	III	4.5	2.14 to 6.84	1.6±0.3	1.8±0.2
F115A ^{3,35}	III	3.2	2.50 to 3.89	1.8±0.2	2.0±0.3
A160V/F114A [†]	IV/III	-			
G164V/F107A [†]	IV/III	-			
G164V/V110A [†]	IV/III	-			
A160V/F115A	IV/III	5.0	1.83 to 8.13	2.1±0.4	43.5±1.5

*The values are expressed as the mean ± S.E (n = 2 to 5 experiments in duplicate), and the experiments are performed using the TP antagonist [³H] SQ 29,548 as the radioligand (NET936250UC, PerkinElmer).

[†]No significant specific binding to [³H] SQ 29,548 detected for these mutant receptors under the assay conditions.

¹ K_d , Affinity of the antagonist SQ 29,548 for the receptor.

² B_{max} , Binding maximum of the ligand SQ 29,548 for the receptor. Usually expressed as picomoles of TP per milligram of total membrane protein.

³ EC_{50} , the molar concentration of agonist U46619 that produces 50% of the maximal possible effect (calcium mobilized) for TP and mutant receptors.

doi:10.1371/journal.pone.0029996.t001

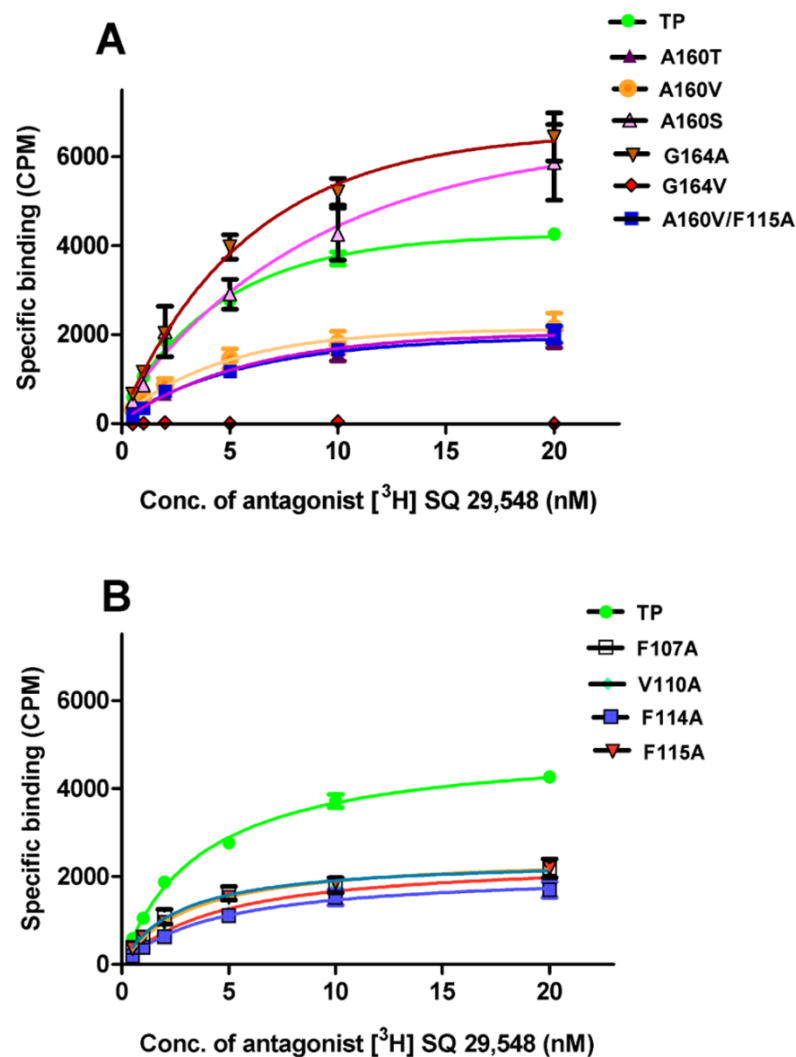


Fig. 2b.3. Saturation binding assays of wild type TP and the mutant receptors using the TP antagonist [^3H] SQ 29,548.

Saturation assays with membrane bound TP and the mutant receptors, TM4 mutants in panel A and TM3 mutants in panel B, were performed with different concentrations of [^3H] SQ 29,548. A one way ANOVA analysis without any post hoc test showed that except for G164V in TM4 there were no significant difference between TP and the mutants at significance level of $p < 0.05$. The data is from a minimum of three independent experiments, with each point in duplicate.

the intracellular compartments (**Fig. 2b.2**). This result shows that the G164V/V110A mutant was properly trafficked to the cell surface but was unable to bind to the antagonist, resembling the G164V phenotype.

2B.3.2 Agonist competition binding assays

We carried out competition radioligand binding assays using the unlabeled agonist U46619 and antagonist [³H] SQ 29,548 on TM4 mutants. Results from the heterologous competition assays showed that A160S and A160V mutants displayed moderately increased affinity towards the agonist U46619 with K_i values of 1.52 μ M (95% CI, 0.89-2.61) and 1.51 μ M (95% CI, 0.89-2.6) compared to 1.80 μ M (95% CI, 1.15 – 2.60) for TP (**Fig. 2b.4**). Significant changes were observed for the A160T and G164A mutants that displayed K_i values of 0.72 μ M (95% CI, 0.30-1.66) and 1.17 μ M (95% CI, 0.54-2.52). A one way ANOVA showed there is a significant difference between the mutants and TP at significance level of $P < 0.01$ ($n=3$). The K_i value of G164V mutant could not be determined because it did not bind to the antagonist under our assay conditions. While Gly164^{4.57} is more than 7Å from the TP receptor ligand binding pocket, the changes observed for the G164A and G164V mutants can be attributed to the structural influence of amino acid at position 4.57 on the ECL2 (see modeling). The increase in affinity of A160T for the agonist U46619 might be due to indirect effects, i.e., changes in ligand binding pocket or the receptor adopting an active state conformation, due to perturbation of helical packing by the A160T mutant. To elucidate the observed differences in agonist affinities, and decipher whether the mutations affected G protein coupling or activation, measurement of changes in intracellular Ca^{2+} upon agonist stimulation were pursued.

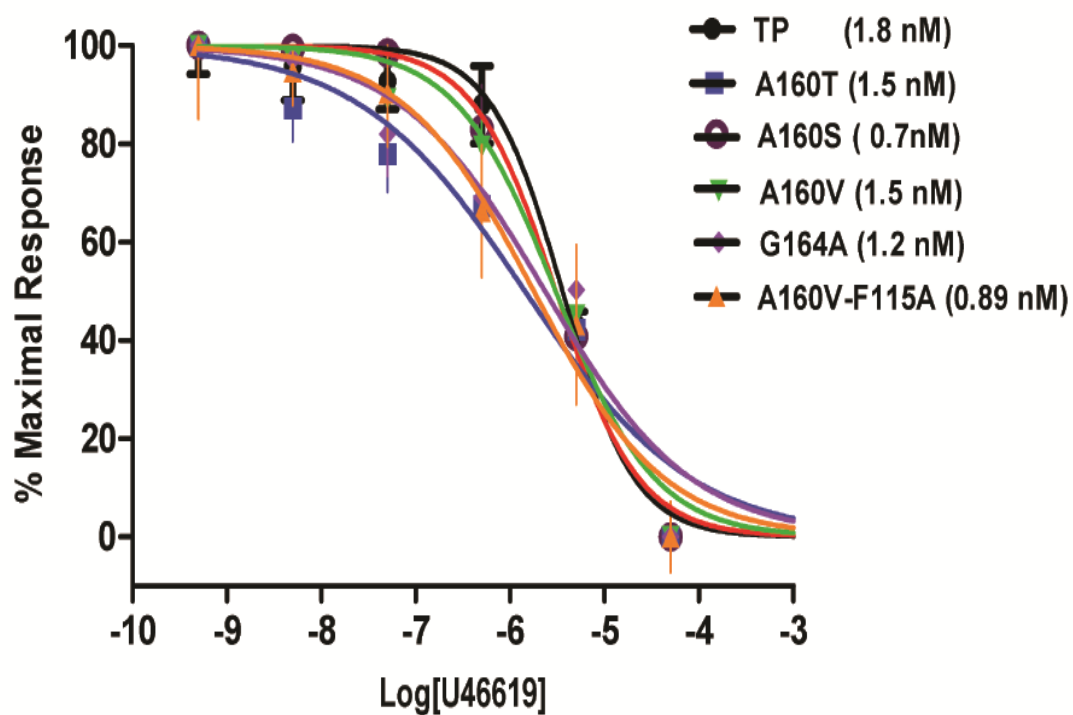


Fig. 2b.4. Heterologous competition curves of TP and select mutants using the unlabeled agonist U46619 and antagonist [³H] SQ 29,548.
The Ki values (μM) are shown in parenthesis.

2B.3.3 Intracellular Ca²⁺ signaling

Characterization of Gαq mediated signaling of the TP receptor and mutants were carried out by measuring the intracellular Ca²⁺ flux upon stimulation with agonist U46619 (**Fig. 2b. 5**). No significant variation was found in the EC₅₀ values of A160S and A160V compared to wild type (**Table 2b.1**). In agreement with the agonist competition assays, the A160T mutant showed moderate increased potency towards the agonist U46619, as illustrated by a left shift in the dose response (**Fig. 2b.5A**). Significant changes were seen with the Gly164^{4,57} mutants, the G164A demonstrated increased potency, and G164V mutant showed reduced potency as demonstrated with a right shift in dose response with agonist U46619 (**Fig. 2b.5B**). Nevertheless, the G164V mutant reached up to 90% of wild type activation upon stimulation with higher doses of agonist U46619. The TM3 mutants F107A, V110A, F114A and F115A upon stimulation with agonist U46619 showed an increase in intracellular Ca²⁺ flux equivalent to 60-80 % of TP generated signal (**Fig. 2b.5C**). Interestingly, the TM3 mutants displayed a left shift in the dose response curves, showing that these mutants had increased potency. Indeed, these mutants displayed 4 to 7 fold increase in U46619 potency (decrease in EC₅₀ concentration for half-maximum response) with EC₅₀ values from 1.8 nM to 2.7 nM compared to EC₅₀ of 13 nM for TP (**Table 2b.1**). Two double mutants, one each at positions 4.53 and 4.57 showed agonist dependent signaling but differed in their dose response characteristics. The double mutant A160V/F115A showed reduced potency as demonstrated by a right shift in dose response (**Fig. 2b.5D**). The G164V/V110A mutant lost SQ 29,548 binding and exhibited a reduced calcium response which could be restored upon stimulation with elevated levels of U46619.

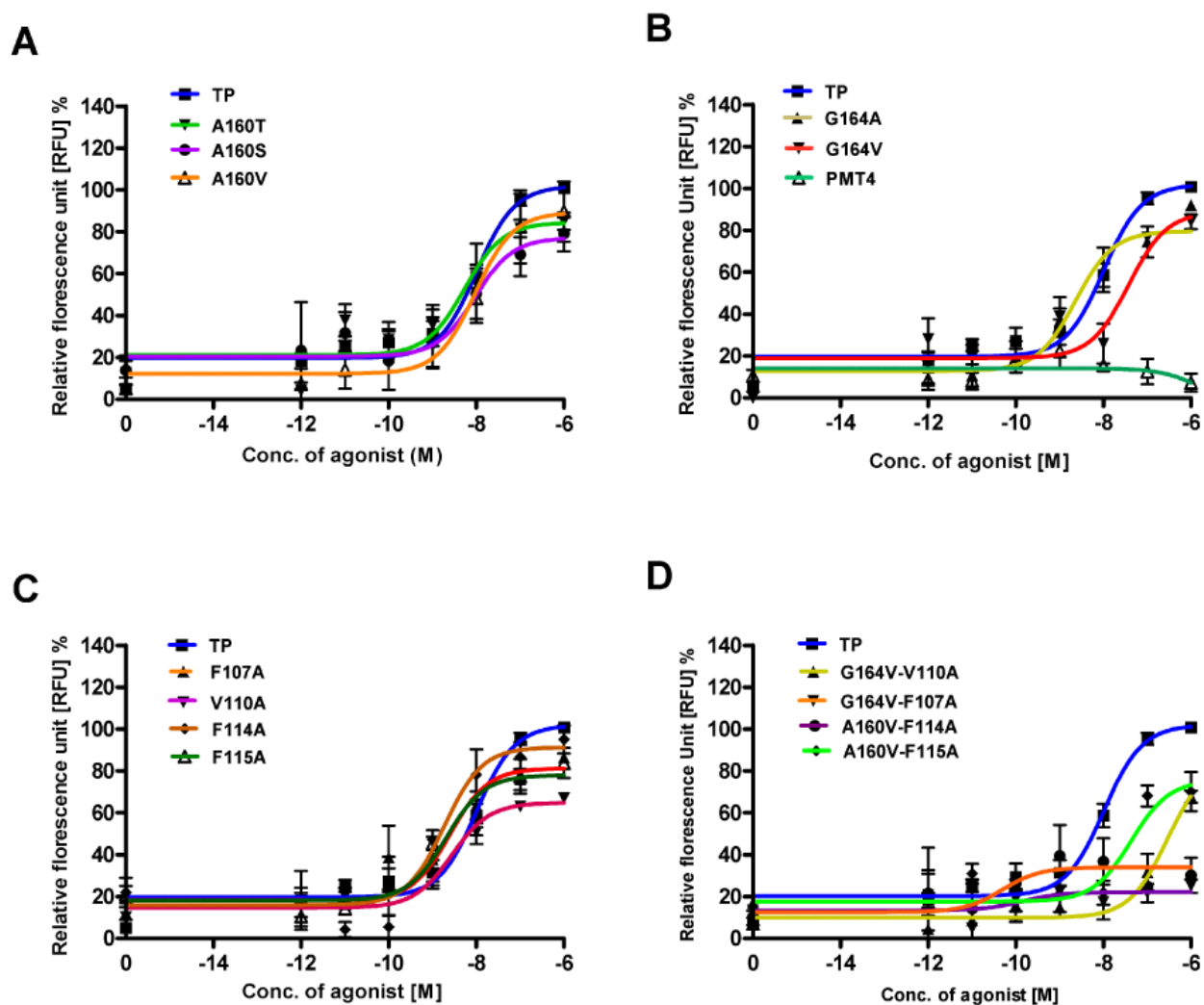


Fig. 2b.5. Characterization of *Gαq*-mediated signaling of the TP and mutant receptors.

The data shows agonist U46619 induced calcium release for TP, mutants and mock transfected (vector pMT4) HEK293T cells, and is expressed as a percentage of the TP activity. Ala160 mutants (panel A), Gly164 mutants and mock transfected cells (panel B), TM3 mutants (panel C) and double mutants (panel D). The results are from at least three independent experiments performed in duplicate.

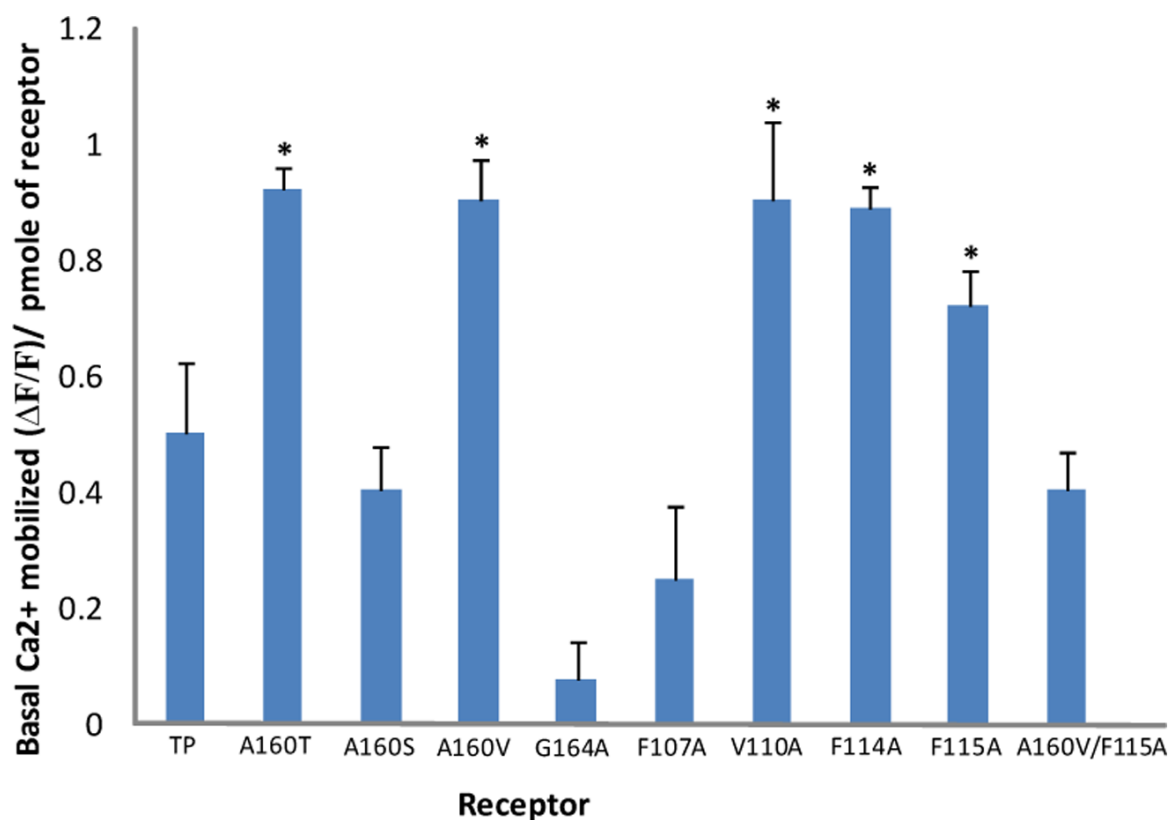


Fig. 2b.6. Characterization of basal or agonist-independent activity.

Bar plot representation of the basal amount of calcium released by TP and mutants per picomole of functional protein expressed (see methods). The G164V mutant could not be assessed as it did not bind to the antagonist, and the amount of functional receptor could not be calculated. Results are obtained from a minimum of two independent experiments done in duplicate. The single astrix indicate there is a significant difference in the amount of calcium released at zero concentration of agonist with respect to wild type TP at significance level $p < 0.05$. Error bars indicate mean $\pm$ SD.

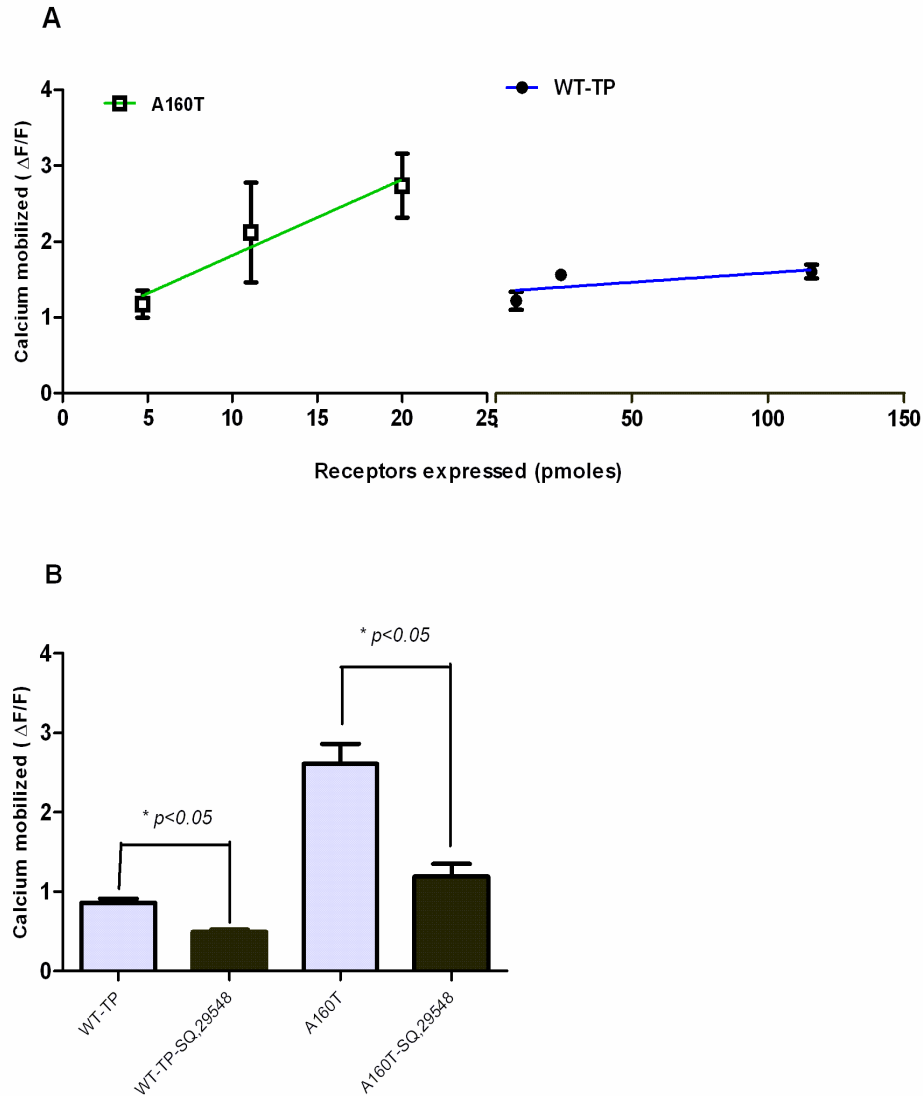


Fig. 2b.7. A. Effect of receptor density on basal Ca^{2+} mobilization.

WT-TP (blue line) and A160T (green line) constructs were expressed in HEK293T cells at different receptor densities by varying amounts of DNA used in each transfection (3 μg to 9 μg DNA per 10^5 cells). Receptor expression levels were determined by radioligand binding assays using a single saturating concentration (20nM) of [^3H] SQ 29,548. The slope of WT-TP was 0.002732 ± 0.001595 of calcium mobilized ($\Delta F/F$) /pmol of receptor while that of the A160T was 0.1001 ± 0.04041 . **B. Reversal of basal activation.** Agonist-independent calcium mobilization for WT-TP and A160T (grey bars), and calcium release after cells were pre-treated with 1 μM of unlabelled antagonist SQ 29,548 (black bars). Unpaired students *t* test between A160T basal calcium mobilization and A160T pre-treated with SQ 29,548 showed a significant decrease in basal activity at $p < 0.05$. Similar results were obtained for WT-TP. The results are from 2 independent experiments done in triplicate and are represented as Mean $\pm$ SD.

Basal Ca^{2+} levels of the TP mutants corrected for receptor expression levels were measured to assess constitutive activity. Among the TM4 mutants, only bulky β -branched replacements at position 4.53, A160T and A160V displayed a 2-fold increase in agonist-independent activity (**Fig. 2b.6**). The constitutive activity and the reversal of basal activity for the polymorphic variant A160T was characterized (**Fig. 2b.7**). In addition, three TM3 mutants V110A, F114A and F115A displayed constitutive activity (**Fig. 2b. 6**). This is not surprising as the mutated residues are towards the extracellular side of TM3 that is known to play a pivotal role in ligand binding and activation in many Class A GPCRs [148, 149].

2B.3.4 Thermal sensitivity assays

To examine the stability of the TM4 mutants, we monitored the ability of the mutants to retain antagonist affinity after incubation at 25°C, 37°C and 42°C as a function of time (**Fig. 2b.8**). Thermal sensitivity of the wild-type or TM4 mutant receptors was compared to distinguish the contribution of residues at positions 4.53 and 4.57 to stability of the helical core. The wild-type and mutants A160T, A160S and A160V were stable for 5 hrs at 25°C, whereas the G164A mutant showed about a 30-40% loss in antagonist binding (**Fig. 2b.8A**). Thus, differences between the stabilities of different Ala160 mutants could not be readily discerned at 25°C. However, thermal stability differences between the Ala160 mutants were more apparent at higher temperatures of 37°C and 42°C (**Fig. 2b.8B and C**). Within 1 hr at 42°C, mutants A160T and A160V showed 50-60% loss in total binding similar to G164A, while wild type and A160S showed only 30% loss in total binding.

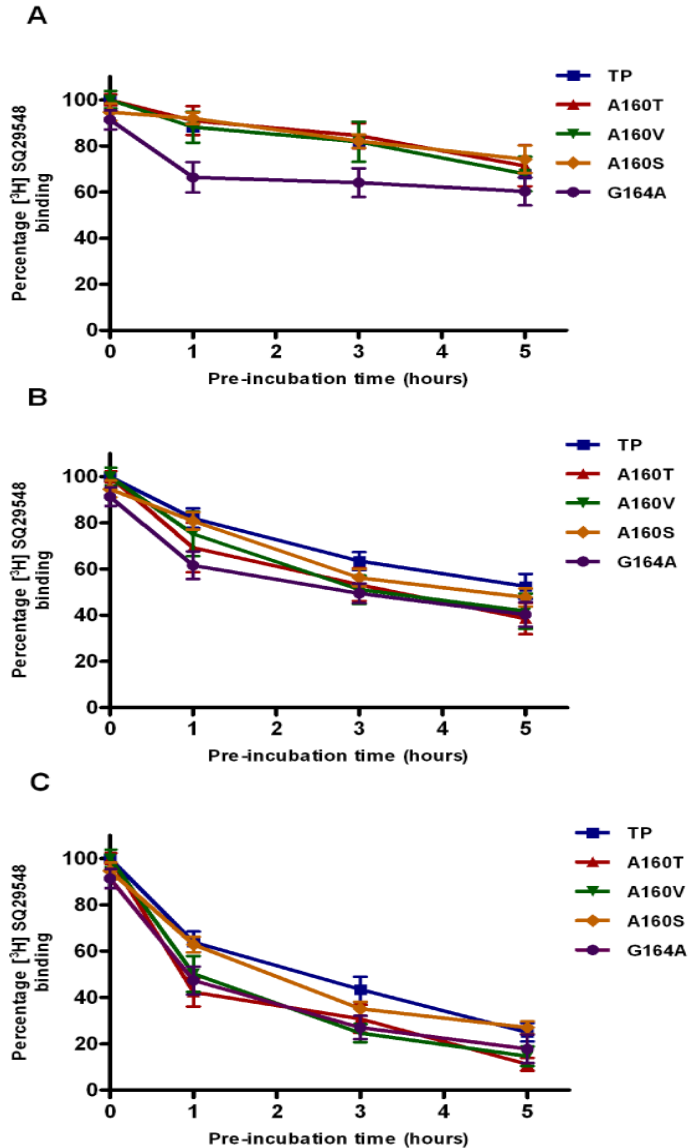


Fig. 2b.8. Thermal sensitivity of wild type TP and select mutants.

It was measured in terms of the ability of the TP and mutants at positions 4.53 and 4.57 to retain antagonist binding after incubation at 25°C (panel A), 37°C (panel B) and 42°C (panel C) as a function of time. The receptor activity decreased in the order, TP>A160S>A160V>A160T>G164A, with G164A being the least stable. The results are mean $\pm$ SE and are from minimum of three independent experiments done in duplicate.

Therefore, it appears that the contribution of Gly164 to receptor stability is very important, and replacement by amino acids with small molecular volumes such as alanine (G164A) causing loss of receptor stability. Interestingly, G164A mutant showed close to two-fold increase in expression of functional receptor compared to wild type (**Fig. 2b.3**). This result was surprising as decreased protein stability normally leads to decreased expression, as is the case with A160T and A160V mutants. We speculate that the G164A mutant might be resistant to proteolysis. Data were determined to be statistically significant using the unpaired student *t* test at significance level of $p < 0.05$.

2B.3.5 Molecular modeling

Molecular models of TP and the TM4 mutants were constructed to interpret the results in structural terms. (**Fig. 2b.9A**) shows the homology model of SQ 29,548 bound TP model superimposed with the structures of rhodopsin (PDB ID, 1U19) and antagonist bound β_2 -AR (PDB ID, 2RH1). The SQ 29,548 bound TP model shows very good homology to rhodopsin and β_2 -AR crystal structures with backbone C α RMSD of 0.4 Å and 1.5 Å for TM4 and TM3 regions analyzed in this study.

The ECL2 has been shown to play an important role in ligand binding and activation in the TP [13, 15] and other Class A GPCRs [150, 151]. Gly164^{4,57} occupies a crucial position on the extracellular side of TM4, and is located at the base of the N-terminal end of ECL2. In wild type TP and G164A models, Ser191 and Asp193 were found within the 4 Å region of the antagonist molecule SQ 29,548. While in G164V model, Asp193 is within the 4 Å region but the crucial hydrogen bond contact between Ser191 and SQ 29,548 was absent. Mutational studies by Kasawneh *et al.*, [15] have shown that

replacement of Ser191 on ECL2 resulted in mutants that were incapable of binding to SQ 29,548 but retained the functional response to treatment with U46619. This is similar to the G164V phenotype we observed, and our models validate this interaction (**Fig.2b.9B**). In wild type, Ala160^{4.53} is packed between Val111^{3.31}, Phe114^{3.34} and Phe115^{3.35}. Interestingly, in the A160S mutant the side chain β -OH of Ser160 interacts with the main chain carbonyl oxy-group of Trp157^{4.50} and no steric hindrance from residues on TM4 was observed (**Fig.2b.10**). In A160V and A160T mutants, the bulky β -branched side chains are intercalated between Phe114^{3.34} and Phe115^{3.35}. In agreement with the molecular model, the F115A mutant was able to rescue, albeit partially, the expression of A160V in the A160V/F115A double mutant (**Table 2b.1**). In our model of the A160T mutant, the Thr160 is more than 8 Å from the ligand binding pocket and is not in a position to directly interact with the ligand (**Fig. 2b.9** and **Fig. 2b.10**), hence the increase in potency observed is due to its packing interactions with the Phe114^{3.34} and Phe115^{3.35} on TM3, which themselves are very sensitive to replacements and show increased agonist affinity and constitutive activity. Therefore, the agonist-independent activity observed for the A160T and A160V mutants is due to the steric clash with Phe114^{3.34} and Phe115^{3.35} on TM3 resulting in the receptor adopting an active state conformation.

2B.4 Discussion

Role of Ala160^{4.53} and Gly164^{4.57} in TP. The results presented in this report provide important new insights into the role of TM4 in prostanoid receptors and in particular the two conserved residues Ala160^{4.53} and Gly164^{4.57} present in TM4. The residues at positions 4.53 and 4.57 in the TP perform predominantly a structural role in packing of TM3 and TM4 helices. Recently several structures have been reported for agonist-bound Class A

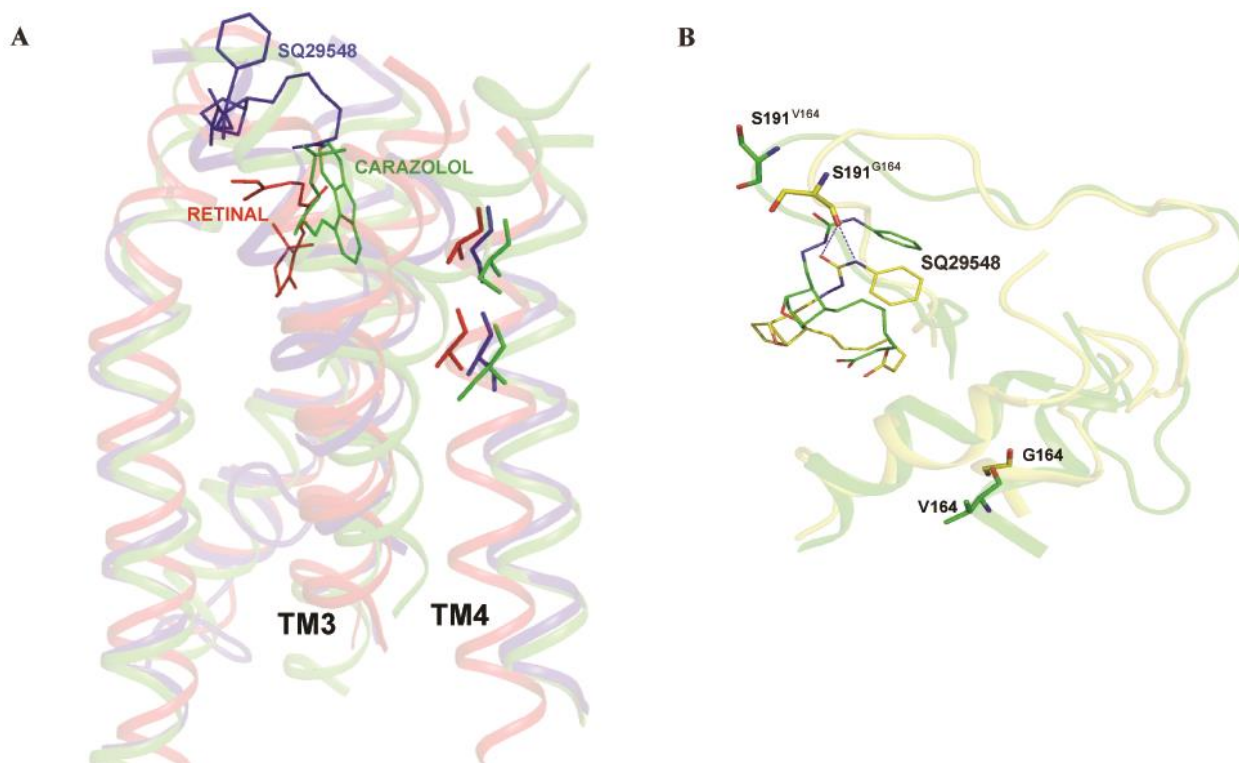


Fig. 2b.9. Molecular models of antagonist bound TP and G164V mutant and comparison with the crystal structures of rhodopsin and antagonist bound β_2 -AR. **Panel A,** Molecular model of TP bound SQ 29,548 superimposed with the structures of rhodopsin (PDB ID 1U19) and antagonist bound β_2 -AR (PDB ID 2RH1). The two residues at positions 4.53 and 4.57 on TM4 in both rhodopsin and β_2 -AR were previously studied. For structural comparison all the three structures were superimposed. The color coding is as follows; Ala164 and Ala168 in rhodopsin (red), Ala160 and Gly164 in TP (blue) and Ser161 and Ser164 in β_2 -AR (green). **Panel B,** Molecular models of TP (yellow) and G164V (green) superimposed. The important amino acids Gly164, Val164 and Ser191 are shown. Notice that Ser191 loses interaction with the antagonist SQ 29,548 in the G164V mutant.

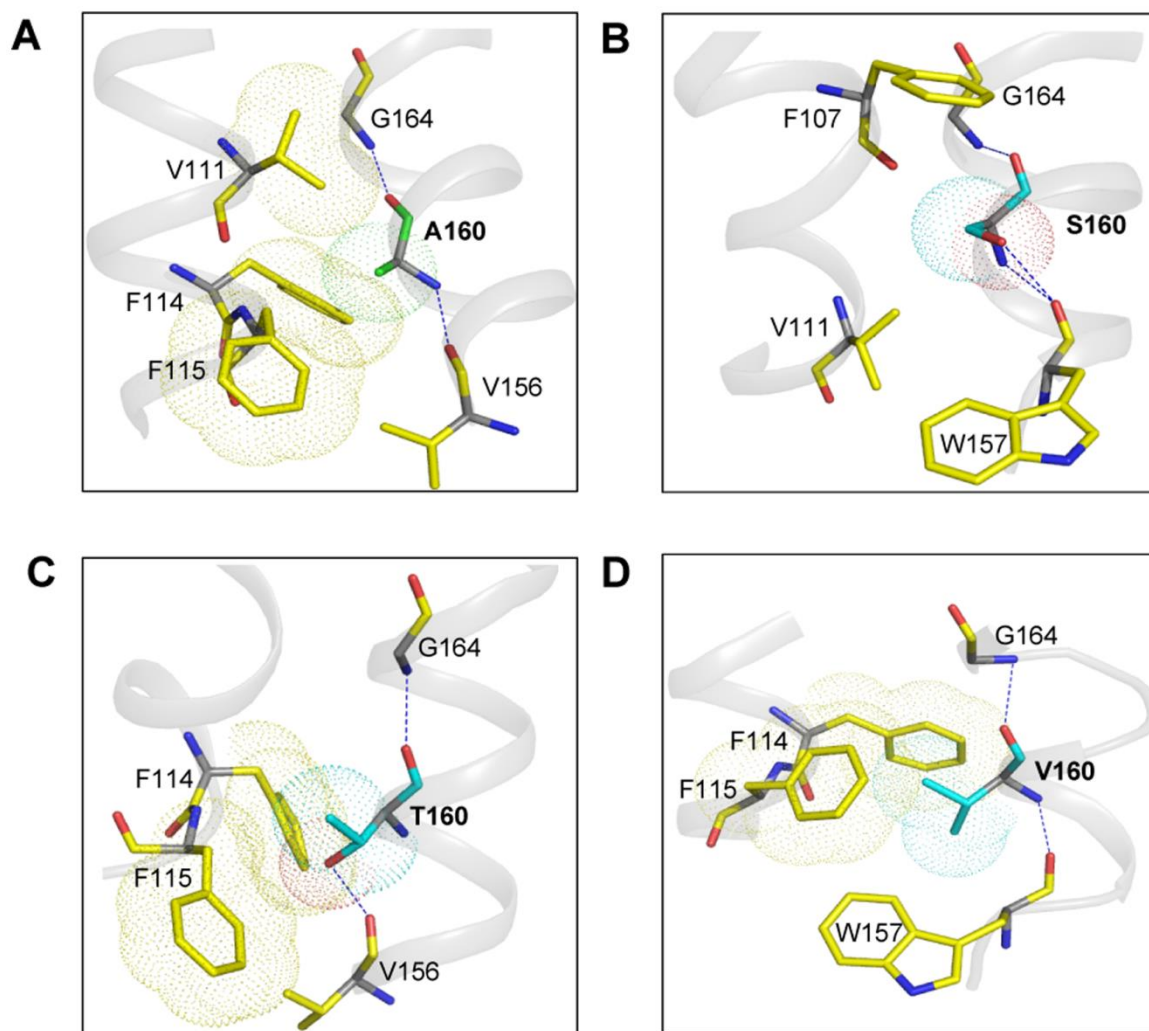


Fig. 2b.10. Molecular models of agonist bound TP and Ala160 mutants. Wild type (panel A), A160S (panel B), A160T (panel C) and A160V (panel D), and the important amino acids are shown in each model.

GPCRs, which showed that agonist induced conformational changes involves rearrangement of the TM3-TM5-TM6 interface. The recent structural elucidation of a constitutively active rhodopsin mutant, E113Q present on TM3, reinforces the central role of TM3 in GPCR activation [129]. However studies that give insights into the structural requirements for the constitutive and the agonist-induced conformational changes in TM3 among the prostanoid receptors are unavailable.

In the TP, the G164V replacement was unable to bind to the antagonist but showed an agonist dose dependent calcium increase. This phenotype was not observed in rhodopsin and β_2 -AR mutated at the same position (4.57). In the β_2 -AR, compensatory mutants designed to rescue the expression of S161V^{4.53} were unsuccessful but V114A^{3.33}/S165V^{4.57} double mutant rescued the expression of S165V^{4.57}, while in rhodopsin L119A^{3.34}/A164V^{4.53} rescued the defect in chromophore formation caused by the retinitis pigmentosa mutant A164V^{4.53} [90, 136]. However, in the TP we did not observe any protein misfolding upon mutation of either Ala160^{4.53} or Gly164^{4.57} residues. This is in contrast to rhodopsin, where the nsSNP A164V destabilizes helix packing resulting in protein misfolding and retinitis pigmentosa [136]. Interestingly, the human red cone opsin polymorphic variant Ser180Ala (homologous to the rhodopsin nsSNP A164V) accounts for the subtle difference in normal color vision and influences the severity of red-green color vision deficiency [152]. We can speculate as to why the compensatory mutants in TP were not as successful compared to rhodopsin and β_2 -AR. In this study, the compensatory mutations were designed based on proximity between two amino acids in the molecular model, it is possible that there is a network of interhelical hydrophobic interactions involving Ala160^{4.53}, Phe114^{3.34} and Phe115^{3.35}, while subtle changes are tolerated

(mutations to smaller amino acids) any major change (replacement with larger residues or double replacements) would disturb this hydrophobic network causing changes to the ligand binding pocket in the TP.

Polymorphic variant Ala160Thr. Studies have shown that more than 80% of the diseases causing mutations affect protein stability [153]. Similar to the decreased thermal stability and constitutive activity observed for the nsSNP A160T, a recent study on the rhodopsin retinitis pigmentosa mutant, G90V, shows that it has low thermal stability in the dark state and is constitutively active [154]. Analysis of the protein sequences revealed that disease causing SNPs tend to occur at conserved sites [155]. The transmembrane region from residues 4.53 to 4.57 in Class A GPCRs, consist of amino acids with small molecular volumes that are highly conserved. While this region is well studied in the opsin and amine subfamilies of Class A GCPRs, and the disease causing nsSNPs have been characterized, comparable studies in the prostanoid subfamily have not been pursued thus far. Amino acid sequence analyses of 46 TP sequences showed 81% to have alanine at 4.53 and 85% have glycine at position 4.57. The other residues were serine (16%) at 4.53 and alanine (13%) at 4.57, both amino acids with small molecular volumes. Our study reveals a structural role for the nsSNP A160T variant, while the clinical significance of this nsSNP remains to be determined.

Chapter 3

New Insights into Structural Determinants for Prostanoid Thromboxane A₂ Receptor- and Prostacyclin Receptor-G Protein Coupling

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3.1 Introduction

G protein-coupled receptors (GPCRs) contain seven transmembrane (TM) helices and signal predominantly through heterotrimeric G proteins in response to diverse extracellular stimuli, including neurotransmitters, light, taste, and smell. GPCRs form the largest group of membrane receptors and are divided into four classes, with the pharmacologically important class A comprising more than 70% of the GPCRs present in humans [5]. The prostanoid receptors belong to the class A GPCR family and are poorly characterized in their structural aspects. The prostanoids thromboxane A₂ (TxA₂) and prostacyclin (PGI₂) have been shown to play crucial but opposing vascular roles, with TxA₂ stimulating platelet aggregation and vasoconstriction and PGI₂ inhibiting platelet aggregation and causing vasodilation [2,121,156]. The recent withdrawal of selective cyclooxygenase 2 (COX-2) inhibitors (e.g., Vioxx) due to increased numbers of cardiovascular events [157] and cardiovascular concerns with nonsteroidal anti-inflammatory drugs (NSAIDs) (e.g., ibuprofen) [158, 159] highlight the importance of understanding the G protein specificity of these opposing receptors.

The thromboxane A₂ receptor (TP) activates its cognate G protein (G_{αq}) in response to binding to TxA₂, while the prostacyclin receptor (IP) mediates signaling in response to the binding of PGI₂, primarily through the G_{αs} based effector system. In addition, previous studies have shown that the IP is capable of coupling to multiple G proteins in a species- and/or tissue-specific manner, although the molecular basis behind this coupling to different G proteins is not properly understood [160-162]. The activation of a GPCR leads to structural protein changes in the cytoplasmic loops to activate the G protein. The recent structural elucidation of the metarhodopsin II bound to a peptide [163] and the β₂-adrenergic receptor (β₂-AR)-Gs (the stimulatory protein for adenylyl cyclase) protein

complex [164] give new insights into how GPCR-G protein complex formation occurs. These two structures now set the stage to identify sequence and structural features on GPCRs that may define specificities for particular G proteins. In the prostanoid receptor subfamily, there is limited structural and functional information on the role of intracellular loops (ICLs) in the binding and activation of G proteins. Previous site directed mutagenesis studies on the TP hypothesized that all three ICL regions are involved in determining G protein selectivity and specificity [47,48,141]. The dominantly inherited bleeding disorder variant R60L in ICL1, the F138D mutation in ICL2, and the C223S mutation at the TM5/ICL3 boundary affected agonist-induced calcium signaling [16, 49].

In this study, by using a chimeric receptor approach combined with molecular modeling and site-directed mutagenesis, we analyzed the role of ICL regions among the prostanoid receptors TP and IP that determine the binding and activation of $G_{\alpha q}$ and $G_{\alpha s}$, respectively. We systematically replaced each of the three TP ICL regions with the ICL regions of the IP. In the case of larger ICLs, such as ICL2 and ICL3, multiple chimeras were required in order to determine the minimal structural region of the loop that is required for optimal G protein coupling and signaling. Therefore, sequential replacement of the ICL2 and ICL3 of the TP with those of the IP was also pursued. In these replacements, the amino acid sequences and lengths of ICL2 and ICL3 influenced G protein activation, receptor folding, and the level of constitutive activity. To elucidate the structural constraints in the loops of each of the chimeric receptors that might be responsible for the observed activities, molecular modeling studies were pursued on the chimeric receptors that are bound to a C-terminal peptide fragment of the G_{α} subunit. The most interesting chimeric receptor is TP ICL2B-3B-IP; with an intermediate number of TP ICL2 and TP ICL3 amino acids replaced by IP ICL2 and IP ICL3, it showed TP agonist U46619-induced

cyclic AMP (cAMP) accumulation of up to 90% of the wild-type (WT) IP induced with iloprost and Gαq mediated inositol-1, 4, 5-trisphosphate (IP₃) and Ca²⁺ signaling of up to 90% of the wild-type TP. Next, to validate our molecular models, site-specific mutations were constructed. To test the stringency of our chimeric constructs, we introduced naturally occurring mutations present in the ICL regions into select chimeras. The TP ICL2B-3B-IP chimera was able to rescue the signaling of the dysfunctional genetic variant R60L to that of wild-type TP levels. Our results show that the predominant ICLs that determine G protein specificities among the prostanoid receptors are ICL2 and ICL3. We discuss our findings in the context of the recent structures of GPCR-G protein complexes.

3.2 Materials and methods

3.2.1 Materials

The TP antagonist [³H]-labeled SQ 29,548 was purchased from PerkinElmer (product no. NET936250UC). The TP agonist U46619 and the IP agonist iloprost were purchased from the Cayman Chemicals Company (Michigan). Protease inhibitors and common chemicals were purchased from either Fisher or Sigma. The buffers and detergents were the same as those used previously [165].

3.2.2 Synthesis of chimeric TP constructs, site-directed mutagenesis, and cell cultures

The wild-type TP, chimeric TP-IP genes carrying the rho-1D4 epitope tag at the C terminus, and specific site-directed mutations were synthesized commercially (GenScript) and incorporated into the plasmid expression vector pMT4 [76,90,165]. The design and analyses of these constructs are described in Results. The genes were transiently expressed in heterologous cell lines, and the membranes were prepared according to previously

published protocols [76, 165]. To minimize variations in transfection efficiency, the chimeric sequences were codon optimized for their expression in mammalian cells, and equal amounts of DNA (6 µg per 5×10^6 cells) were used. Following the transient transfection, cell viabilities were determined, and viable cells were used for the assays.

3.2.3 Radioligand binding assays

Saturation binding assays were carried out using [^3H]-labeled SQ 29,548 (product no. NET936250UC; Perkin- Elmer) and as described previously [165].

3.2.4 Determination of receptor expression by flow cytometry analysis

The cell surface expressions of TP, IP, and different constructs were determined using a BD FACSCanto flow cytometer. HEK293T cells were transfected with 6 µg of DNA per 5×10^6 cells using Lipofectamine 2000. Twenty-four hours after the transfections, 1×10^5 viable cells were taken into a flow cytometry tube and washed 2 to 3 times with fluorescence activated cell sorter (FACS) buffer (phosphate-buffered saline [pH 7.4] containing 0.5% bovine serum albumin) by centrifugation for 4 min at 1,500 rpm. The cells were incubated for 60 min on ice with a 1:100 dilution of the polyclonal antibody TBXA2R (0.5 mg/ml) (catalog no. LS-B4842; LifeSpan BioSciences) and IP antibody (item no. 10005518; Cayman Chemicals), which target the N termini of the human TP and IP, respectively. The cells were washed 2 to 3 times with FACS buffer and incubated in the dark with a 1:500 dilution of the secondary antibody Alexa Fluor488 for 60 min on ice. The cells were washed 2 to 3 times with FACS buffer and resuspended in 200 µl of FACS buffer. The fluorescence signals of 1×10^4 cells/ tube were measured using single-color analysis by the BD FACS Canto analyzer using settings of 159V for forward scatter (FSC),

379 V for side scatter (SSC), and 385 V for Alexa Fluor 488. The results were analyzed using the FACS Diva and FlowJo software programs. The cell surface receptor expression was calculated as a percentage of the wild-type TP expression level, which was set to 100%. The nonspecific signal from mock-transfected cells was $10\% \pm 2\%$.

3.2.5 Determination of intracellular cAMP

The cAMP assays were carried out in HEK293S cells using a commercially available cAMP assay kit (Hit- Hunter cAMP XS; DiscoverX, Fremont, CA) and as described previously [76]. In brief, 48 h after transient transfection, the cells were stimulated with various concentrations (from 10^{-6} M to 10^{-12} M) of the agonist iloprost for the IP (positive control) and the agonist U46619 for the TP and chimeric TP-IP receptors. Luminescence was measured after overnight incubation using a Flex Station 3 microplate reader (Molecular Devices, CA). The assays were carried out 3 to 5 times each in duplicate, and the data were analyzed using PRISM software version 5 (GraphPad Software Inc., San Diego, CA). The cAMP values of the chimeric TP-IP receptors, expressed in relative luminescence units (RLUs), were normalized to that of the WT IP.

3.2.6 Determination of intracellular IP₃ and calcium

The changes in intracellular calcium levels were measured by using the fluorescent calcium sensitive dye Fluo-4NW (Invitrogen) and as described previously [165]. Receptor activation was determined by measuring the changes in intracellular calcium levels after the application of different concentrations of the agonist U46619 for the TP and chimeric TP-IP receptors and of iloprost for IP receptors using a FlexStation-3 fluorescence microplate reader (Molecular Devices, CA) at 525 nm, following excitation at 494 nm. Dose-response

curves were generated and 50% effective concentrations (EC50s) were calculated by nonlinear regression analysis using PRISM software version 5.0 (GraphPad Software Inc., San Diego, CA) after subtracting the responses of mock-transfected cells that were stimulated with the same concentrations of the agonists. For an estimation of the calcium that was mobilized using the non ratiometric calcium indicator dye Fluo-4NW, we used the $\Delta F/F$ ratio, which calculates approximate calcium levels using the equation $\Delta F/F = (F - F_{base}) / (F_{base} - B)$, where F is the measured fluorescence intensity of Fluo-4NW, F_{base} is the fluorescence intensity of Fluo-4NW in the cell before stimulation, and B is the background signal determined from areas adjacent to the cell [139]. The inositol-1,4,5-trisphosphate (IP₃) assays were carried out in HEK293T cells using a commercially available IP₃ assay kit (HitHunter IP₃ fluorescence polarization [FP] assay; DiscoverX, Fremont, CA) according to the instructions supplied by the manufacturer.

3.2.7 Statistical analysis

Statistical analyses using one-way analysis of variance (ANOVA) with Tukey's *post hoc* test from at least 3 independent experiments (performed in duplicate) were done to determine which chimeric receptor or mutant exhibited a response that was statistically different from that of the wild-type TP or IP, with a P value of 0.05 considered to be statistically significant (**Table 1; Fig.3.6, 3.7, 3.8**).

3.2.8 Immunofluorescence analyses

HEK293T cells were seeded into six well tissue culture plates containing glass coverslips treated with sterilized poly-L-lysine (Sigma). The cells were transiently transfected with the wild type TP or the chimeric receptors using Lipofectamine 2000. The

procedure was similar to previously published protocols [166]. Representative cells were visualized using an Olympus IX81 microscope for cytoplasmic or plasma membrane localization of the receptors.

3.2.9 Protein molecular modeling

We have used the homology model of the TP alpha isoform (TP α) that was built and validated by our group previously [165]. The chimeric receptors TP ICL1-IP, TP ICL2-IP, TP ICL2B-IP, TP ICL3B-IP, and TP ICL3C-IP were also modeled and docked with the TP agonist ligand U46619 following a published protocol [165].

3.2.10 Receptor-G α peptide docking

The active conformation of the TP receptor was docked with the 14 C-terminal amino acids of human G α q (GenBank accession no. AAB39498.1). The 14 C-terminal amino acids of the human G α q were modeled using rat G α q (3AH8) as a template. The receptor and G α peptide were docked using the ZDOCK server [167]. The docking calculations were carried out using the fast Fourier transform based protein-docking method using ZDOCK. It involves searches of all possible binding modes in the translational and rotational spaces between two proteins and evaluates each using an energy-scoring function [168]. The poses with the best energy scores were chosen for further analysis. Our docking studies revealed that the 14-amino-acid G α peptide bound to the TP in two positions, one between ICL1 and ICL2 and the other between ICL2 and ICL3. To determine which of the two positions the correct one was, we utilized the recently solved structure of the β 2 adrenergic receptor (β ₂-AR)-G α s (3SN6) protein complex. We built the human G α q (35 to 359 residues) model by homology modeling using the mouse

Gαq (2RGN) crystal structure as a template, and using the TP model from above, both were threaded on the β₂-AR-Gas (3SN6) complex. Molecular dynamic simulations (10 ns) using the Tripos force field were performed on the TP-Gαq complex using the SYBYL-X v2.1 molecular modeling suite (Tripos Inc.). In this model, the C-terminal region of Gαq was located between the ICL2 and ICL3 of the TP. Based on this, the model with the C-terminal Gαq peptide bound between ICL2 and ICL3 of the TP was used for further analysis, and the models were visualized using PyMOL.

3.3 Results

3.3.1 Design and construction of chimeric TP-IP receptors

The amino acid sequences of the TP and IP receptors were obtained from the G protein-coupled receptor database (GPCRDB), and alignment was performed using ClustalW [120, 169]. From the multiple-sequence alignment, the TP chimeras containing intracellular IP loops were designed in three steps. In the first step, TM sequence prediction servers were used in predicting the TM regions (**see Table 3.1**). In the second step, a total of four chimeric TP-IP receptors were designed as follows: three TP chimeric receptors were constructed containing each of the three ICLs of the IP and the other one (TP-ICL1,2,3- IP) with all the loops replaced (**Fig. 3.1A**). In the final step, the sequential replacement of ICL2 and ICL3 of the TP with the IP sequence was carried out by replacing six amino acids at a time (i.e., three amino acids from the N termini and three amino acids from the C termini of the loop regions). Following the procedure described above, two ICL2 chimeric receptors, TP ICL2A-IP and TP ICL2B-IP, and three chimeric receptors, TP ICL3A-IP, TP ICL3B-IP, and TP ICL3C-IP, were constructed for ICL3 (**Fig. 3.1B**).

Table 3.1 Analysis of the transmembrane (TM) regions of both the human TP and IP receptors

RECEPTOR			ICL1 Amino acid sequence	ICL2 Amino acid sequence	ICL3 Amino acid sequence
OP SIN	Crystal structure (PDB 1U19)		65-71	140-149	225-240
	Algorithm	SPLIT	62-72	140-152	229-250
		TMPred	60-73	140-152	228-252
		TMHMM	62-73	134-152	225-253
		HMMTOP	72-83	142-161	231-261
β_1 -AR	Crystal structure (PDB 2VT4)		39-46	112-124	207-254
	Algorithm	SPLIT	40-47	105-126	201-230
		TMPred	42-52	112-132	204-235
		TMHMM	38-57	118-129	199-230
		HMMTOP	41-52	110-133	201-234
β_2 -AR	Crystal structure (PDB 2RH1)		61-66	137-146	231-263
	Algorithm	SPLIT	61-68	133-151	223-241
		TMPred	63-74	135-156	224-246
		TMHMM	59-69	130-149	221-242
		HMMTOP	62-73	134-155	224-246
A2A	Crystal structure (PDB 3EML)		49-55	122-132	220-240
	Algorithm	SPLIT	49-51	117-135	219-238
		TMPred	56-62	113-142	216--245
		TMHMM	47-58	114-135	213-242
		HMMTOP	54-63	121-142	219-246
TP	Algorithm	SPLIT	56-65	128-152	229-248
		TMPred	50-70	132-153	220-250
		TMHMM	52-63	131-148	226-247
		HMMTOP	53-66	133-156	228-251
IP	Algorithm	SPLIT	41-47	124-134	213-235
		TMPred	38-47	116-137	203-237
		TMHMM	38-48	118-135	211-237
		HMMTOP	41-50	114-137	205-237

Analysis of the transmembrane (TM) regions of both the human TP and IP receptors using TM sequence prediction softwares [170-172]. The loop regions from crystal structures of GPCRs (opsin, β_1 -AR, β_2 -AR, A2A) were used as controls to test the stringency of the software programs in predicting TP and IP loop regions. The TMHMM program (highlighted in bold) showed a high degree of specificity in predicting the TM regions of the GPCRs analyzed and the predicted loop regions were used in the design of the chimeric receptors.

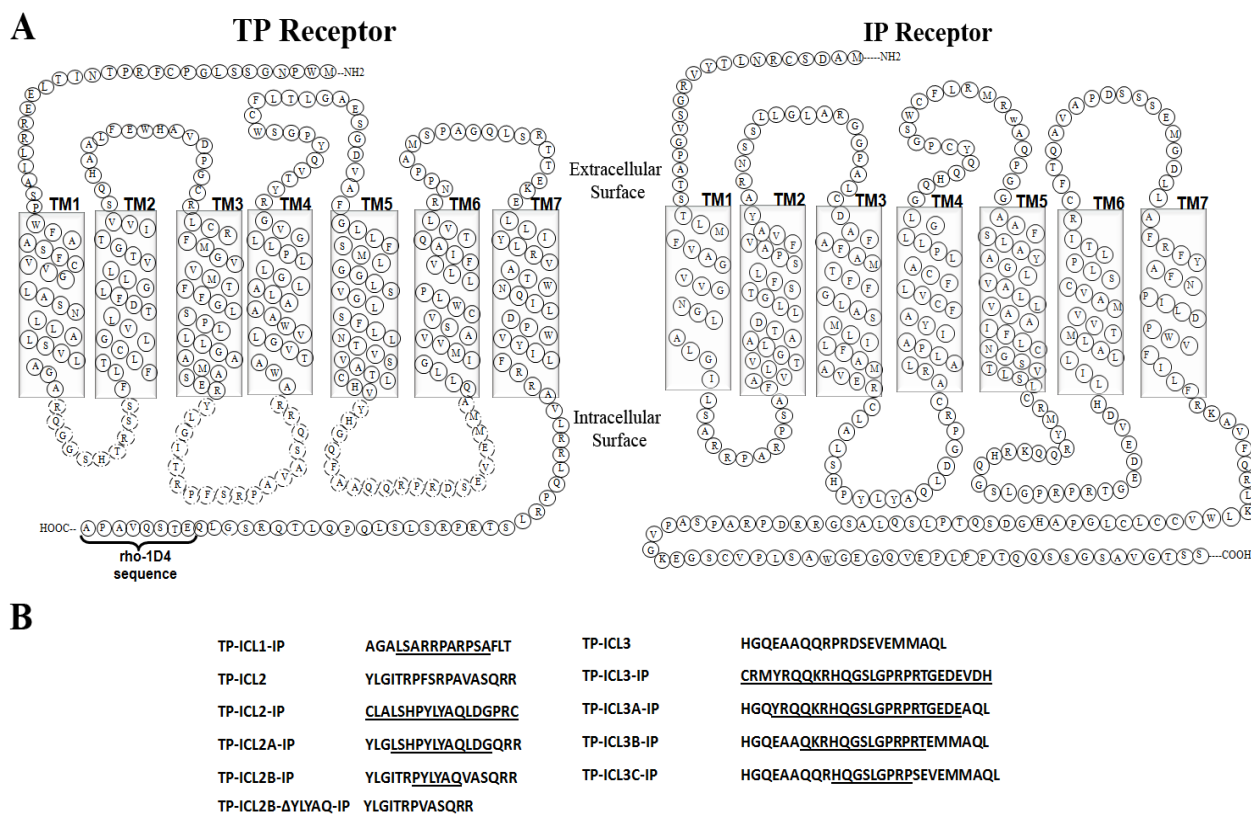


Fig. 3.1 Schematic representation of the amino acid sequence of the TP, IP, and chimeric receptors.

(A) Secondary structure representation of the TP and IP amino acid sequences. The intracellular loop (ICL) regions replaced in the TP are indicated by dashed lines. (B) The amino acid sequences of the TP replaced by the IP sequence are underlined. TP ICL1-IP represents the TP with its first ICL replaced by the IP ICL1. Similarly, TP ICL2-IP represents the TP with its second ICL replaced by the IP ICL2; TP ICL3-IP represents the TP with its third ICL replaced by the IP ICL3; TP ICL2B- ΔYLYAQ-IP represents the TP ICL2B-IP receptor with the pentapeptide YLYAQ sequence deleted.

3.3.2 Effect of loop replacements on TP expression, antagonist binding, and internalization

The levels of functional receptor expression were quantified by saturation ligand binding assays on the WT- TP and the chimeric receptors expressed in COS-1 cells using the radiolabeled TP antagonist SQ 29,548 (**Table 3.2**; see also **Fig. 3.2**). Among the complete loop replacements, only the TP ICL1-IP receptor with the replacement of ICL1 of the TP with that of the IP bound to the antagonist with a dissociation constant (K_D) of 4.8 ± 1.1 nM, which is close to the K_D of 5.8 ± 1.3 nM for the WT-TP. However, the complete replacement of ICL2 and ICL3 of the TP with IP ICLs failed to show any specific binding to SQ 29,548 (**Fig. 3.2**). To elucidate whether this loss in antagonist binding was due to major structural changes in the receptor that perturb the ligand binding pocket or due to poor cell surface expression and/or misfolding of the receptor, analyses of receptor expression by FACS, Western blotting, and immunofluorescence microscopy were carried out on these chimeric receptors. The WT-TP, IP, and the chimeric constructs, with the exception of the TP ICL3-IP and TP ICL3A-IP chimeras, were expressed at similar levels based on FACS analyses (**Table 3-2**). Immunofluorescence analyses showed TP ICL3-IP and TP ICL3A-IP to be partly retained in the intracellular compartments (**Fig. 3.3**). The functional receptor expression observed with the saturation radioligand binding, cell surface receptor expression by FACS analysis, and cellular localization by immunofluorescence was further supported by Western analysis (**Fig. 3.3**). The ICL2 chimeric receptors TP ICL2A-IP and TP ICL2B-IP bound to the antagonist with affinities similar to that of the WT-TP, but TP ICL2B-IP showed a 2-fold-higher B_{max} (binding maximum of the ligand SQ 29,548) than that of the WT-TP (**Table 3.2**). In comparison, among the ICL3 chimeric receptors, only TP ICL3CIP, with the shortest amino acid

replacement, showed specific binding to the TP antagonist SQ 29,548. Interestingly, the TP ICL3B-IP chimeric receptor showed no significant specific binding to SQ 29,548 (**Table 3.2**). The chimeric receptor with all three TP ICLs replaced with IP ICLs failed to show any specific binding to SQ 29,548, and immunofluorescence analyses showed that a majority of the receptors expressed were not targeted to the cell surface and were retained in the intracellular milieu (data not shown). To elucidate whether any of the chimeric receptors undergo internalization in the absence or presence of an agonist, the TP and the chimeras were treated with the agonist U46619, while the IP was treated with its agonist, iloprost. Interestingly, the TP and the chimeric receptors did not show any significant agonist-dependent or -independent internalization, whereas the IP showed a significant agonist-dependent internalization, with only ~40% of the receptor on the cell surface (**Fig. 3-3**).

3.3.3 Characterization of intracellular Ca^{2+} signaling of chimeric receptors

The characterizations of $\text{G}\alpha_q$ -mediated signaling of the WT-TP and chimeric receptors were carried out by measuring the intracellular Ca^{2+} flux upon stimulation with the agonist U46619 (**Fig. 3.4**). Interestingly, the TP ICL1-IP chimeric receptor with the complete replacement of ICL1 of TP with IP showed an increase in U46619 potency (a decrease in the EC_{50} for half-maximum response), with an EC_{50} of 5 nM compared to an EC_{50} of 13 nM for the WT-TP (**Table 1**). The TP ICL2-IP chimeras showed a left shift in dose response (**Fig. 3.4**), with the restoration in EC_{50} s to wild-type TP levels, as the TP sequence increased in the ICL2 region (**Table 1**). The TP ICL3-IP and TP ICL3A-IP chimeric receptors did not show any dose-dependent increases in intracellular Ca^{2+} (data not shown);

Table 3.2 Summary of ligand binding properties and expression of the wild-type TP, IP, and chimeric receptors

Receptor	Intracellular loop (s)	$K_d^{a,b}$ (nM)	$B_{max}^{a,c}$ (pmol/mg)	Cell surface ^d expression (%)	EC_{50}^e Ca ²⁺ (nM)	EC_{50}^f cAMP (nM)
Wild-type TP		5.8 ± 1.3	5.1 ± 0.3	100	13.1 ± 0.8	ND
Wild-type IP				108 ± 1	ND	7.7 ± 0.3
TP ICL1-IP	ICL1	4.8 ± 1.1	8.9 ± 0.4	96 ± 7	4.6 ± 1.1	ND
TP ICL2-IPg	ICL2	ND		93 ± 7	8.3 ± 0.5	22.7 ± 0.3
TP ICL2A-IP	ICL2	4.3 ± 0.6	3.1 ± 0.3	76 ± 6	11.0 ± 0.4	51.2 ± 1.3
TP ICL2B-IP	ICL2	5.3 ± 0.8		108 ± 7	15.6 ± 2.0	62.2 ± 1.2
TP ICL3-IPg	ICL3	ND		42 ± 4		
TP ICL3A-IP	ICL3	ND		49 ± 7		
TP ICL3B-IPg	ICL3	ND		92 ± 2		
TP ICL3C-IP	ICL3	5.8 ± 1.2	3.4 ± 0.4	89 ± 1	32.4 ± 1.3	39.8 ± 1.1
TP ICL2B-ICL3B-IP	ICL2/3	8.1 ± 3.9	0.9 ± 0.2	97 ± 2	61.6 ± 2.1	ND
TP ICL2B- Δ YLYAQ ^h	ICL2	7.7 ± 4.0	0.7 ± 0.2	81 ± 9	30.7 ± 0.2	18.3 ± 0.2
R60L	ICL1	9.8 ± 1.3	1.3 ± 0.2	85 ± 2	37.5 ± 0.2	ND
TP ICL2B-R60L	ICL1/2	19.7 ± 2.0	2.5 ± 0.2	96 ± 2	44.9 ± 0.4	
TP ICL2B-3B-IP-R60L	ICL1/2/3	21.7 ± 3.9	4.1 ± 0.2	92 ± 1	12.1 ± 0.4	
T135A	ICL2	7.6 ± 2.6	5.7 ± 0.8	72 ± 12	17.0 ± 0.2	
R136A	ICL2	8.7 ± 1.9	5.5 ± 0.5	81 ± 17	ND	
R147A	ICL2	9.2 ± 2.3	6.0 ± 0.6	82 ± 17	14.2 ± 0.4	
R148A	ICL2	7.3 ± 2.1	9.8 ± 0.1	46 ± 12	ND	
H224A ^g	ICL3	ND		49 ± 10	12.2 ± 0.3	
V225A ^g	ICL3	ND		88 ± 8		
E230A ^g	ICL3	ND		72 ± 2	8.0 ± 0.2	
Q252A ^g	ICL3	ND		97 ± 19	2.1 ± 0.2	

a The values are expressed as the mean ± standard error (SE) of 3 to 5 experiments in duplicate performed using the TP antagonist 3H-labeled SQ 29,548 as the Radioligand (product no.NET936250UC, PerkinElmer). ND, not determined.

b K_d , affinity of the antagonist SQ 29,548 for the receptor.

c B_{max} , binding maximum of the ligand SQ 29,548 for the receptor, expressed as pmol of the TP receptor per mg of total membrane protein.

d Cell surface expression of the receptor determined using flow cytometry (see Materials and Methods), represented using the wild-type TP set at 100%.

e EC_{50} s in this column indicate the molar concentrations of the agonist U46619 that produce 50% of the maximal possible effect (calcium mobilization) for the TP and chimeric or mutant receptors. A one-way ANOVA of the EC_{50} values for calcium mobilized between the wild-type TP and the chimeric receptors and mutants showed a significant difference ($P < 0.05$).

f EC_{50} s in this column indicate the molar concentrations of the agonist iloprost for IP and of U46619 for the TP and chimeric or mutant receptors that produce 50% of the maximal possible effect (cAMP production).

g No significant specific binding to the antagonist 3H-labeled SQ 29,548 and/or dose-dependent response to the agonist for these receptors was detected under our assay conditions.

h TP ICL2B-IP with the YLYAQ sequence in the middle of ICL2B removed.

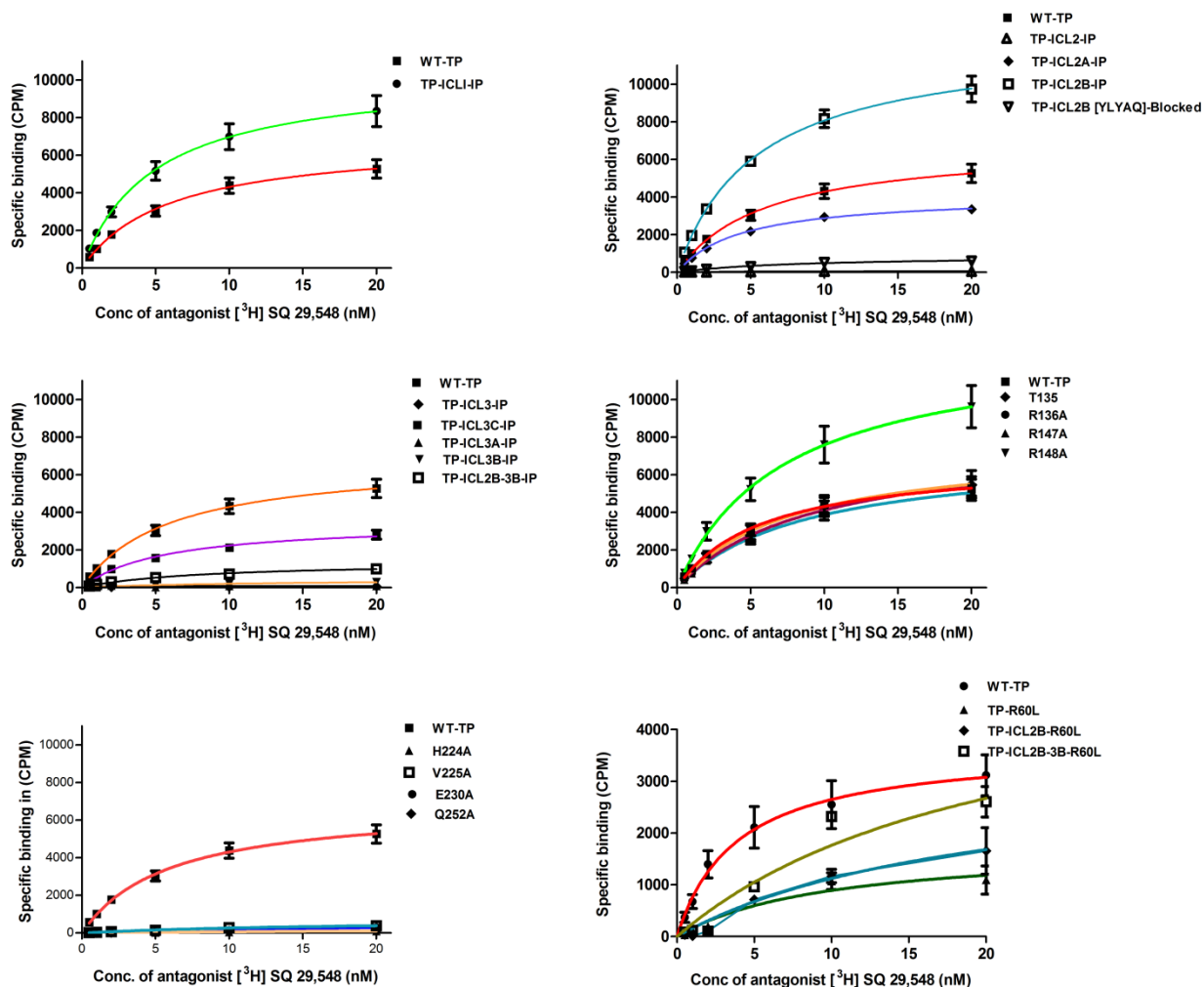


Fig. 3.2. Saturation binding assays of wild type (WT) TP and the chimeric receptors using the TP antagonist $[^3\text{H}]$ SQ29, 548.

Saturation assays with membrane bound WT -TP and the chimeric receptors were performed with different concentrations of $[^3\text{H}]$ SQ 29, 548 (0.5 nM - 20 nM). Specific binding was obtained by removing nonspecific binding from total binding observed. Binding of $[^3\text{H}]$ SQ 29,548 in the presence of 10 μM SQ 29,548 was used as a measure of nonspecific binding. The data is from a minimum of three independent experiments, with each point in duplicate.

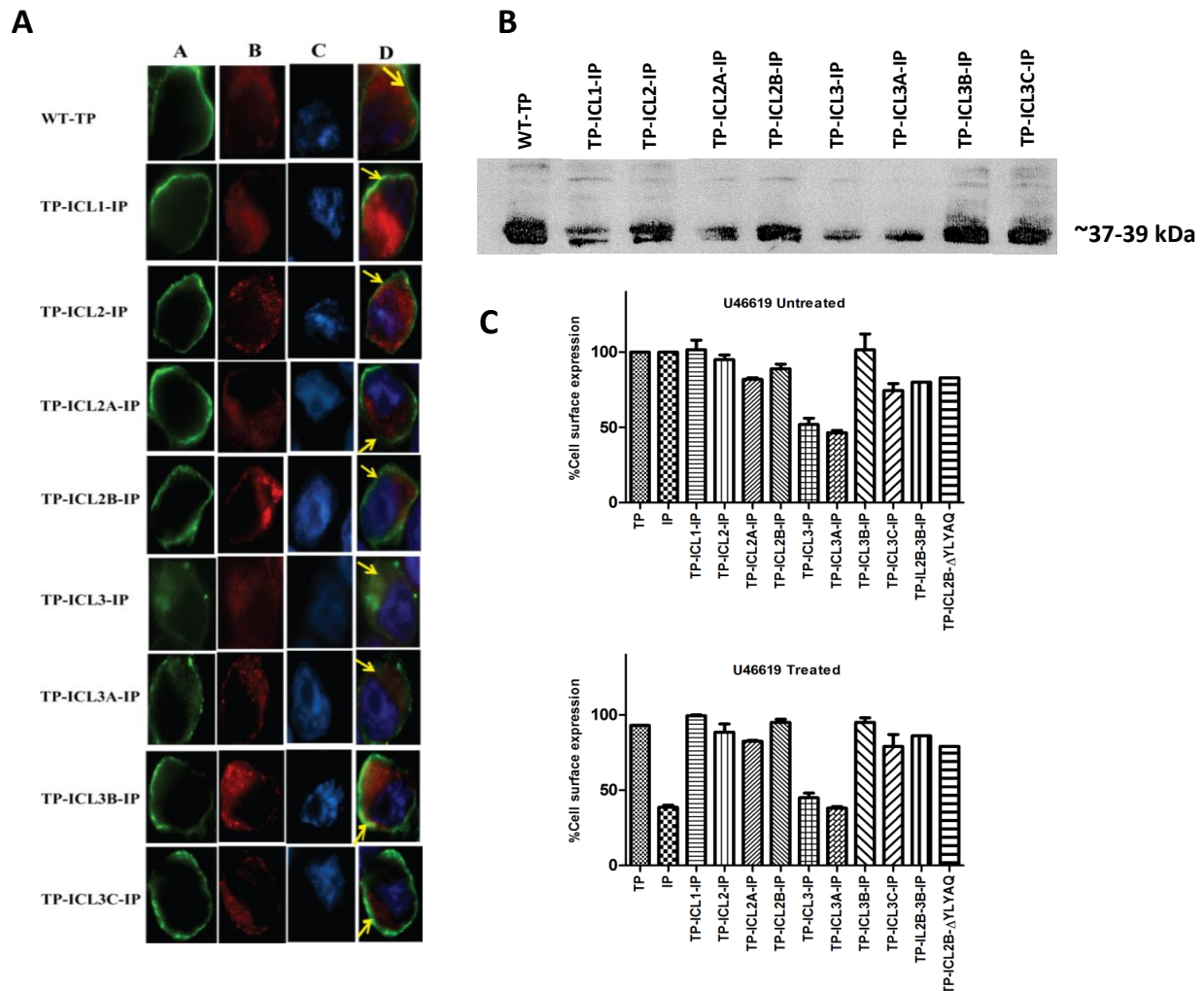


Fig. 3.3. Immunofluorescence microscopy and western blot analysis of WT-TP and chimeric receptors in HEK293T cells. **A)** Double-label immunofluorescence was performed using mouse monoclonal anti-rho-1D4 antibody which recognizes the C-terminal octapeptide tag on the expressed receptors, and rabbit polyclonal anti-calnexin antibody which localizes to the endoplasmic reticulum (ER). The WT-TP and TP chimeric receptors were visualized using goat anti-mouse Alexafluor 488 secondary antibody (**panel A**) and the ER was visualized with goat anti-rabbit Alexafluor 594 secondary antibody (**panel B**). The nucleus stained with Hoechst-33342 dye is shown in blue (**panel C**). The overlay of the receptor, ER and nucleus is shown in **panel D** (location of the expressed receptor is indicated by an arrow). **B)** Western blot analysis was performed using 5µg of total solubilized membrane protein and the protein detected using monoclonal anti-rho-1D4 antibody, as described previously [156, 159]. A representative blot is shown. Mol. wt range is indicated next to the gel. **C)** Agonist independent (untreated) and dependent (treated for 2hrs) internalization of TP, IP and TP IP chimeras. FACS analysis to determine the receptors on the cell surface was performed using antibodies specific for the extracellular region of TP (and for TP -IP chimeras) and IP. TP and the chimeras were treated with the TP agonist U46619, while IP was treated with iloprost. The results are from two independent experiments in duplicate. Error bars represent mean $\pm$ SD

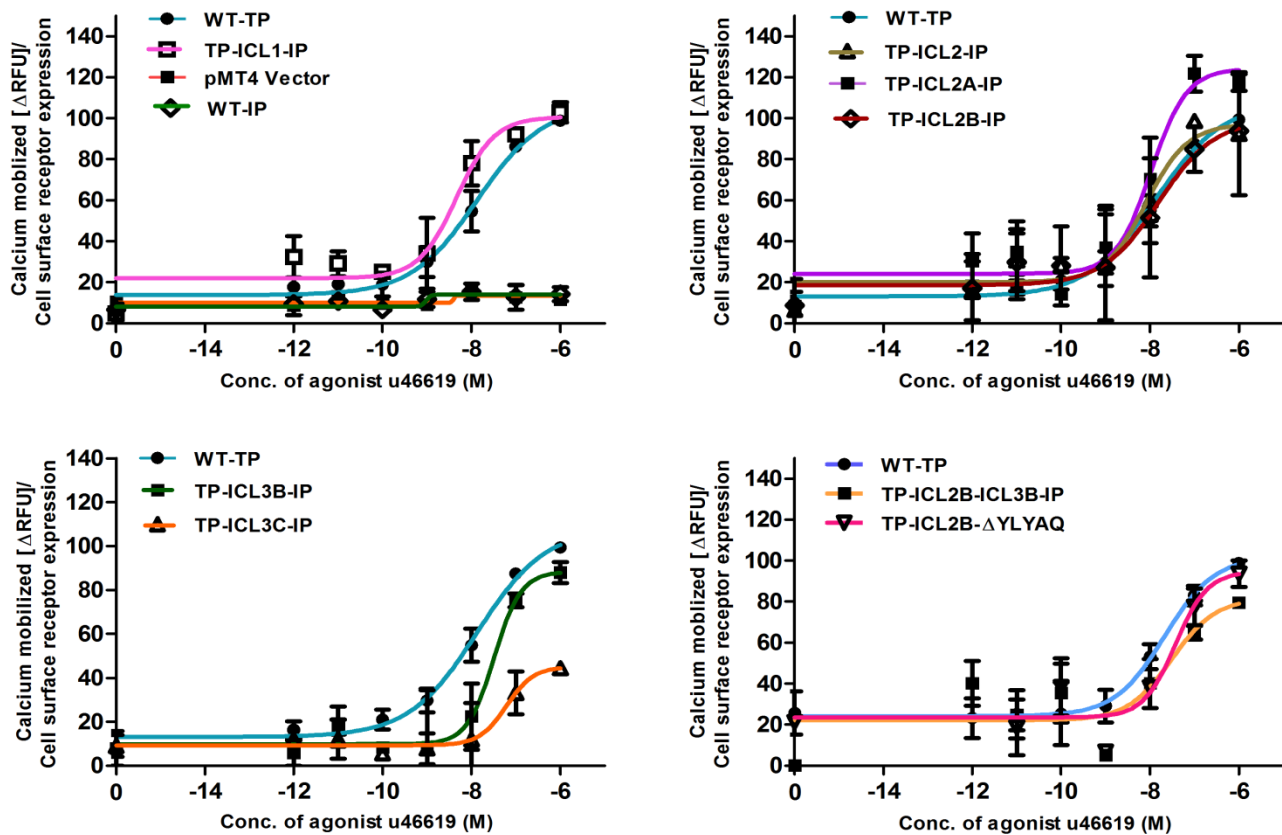


Fig. 3.4. Characterization of *Gaq*-mediated signaling of the wild-type TP and chimeric receptors.

Receptor activity was determined by measuring the agonist independent (basal) and dependent changes (Intrinsic) in intracellular calcium levels using transiently transfected HEK293T cells. The data show the basal (zero concentration of agonist or water alone) and agonist U46619-induced calcium mobilization for the WT-TP, chimeric receptors, and mock-transfected (vector pMT4) HEK293T cells. The results are expressed as a percentage of the WTTP activity and are from at least three independent experiments performed in duplicate. The results were normalized to calcium mobilized (Δ RFUs) relative to cell surface expression of the receptors as determined by FACS.

this is in agreement with our expression data, which showed that a majority of these two chimeras were retained in the intracellular milieu. The TP ICL3B-IP, with an intermediate amount of amino acids replaced, and TP ICL3CIP, with the least number of amino acids replaced, showed intracellular Ca^{2+} mobilizations equivalent to ~70% and 25% of the TP-generated signals, with EC_{50} s of 32 nM and 61 nM, respectively (**Fig.3.4**)

3.3.4 Characterization of intracellular cAMP signaling of chimeric receptors

To determine whether the chimeric TP-IP receptors can couple to $\text{G}\alpha\text{s}$ and stimulate cAMP production, we analyzed the chimeric receptors for their ability to generate cAMP upon stimulation with the TP agonist U46619 (**Fig. 3.5**). The WT IP activated by its agonist iloprost was used as a positive control, and the WT-TP stimulated with U46619 was used as a negative control (**Fig. 3.5A**). The WTTP, TP ICL1-IP, and all the TP ICL3-IP chimeras, except TP ICL3B-IP, did not generate any dose-dependent cAMP response upon stimulation with the agonist U46619 (**Fig. 3.5**). In contrast, all the TP ICL2 chimeras with complete and sequential replacements of the amino acids of ICL2 showed $\text{G}\alpha\text{s}$ mediated cAMP signaling (**Fig. 3.5B**). Among the ICL3 chimeras, only TP ICL3B-IP with an intermediate number of amino acids replaced showed an agonist concentration-dependent increase in cAMP signaling (**Fig. 3.5**).

3.3.5 Characterization of the intrinsic and basal signaling of the chimeric receptors

Both intracellular Ca^{2+} and cAMP assays were carried out with the receptors stimulated with water alone to determine the basal or agonist-independent signals and with a single saturating concentration (1 μM) of agonists to determine the maximum agonist-

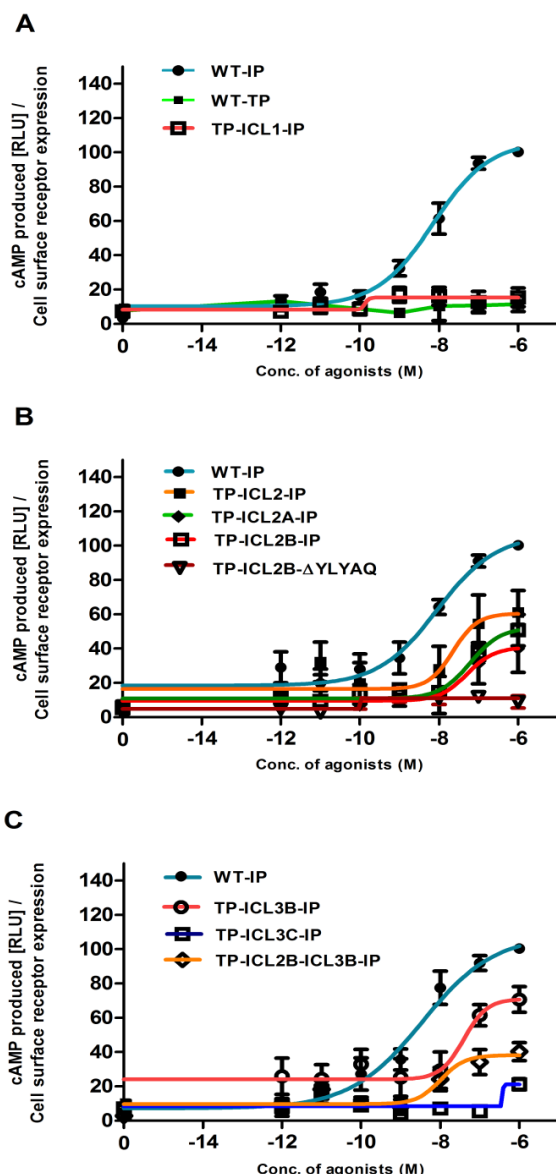


Fig.3.5. Agonist-induced cAMP production by the IP, TP, and TP-IP chimeric receptors.

Receptor activity was assessed by measuring the cAMP produced in a dose-dependent manner by the receptors expressed in HEK293S cells as described in Materials and Methods. (A) Basal (zero concentration) and agonist (U46619 or iloprost)-induced cAMP production for the WT IP, WT-TP, and TP ICL1-IP chimeric receptors. The WT IP and ICL2 chimeric receptors (B) and the WT IP and ICL3 chimeric receptors (C) are shown. The cAMP values of the chimeric TP-IP receptors, measured in relative luminescence units (RLUs), are expressed as a percentage of the WT IP activity and are from at least three independent experiments performed in duplicate. The results were normalized to cAMP produced (RLUs) relative to cell surface expression of the receptors as determined by FACS.

induced intrinsic signals (**Fig. 3.6**). The complete loop replacement chimeras, TP ICL1-IP and TP ICL2- IP, and the ICL2 and ICL3 chimeras TP ICL2A-IP, TP ICL2B-IP, and TP ICL3B-IP showed intrinsic activities for intracellular Ca^{2+} that were 60% to 90% of that of the WT-TP (**Fig.3.6A**). To obtain more direct evidence of phospholipase C β (PLC β) activation, agonist-stimulated IP_3 generation was measured (**Fig. 3.7**). The levels of intrinsic activity obtained with the IP_3 assay were similar to those observed for intracellular Ca^{2+} . Interestingly, the TP ICL2A-IP and TP ICL2B-IP chimeric receptors showed constitutive IP_3 and Ca^{2+} signaling to various degrees (**Fig. 3.7 and 3.8**). Surprisingly, the TP ICL3B-IP and TP ICL2A-IP chimeric receptors showed U46619-induced cAMP signaling equivalent to that of the WT IP-generated signal (IP stimulated with iloprost) (**Fig. 3.6B**). Only the TP ICL1-IP and TP ICL3C-IP chimeric receptors showed reduced cAMP signaling.

3.3.6 Protein molecular modeling

Previous studies have shown that there are multiple regions on the $\text{G}\alpha$ subunit that are involved in mediating GPCR-G protein interactions [173, 174]. Recent mutational studies indicated that the $\alpha 4$ - $\beta 6$ loop of $\text{G}\alpha_q$ is important, while structural studies have shown that the carboxy-terminal residues, including the $\alpha 4$ -5 helix and the αN - $\beta 1$ junction of the $\text{G}\alpha_s$ subunit, are important in mediating GPCR-G protein coupling [175, 176]. Molecular models of the TP and the chimeric receptors bound to the agonist U46619 and docked with the 14 C-terminal amino acids of human $\text{G}\alpha_q$ (Gen Bank accession no. AAB39498.1) were constructed to interpret the results in structural terms. The intermolecular interactions between the TP chimeric models and $\text{G}\alpha$ peptide sequence are listed in **Table 3.3**.

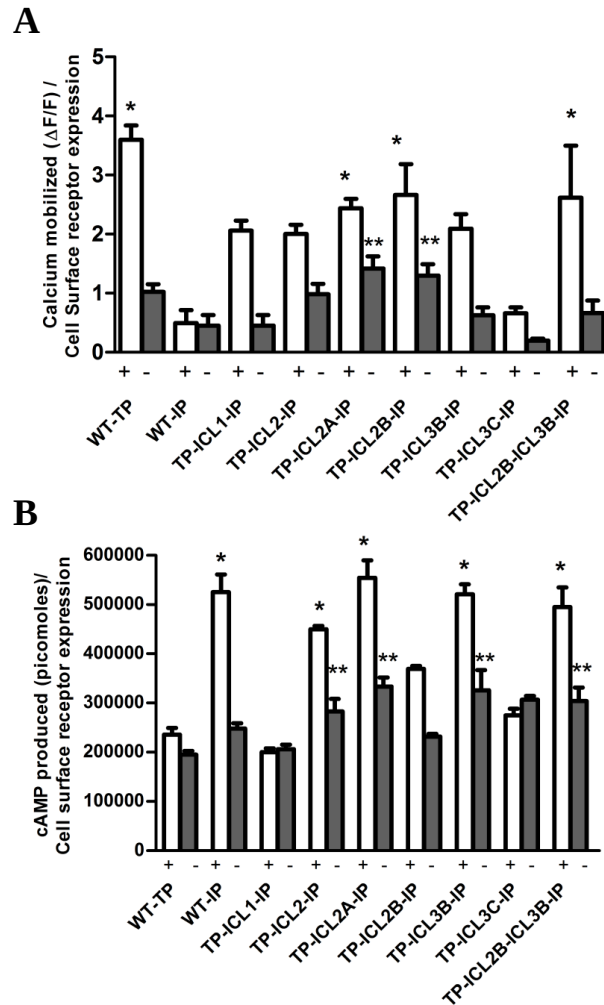


Fig. 3.6. Bar plot representation of the calcium mobilized (A) and cAMP produced (B) upon activation of the TP or IP and chimeric receptors. The cells expressing the IP were stimulated with the IP agonist iloprost, while cells expressing the TP and TP-IP chimeras were stimulated with the TP agonist U46619. Shown are the agonist-independent or basal activities (-) and the activities after stimulation (+) with a single saturating concentration (1 μ M) of agonists to determine the maximal agonist-induced or intrinsic signals. The results were normalized to the amounts of calcium mobilized and cAMP produced relative to cell surface expression of the receptors as determined by FACS. The results are from a minimum of 3 independent experiments performed in duplicate. A one-way ANOVA with Tukey's *post hoc* test was used to check the significance levels of the amounts of calcium mobilized or cAMP produced. The single asterisks indicate a significant difference in the amount of cAMP produced or calcium mobilized at the highest concentration of agonist with respect to the WT-TP (for cAMP produced) or WT IP (for calcium mobilized) ($P < 0.05$). The double asterisks indicate calcium mobilization at a basal level compared to WT IP basal level activity and cAMP produced at a basal level compared to WT-TP basal activity ($P < 0.05$). The bar plots do not include the chimeras TP ICL3-IP and TP ICL3A-IP, as they failed to show any dose-dependent response. The error bars represent means $\pm$ standard deviation (SD).

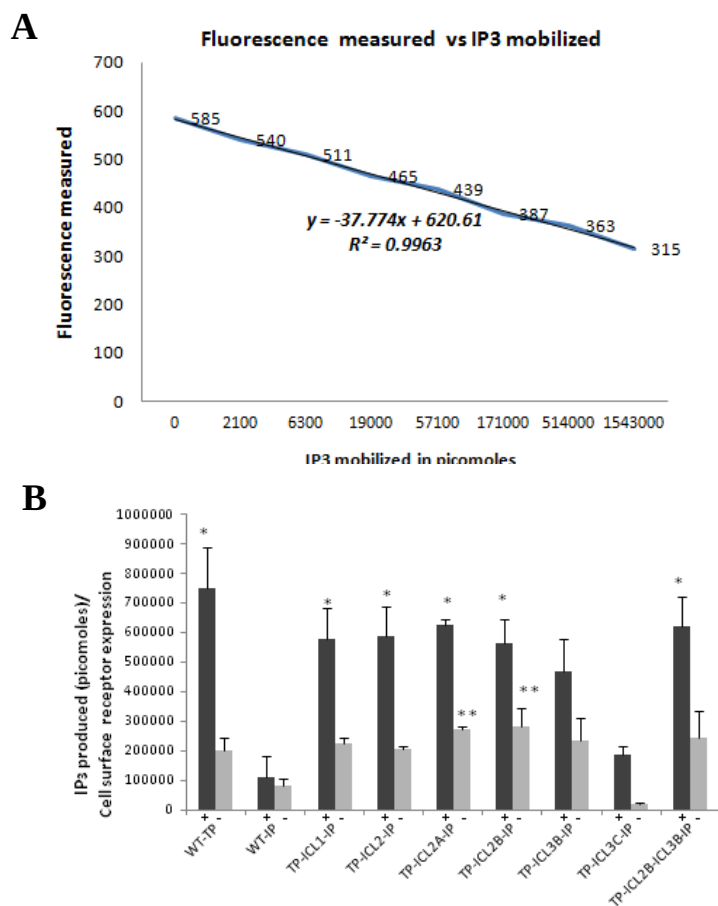


Fig. 3.7. Analysis of IP₃ accumulation by WT -TP, WT-IP and chimeric receptors.

A) IP₃ standard curve. An IP₃ standard curve was constructed by titrating known amounts of IP₃ and measuring the fluorescence according to instructions supplied by the manufacturer (HitHunter IP₃ assay kit, DiscoverX, USA). The Fig shows a linear relationship between the fluorescence measured (Y axis) with the amounts of IP₃ in picomoles (X axis). The slope value obtained $y = -37.774x + 620.61$ was used to measure the amount of IP₃ released when cells expressing the receptors were treated with a single saturating concentration of agonist (10^{-6} M), and also the basal values without any agonist treatment. B) IP₃ mobilized by WT and chimeric receptors. Cells expressing the IP are stimulated with IP agonist Iloprost, while cells expressing the TP and TP -IP chimeras were stimulated with TP agonist U46619. Shown are the agonist independent or basal activity(-), and activity after stimulation (+) with a single saturating concentration (1μ M) of agonists to determine the maximal agonist -induced or intrinsic signal. Results are normalised to IP₃ mobilized by cell surface expression of the receptor as determined by FACS. Results are from a minimum of 3 independent experiments performed in duplicate. A one way ANNOVA with *Tukey's post hoc* test was used to check the significance level of the amount of IP₃ mobilized. The single asterisk indicate there is a significant difference in the amount of IP₃ mobilized at the highest concentration of agonist with respect to WT -IP at significance level of $p < 0.05$. Whereas double asterisk indicate IP₃ mobilization at basal level compared to WT-IP basal level activity and at significance level of $p < 0.05$. The bar plots does not include the chimeras TP -ICL3- IP, TP-ICL3A-IP as they failed to show any dose dependent response. Error bars represent mean $\pm$ SD.

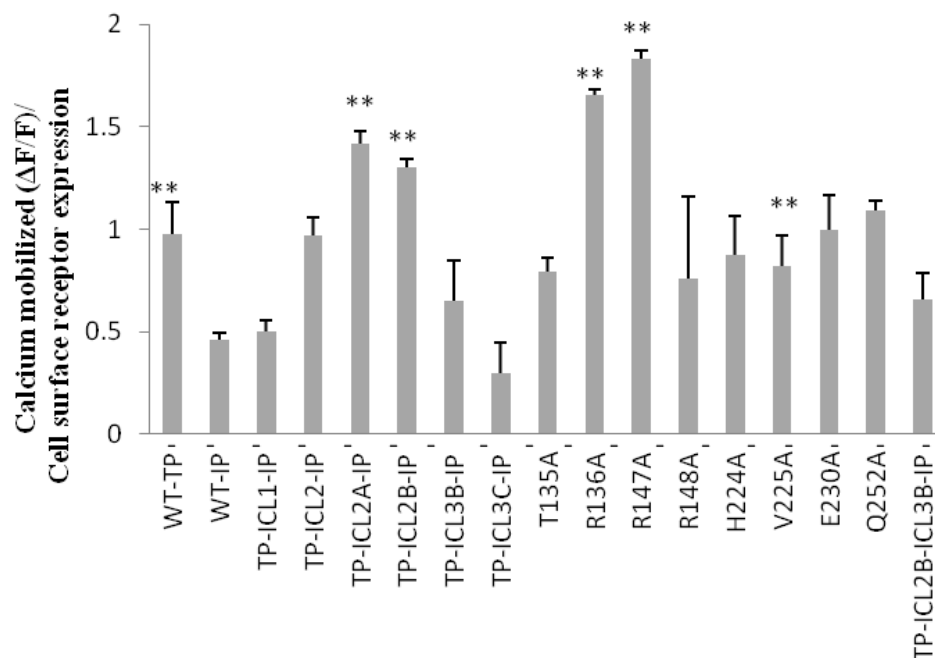


Fig. 3.8. The bar plot represents the basal or agonist-independent calcium signaling by WT-TP, WT-IP, chimeric receptors and loop mutants. HEK293T cells expressing the WT-TP, WT-IP, loop mutants and the TP-IP chimeras are used in the assay. Shown are the agonist-independent or basal activities of these cells. Results are normalised to calcium mobilized by cell surface expression of the receptor as determined by FACS. Results are from a minimum of 3 independent experiments performed in duplicate. A one way ANNOVA with Tukey's *post hoc* test was used to check the significance level of the amount calcium mobilized. The double asterisk indicate there is a significant difference in the amount of calcium mobilized with respect to WT-IP receptor at significance level $p < 0.05$. Error bars represent mean $\pm$ SD.

Docking simulations between the TP ICL2B-IP chimeric receptor and the $G\alpha$ C-terminal sequence revealed a series of H bonding and hydrophobic interactions that differed from those of TP ICL2 (**Fig. 3.9**). In the WT-TP model, Glu1293.49 from the conserved E (D) RY motif on TM3 is involved in a salt bridge with Arg148 of ICL2, possibly restraining the ICL2 loop in an inactive conformation. In addition, there is a network of hydrogen bonds involving Glu355 and Tyr356 from the $G\alpha$ peptide and Thr135 and Arg136 from TP ICL2. In the TP ICL2B-IP chimeric model, the salt bridge is missing, and new H-bond interactions involving Arg136 on ICL2B, Glu230 on ICL3 with Glu355, and Lys354 of the $G\alpha$ peptide were observed. TP ICL2B-IP differs from the WT-TP in the absence of pentapeptide FSRPA sequence in the middle of the ICL2 loop (**Fig.3.1**). This sequence is replaced by another pentapeptide sequence, YLYAQ, in the TP ICL2B-IP chimera. To elucidate whether the presence of the YLYAQ sequence or the absence of the FSRPA sequence is responsible for the TP ICL2B-IP phenotype, molecular models of the TP- $G\alpha$ peptide complex with the FSRPA sequence removed and TP ICL2B-IP- $G\alpha$ peptide complex with the YLYAQ sequence removed were analyzed. Removing the FSRPA sequence from docking simulations in the WT-TP revealed no major changes (**Fig. 3.9**). The $G\alpha$ peptide-bound TP ICL3-IP chimeric receptors revealed fewer H-bond and hydrophobic interactions than the TP ICL2-IP chimeric receptors (**Fig. 3.9**). In the TP ICL3B-IP model, we observed interactions involving His224 and Val225 at the cytoplasmic end of TM5 and Glu230 and Gln252 on TP ICL3B-IP with the Tyr356 and Val358 on the $G\alpha$ peptide sequence. Interestingly, a triad of hydrogen bonds connected the side chain -OH of Tyr356 with the amide of His224 and backbone of Val225. Another interesting feature is the extensive loss of secondary structure in ICL3 of the TP ICL3B-IP chimeric receptor compared to that of the WT-TP (**Fig. 3.9**).

3.3.7 Amino acid replacements guided by molecular modeling

To validate the predictions from our molecular models, amino acids and loop regions that were shown by the models to be important for receptor activation and/or $G\alpha$ peptide binding were replaced, and the mutants were characterized. Molecular models indicated that the YLYAQ sequence of TP ICL2B-IP was important in determining the G protein specificity of the TP. Therefore, a TP ICL2B-IP receptor with the pentapeptide YLYAQ sequence deleted (TP ICL2B- Δ YLYAQ-IP) was constructed and characterized (**Table 3.2**). In addition, the residues Thr135, Arg136, Arg147, and Arg148 on TP ICL2B-IP and His224, Val225, Glu230, and Gln252 on TP ICL3B-IP were replaced with alanine, and the mutants were characterized (**Table 3.2**). The functional characterization of the TP ICL2B- Δ YLYAQ-IP chimeric receptor revealed that it is expressed at a lower level, compared to WT-TP, as shown by both the B_{max} and FACS analysis, but it has an affinity toward the TP antagonist SQ 29,548 that is similar to that of the WT-TP (**Table 3.2**). Though it displayed a small right shift in the U46619 dose response with an EC_{50} of 37 nM (**Fig. 3.4**), as expected, this chimeric receptor did not generate cAMP upon stimulation with the TP agonist U46619 (**Fig. 3.4**). This validates our molecular model and revealed that the YLYAQ amino acid sequence in ICL2 is responsible for the $G_{\alpha s}$ -coupling specificity of the TP ICL2B-IP chimeric receptor. Since the TP ICL2B-IP and TP ICL3B-IP chimeric receptors have intermediate IP ICL2 and ICL3 sequence lengths and showed 60% and 100%, respectively, of cAMP generation upon stimulation with the TP agonist, we wanted to test whether combining these two loops (ICL2B and ICL3B) in one chimeric construct

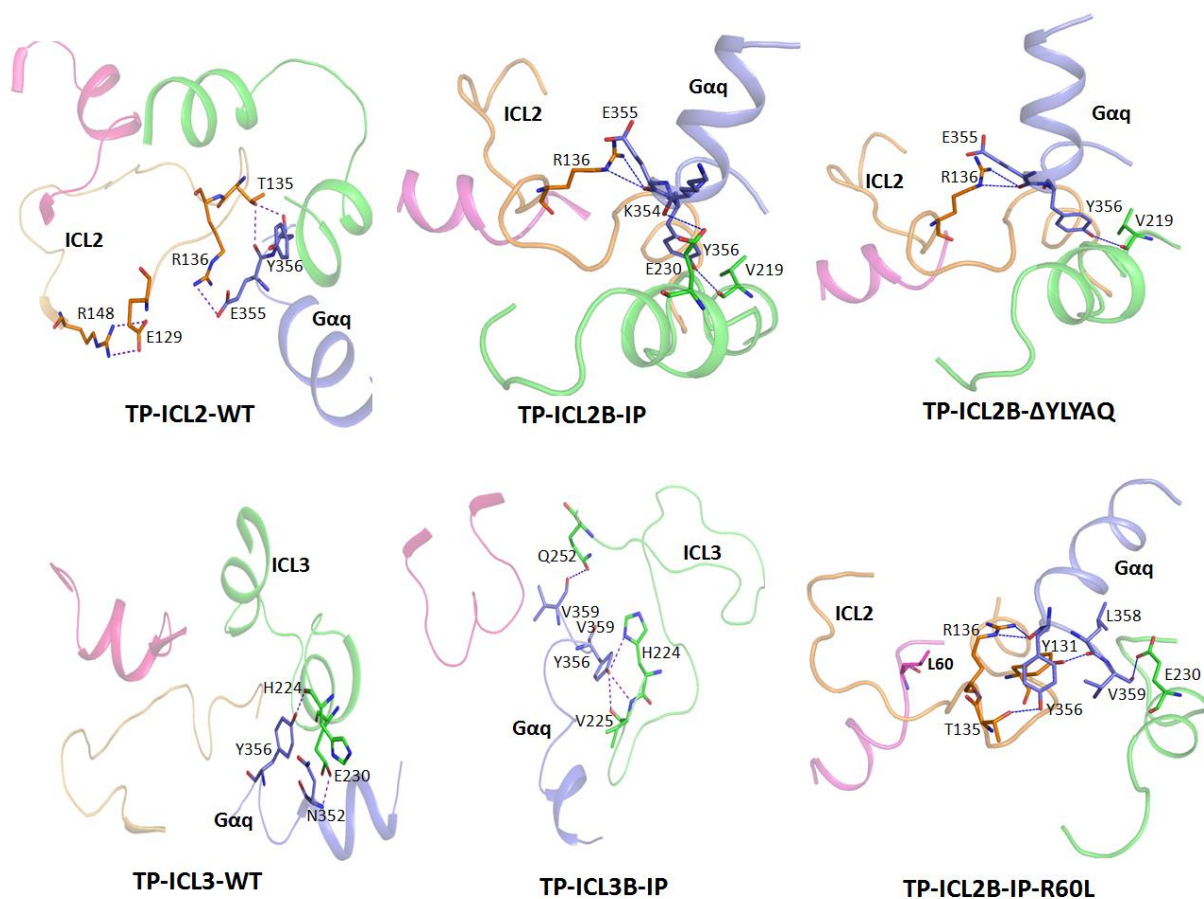


Fig. 3.9. Molecular models of the TP and TP-IP chimeric receptors bound to the $G\alpha$ C-terminal peptide. The residues involved in the interactions are represented as sticks; hydrogen bonds are shown as blue lines. The color representations of the different ICLs are as follows: ICL1, magenta; ICL2, light orange; ICL3, green; and $G\alpha$ peptide, blue. For clarity, only the intracellular loops (ICLs) are shown. The interactions observed in TP ICL2-WT and TP ICL3-WT and the chimeric TP ICL2B-IP, TP ICL2B-IP-R60L, TP ICL2B- Δ YLYAQ-IP, and TP ICL3B-IP receptors bound to $G\alpha$ peptide are shown. In the TP ICL2B-IP-R60L model, Leu60 on ICL1 is pointing away from the $G\alpha$ peptide binding region located between ICL2 and ICL3. Note the loss of the secondary structure in ICL3 of the TP ICL3B-IP chimera compared to the wild-type TP ICL3.

would result in a super chimera. Therefore, the chimeric TP ICL2B-ICL3B-IP receptor was constructed and analyzed. This chimeric receptor showed good intrinsic Ca^{2+} mobilization (~90%) and cAMP production, indicating that there is a cumulative effect upon adding two loops, ICL2B and ICL3B (**Fig. 3.5**). The R148A mutant on ICL2 was expressed at low levels but showed hyperactivity with a large upward shift in the U46619 dose response (**Fig. 3.10**). This shows that Arg148 in ICL2 restrains the activity of the receptor, by either forming a salt bridge with Glu129, as in the WT-TP model, or being a major contributor to the H-bond network, as observed in the TP ICL2B-IP chimeric receptor. The R136A mutant behaved like the WT-TP in its ligand binding properties (**Fig. 3.10**) but showed a slightly elevated level of basal Ca^{2+} signaling (**Fig. 3.8**). The T135A and R147A mutants both bound to the antagonist SQ 29,548, but R147A showed a reduced affinity toward the antagonist and twice the basal Ca^{2+} signaling of either the WT-TP or the WT IP (**Table 3.2; Fig.3.8**). This result shows that the H-bonding capabilities of Thr135, Arg136, and Arg147 are crucial for $\text{G}\alpha_q$ coupling, while Arg148, through its interaction with the ERY motif on TM3, is important for receptor activation.

3.3.8 Natural variants in the intracellular loops and predicting their roles using chimeric receptors

To test the stringencies of our molecular models and chimeric constructs, we introduced naturally occurring polymorphic variants into select chimeric constructs. Currently, there are two known signaling-deficient genetic variants in the TP and IP within the ICL regions: the TP R60L variant present in the ICL1 and the IP R212C variant (by amino acid sequence analysis, Arg212 in the IP corresponds to His224 in the TP) (**Fig. 3.11**) in ICL3, which was recently shown to cause cardiovascular disease progression [54]. Molecular modeling studies revealed Arg60 in TP to interact with Met126 and Arg130 of

the ERY motif on TM3 through hydrogen-bond interactions (**Fig. 3.12**). In addition, the salt bridge between Glu129 of the ERY motif on TM3 and Arg148 was also present. Interestingly, the introduction of R60L in the TP led to the disappearance of interactions with Met126 and Arg130, but the Glu129 and Arg148 interaction was found to be intact. However, in the case of TP ICL2B-IPR60L, the interactions between Met126 and Arg130, as well as salt bridge interactions between Glu129 and Arg148, were absent, mimicking a more active conformation. As predicted from the molecular models, the functional characterization of the TP ICL2B-IP-R60L and TP ICL2B-ICL3B-IP-R60L chimeras showed that both were able to rescue the Ca^{2+} mobilization of the signaling-deficient R60L to wild-type TP levels (**Table 3.2**; see also **Fig. 3.10**). The molecular model of the TP ICL3B-IP receptor predicted interactions involving His224 (which corresponds to Arg212 in the IP) and Val225 at the boundary of TM5 and ICL3 and Glu230 and Gln252 on TP ICL3B-IP with Tyr356 and Val359 on the $\text{G}\alpha$ peptide sequence (**Table 3.3**). The H224A and V225A mutations were introduced into the TP ICL3B-IP and characterized. As expected, the H224A and V225A mutations caused a complete loss of signaling by the TP ICL3B-IP chimera (**Table 3.2**). No specific binding to the antagonist was observed even for the E230A and Q252A mutants, but they showed increased potencies toward the agonist U46619; this was illustrated by a left shift in the dose response (**Fig. 3.10**) and with EC_{50} s that were 2- to 3-fold lower than that of the WT-TP (**Table 3.2**). Surprisingly, both the E230A and Q252A mutants displayed statistically significant increases in agonist-independent activities (**Fig.3.8**).

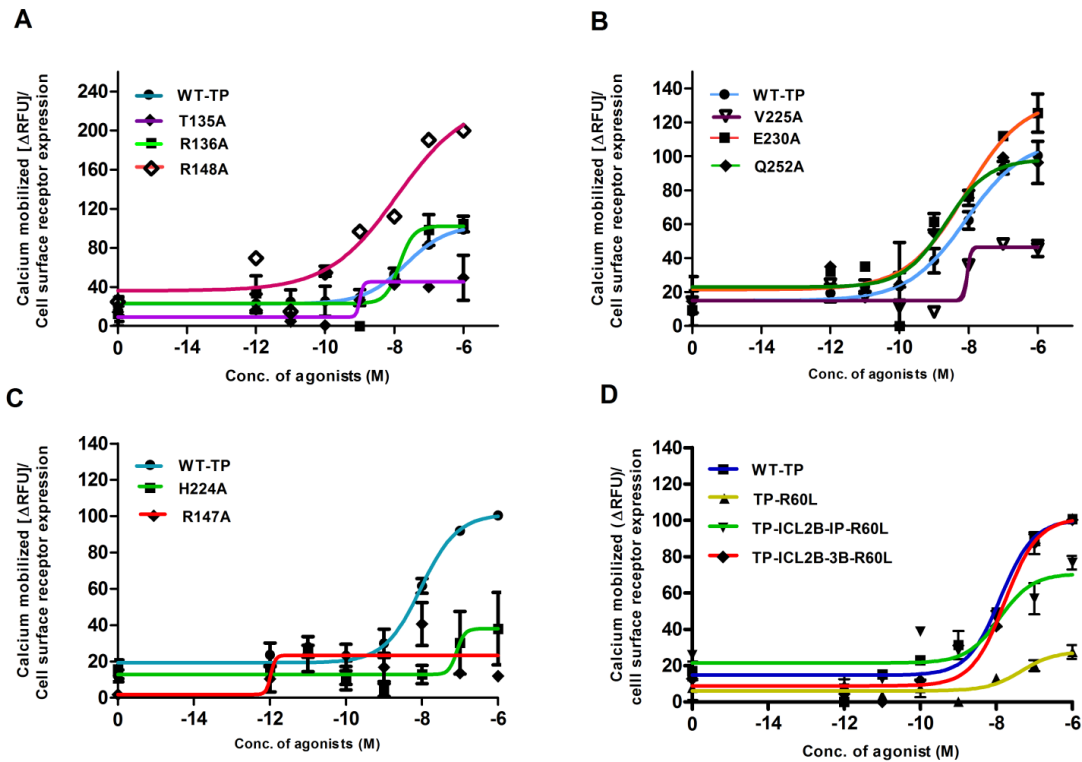


Fig. 3.10. Characterization of $G_{\alpha q}$ mediated calcium signaling of the WT-TP, mutant receptors and chimeras containing the R60L variant.

The data shows agonist U46619 induced calcium mobilization for WT-TP and mutants and normalized to wild type TP cell surface expression as determined by FACS. In panel A, ICL2 mutants; in panel B & C, ICL3 mutants and in panel D, the TP genetic variant R60L and the chimeras containing this genetic variant are displayed. TP-ICL2B-3B-IP-R60L rescues the signaling of the R60L to wild type TP levels (panel D).

Table 3.3 Potential intermolecular interactions within the TP-Gαq peptide interface

TP region	Atoms in TP receptor			Atoms in Gαq C-terminal peptide
	TP-WT (model)	TP-ICL2B-IP (model)	TP-ICL3B-IP (model)	
TM3 (cytoplasmic end) and ICL2	R136-NH2	R136-NE R136-NH2		E355-OE2 E355-O E355-O
TM5 (cytoplasmic end) and ICL3	H224-ND1 E230-OE1	V219-O E230-OE1	H224-ND1 V225-O Q252-OE1	N352-ND2 N352-N Y356-OH K354-O Y356-OH Y356-OH V359-O

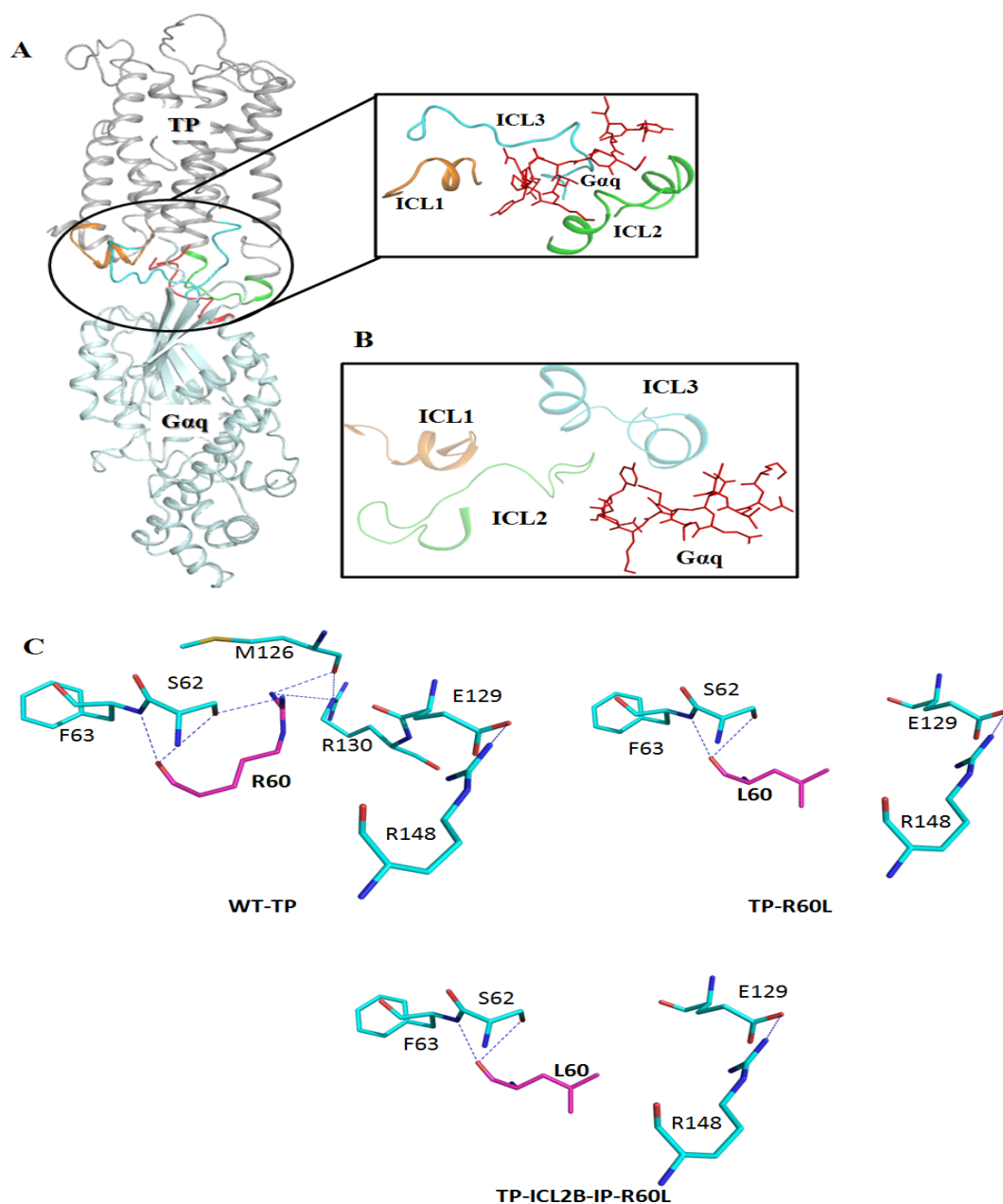


Fig.3.12. Panel A, Molecular model of Gαq bound to TP predicted by superimposing the TP-Gαq 3D models on the β_2 -AR-Gas crystal structure followed by MD simulations for 10ns using SYBYLX v2.0. **Panel B**, represents a molecular model of Gαq C-terminal peptide bound to the TP predicted using Z dock server. In both the models, the Gαq C-terminal peptide is found bound between ICL2 and ICL3 regions. **Panel C**, In TP-wild type, R60 is interacting with M126 and R130 of ICL2 by H bond interactions, and the salt bridge between E129 of D/ERY motif and R148 is present. In case of TP-R60L mutant, interactions with M126 and R130 disappear but the salt bridge was still present, restraining the activity of the receptor.

3.4 Discussion

In this work, we utilized a chimeric receptor and molecular modeling-based mutagenesis approach for elucidating the structural basis of prostanoid receptor-G protein interactions. In the prostanoid family of GPCRs, amino acid sequence analyses of ICLs show diversity in both amino acid composition and sequence length (**Fig. 3.11**).

Interestingly, IP receptors showed a high degree of sequence conservation compared to TP receptors within the ICL1 and ICL2 regions. We have identified the ICL regions on the prostanoid receptors TP and IP, which contribute toward their G protein specificities and lead to well-documented completely opposite pathophysiological effects. The ICL2 region, specifically the pentapeptide YLYAQ sequence in the middle of the ICL2 of IP, plays an important role in determining G α s specificity. In addition, ICL3 of the IP also has an important role in G α s coupling, as demonstrated by our TP ICL3B-IP chimeric receptor, while both the ICL2 and ICL3 regions of the TP play important roles in G α q-effector coupling. Using the ICL2 and ICL3 chimeras, we were able to rescue signaling of the dysfunctional bleeding disorder genetic variant R60L present in ICL1 to the wild-type TP levels.

Chimeric receptor approach for elucidating GPCR structure and function. Since the introduction of chimeric receptors for delineating the domains involved in ligand binding specificity of class A GPCRs by Kobilka *et al.*, [177], their approach has been used successfully on a number of GPCR systems [178]. Studies on chimeric α_1/β_2 -adrenergic receptors (α_1 AR/ β_2 -AR) with the third ICL of the β_2 -AR replaced with that of the α_1 AR demonstrated ligand binding capabilities similar to those of the β_2 -AR and showed downstream signaling as an α_1 AR [179]. Among the dopamine receptors, D₁ receptors

activate adenylyl cyclase, resulting in the production of second messenger cAMP, whereas the D2 receptors inhibit adenylyl cyclase. D1 chimeric receptors with TM6, TM7, and the c-terminus replaced with those of the D2 receptors showed enhanced binding of the D2 agonist and inhibited adenylate cyclase [180]. Similar results were also obtained with chimeric receptors composed of gonadotropic luteinizing hormone (LH) and follicle-stimulating hormone (FSH) receptors. The N terminus of the FSH receptor replaced with that of the LH receptor resulted in IP₃ production when stimulated with FSH [181]. A chemokine receptor (CCR5) has been shown to interact with HIV and play a crucial role in its entry. The chimeric receptors constructed between bacteriorhodopsin and the CCR5 extracellular segments provided specific insights into the regions in CCR5 that confer HIV co-receptor function [182]. The uniqueness of the current study lies in our three-pronged approach to elucidate GPCR-G protein interactions in a receptor family that has been shown only recently to play an important role in the development of human cardiovascular disease. First, we constructed and characterized chimeric receptors with complete and sequential replacements of ICL loops. Next, to interpret the results in structural terms, we built molecular models of the chimeric receptors bound to the G α peptide. Finally, we used mutagenesis to validate the proposed models of prostanoid receptor-G protein interactions.

ICL1. The TP ICL1 has been implicated in both G protein dependent and -independent signaling. Earlier studies showed that the dominantly inherited bleeding disorder variant R60L impaired calcium signaling [49], and recent studies have shown that ICL1 in the TP couples with and inhibits the large conductance voltage- and calcium-activated potassium channels (MaxiK channels) [183]. It has been proposed that this direct interaction with MaxiK channels facilitates the G protein-independent TP to MaxiK transinhibition, which

would promote vasoconstriction [183]. Interestingly, the results from our studies show that except for a conserved Arg that is present at analogous positions in ICL1 of prostanoid receptors (Arg56 of the TP ICL1-IP construct corresponds to Arg45 of the IP or Arg60 in the TP), the remaining part of the TP ICL1 is not important for activation, as the TP ICL1-IP chimeric receptor showed elevated levels of Ca^{2+} in response to treatment with agonist U46619 (**Table 3.2**). cAMP accumulation assays to determine whether ICL1 of the IP can signal through $\text{G}\alpha\text{s}$ in the TP ICL1-IP failed to show any response when the chimeric receptor was stimulated with the TP agonist, indicating that it may not be crucial for $\text{G}\alpha\text{s}$ -mediated effector signaling. Our mutational studies using the genetic variant R60L, and chimeras containing this variant, showed that R60L is important for receptor activation by mediating interactions with the ERY motif in TM3 rather than by binding to G proteins.

ICL2. All the TP ICL2-IP chimeric constructs, except TP ICL2B- ΔYLYAQ -IP, showed agonist U44619-induced cAMP accumulation of more than 60% of that of the wild-type IP induced with iloprost (**Fig. 3.5**). The deletion of the pentapeptide YLYAQ sequence from the TP ICL2B-IP receptor drastically reduced the ability of this chimeric receptor to generate $\text{G}\alpha\text{s}$ -mediated cAMP signaling from the wild-type IP levels to that of the wild-type TP levels (**Fig. 3.5**). Interestingly, the TP ICL2B- ΔYLYAQ -IP receptor showed very good Ca^{2+} mobilization. In general, the ICL2 chimeric receptors showed significant constitutive activity with respect to a $\text{G}\alpha\text{q}$ -based assay (**Fig. 3.4**), and there was a gradual restoration of Ca^{2+} signaling (EC_{50}s) to the WTTP values, with an increase in the TP sequence in the loop. From a site-directed mutational analysis of the ICL2 region, we were able to identify Thr135, Arg136, and Arg147 as forming hydrogen bonds crucial for $\text{G}\alpha\text{q}$ coupling and Arg148 to restrain the receptor activity by forming a salt bridge with Glu129

(of the ERY motif) in TM3. The involvement of ICL2 in determining $G_{\alpha s}$ specificity might be a general mechanism of GPCR-G protein interaction, as shown by the recent structure of the β_2 -AR-Gs protein complex [164]. This is the first crystal structure of a GPCR transmembrane signaling unit consisting of an agonist-occupied β_2 -AR and nucleotide-free Gs heterotrimer, and it revealed extensive interactions between ICL2, TM5, and TM6 of the β_2 -AR and the N- and C-terminal regions of the $G_{\alpha s}$ subunit. Even though we have used only the C-terminal G_{α} peptide for analysis, our data nevertheless dovetail with the published structural data using the Gs heterotrimer on the importance of ICL2 in determining $G_{\alpha s}$ specificity.

ICL3. The large ICL3 has very low sequence homology among members of the GPCR family, including the prostanoid receptors. It was previously shown in different class A GPCRs that the proper conformation of ICL3 is important for receptor folding [16]. Interestingly, the TP ICL3B-IP showed $G_{\alpha q}$ -dependent intrinsic signaling of up to 70% of that of the WT-TP and $G_{\alpha s}$ -dependent intrinsic signaling similar to that of the WT IP (**Fig. 3.4**). Results from molecular modeling showed a loss of secondary structure in ICL3 of the TP ICL3B-IP chimera compared to the wild-type TP ICL3-IP, resulting in increased flexibility and fewer constraints in the ICL3 region, presumably favoring an active state structure and/or enhanced binding with the G protein. The TP ICL3B-IP sequence seems to be the optimum for both $G_{\alpha q}$ - and $G_{\alpha s}$ -dependent signaling, as the further reduction of the IP ICL3 sequence, as in TP ICL3C-IP, causes a drop in both cAMP and Ca^{2+} signal levels. Furthermore, the TP ICL3C-IP chimeric receptor regains binding to the TP antagonist SQ 29,548 with affinity and expression levels similar to those of the wild-type TP. Previous site-directed mutational studies of residues at the cytoplasmic end of TM5 and the start of

ICL3 in the TP revealed that Cys223 is important for Gαq signaling [16]. The studies on the IP have reported that the genetic variant R212C (corresponding to His224 in the TP) displays defective signaling leading to cardiovascular disease progression [54]. TP-IP heterodimerization studies have shown that R212C exerts a dominant negative action of the TP-IP heterodimer [75]. Interestingly, an H224R mutation in the wild-type TP was shown previously to have normal ligand binding and calcium-signaling characteristics [16]. Our studies show that introducing the H224A mutation into the TP ICL3B-IP chimera disrupts the signaling by this chimera, supporting our observation that the hydrogen-bonding capability of His244 is required for Gαq-mediated calcium signaling. Our data from molecular models, validated by site-directed mutational analysis, point to the importance of the hydrogen-bond triad connecting His224 and Val225 with Tyr356 of the Gα peptide.

Interplay between ICL1, ICL2, and ICL3. In our models, the C-terminal Gα peptide bound to the TP at two different sites (see Materials and Methods), one between ICL1 and ICL2 and the other between ICL2 and ICL3. This is predominantly due to the small size of the peptide used in our studies. Based on the recent structural studies of metarhodopsin II that was bound to a peptide [163], the β₂-AR-Gs protein complex [164], and our data, the position between ICL2 and ICL3 is more likely the accurate one. It is possible that the ICL1 region binds to some other sequence of the G protein, such as the amino-terminal region, although the recent crystal structure of β₂-AR-Gs does not show ICL1 to be involved in G protein binding. In our data, the most interesting changes were observed for the ICL2 and ICL3 regions. The loop region that has the best basal activity and determines the EC₅₀ is ICL2, with ICL3B also playing an important role in conferring intrinsic activity. This shows that both ICL2 and ICL3 in the prostanoid receptors are predominantly

involved in G protein coupling and signaling. This differs from the β_2 -AR-Gs structure, which has shown only the ICL2 region to be involved in Gs coupling. This possible discrepancy is because in our studies, we have used entire loop regions, while the β_2 -AR-Gs structure is missing the ICL3 region between Arg239 and Cys265. A length of 26 amino acids in ICL3 of the β_2 -AR is disordered in the structure. The absence of ICL3 might be responsible for the enhanced interactions observed between TM5, which is extended by two helical turns on the cytoplasmic side (compared to the inactive β_2 -AR structure), and the $\alpha 5$ helix at the C-terminal end of Gs in the β_2 -AR-Gs structure. In conclusion, delineation of the interacting domains on the prostanoid receptors TP and IP, and their associated G proteins, was achieved by the construction of chimeric signaling molecules. Detailed analyses of the critical regions responsible for the specificities of prostanoid receptor-G protein interactions provide a framework for understanding the fidelity of prostanoid signaling and the creation of novel tools for drug discovery.

Chapter 4

High-level expression, purification and characterization of a constitutively active thromboxane A₂ receptor polymorphic variant

Xu B, **Chakraborty R**, Eilers M, Dakshinamurti S, O'Neil JD, Smith S.O, Bhullar RP, Chelikani P (2013) *PLoS ONE* 8(9): e76481.

4.1 Introduction

G protein-coupled receptors (GPCRs) comprise the largest family of membrane proteins encoded by the human genome. On binding to extracellular stimuli, these receptors activate intracellular proteins thereby providing an important link between the cell and its environment [120]. A substantial number of GPCRs in humans harbor genetic variants [133] including nucleotide insertion or deletion, as well as single nucleotide changes referred to as single nucleotide polymorphisms (SNPs). Some of these SNPs lock the GPCR in an active form, and initiate intracellular signaling even in the absence of extracellular stimuli, these are referred to as constitutively active mutants (CAMs). The structural characterization of these CAMs is impeded by the lack of proper expression systems, as most often high-level expression of these CAMs appear to be toxic to the cells [184]. An approach to circumvent this hurdle is the use of a tetracycline-inducible HEK293 cell line [185]. Recently the structures of two CAM GPCRs were reported (PDB ID: 2X72 and 4A4M) using this cell line, although the CAMs required stabilization using an engineered disulfide bond [163, 186].

The human thromboxane A_2 receptor (TP) belongs to the prostanoid subfamily of GPCRs. The receptor mediates vasoconstriction and thrombosis on binding to thromboxane (TxA_2) thereby playing an important role in cardiovascular disease and stroke [2]. TP was first cloned in 1991 and shown to exist in two isoforms in humans, $TP\alpha$ and $TP\beta$, differing only in their C-terminus [10]. Recently, we reported the first CAM in $TP\alpha$ (henceforth referred to as TP or WT-TP), the genetic variant A160T present in transmembrane (TM) helix 4 [165]. Though the clinical relevance of this CAM in TP is yet to be elucidated, based on CAMs at similar positions in rhodopsin that lead to retinitis pigmentosa, it is likely A160T mutation causes cardiovascular disease progression. A high-resolution

structure of a prostanoid receptor has not been determined. Recently, glycosylated human TP was expressed in Sf-9 cells using an optimized baculovirus expression system [187]. From heterologous expression in HEK293 cells, TP protein levels of 0.5-2.0 pmol/mg of membrane protein have been reported [46, 188]. The main goal of the present work was to improve the expression levels of both the TP and CAMs for high-resolution structural studies. Towards this aim, codon-optimized TP and the A160T mutant were synthesized, and transiently expressed in both COS-1 and HEK293 cells. Expression of these constructs resulted in yields of 3.8 ± 0.3 picomoles of WT-TP and 1.8 ± 0.4 picomoles of A160T per milligram of membrane protein, respectively. Next, expression of these genes in HEK293S-TetR cells resulted in a 4-fold increase in expression, resulting in yields of 15.8 ± 0.3 pmol of receptor/mg of membrane protein. To date, this expression level is the highest reported for any diffusible ligand activated GPCR CAM. The WT-TP and the A160T mutant expressed in the HEK293S (GnTI⁻)-TetR cell line showed homogenous and restricted N-glycosylation. Secondary structure analysis of the purified receptors was pursued by circular dichroism (CD) spectropolarimetry.

4.2 Materials and methods

4.2.1 Materials

Anti-FLAG M2 affinity gel (Cat # A2220), FLAG peptide (Cat# F3290), and FLAG antibody were from Sigma. All of the lipids and detergents, including n-dodecyl- β -D-maltoside were purchased from Anatrace. Common chemicals and reagents were purchased from either Sigma or Fisher. Restriction enzymes were from NEB, and cell culture supplies were purchased from Invitrogen. The radiolabeled ligand [³H] SQ 29,548 was purchased from PerkinElmer (NET 936), and cold SQ 29,548 was obtained from Cayman Chemicals

(Cat # 19025). Buffers used were as follows: PBS buffer, 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4 , 10 mM Na_2HPO_4 (pH7.4); Buffer A (lysis buffer), 10 mM Tris-HCl, pH 7.4, containing protease inhibitors (1mM EDTA, 10 $\mu\text{g}/\text{ml}$ benzamidine, 10 $\mu\text{g}/\text{ml}$ leupeptin, 20 $\mu\text{g}/\text{ml}$ trypsin inhibitor, and 0.2mM phenylmethylsulfonyl fluoride); Buffer B (storage buffer), 50 mM Tris-HCl, pH 7.4, 12.5 MgCl_2 , containing protease inhibitors as in Buffer A; Buffer C (binding buffer), 75 mM Tris-HCl, pH 7.4, 12.5 mM MgCl_2 , containing protease inhibitors as in Buffer A; Buffer D (solubilization buffer) 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA, containing protease inhibitors as in Buffer A; Buffer E (Elution buffer) 50 mM Tris- HCl, pH 7.4, 150 mM NaCl.

4.2.2 Construction of tetracycline-inducible HEK293S stable cell lines expressing TP and A160T

The genes FLAG-TP-1D4 and FLAG-TP-A160T-1D4 in plasmid pUC57 and codon-optimized for expression in mammalian cells were synthesized commercially (GenScript Inc, USA). These two genes in pUC57 and pACMVtetO were digested with restriction enzymes Kpn1 and NotI for 2 h at 37 °C. After removal of the enzyme using Qiagen gel purification kit, the TP fragment was ligated into the plasmid pACMVtetO, and transformed into competent *E. coli* DH5 α . The transformants were screened for the presence of the 1.2 kb TP and A160T genes following digestion with Kpn1 and NotI. Their identity was confirmed by DNA sequencing. The plasmids with the correct gene sequence were then transfected into HEK293S-TetR and HEK293S-TetR (GnTI^-) cells using lipofectamine 2000. The expression and selection were carried out as described previously [98]. The expression of the clones using slot blot was detected using the anti-FLAG-antibody and visualized by chemiluminescence (ECL, Amersham).

4.2.3 Systematic detergent screening

Detergent screening was carried out using the solution master detergent kit from Anatrace containing 88 detergents. WT-TP was solubilized in lysis buffer containing various detergents for 1 h at 4 °C, and samples were analyzed on a dot blot. The ability of each detergent to solubilize WT-TP was quantified using ImageJ software.

4.2.4 Purification of WT-TP and A160T

Cell pellets from two dishes (15 cm each) were resuspended using 100 ml of Buffer A. The suspension was homogenized using a dounce homogenizer (20 strokes), and centrifuged at 48,000 xg for 30 min. After weighing the membrane pellet, each gram of membrane pellet were suspended in 10 ml of buffer D containing 1% DM and 0.2% CHS using a dounce homogenizer (20 strokes). The suspension was mixed by nutation at 4 °C for 1 h, and centrifuged at 48,000 xg for 30 min to remove any insoluble particulate material. Solubilized TP or A160T were incubated with FLAG-resin in batch mode (binding capacity of FLAG resin is 0.6 mg/ml) with slow nutation for 2 h at 4°C. The receptor bound resin was then collected by centrifugation at 1,500 xg and washed with Buffer D containing 0.05% DM and 0.01% CHS until the absorbance of the wash at 280 nm was < 0.01. Elution was carried out with Buffer E containing 0.05% DM, 0.01% CHS and 0.1 mM FLAG peptide. The fractions obtained were assayed for receptor binding using [3H] SQ 29,548 and/or the protein concentration was determined by a Biorad DC protein assay. Radioligand binding assays were as described previously [165, 189].

4.2.5 Immunoblot analysis

One to five micrograms of the protein sample were resolved by using a 10% gel by SDS-PAGE. The protein was then transferred from the gels onto a nitrocellulose membrane by electroblotting. The WT-TP and the A160T mutant receptor were visualized by immunodetection with the anti-FLAG antibody or rho-1D4 antibody.

4.2.6 Circular dichroism (CD) spectropolarimetry

CD spectra were recorded on a JASCO J-810 spectropolarimeter at the indicated temperatures over the wavelength range of 190 nm to 260 nm with a step size of 1nm. Spectra were collected with a cylindrical quartz sample cell with a path length of 0.05 cm. Spectra of purified receptors were baseline corrected by subtracting the spectra of buffer solutions. The intensity and wavelength of the spectropolarimeter were calibrated using solutions of d-10- camphorsulfonic acid. Mean Residue Ellipticities (10^{-3} deg.cm².dmole⁻¹) were calculated using the equation: $[\Theta] M = (Mr) (\Theta) / (10) (l) (c) (n)$, where Mr is 39,212.8 grams per mole for WT-TP and 39,242.8 grams per mole for the A160T mutant, l is the cell path length in cm, Θ is the measured ellipticity in millidegrees, c is the protein concentration in g/L, and n = 359. CD spectra were deconvoluted using the K2D3 algorithm ([http:// www.ogic.ca/projects/k2d3/](http://www.ogic.ca/projects/k2d3/)) [190]. The CD spectra were processed using Wolfram Mathematica 9 (Wolfram Research, Inc., IL, USA).

4.3 Results and Discussion

4.3.1 Expression of TP and the A160T CAM in HEK293S-TetR and HEK293S-TetR (GnTI-) cell lines

The WT-TP and A160T genes that were synthesized and used in the current study had the same salient features as those previously described for the β_2 -AR gene [98]. In

addition to simplify detection of the full-length protein and purification, a FLAG-epitope tag (DYKDDDDK) and rho-1D4 octapeptide (ETSQVAPA) tag were added to the N-terminus and c-terminus, respectively (**Fig. 4.1**). Transient expression of these genes in either COS-1 or HEK293S cells resulted in expression levels of 3.8 ± 0.3 pmol TP/mg and 1.8 ± 0.4 pmol A160T/mg of membrane protein [165, 189]. To increase the expression levels, construction of stable cell lines using the HEK293S-TetR inducible system was explored. The expression was optimized by varying the concentrations of the inducers, both tetracycline and sodium butyrate, and the results quantified by western blotting and spot densitometry. The addition of sodium butyrate and tetracycline had a cumulative effect, with 7.5 mM sodium butyrate found to be the optimum (**Fig. 4.2**). Interestingly, tetracycline alone was able to induce up to 60%, of the level of expression of the WT-TP. However, after induction with both tetracycline and sodium butyrate, WT-TP and A160T were expressed at 15.8 ± 0.3 pmol/mg and 2.1 ± 0.3 pmol/mg of membrane protein, respectively, as determined by radiolabeled antagonist [^3H] SQ 29,548 binding. The expression level determined from the radioligand assay for the A160T CAM is not a true indicator of its expression. This is because the radioligand used [^3H] SQ 29,548 is an antagonist for TP and CAMs being in an active state have low affinity for antagonists. Active state stabilizing mutations of the A2A adenosine receptor used for crystallization showed greatly reduced binding of five antagonists [191]. Indeed, based on intensity of the immunoblots (**Fig. 4.3**) and functional yield obtained after purification, both the WT-TP and A160T CAM are expressed at similar levels (please see purification section).

Immunoblot analysis showed the WT-TP and A160T mutant expressed in HEK293S-TetR stable cell lines consist of two major bands with molecular masses in the range of 30-55 kDa (**Fig. 4.3**). Previous studies have reported that TP heterogeneously

expressed in different cell lines appears to be N-glycosylated. TP expressed in SF9 cells shows only one band at ~50kDa [10], and that expressed in HEK293 produced two major bands, a 60-66kDa band of presumably fully N- glycosylated receptor and a lower molecular mass nonglycosylated protein of 30–46 kDa [192]. Previously, it was shown that opsin and β_2 - adrenergic receptor (β_2 -AR) expressed in the HEK293S (GnTI⁻)-TetR showed homogenous and restricted N glycosylation [98, 184]. The HEK293S (GnTI⁻) cell line is resistant to ricin as a consequence of loss of N-acetylglucosamine transferase 1 (GnTI⁻) activity [184]. Therefore, we also constructed HEK293S (GnTI⁻)-TetR inducible stable cell lines expressing WT-TP and A160T. WT-TP and A160T expressed in the HEK293S (GnTI⁻)-TetR cell line showed homogenous glycosylation and migrated predominantly as a single band with a molecular mass of ~ 37kDa (**Fig. 4.3**). We have also evaluated the glycosylation status of WT-TP and A160T expressed in the HEK293S-TetR by pursuing PNGaseF treatment. Treatment with the N-glycosidase resulted in disappearance of the higher molecular weight band, confirming the N-glycosylation status of TP (**Fig. 4.4**).

4.3.2 Detergent screening

The solubilization of receptors from membranes is a critical step in purification of membrane proteins, thus the detergent used for solubilization is important. Previously, the detergents octyl- β -D-glucoside (OG), n-dodecyl- β -D-maltoside (DM), and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) have been used in WT-TP purification [187, 193]. However, we found 80% of WT-TP ligand binding activity was lost when 1% DM was used to solubilize TP from HEK293STetR cells (data not shown). This loss of activity was surprising, as 1% DM is routinely used in the purification of

GPCRs, including the β_2 -AR [98]. A combination of detergent and cholesterol hemisuccinate (CHS) was used with much better success in the purification of a number of GPCRs [194]. To investigate which detergent might be appropriate for obtaining a higher yield at the solubilization step, we carried out a systematic detergent screen. We screened 88 detergents including nonionic, anionic, cationic and zwitter-ionic detergents. We also carried out solubilization using different percentages of DM and CHS mixtures (**Table 4.1**). There were clear differences in efficiency of WT-TP solubilization by different detergents as determined by slot blot analysis. However, for those detergents that display a higher level of WT-TP solubilization there was no statistically significant difference in the functional yield of receptor (**Table 4.1**). We tried different combinations of DM and CHS and found that addition of 0.2% CHS to 1% DM led to an increase in functional yield of the solubilized WT-TP to 40-45% (**Table 4.2**). This result suggested that membrane cholesterol might be required for TP stability and/or function. However, it remains to be determined whether the modulation of receptor activity observed is due to the direct interaction between cholesterol and TP, or indirect effects caused by the influence of cholesterol on membrane structure or detergent micelle morphology.

4.3.3 Purification of TP and the A160T CAM

For receptor purification, membranes were prepared from 1.5×10^7 HEK293S-TetR cells grown as monolayers in 15 cm dishes.

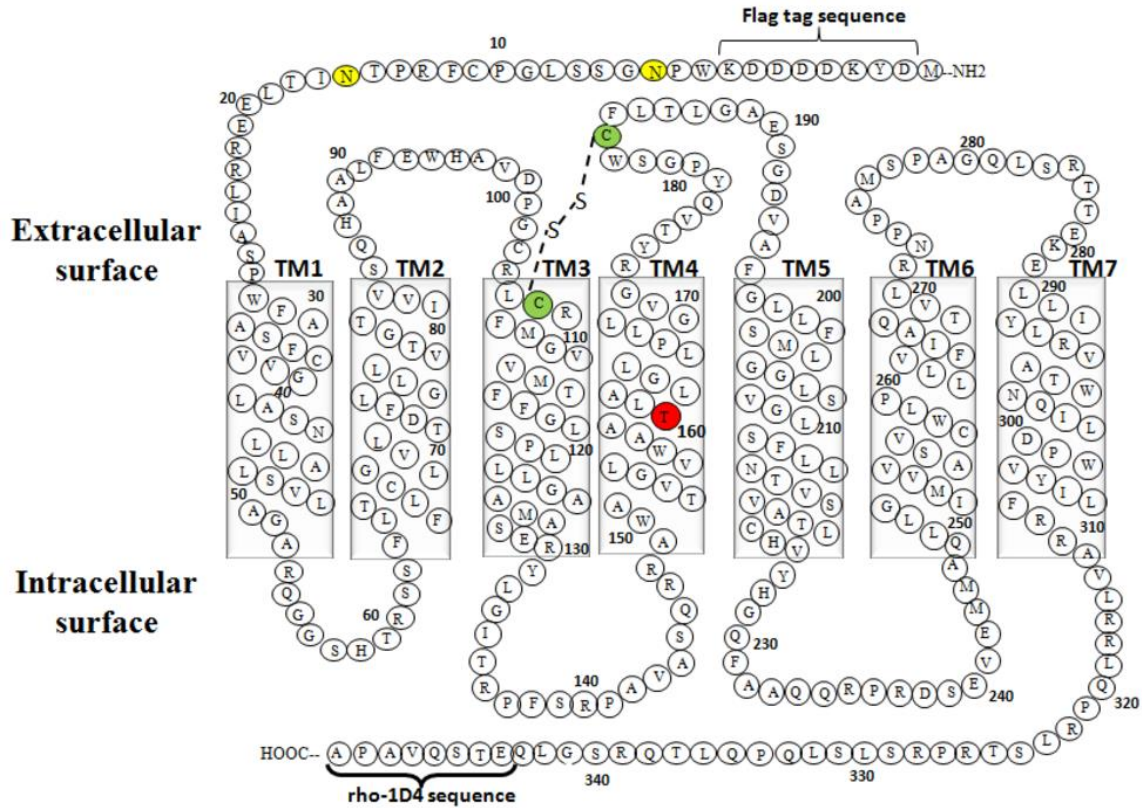


Fig. 4.1. Secondary structure representation of the TP α amino acid sequence with the genetic variant A160T.

Amino acids are shown in single lettered codes, and the residue numbering excludes the epitope tags at both ends. Shown are the seven transmembrane helices (TM1-7), the FLAG sequence at the N-terminus, the N-glycosylated residues Asn4 and Asn16 (yellow colored residues), the disulphide bond between Cys 105 and Cys183 (green colored residues), and the rho-1D4 octapeptide epitope tag at the C-terminus. The genetic variant A160T (residue 4.53 using Ballesteros-Weinstein numbering) on TM4 is highlighted in red.

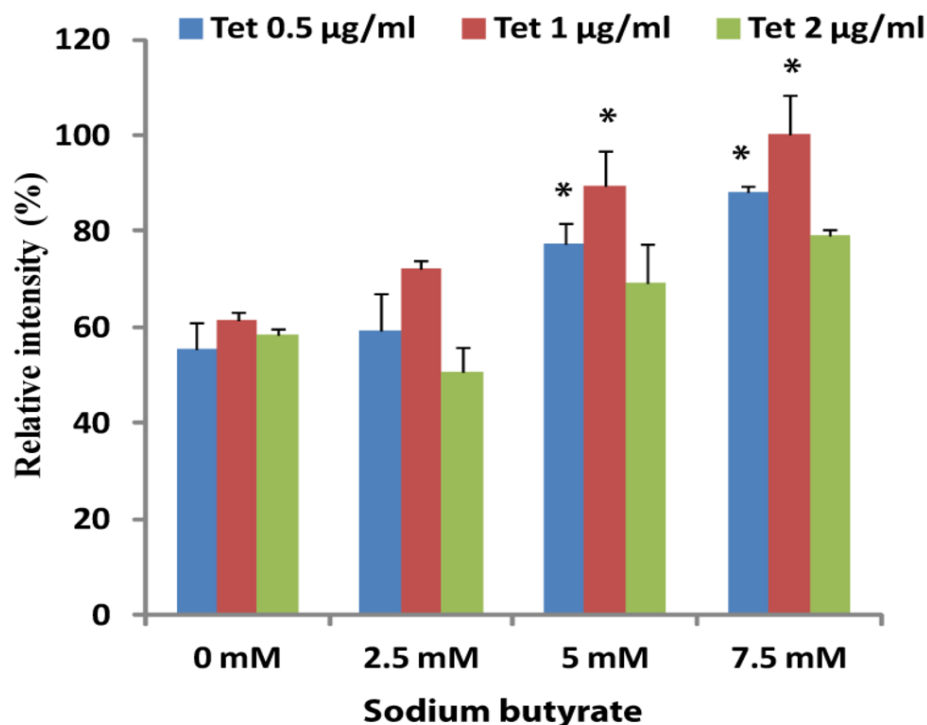


Fig. 4.2. Optimization of TP expression in HEK293S-TetR inducible cells.

The expression of TP in the HEK293S-TetR was induced by tetracycline and/or sodium butyrate as shown. Following induction, the samples were harvested, solubilized, and analyzed using the dot blot technique and probed with FLAG antibody. The results were quantified using spot densitometry (ImageJ software) and normalized to 100% of relative intensity. A one way ANOVA with tukey's post hoc test was done where the samples obtained from 5 and 7.5 mM of sodium butyrate with 0.5 and 1 µg of Tet/ml showed statistical significance at * $p < 0.05$ compared to 0 mM sodium butyrate and 0.5 and 1 µg of Tet/ml.

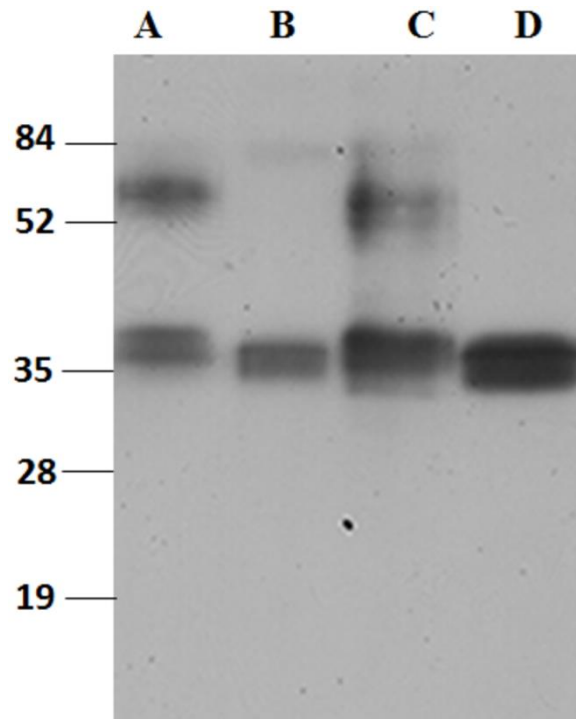


Fig. 4.3. Immunoblot analysis of TP and A160T using the monoclonal FLAG antibody.

TP and the A160T variant expressed in HEK293S-TetR stable cell line (lanes A and C). By using a HEK293S (GnTI⁻) cell line defective in N-acetylglucosamine transferase I, TP and A160T were expressed with restricted and homogeneous N-glycosylation (lanes B and D). 5 μ l of protein were loaded into all wells, and western blot analysis carried out using the FLAG antibody. Mobility of molecular weight standards is indicated next to the gel. The epitope tag for the monoclonal FLAG antibody was added to the N-terminus of the TP receptor.

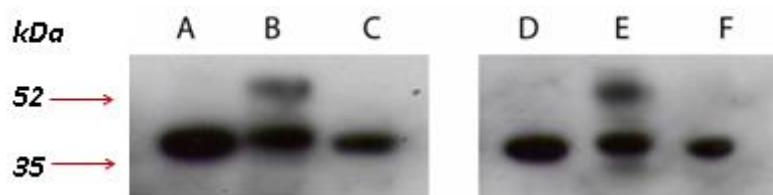


Fig. 4.4. Immunoblot analysis of TP and A160T variant digested with PNGaseF and detected using the monoclonal FLAG antibody.

TP and the A160T variant were expressed in HEK293S (GnTI-) cell line with restricted and homogeneous N-glycosylation (lanes A and D). TP and the A160T variant were expressed in the HEK293S-TetR stable cell line (lanes B and E). TP and the A160T variant expressed in the HEK293S-TetR stable cell line were digested with PNGase F (New England Biolabs) (lanes C and F). TP and the A160T variant were treated with 0.5% SDS and 40 mM DTT at 100 °C for 10 min and then add 50 mM Na₃PO₄ buffer (pH 7.5), 1% NP-40 and 2µl PNGase F, and incubated at 37 °C for 1 h. Equal amount (10 µg) of protein were loaded in all wells. The size of the molecular weight standards is indicated next to the gel.

The membranes were solubilized using 1% DM and 0.2% CHS, and the yield of the WT-TP as determined by ligand binding assay was found to be ~40% (**Table 4.2**). In the next step, anti-FLAG M2 agarose was used to purify WT-TP as well as the TP-A160T mutant. The receptors were found to be more than 90% pure, as analyzed by 10% SDS-PAGE (**Fig. 4.5**). The receptors produced in HEK293S-TetR were glycosylated, and migrated as two bands with the major band around ~35 kDa and a minor band of ~55 kDa. Previously we have shown by thermal sensitivity assays that the A160T mutant exhibits a 30-40% decrease in stability as compared to WT-TP [165]. In line with this observation, we found that the A160T is less stable during the purification, displaying a prominent band at ~25 kDa (**Fig. 4.5, lane E**) a proteolysis/degradation product of the C- terminus of A160T, that was detected by the rho-1D4 antibody (data not shown). The overall recovery of WT-TP obtained after purification using anti-FLAG M2 agarose beads was ~11%. The functional yield of the WT-TP using the single step affinity purification was 45 $\mu\text{g}/10^6$ cells. This corresponds to a yield of ~1mg of purified WT-TP from a liter (4.4×10^7 cells) of induced HEK293S cells cultured in a bioreactor. We did not carry out ligand binding assays during the purification of the A160T CAM, as it had low affinity for the antagonist [3H] SQ 29,548 which is expected for a CAM. The yield of the purified A160T mutant as determined from the total protein eluted after the FLAG affinity purification was comparable to that of WT-TP (**Table 4.3**).

4.3.4 Secondary structure analysis of purified TP and A160T mutant

Previously we showed that the A160T mutant exhibits loss of thermal stability [165]. In that study, as only membrane preparations of the mutant and wild type were used, the structural changes could not be characterized. Now, we present the temperature-

dependent secondary structure changes of the purified WT-TP and A160T receptors using CD spectropolarimetry. Based on the data from our previous thermal sensitivity assays, we chose to study the proteins at 25°C and 47°C and at 0 hrs and 3 hrs as time points. Far-UV CD spectropolarimetry confirmed that both the WT-TP and A160T mutant are predominantly α -helical. The spectra were analyzed using the K2D3 algorithm, which predicted an α -helix content of ~70% for both the WT-TP and A160T mutant. The results suggest that the A160T mutant secondary structure is less stable than the WT. Heating for 3 hr has only a very small effect on the WT-TP and causes a measurably larger loss of secondary structure in the mutant (**Fig. 4.6**). This suggests that the loss of activity or thermal sensitivity that was previously observed for the A160T is not owing to large unfolding of the protein but rather to a more subtle effect. Our results suggest that very little change occurs between 0 hrs and 3 hrs at room temperature for both the A160T mutant and the WT-TP (data not shown). For the mutant, it appears that no change occurs over the 3 hr incubation at 47 °C (**Fig. 4.6B**). This suggests that all the change in conformation took place during the 5 minutes that we allowed for the sample to warm up from 25 °C to 47 °C. In contrast, for the WT-TP the difference between 0 hr and 3 hr spectra suggests there is very little change in the first 5 minutes of heating (data not shown). Recently, the purification of glycosylated WT-TP in milligram amounts using a baculovirus expression system was reported [187]. However, none of the studies on the prostanoid receptors reported the ability to express and purify a homogenously glycosylated receptor, or a CAM at high-levels. The effect of various detergents on prostanoid receptor function or stability was also not tested.

Table 4.1 Detergent screen for solubilization of FLAG-TP expressed in HEK293S-TetR stable cell line.

Number	Detergent	CMC(%)	Concentration	Type	Normalized spot intensity (%)
1	ANAMEG-7	0.65	2%	N	
2	ANAPOE-C12E10	0.013	1%	N	
3	ANAPOE-35	0.001	1%	N	
4	ANAPOE-58	0.0045	1%	N	
5	ANAPOE-C10E6	0.025	1%	N	
6	ANAPOE-C10E9	0.053	1%	N	
7	ANAPOE-C12E8	0.048	1%	N	
8	ANAPOE-C12E9	0.003	1%	N	
9	ANAPOE-C13E8	0.0055	1%	N	
10	ANAPOE-20	0.0072	1%	N	
11	ANAPOE-80	0.0016	1%	N	
12	ANAPOE-X-100	0.015	1%	N	
13	ANAPOE-X-114	0.011	1%	N	
14	ANAPOE-X-305	-	1%	N	
15	ANAPOE-X-405	0.16	1%	N	
16	ANZERGENT 3-8	10.9	2%	Z	
17	ANZERGENT 3-10	1.2	2%	Z	
18	ANZERGENT 3-12	0.094	1%	Z	
19	ANZERGENT 3-14	0.007	1%	Z	
20	BIG CHAP DEOXY	0.12	1%	N	
21	BIG CHAP	0.25	1%	N	
22	CHAPS	0.49	2%	Z	
23	CHAPSO	0.5	1%	Z	
24	CYGLU-3	0.86	2%	N	
25	CYMAL-1	15%	2%	N	
26	CYMAL-2	5.4	2%	N	
27	CYMAL-3	0.86	1%	N	
28	CYMAL-4	0.37	1%	N	
29	CYMAL-5	0.12	1%	N	
30	CYMAL-6	0.028	1%	N	
31	CYMAL-7	0.0099	1%	N	
32	CYCLOFOS-2	7.5	2%	Z	
33	CYCLOFOS-3	1.3	2%	Z	
34	CYCLOFOS-4	0.45	2%	Z	
35	CYCLOFOS-5	0.15	1%	Z	
36	CYCLOFOS-6	0.094	1%	Z	
37	CYCLOFOS-7	0.022	1%	Z	
38	N-DODECYL-β-D-MALTOSE	0.0087	1%	N	
39	N-DODECYL-α-D-MALTOSE		1%	N	
40	N-DECYL-β-D-MALTOSE	0.087	1%	N	
41	N-DECYL-α-D-MALTOSE	0.076	1%	N	
42	N-DECYL-β-D-THIOMALTOSE	0.045	1%	N	
43	N-DODECYL-β-D-THIOMALTOSE	0.0026	1%	N	
44	DODECYLDIMETHYLGLYCINE	0.041	1%	Z	
45	DECYLDIMETHYLGLYCINE	0.46	2%	Z	
46	DODECYLDIMETHYLAMINEOXIDE	-	1%	Z	
47	DEOXYCHOLIC ACID, SODIUM SALT	0.24	1%	A	
48	DIMETHYLHEPTYL-β-D-MALTOSE	1.2	2%	N	
49	FOS-MEA-8	0.59	2%	Z	
50	FOS-MEA-10	0.15	1%	Z	
51	FOS-CHOLINE-8	3.4	2%	Z	
52	FOS-CHOLINE-9	1.2	2%	Z	
53	FOS-CHOLINE-10	0.35	2%	Z	
54	FOS-CHOLINE-11	0.062	1%	Z	
55	FOS-CHOLINE-12	0.047	1%	Z	
56	FOS-CHOLINE-13	0.027	1%	Z	
57	FOS-CHOLINE-14	0.046	1%	Z	
58	FOS-CHOLINE-15	0.027	1%	Z	
59	FOS-CHOLINE-16	0.0027	1%	Z	
60	FOS-CHOLINE-ISO-9	0.99	2%	Z	
61	FOS-CHOLINE-ISO-11	0.9	2%	Z	
62	FOS-CHOLINE-UNSAT-11-10	0.21	1%	Z	
63	N-HEPTYL-β-D-GLUCOSIDE	1.9	2%	N	
64	N-HEPTYL-β-D-THIOGLUCOSIDE	0.85	2%	N	
65	N-HEXYL-β-D-GLUCOSIDE	6.6	2%	N	
66	N-HEXYL-β-D-MALTOSE	8.9	2%	N	
67	C8E6 ANAGRADE	0.39	2%	N	
68	MEGA-8	2.5	2%	N	
69	N-NONYL-β-D-GLUCOSIDE	0.2	1%	N	
70	N-NONYL-β-D-MALTOSE	0.28	2%	N	
71	N-NONYL-β-D-THIOMALTOSE	0.15	1%	N	
72	N-OCTYL-β-D-MALTOSE	0.89	2%	N	
73	N-OCTYL-β-D-GLUCOSIDE	0.53	2%	N	
74	N-OCTYL-β-D-THIOMALTOSE	0.4	2%	N	
75	C12E8 ANAGRADE	0.084	1%	N	
76	2-PROPYL-1-PENTYL-B-D	1.9	2%	N	
77	C10E5 ANAGRADE	0.031	1%	N	
78	PMAL-C8		1%	Z	
79	SODIUM CHOLATE	0.41	2%	A	
80	SODIUM LAUROYL SARCOSINE	0.42	2%	A	
81	SUCROSE MONODODECANOATE	0.016	1%	N	
82	N-TETRADECYL-β-D-MALTOSE	0.00054	1%	N	
83	N-TRIDECYL-β-D-MALTOSE	0.0017	1%	N	
84	C8E4 ANAGRADE	0.25	1%	N	
85	TETRADECYLDIMETHYLAMINE OXIDE	0.0075	1%	Z	
86	N-UNDECYL-β-D-MALTOSE	0.029	1%	N	
87	N-UNDECYL-α-D-MALTOSE	0.029	1%	N	
88	N-UNDECYL-β-D-THIOMALTOSE	0.11	1%	N	
89	1%DM		1%	N	
90	0.5% DM		0.5%	N	
91	0.5% DM+0.1% CHS		0.5%	N	
92	1% DM+0.2% CHS		1%	N	
93	MEMBRANE				

Table 4.2 Purification of thromboxane A₂ receptor.

Purification step	Total protein (mg)	Specific Activity (pmol/mg)	Activity (pmol)	Yield (%)
Membrane fraction	4.19 ±0.27	16.89 ±1.16	70.37 ±6.18	
Solubilized receptor	3.54 ±0.36	6.71 ±1.28	24.48 ±5.91	39.32 ±3.54
FLAG-affinity purified	0.18 ±0.03	36.46 ±3.57	6.8 ±1.68	10.35 ±0.77

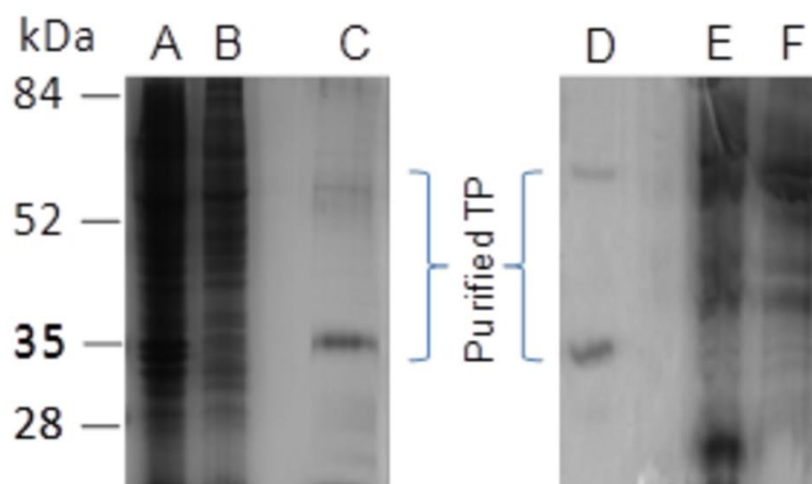


Fig. 4.5. SDS-PAGE (10%) analysis of TP and A160T purification.

Membrane preparations (lanes A and E), solubilized (lanes B and F) and FLAG-affinity purified TP and A160T (lanes C and D) from protein expressed in HEK293S-TetR cells. Proteins were detected by Coomassie staining. 5 μ g of protein were loaded into all wells. Mobility of molecular weight standards is indicated next to the gel. The receptor produced in HEK293S-TetR was glycosylated and migrated as two bands on the SDS-PAGE with the minor band of ~55 kDa and the major band showing an apparent molecular mass of ~37kDa.

Table 4.3. Purification of the CAM A160T from HEK293S-TetR and HEK293S (GnTI) -TetR stable cell lines*

Purification step	HEK293S-TetR Total protein (mg)	HEK293S (GnTI)-TetR Total protein (mg)
Membrane fraction	6.21	5.27
Solubilized receptor	5.82	4.84
FLAG-affinity Purified receptor	0.31	0.35

*Radioligand assays were not performed as the CAM A160T displayed weak affinity for the antagonist radiolabel [³H] SQ 29,548. Hence, the amount of receptor obtained in terms of protein concentration is displayed at each purification step.

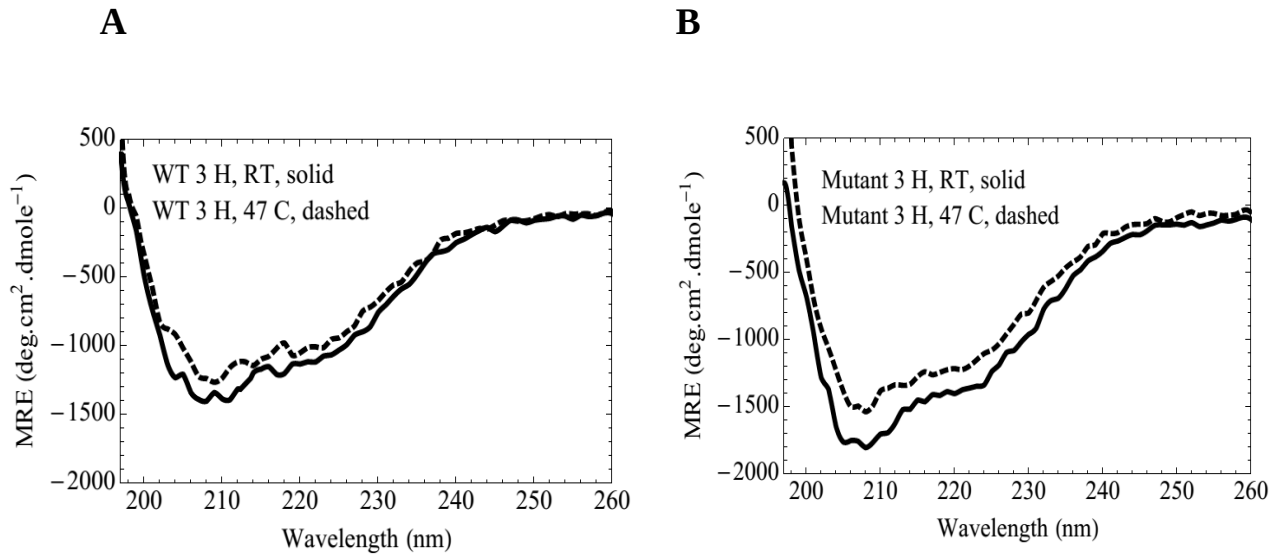


Fig. 4.6. Circular dichroism spectra of purified TP and A160T mutant at different temperatures.

The spectra show typical α -helical profiles with minima at 208 nm and 222 nm. These results suggest that WT-TP (panel A) and A160T (Panel B) are folded correctly. The A160T mutant is thermally more sensitive and shows a subtle loss of secondary structure compared to WT-TP.

In conclusion, our studies revealed that using the HEK293STetR- inducible system both WT-TP and the A160T CAM are expressed at up to 4-fold higher levels, and they showed homogenous glycosylation when expressed in the HEK293S (GnTI⁻)-TetR cell line. The yield of the functional receptor obtained from the one-step purification now makes it feasible to purify WT-TP and CAMs in milligram amounts from mammalian cells. Furthermore, additional approaches can be used to improve the stability and yield of TP and mutants. These include addition of antagonist to cultures expressing TP or agonist expressing the CAMs, and inclusion of ligands during the purification process might also increase stability of the protein. Scale up of the expression using suspension cultures in a bioreactor using established techniques should allow production of TP, and importantly the A160T CAM at levels suitable for the application of high resolution biophysical studies, such as crystallography. This is the first study to report on the successful high-level expression, purification and biophysical characterization of a naturally occurring, diffusible ligand activated GPCR variant that exhibits constitutive activity.

Chapter 5

Inverse agonism of SQ 29,548 and Ramatroban on thromboxane A₂ receptor

Chakraborty R, Bhullar RP, Dakshinamurti S, Hwa J, Chelikani P (2014) *PLoS ONE* 9(1): e85937. doi:10.1371/journal.pone.0085937

5.1 Introduction

Thromboxane A₂ (TxA₂) is a major product of arachidonic acid metabolism and is known to be the key mediator of platelet aggregation and smooth muscle contraction [2,195, 196]. The action of TxA₂ is mediated by its cognate G protein-coupled receptor (GPCR) thromboxane A₂ receptor (TP), which exists in two isoforms, TP α and TP β , differing only in their C- terminal region. The TP α has a wide spread tissue distribution in humans and is implicated in pathophysiological conditions such as platelet aggregation, bleeding disorders, cardiovascular diseases, atherosclerosis, and asthma [49, 156, 196].

GPCRs are known to function even in the absence of an agonist molecule and this phenomenon is known as constitutive receptor activity. It can be explained using the multiple state model of receptor activation [197, 198]. Over the last decade considerable numbers of GPCRs were shown to have constitutive activity [79, 80]. This phenomenon of GPCRs became the most important tool in discriminating between inverse agonists and neutral antagonists [81, 82]. Inverse agonists are compounds or drugs known to reduce the constitutive GPCR activity and are often defined to have a (-1) efficacy whereas neutral antagonists do not affect the basal GPCR activity and have (0) efficacy [83]. Interestingly, a number of drugs currently in use that target GPCRs are inverse agonists rather than neutral antagonists. For example, the antagonist metoprolol for β -adrenergic receptors, losartan for Angiotensin receptors, haloperidol for Dopamine receptors and cetirizine and cimetidine for Histamine H₁ and H₂ receptors are now classified as inverse agonist for their respective targets [83, 198-200].

TP exhibits basal or constitutive activity in the absence of any ligand [165]. Previously, we have discovered constitutively active mutants (CAMs) in transmembrane

(TM) 3 and 4 of TP (**Fig. 5.1**). The mutants V110A, F114A in TM3 and the genetic variant A160T in TM4 displayed constitutive activity to varying levels [165]. Due to the excessive agonist independent activity of A160T, we speculated that this genetic variant might cause cardiovascular disease (CVD) progression [53]. For effective therapeutic intervention, an inverse agonist would be required to lower the activity of the constitutively active receptor.

TP antagonists are known to be beneficial for treating cardiovascular diseases, platelet disorders, and asthma [201, 202]. The discovery of CAMs in TP provided a unique opportunity to screen well known TP antagonists for inverse agonist activity. In this work, we chose four compounds, SQ 29,548, Ramatroban (BAY-u3405), Diclofenac and L-670596 to test for inverse agonism based on their potency and selective effects on human platelets. SQ 29,548 is a selective TP antagonist recognized for its well-established effect to antagonize platelet aggregation and contraction in respiratory smooth muscle cells [203]. Ramatroban, a TP antagonist recognized to inhibit platelet aggregation induced by collagen and U46619 [204]. Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), concentration dependently and selectively inhibited TP mediated contraction in smooth muscles as well as human platelet aggregation [205]. The fourth compound we tested was L-670596, a potent TP antagonist in human platelets and shown to inhibit contraction in guinea pig tracheal rings in a concentration dependent manner [206]. We tested the pharmacological profiles of these compounds using the CAMs, in both, HEK29T cells, and in a novel megakaryocyte based system to evaluate their effects on human platelet function.

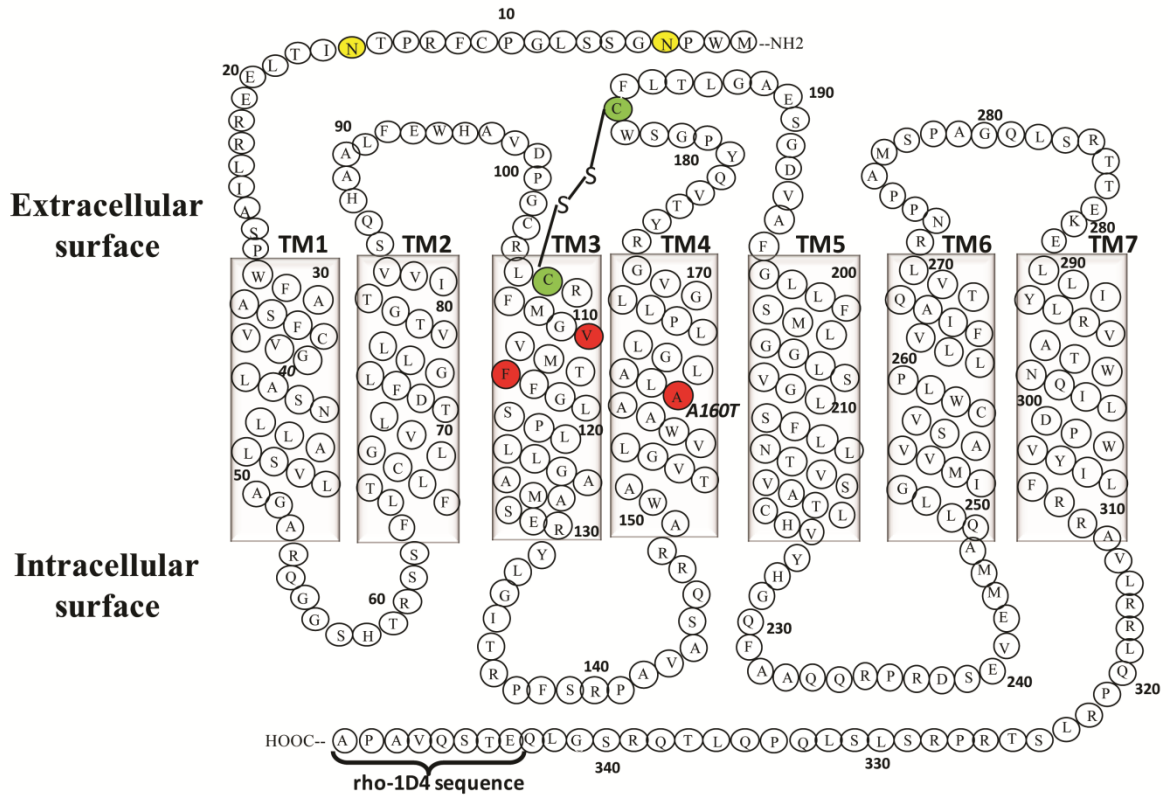


Fig. 5.1. Secondary structure representation of TPa amino acid sequence.

Amino acids are shown in single letter codes, and the residue numbers exclude the epitope tag (FLAG-tag) at the N-terminus. Shown are the seven transmembrane helices (TM1-7), the N-glycosylated residues Asn4 and Asn16 (yellow colored residues), the disulphide bond between Cys 105 and Cys 183 (green colored) and the rho-1D4 tag at the C-terminus. The genetic variant A160T^{4.53} on TM4 and V110^{3.30}, F114^{3.34} on TM3 (Ballesteros-Weinstein numbering in the superscript) is highlighted in red.

Our results suggest that SQ 29,548 and Ramatroban are inverse agonists for TP, whereas, L-670596 and Diclofenac are neutral antagonists.

5.2 Materials and methods

5.2.1 Materials

The TP antagonist SQ 29,548 was purchased from Sigma (product no. NET936). Ramatroban and Diclofenac were purchased from Cayman Chemicals (Michigan, USA), L-670596 from Tocris Bioscience (Bristol, UK). The TP agonist U46619 was purchased from the Cayman Chemicals Company. Protease inhibitors and common chemicals were purchased from either Fisher or Sigma. The buffers and detergents were the same as those used previously [165]. Polyclonal antibody directed towards 1215 amino acids of the N-terminal in TP α was purchased from Lifespan BioSciences (Washington, USA). PE-anti-CD41 antibody and FITC-anti-CD62P were purchased from Biolegend (California, USA). Nucleofection kit for Meg 201 cells was purchased from Lonza (Texas, USA).

5.2.2 Molecular biology and cell culture

The TP α mutants used in this study were described previously [165]. To minimize variations in transfection efficiency, the total amount of transfected DNA was kept constant in all cases at 6 μ g of DNA per 5×10^6 cells. For transient transfections of HEK293T cells using the plasmid pMT4, lipofectamine 2000 (Invitrogen) mediated transfection was used as described by the manufacturer.

5.2.3 Flow cytometry analysis of cell surface receptor expression

Cell surface expression of the WT-TP, V110A, F114A and A160T mutants transfected with different concentrations (3- 9 μg) of DNA per 5×10^6 cells was determined using BD FACS Canto flow cytometer, and as described previously [165]. The results shown are from a minimum of three sets of experiments. FACS data was normalized to WT-TP DNA of 3 μg which was taken as 100%.

5.2.4 Determination of Ca^{2+} mobilization

The DNA coding for the WT-TP, V110A, F114A and A160T were expressed in HEK 293T cells using 6 μg of DNA per 5×10^6 cells. Changes in intracellular calcium were measured by using the fluorescent calcium sensitive dye Fluo-4NW (Invitrogen) as described previously [165,189,207]. Mock transfected (with vector pMT4) cells were used as a negative control. Determination of basal Ca^{2+} levels for agonist-independent signaling was carried out using Flexstation-3 fluorescence plate reader (Molecular Devices, CA, USA) at 525 nm following excitation at 494 nm. To determine whether the drugs decrease the basal activity of WT-TP and the mutants, cells expressing the receptors were incubated with 1 μM concentration of each of the compounds, SQ 29,548, Ramatroban, L-670596 and Diclofenac separately for 15-20 mins and the changes in intracellular calcium mobilization was determined. Ca^{2+} mobilized (ΔRFU) was corrected for receptor expression levels using FACS data. Similarly, a concentration dependent Ca^{2+} response was also measured with highest concentration of the drugs (10 μM) and blank or background being buffer or water alone.

5.2.5 Determination of inositol-1, 4, 5-trisphosphate (IP₃) mobilization

IP₃ assays were carried out in HEK293T cells using a commercially available IP₃ assay kit (HitHunter IP₃ fluorescence polarization [FP] assay; DiscoverX, Fremont, CA) according to the instructions supplied by the manufacturer and as described previously [189]. A standard graph was constructed using different concentrations of IP₃ provided by the manufacturer, and this graph was used to calculate the amount of IP₃ released by the wild type and mutant receptor, as previously described [189]. Briefly, to determine whether the drugs decrease the basal activity of WT-TP and the mutants, cells expressing the receptors were incubated with 1 μ M concentration of the compounds, SQ 29,548, Ramatroban, L-670596 and Diclofenac separately for 15-20 mins and the basal level of IP₃ mobilization was determined and corrected for receptor expression levels using FACS data.

5.2.6 Flow cytometry analysis of P-selectin (CD62P)

Human megakaryocytes (Meg-01, ATCC: CRL-2021), were nucleofected using Lonza kit C with 3 μ g of WT-TP or A160T per 100,000 cells following recommended manufacturers protocol and as described previously [53]. Briefly, nucleofected cells were incubated for 24 hours at 37°C. Then platelet like particles (PLPs) was collected from the media of the nucleofected megakaryocytes. PLPs were incubated in vehicle (buffer or water alone) for 15 mins at room temperature. The PLPs were incubated in PBS containing PE-anti-CD41 to label all PLPs and FITC-anti-CD62P to label activated particles and incubated for 1 hour at 4°C. The samples were washed 2 times with PBS spun down and resuspended in PBS for Flow cytometry analysis. Similarly, to assess whether the drugs decrease CD62P activation in PLPs, 1 μ M concentration of each compound, SQ 29,548, Ramatroban, L-670596 and Diclofenac on WT-TP and A160T were tested.

5.2.7 Statistical analysis

Statistical analysis using one-way analysis of variance (ANOVA) with Tukey's *post hoc* test from at least 3 independent experiments was done to determine statistical significance wherever applicable.

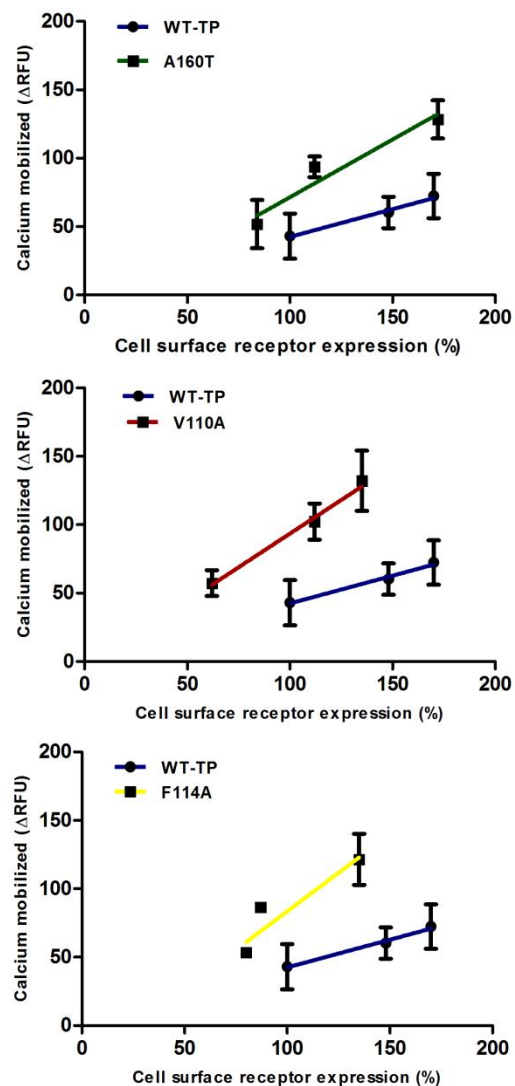
5.3 Results

5.3.1 Characterization of constitutive activity of WT-TP, V110A and F114A

In our previous report we characterized the constitutive activity of only the A160T genetic variant, in detail [165]. We now provide detailed characterization of the constitutive activity of V110A and F114A, demonstrating the effect of receptor density on Ca^{2+} mobilization (**Fig. 5.2**). Analysis of the expression of the WT-TP and mutants was pursued using flow cytometry, and the specificity of the antibodies used was reported in our previous study [189]. We observed a positive linear correlation between the amounts of receptor expressed and the basal Ca^{2+} mobilization for all the mutants compared to WT-TP (**Fig. 5.2**). The slope of expression vs. basal activity for the V110A, F114A and A160T mutants showed high basal signaling, with constitutive activity ranging from 2 to 3 fold over WT-TP (**Fig. 5. 2**).

5.3.2 Effect of TP antagonists on constitutive intracellular Ca^{2+} signaling

The concentration dependent effects of the four TP antagonists, on calcium mobilization was tested on both WT-TP and A160T, and TP agonist U46619 was used as a positive control (**Fig. 5.3**). SQ 29,548 was able to reduce the basal activity of WT-TP at only the two higher concentrations of 1 μM and 10 μM , whereas Ramatroban, Diclofenac, and L-670596 did not affect the basal activity of WT-TP (**Fig. 5.3A**).



Cell surface expression

Receptor	DNA (3μg)	DNA (6μg)	DNA (9μg)	Slope	(R ²)
WT-TP	100	148 ± 28	170 ± 11	0.40 ± 0.27	(0.18)
V110A	62 ± 32	112 ± 30	135 ± 26	0.98 ± 0.26	(0.66)
F114A	80 ± 20	87 ± 15	135 ± 21	1.12 ± 0.28	(0.70)
A160T	84 ± 25	112 ± 23	172 ± 9	1.02 ± 0.29	(0.62)

Fig. 5.2. Effect of receptor density on basal Ca²⁺ mobilization.

WT-TP (blue) and A160T (green), V110A (red) and F114A (yellow) constructs were expressed in HEK293T cells at different receptor densities by varying amounts of DNA used in each transfection (3μg to 9 μg DNA per 5x10⁶ cells). Receptor expression levels were determined by FACS analysis using polyclonal antibody specific to the N-terminal sequence between amino acids 1-15 of WT-TP. FACS data was normalized to WT-TP DNA of 3 μg which was taken as 100%. The slopes of WT-TP, A160T, V110A, F114A are 0.40 ± 0.27, 1.02 ± 0.29, 0.98 ± 0.26 and 1.12 ± 0.28 respectively.

With the A160T variant, both SQ 29,548 and Ramatroban were able to reduce the basal calcium mobilization, whereas L-670596 and Diclofenac did not show any effect at higher concentrations (**Fig. 5.3B**). Next, we characterized the effects of the four TP antagonists, on the basal Ca^{2+} mobilization by the three CAMs. Based on our results, we chose 1 μM of the compounds for our studies. The results show, 1 μM of SQ 29,548 decreased the basal Ca^{2+} mobilization of WT-TP by more than 50% whereas the other drugs did not show any significant decrease (**Fig. 5.4A**). The effect of the drugs on basal calcium mobilization of the genetic variant A160T, clearly indicate that 1 μM of SQ 29,548 and 1 μM of Ramatroban were able to decrease the basal Ca^{2+} mobilization by almost 80% and 70% respectively. However, no statistically significant change in the basal calcium mobilization was observed when treated with 1 μM of L-670596 or Diclofenac (**Fig. 5.4B**). Similar to the results obtained with A160T, 1 μM of SQ 29,548 and 1 μM Ramatroban were able to decrease the basal activity of V110A and F114A CAMs significantly, whereas L-670596 or Diclofenac did not change the basal activity of the CAMs (**Fig. 5. 4C and 4D**).

5.3.3 Effect of TP antagonists on intracellular IP_3 mobilization

The genetic variant and CAM A160T was selected for further analysis. The effects of the four TP antagonists on constitutive IP_3 mobilization by WT-TP and A160T were studied. The TP agonist U46619 (1 μM) was used as a positive control. SQ 29,548 was able to decrease the basal activity of WT-TP by 40-50% however no statistically significant effects were observed for the other three compounds (**Fig.5.5A**). Interestingly, 1 μM of SQ 29,548 or Ramatroban, were able to decrease the basal activity of A160T by 50% (**Fig. 5.5B**). However, no significant decrease in the basal IP_3 mobilization was observed when WT-TP or A160T were treated with 1 μM of L-670596 or Diclofenac.

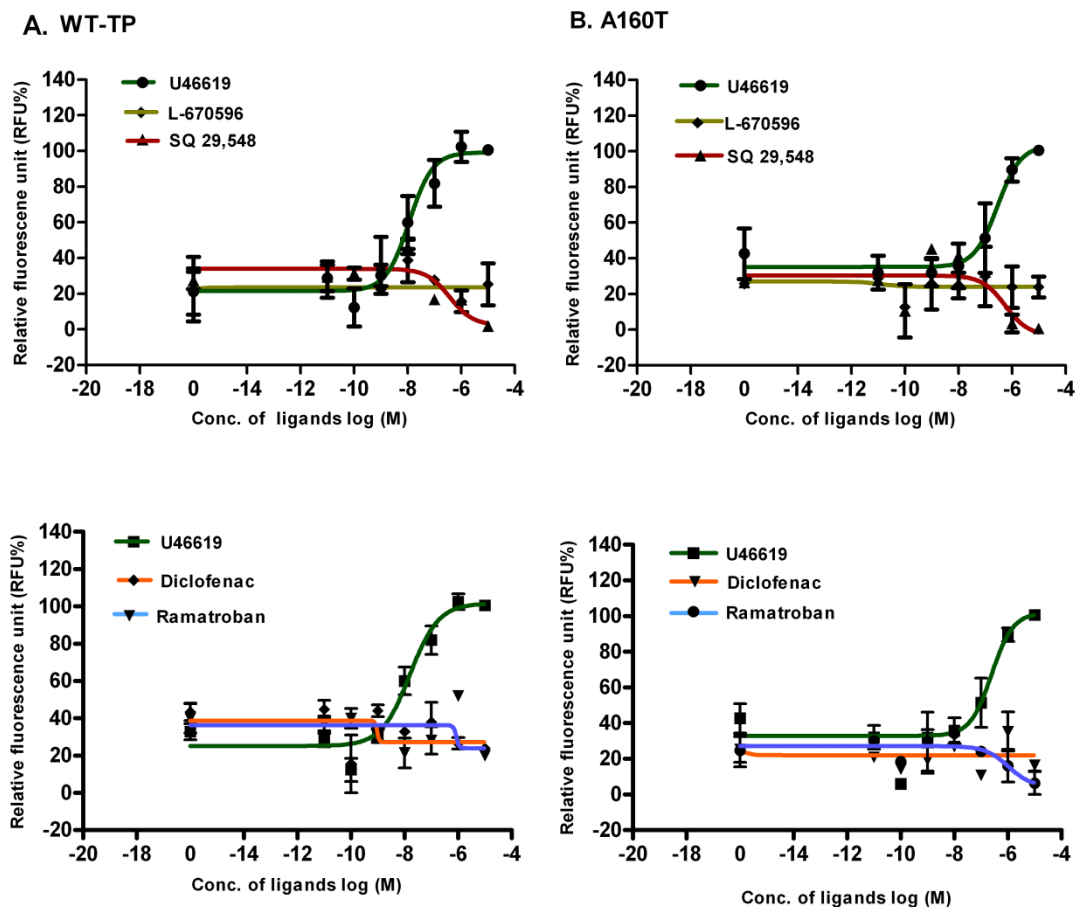


Fig. 5.3. Effect of TP ligands on intracellular Ca^{2+} signaling.

Concentration dependent changes in calcium mobilization of cells expressing WT-TP (A) and A160T (B) after application of different ligands. Ca^{2+} levels were measured as described in materials and methods. Results are presented as % RFU of the maximal response obtained with after stimulation with $10 \mu\text{M}$ of TP agonist U46619. Data are represented as mean $\pm$ SD and are from at least three independent experiments done in duplicate.

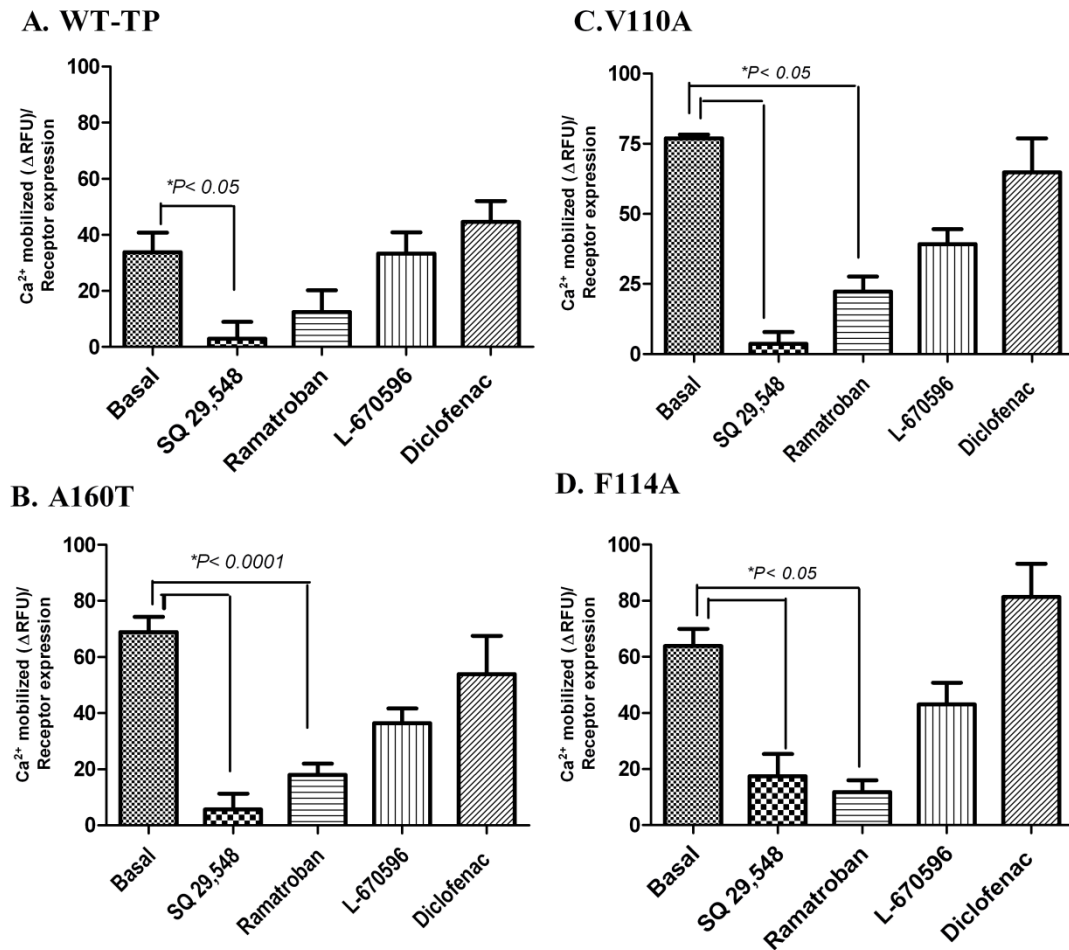
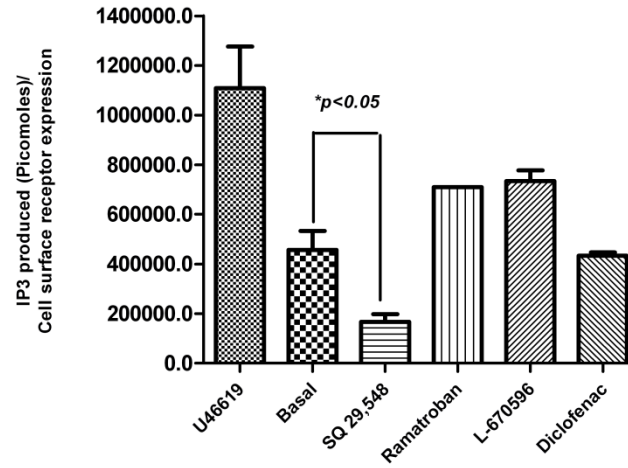


Fig. 5.4. Effect of TP antagonists on constitutive intracellular Ca²⁺ signaling. Agonist-independent calcium mobilization for WT-TP, A160T, V110A and F114A after cells was pre-treated with 1 μ M of SQ 29,548, Ramatroban, L670596 and Diclofenac. Ca²⁺ mobilized (Δ RFU) was corrected for receptor expression levels using FACS data. A one way ANOVA with *tukey's post hoc* test between control and mutant receptors treated with different compounds showed a significant decrease in basal activity at $p < 0.0001$ and $p < 0.05$. Similar results were obtained for WT-TP, basal vs SQ 29,548 at $p < 0.05$. The results are from 3 independent experiments done in triplicate and are represented as Mean $\pm$ SD.

A. WT-TP



B. A160T

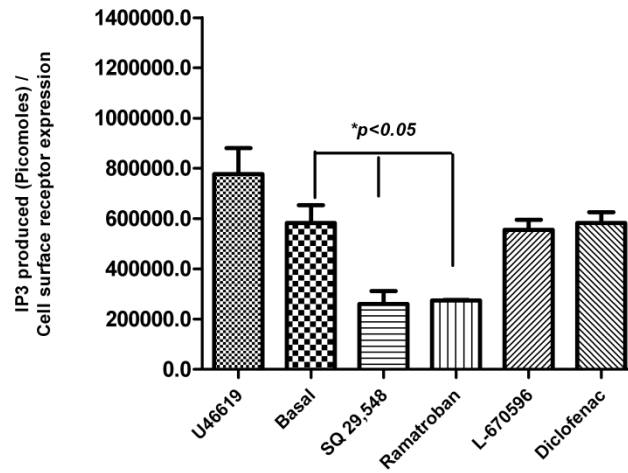


Fig. 5.5. Effect of TP antagonists on intracellular IP₃ mobilization.

The bar plot diagram shows agonist U46619 (1 μ M) induced and agonist-independent IP₃ mobilization for WT-TP and A160T. The graph also shows IP₃ release after cells were pre-treated with 1 μ M of SQ 29,548, Ramatroban, L670596 or Diclofenac. Total IP₃ (picomoles) mobilized was corrected for receptor expression levels. A one way ANOVA with *tukey's post hoc* test between basal and receptor pretreated with different compounds showed a significant decrease in basal activity at $p < 0.05$.

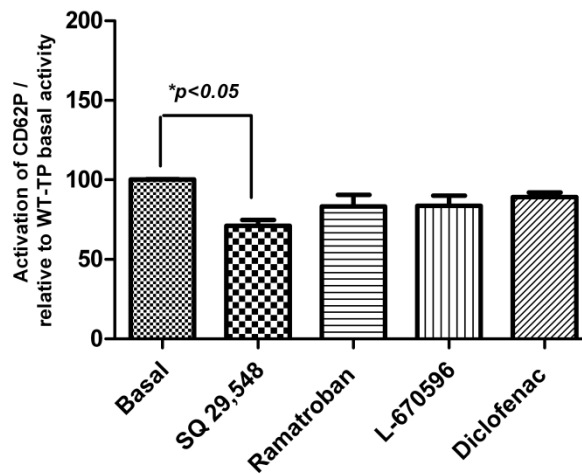
5.3.4 Effect of TP antagonists “In Platelet” functional analysis

Studies involving identification of inverse agonist(s) for GPCRs have routinely used heterologous expression systems. To have a more physiologically acceptable scenario, we used a human platelet like system that can be genetically modified. Recently, we have shown that Meg-01 can be transfected and can produce platelet like particles [53]. To test the inverse agonist effect of the drugs on the constitutive activity of A160T in Meg-01 cells, the genetic variant A160T was transfected into the Meg-01 cells and PLPs collected. WT-TP was used as the negative control. PLPs were measured by flow cytometry for CD62P (P-selectin) after basal activation i.e. in presence of buffer alone (control) and after the use of the drugs SQ 29,548, Ramatroban, L-670596 and Diclofenac. The A160T variant, as expected showed higher baseline activity than WT-TP (**Fig. 5.6**). The results from the heterologous system were once again validated, when it was found that both 1 μ M of SQ 29,548 as well as Ramatroban decreased the basal activity of A160T significantly, whereas 1 μ M of L-670596 and 1 μ M of Diclofenac had no significant effect on the basal activity of A160T (**Fig. 5.6**). This finding is clinically important as it demonstrates the inverse agonist properties in our PLP model, suggesting there may be a similar protective effect against cardiovascular disease *in vivo*.

5.4 Discussion

Overexpression of GPCRs in some cases has been shown to enhance the basal receptor activity, which in turn facilitates understanding of ligand interactions with its receptor and the signal transduction pathway [198, 208]. The concept of constitutive

A. WT-TP



B. A160T

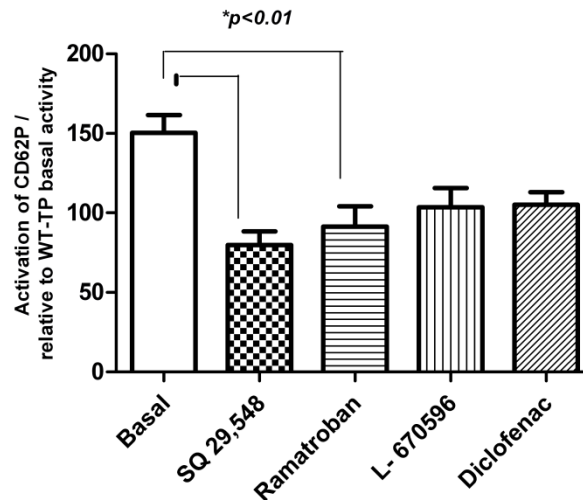


Fig. 5.6. Effect of TP antagonists “In Platelet” functional analysis.

The bar plot represents FACS analysis of P selectin (CD62P) on the surface PLPs liberated from cultured Meg-01 cells. Activity or response under basal conditions was measured. A160T showed a considerable higher basal activity compared to that of WT-TP, which was decreased by addition of each of 1 μ M SQ 29,548 or Ramatroban. A one way ANOVA with *tukey's post hoc test* between WT-TP and SQ 29,548 as well as A160T pretreated with different compounds SQ 29,548, and Ramatroban showed a significant decrease in basal activity at $p < 0.05$ and $p < 0.01$ respectively. The results are from a minimum of 3 independent experiments and are represented as Mean $\pm$ SD.

receptor activity is now well established and is often caused due to the presence of single nucleotide polymorphisms (SNPs) or mutations in TMs or intracellular loop domains. SNPs are defined to occur in at least 1% of the population and are often linked with receptor disorder or human diseases [209]. Some of these SNPs can lead to human diseases, while others can induce multiple signaling states in the receptor leading to distinct signaling pathways. In rhodopsin, the G114V and N184P variant has been shown to cause protein misfolding resulting in retinitis pigmentosa in patients [210]. Similarly, the V103I mutation in the melanocortin receptor has been associated with decreased incidence of obesity in normal individuals [211]. Interestingly, *in vitro* studies of the R347C variant in $\alpha 1A$ -adrenoreceptor showed that it did not affect receptor signaling and trafficking mechanism, which is consistent with *in vivo* data that showed the variant in humans as predicted was not related to any disease [212]. Other examples include melanocortin-4 receptor (MC4R)-CAMs, thyroid stimulating hormone (TSH)-CAMs, and luteinizing stimulating hormone (LSH)-CAMs which are linked to different human diseases , [209, 213]. Our recent studies from sequencing 897 cardiovascular patients did not reveal the presence of the A160T variant [53]. Though the clinical significance of the A160T in TP is not yet elucidated we speculate that this SNP, because of its constitutive activity might cause CVD progression or be involved in other pathophysiological process.

In this report, we confirmed a positive correlation between receptor density and basal calcium released, establishing that V110A and F114A are CAMs. Following transfection of F114A, V110A, A160T and WT-TP, the basal calcium mobilization was decreased when treated with 1 μ M of SQ 29,548 or 1 μ M of Ramatroban demonstrating their role as inverse agonists, whereas no effect was observed for Diclofenac or L-670596

treatment. The inverse agonist activity of SQ 29,548 and Ramatroban was also confirmed by IP₃ mobilization assays.

In the next part, we focused on the SNP variant A160T in TP, as it is clinically relevant. Previous studies have shown that rodent thromboxane system differs significantly from the human system [53, 214]. To investigate the role of the four compounds in an accepted physiological scenario such as human platelet function, we tested their effects on Meg-01 based system for platelet activation. Meg-01 cells spontaneously release PLPs into culture medium which express markers such as CD41 and CD62P on their surface [215]. Since P selectin (CD62P) are only expressed on activated platelets, measuring CD62P expression on platelet surface using flow cytometry assay has been widely employed to characterize platelet activation in various experimental and clinical conditions [216]. Our data using the Meg-01 revealed that there is a spontaneous overexpression of P selectin on the PLPs containing A160T compared to WT-TP without any agonist treatment leading to constitutive activity. Strikingly, the constitutive activity was decreased for A160T when treated with 1 μ M concentration of SQ 29,548 or Ramatroban validating their role as inverse agonists (**Fig. 5. 6**). However, no significant effect was observed for Diclofenac and L-670596 suggesting their role as neutral antagonist. A TP antagonist or inverse agonist would preferably be more acceptable to low dose aspirin in the light of the recent events surrounding COX-2 inhibitors [217]. Previous studies have highlighted the clinical side effects of COX-2 inhibitors, as the TxA₂/PGI₂ balance is critical in maintaining the cardiovascular homeostasis [156, 217]. The pharmacological characterization of TP CAMs, allowed us to revisit some of the potent TP antagonists and classify them under neutral and inverse agonist categories.

Chapter 6

**Role of polymorphic variant A160T in mediating signaling cross-talk between the
prostanoid receptors TP and IP.**

6.1 Introduction

Membrane receptors are attractive drug targets because they mediate the communication between the cell and its environment. GPCR dimerization has been shown to play important roles in receptor trafficking, ligand binding, G protein selectivity towards a particular monomer, and also internalization of two receptors when stimulated with a ligand for one of them. GPCRs structures have been known to contain seven TM helices (TM1-TM7) and have conserved amino acid residues that exhibit important structure and functional roles. Atomic force microscopy studies (AFM) have shown that the prototypical receptor rhodopsin exists as higher order oligomers in native disc membranes [218]. Interestingly, the dimer interface has been shown to involve TM4 and TM5 not only in rhodopsin but also in muscarinic, chemokine receptors etc. Later studies, using site-directed cysteine mutants on TM4 of rhodopsin and chemical cross-linking identified the residues W175C and Y206C to readily form dimers in presence of Cu^{2+} phenanthroline [219]. Subsequent studies using FRET and with low receptor densities reconstituted in asolectin liposomes confirmed that rhodopsin self –associates in the membranes [220]. Further, chemical cross-linking using cysteine substitutions clearly indicated TM4 to be part of the dimer interface in Dopamine receptor (D2R) [132]. Cysteine substitutions also revealed functional crosstalk between two monomers formed by TM4. In presence of an agonist, dimer formation between two monomers of D2R were increased significantly locking the receptor in an active conformation whereas inverse agonist treatment slowed the formation of dimers involving TM4 [132]. Similarly, homodimerization and heterodimerization studies using FRET have identified TM1 and TM4 to be important for dimerization in both chemokine receptors, CCR4 and CCR5 [221]. Interestingly, a CCR4 peptide mimicking the

TM4 reduced homodimerization between CCR4 receptors in cancer cells indicating towards TM4 role in CCR4 dimerization [221].

In normal conditions, it is now well established that heterodimerization of TP with IP resulted in coupling of TP α to cAMP generation and rendered the TP sensitive to regulation by TP or IP agonists via cAMP generation [222]. Interestingly, studies on TP under hypoxic condition, in neonatal porcine pulmonary artery myocytes, in a model of persistent pulmonary hypertension of the newborn (PPHN) showed that TP is hyper responsive [23]. The observed phenomenon has been attributed towards IP inactivation in hypoxic conditions [25]. Therefore, under hypoxic conditions the TP might play a key role whereby the TP-IP heterodimer formation causes a switching the pathway towards TP α coupling to G α q for calcium mobilization. This in turn might contribute to the adverse cardiovascular effects. This is in sharp contrast to the IP check on TP adverse effects under normoxic condition.

Humans with the genetic variant IP-R212C are heterozygous for the mutant [75]. Thus far, most of the studies on TP-IP crosstalk were from the IP perspective and none have examined the crosstalk in the context of TP genetic variants. The polymorphic variant A160T is present in TM4 of TP. As discussed before, TM4 is shown to be important for dimerization in GPCRs. Besides that, A160T is the only genetic variant of TP characterized thus far, that displayed constitutive activity [165]. In addition, the effect of this genetic variant on TP-IP crosstalk under stress and or pathophysiological conditions such as hypoxia is unknown. How A160T modifies WT-TP or WT-IP function in a dimer model has not been examined under normoxic and hypoxic conditions (**Fig. 6.1**). Therefore the objective of this study was to examine the effect of the polymorphic variant TP-A160T on its dimeric partners under normoxic and hypoxic condition **Fig. 6.1**.

6.2 Materials and methods

6.2.1 Cell culture and transfections

Amino acid substitutions were introduced into the synthetic TP α gene carried by the expression vector pMT4 as described previously [165]. Transient transfections of WT-TP or WT-IP or mutants in HEK293T cells using the plasmid pMT4, lipofectamine 2000 (Invitrogen) mediated transfection was used as described by the manufacturer [189]. In HEK293T cells, for transfections of a single receptor, the amount of DNA used was 3 μ g per 5x10⁶ cells referred to as (1x). However, for co-transfections, 3 μ g of DNA per 5x10⁶ cells of both receptors was used to maintain 1:1 stoichiometry and minimize variations. In certain cases to achieve 1:2 stoichiometry of DNA in co-transfections, 6 μ g of DNA per 5x10⁶ cells was used wherever applicable.

6.2.2 Hypoxic and normoxic conditions

HEK293T cells were grown to confluence in DMEM F12 media with L-glutamine, 10% fetal bovine serum, 1% penicillin, 1% streptomycin. The cells were then grown in normoxic (21% O₂, 5% CO₂) or in a hypoxic (10 % O₂, 5% CO₂, balance N₂) culture chamber for 24 hours and as described previously [23].

6.2.3 Determination of calcium mobilization

Changes in intracellular calcium were measured by using the fluorescent calcium sensitive dye Fluo-4NW (Invitrogen). After 6-8 hours of transient transfection of HEK293T cells using lipofectamine 2000, 1 x 10⁵ viable cells were plated into each well of a 96-well

tissue culture treated BD-falcon optilux plates. Cells mock transfected with vector pMT4 were used as a negative control. Following 24 hours of incubation at 37°C under normoxic and hypoxic condition, the media was removed and cells were incubated with Fluo-4NW (Invitrogen) for 1 hour. Intrinsic and basal receptor activation was determined by measuring changes in intracellular calcium after application of highest concentration (1 μ M) of agonist U46619 for TP and water alone for basal for different co-transfected receptors using Flexstation-3 fluorescence plate reader (Molecular Devices, CA, USA) at 525 nm following excitation at 494 nm. Dose–response curves were generated and EC₅₀ values calculated by nonlinear regression analysis using PRISM software version 4.0 (GraphPad Software Inc, San Diego, CA) after subtracting the responses of mock-transfected cells stimulated with same concentrations of agonists.

6.2.4 Determination of cyclic AMP mobilization

The cAMP assays were carried out in HEK293T cells using a commercially available cAMP assay kit (Hit- Hunter cAMP XS; DiscoverX, Fremont, CA) and as described previously [76]. In brief, 48 h after transient transfection, the cells were stimulated with 1 μ M U46619 and /or 1 μ M iloprost and without any agonist (basal) under hypoxic and normoxic condition for 24 hours. Luminescence was measured after overnight incubation using a FlexStation 3 microplate reader (Molecular Devices, CA). The assays were carried out minimum of 3-4 times each in duplicate, and the data were analyzed using PRISM software version 5 (GraphPad Software Inc., San Diego, CA). The cAMP values of the TP-IP receptors, was expressed in relative luminescence units (RLUs).

6.2.5 Flow cytometry

Cell surface expression of TP, IP and different constructs were determined using BD FACS Canto flow cytometer for both hypoxic and normoxic condition. 24 hours after transfection 1×10^5 viable cells were taken into flow cytometry tube, washed 2-3 times with FACS buffer (phosphate-buffered saline pH 7.4, containing 0.5% bovine serum albumin) by centrifugation for 4 min at 1500 rpm. The cells were incubated for 60 min on ice with 1:100 dilution of polyclonal antibodies (0.5 mg/ml), TBXA2R 119 (Cat#LS-B4842, Lifespan Biosciences, USA), and IP antibody (Item# 10005518, Cayman Chemicals, USA) that target the N-terminus of human TP and IP, respectively as labelled (TP AB and IP AB). The cells were washed 2-3 times with FACS buffer and incubated with 1:500 dilution of secondary antibody Alexa-488, in dark for 60 min on ice. The cells were washed 2-3 times with FACS buffer and resuspended in 200 μ l of FACS buffer. The fluorescence signals of 1×10^4 cells/tube were measured using single colour analysis by BD FACS Canto analyzer. The cell surface receptor expression was expressed as percentage of wild type TP, which was set to 100%. The nonspecific signal from mock-transfected cells was $10 \pm 2\%$.

6.2.6 Statistical analysis

All the data were analyzed using GraphPad Prism software 4.0. Statistical analyses were made by ANOVA wherever necessary with suitable post-hoc multiple comparison testing as appropriate.

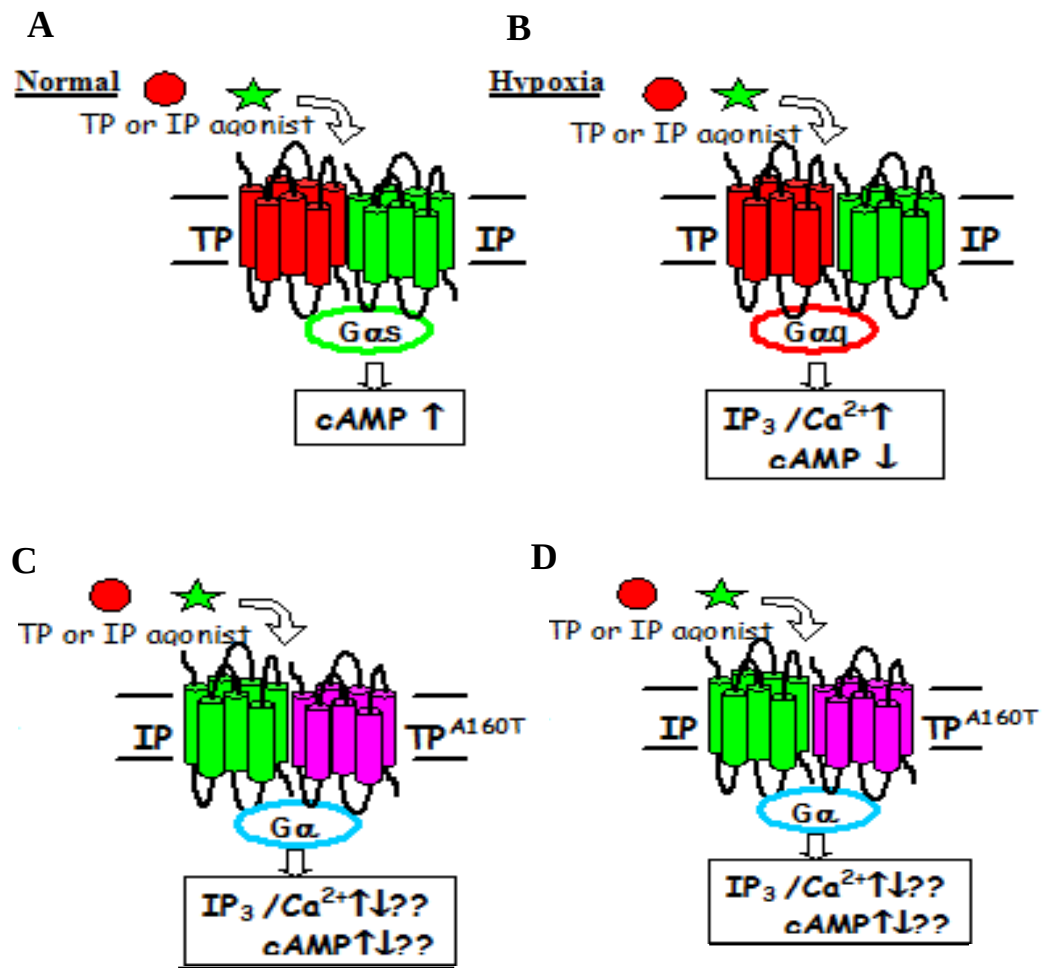


Fig. 6.1 Crosstalk between TP and IP signaling in normal (normoxic) and hypoxia. **Panel A.** In normal conditions, TP α -IP heterodimerization facilitated coupling of TP α to cAMP generation [75]. **Panel B.** In hypoxia, TP-IP cross-signaling causes a decrease in cAMP and increase in IP₃ in PSMCs [23]. The models presented in **Panel C and D** under normal and hypoxic conditions were tested in this chapter.

6.3 Results

6.3.1 Characterization of intracellular Ca^{2+} signaling

The characterization of $\text{G}\alpha_q$ -mediated signaling of the WT-TP, WT-IP and cells co-transfected with TP and IP were carried out by measuring the intracellular Ca^{2+} mobilization upon stimulation with agonist $1\mu\text{M}$ of U46619 (**Fig. 6.2A**). Co-transfection of 1x TP (1x refers to $3\mu\text{g}$ of DNA) or 1x A160T with IP and stimulation with TP agonist U46619 increased the calcium levels under hypoxic condition compared to normoxic conditions (**Fig. 6.2B**). However, co-transfection of 2x IP with TP and stimulation with $1\mu\text{M}$ of U46619 resulted in lowering the calcium response significantly under normoxic as well as hypoxic conditions (**Fig. 6.2A and 2B**).

Similarly, to check the effect of the IP agonist iloprost on TP-IP calcium signaling, the above described receptors and combinations were tested under both normoxic and hypoxic conditions. Under normoxic conditions, iloprost treatment did not cause any significant change in Ca^{2+} flux (**Fig. 6.2C**). In contrast, under hypoxic conditions co-transfection of 1x A160T with 1x IP resulted in significant increase in calcium mobilization compared to WT-TP or WT-IP (**Fig. 6.2D**). However, co-transfection of 2x IP with either TP or A160T resulted in decrease of the calcium flux under hypoxic conditions when stimulated with $1\mu\text{M}$ iloprost (**Fig. 6.2D**). For basal level calcium release for A160T and 1x IP and 1x A160T co-transfection resulted in a significant calcium increase only under hypoxic condition (**Fig 6.2E and F**). The A160T mutant showed a CAM phenotype under hypoxic condition (**Fig 6.2F**).

6.3.2 Characterization of intracellular cAMP signaling

The characterization of G α s-mediated signaling of the WT-TP, WT-IP, A160T and co-transfected receptors was carried out by measuring the intracellular cAMP produced upon stimulation with the agonists U46619 or iloprost (**Fig. 6.3**). Under normoxic condition, co- transfections of 1x TP or 1x A160T (1x refers to 3 μ g of DNA) with 1x IP or even 2 x IP (2x refers to 6 μ g of DNA) and 1 μ M iloprost treatment, increased the cAMP levels compared to WT-TP and with similar levels to IP mobilized cAMP (**Fig 6-3A**). Similar results were found in hypoxic conditions, where IP mediated cAMP was similar to co- transfected TP and /or A160T with IP (**Fig. 6.3B**).

To elucidate the role of the TP agonist U46619 on cAMP signaling, the above receptor combination were assayed. Co-transfection of 1x TP or 1x A160T with IP or 2x IP followed by treatment with 1 μ M U46619 resulted in significant increase in cAMP compared to WT-TP only for A160T-IP or A160T-2xIP and TP-2xIP combinations (**Fig. 6.3C and 3D**). Interestingly, co-transfection of 1x IP with 1x TP did not result in the increase of cAMP response significantly under both hypoxic or normoxic conditions (**Fig 6.3C and 3D**). Similarly, basal level cAMP production for any of the co-transfected receptors did not result in a significant cAMP increase (**Fig. 6.3E**).

6.3.3 Characterization of receptor expression levels

Determination of the expression levels of receptors on cell surface under both normoxic and hypoxic conditions were carried out using Flow cytometry. Compared to normoxic conditions surface expression levels of TP were reduced under hypoxic condition (**Fig. 6.4A and 4B**).

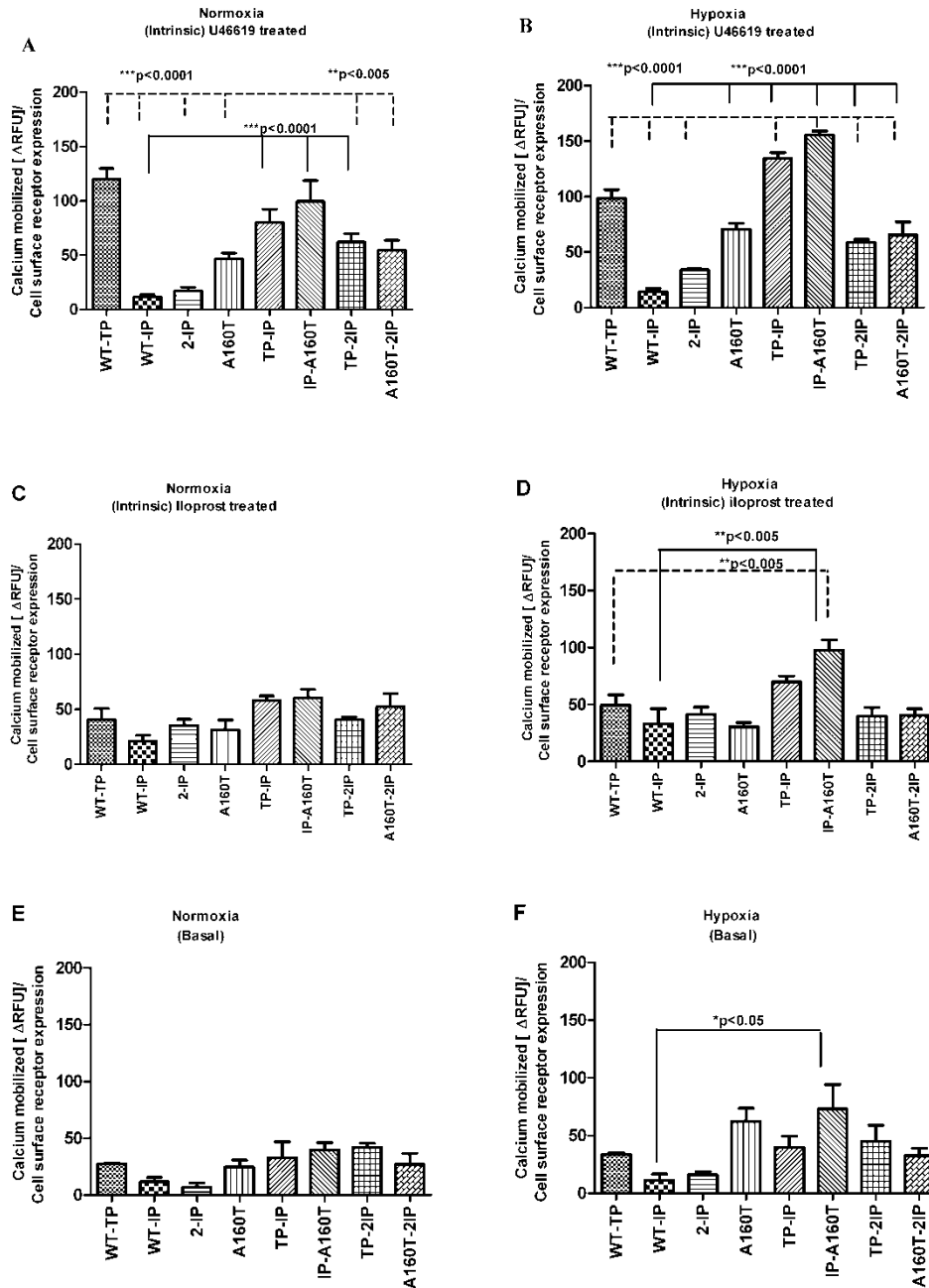


Fig. 6.2. Ca²⁺ mobilization of WT-TP, IP, A160T, and their combinations under hypoxic and normoxic conditions

Agonist-dependent calcium mobilization for WT-TP, WT-IP, A160T, TP-IP, A160T-IP, TP-2IP, and A160T-2IP after cells was treated with 1 μ M of U46619 (**Panel A and B**) or 1 μ M of iloprost (**Panel C and D**) and basal (**Panel E and F**) under normoxic and hypoxic condition. Ca²⁺ mobilized (Δ RFU) was corrected for receptor expression levels using FACS data. A one way ANOVA with *tukey's post hoc* test between WT-TP and/ or WT-IP with different transfected combination of receptors showed a significant difference at * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.0001$

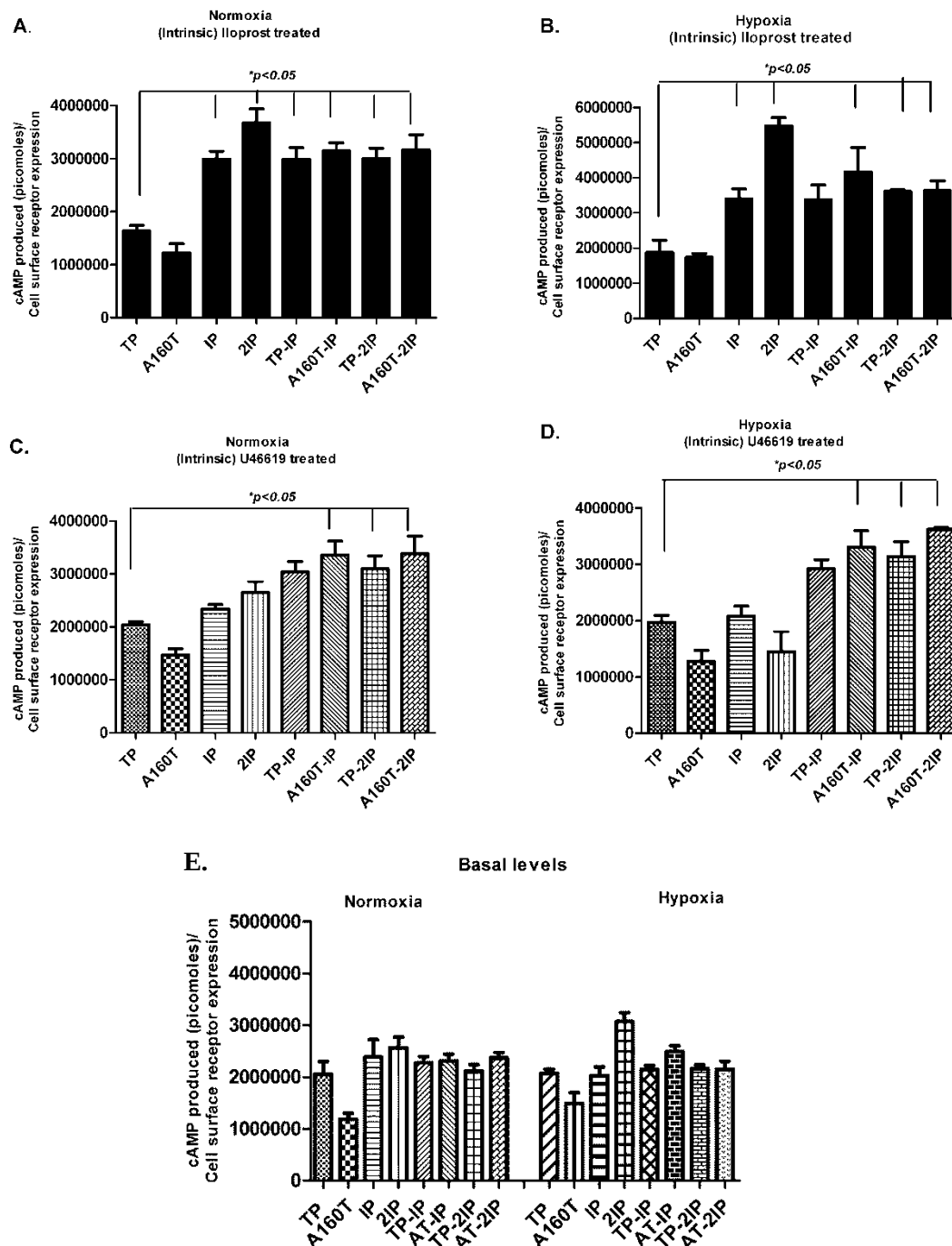


Fig 6.3. cAMP signaling of WT-IP, WT-TP, A160T and their combinations in hypoxic and normoxic conditions.

Agonist-dependent cAMP mobilization for WT-TP, WT-IP, A160T, TP-IP, A160T-IP, TP-2IP, and A160T-2IP after cells was treated with 1 μ M of iloprost (**Panel A and B**) or 1 μ M of U46619 (**Panel C and D**) and basal (**Panel E**) under normoxic and hypoxic condition. cAMP mobilized was corrected for receptor expression levels using FACS data. A one way ANOVA with *tukey's post hoc* test between WT-TP and different transfected combination of receptors showed a significant difference at $*p < 0.05$.

However, IP expression levels were unaffected in hypoxia. Interestingly, co- transfection of TP with IP showed an overall increase of both the receptors compared to TP or IP alone under hypoxia (**Fig. 6.4A and 4B**). However, no such predominant effect was observed in normoxic conditions.

6.4 Discussion

The interplay between TP and IP in maintaining cardiovascular homeostasis is now well established and dimerization has been shown to play important roles [70, 75]. It has been shown in different studies that IP dependent sequestration of TP did not depend on second messengers but instead proceeds through formation of TP α /IP heterodimer [70]. Under hypoxic conditions recent studies have shown that TP α undergoes internalization (Fedui, *et al.*, 2014 unpublished data). In this study TP alone under hypoxia also showed poor surface expression of only ~60-70% compared to TP in normoxic condition (**Fig.6.4**). In previous studies under normoxic conditions, it has been proposed that different and distinct pathways contribute towards homologous regulation of TP and IP [70]. However, in the heterodimer state, regulation is biased towards one monomer which is in an activated state. Interestingly, in this present study it was found that under hypoxic condition the receptor trafficking of TP α and IP was altered compared to the normoxic condition (**Fig. 6.4**).

It has been shown previously that under normoxic conditions heterodimerization of TP with IP resulted in TP α -IP to cAMP generation and rendered the TP sensitive to regulation by IP agonists [70, 75, 223]. These studies showed that co-transfection of TP or A160T with IP resulted in a significant increase in calcium under both hypoxic and

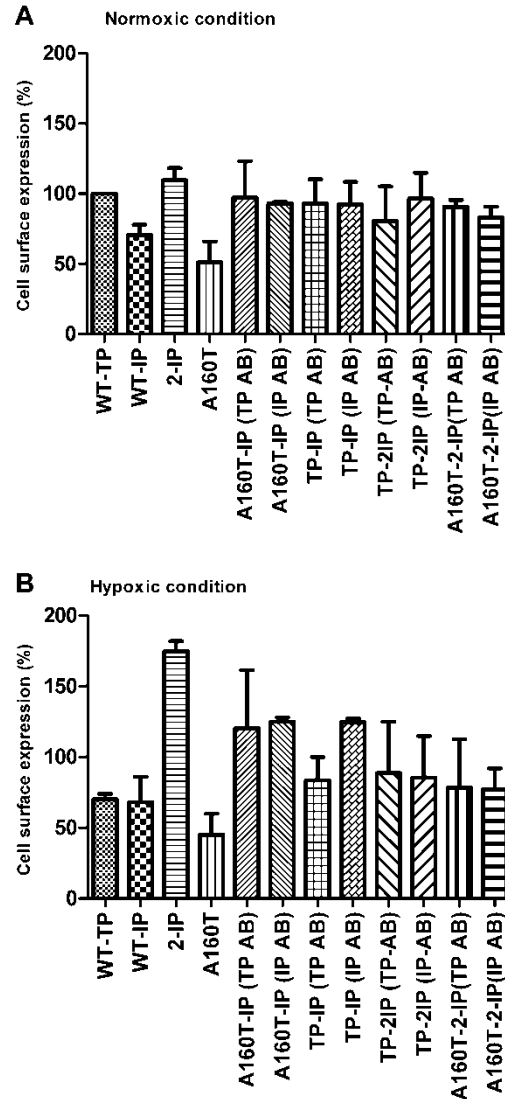


Fig. 6.4. Flow cytometry analysis of WT-TP, WT-IP and A160T.

FACS analysis were performed using polyclonal antibodies TP AB and IP AB that are specific to the N-terminal sequence between amino acids 1-15 of WT-TP and WT-IP respectively. **Panel A** represents the surface receptor expression levels in normoxic condition and **Panel B** represents the surface receptor expression levels in hypoxic condition. FACS data was normalized to WT-TP DNA of 3 μ g which was taken as 100% in normoxic condition.

normoxic conditions compared to IP or TP control. However, under normoxic conditions the amount of calcium mobilized after co- transfection of TP or A160T with IP showed no statistically significant difference compared to WT-TP alone (**Fig. 6.2A**). Interestingly, under hypoxic condition co- transfection of TP and/ or A160T with IP resulted in a statistically significant increase in calcium concentrations compared to TP or IP alone control (**Fig. 6.2B**). The observed phenomenon could be because of two possible reasons, first TP α and IP dimerization could be affected under hypoxic condition resulting in TP hyper responsiveness under hypoxia, and second explanation could be IP mediated up-regulation or co-trafficking of TP to the cell surface under hypoxic condition. Previous studies have indicated that IP mediated TP sequestration is independent of the second messenger system and would require physical association for trafficking to cell surface as single entity [70]. From this study, it was found that co- transfection of 2x IP with 1x TP or A160T could bring down the amount of calcium mobilized on TP activation by U46619 (**Fig. 6.2**). The results clearly point towards the increase in chances of IP/IP dimerization, and the resulting homodimerization of the receptors might play a role in the observed effect of decreased calcium under both hypoxia and normoxia. Further 2x IP transfection with that of either TP or A160T could not bring up the expression levels of 2x IP compared to 2x IP alone indicating towards altered receptor trafficking and hence reduced calcium mobilization.

To better understand the role of IP mediated signaling in the context of TP α /IP heterodimer, cAMP production was measured. In both normoxic and hypoxic conditions, and separate activation by both IP agonist (Iloprost) and TP agonist (U46619) on transfected TP and / or A160T with IP did not affect the cAMP production (**Fig. 6.3**). Interestingly, under hypoxic condition transfection with 2x IP resulted in a significant

increase in the amount of cAMP mobilized, and co-transfection of 2x IP with either TP or A160T could not bring it to the 2x IP levels but was never lower than that of IP mediated cAMP production. The results clearly indicate that possible dimerization could be affected under hypoxic condition, and secondly, when co-transfected together in 1:2 ratios of 1x TP and or A160T with 2x IP the number of receptors trafficked to the surface as a single entity does not get affected pointing towards proper receptor trafficking. Interestingly, the results also indicate a different signaling pathway in TP α /IP heterodimer signaling under hypoxic condition. The results will be strengthened if dimerization data can be validated under hypoxia or normoxia using biophysical approaches such as BRET or FRET.

Chapter 7

Conclusion and future directions

GPCRs have been shown to contain certain amino acids in the TM helices referred to as “signature” residues with > 70% identity which are conserved across class A GPCRs [76]. Interestingly, studies by *Liu et al.*, revealed a second set of sites in the TM helices, where amino acids with small side chains, such as Gly, Ala, Ser, Thr, and Cys, are highly conserved (>90%) when considered as a group, hence referred to as “group-conserved” residues [77]. GPCRs are designed to fold and function in a lipid bilayer environment. The group-conserved amino acids in class A GPCRs could be involved in stabilizing receptor structure-function properties not only through correct folding but also by facilitating receptor activation mechanism(s). In this thesis group-conserved residues in two class A GPCRs, β_2 -AR and TP were targeted. The two group-conserved amino acid residues on TM7, Gly315^{7.42} and Ser319^{7.46} play important structural and functional roles in the β_2 -AR. Gly315^{7.42} stabilizes Trp286^{6.48} in the inactive conformation, while Ser319^{7.46} is in the center of a hydrogen bonding network that stretches from Trp286^{6.48} to Asn322^{7.49}. The hydrogen-bonding interactions between Trp286^{6.48} and Asn322^{7.49} appear to be strongly coupled. The structurally conservative mutation to alanine of either Gly315^{7.42} or Ser319^{7.46} results in an increase in the basal activity of the receptor. From these results, it was proposed that the group-conserved residues on TM7 along with conserved structural waters create an extended interface between the TM helices that facilitates the motion of TM6 relative to TM7 upon receptor activation. These changes would be encompassed within the global toggle switch mechanism proposed by Schwartz and colleagues [224].

Next, the work on the two group-conserved residues in TM4 of TP Ala160^{4.53} and Gly164^{4.57} shows that they perform a structural role by enhancing interhelical packing, and in stabilizing the inactive conformation of the receptor. A bulky residue at position 4.57 as

in the G164V mutant perturbed helical packing causing decreased protein stability but also influenced the interaction between amino acids on ECL2 and the antagonist. This result implies that Gly164^{4,57} indirectly affects ligand binding in TP. Interestingly, these results also reveal the molecular mechanism of the nsSNP variant A160T. The nsSNP A160T has a destabilizing effect on the TP protein structure and causes the receptor to adopt an active state conformation. Together, the results presented above indicate that the study of naturally occurring mutations in conjunction with site-directed mutagenesis can serve as powerful tools in assessing the importance of regional helix-helix interactions in GPCRs and other integral membrane proteins.

Receptor activation has been shown to activate downstream G-protein signaling. Structural studies using TP-IP chimeras to delineate the binding pocket of G-proteins were carried out. The studies with TP-IP chimeras allowed the delineation of the interacting domains on the prostanoid receptors TP, IP and their associated G- proteins. Detailed analysis of the critical regions responsible for the specificity of prostanoid receptor-G protein interactions provided a framework for understanding the fidelity of prostanoid signaling and the creation of novel tools for drug discovery. In the absence of crystal structures of GPCR-interacting protein complexes other than β_2 -AR -Gs complex, the chimeric receptors could be useful tools for identifying binding sites and provide mechanistic insights for GPCR modulating proteins such as β arrestins and GRKs under different signaling states. The chimeras could be used as bait to identify novel regulatory proteins in prostanoid receptors.

Over the last few years there has been a flurry of GPCR crystal structures reported. However not a single structure from the important class of prostanoid receptors was

elucidated thus far. In this thesis, purification of TP and A160T genetic variant of TP were pursued. WT-TP and the A160T CAM using the HEK293S-TetR-inducible system showed expression levels of up to 4-fold higher compared to the transient expression system. Homogenous glycosylation was observed when expressed in the HEK293S (GnTI⁻)-TetR cell line. Using tetracycline inducible HEK293S system, A160T was expressed at high levels and CD spectropolarimetry was pursued on the purified CAM receptor. The CD spectra showed that the loss of thermal stability of the A160T mutant is due to the subtle changes in the secondary structure of the A160T protein.

This is the first study to report on the successful high-level expression, purification and biophysical characterization of a naturally occurring, diffusible ligand activated GPCR variant that exhibits constitutive activity. The yield of the functional receptor obtained from the one-step purification now makes it feasible to purify WT-TP and CAMs in milligram amounts. Furthermore, additional approaches can be used to improve the stability and yield of TP and mutants. These include addition of antagonist to cultures expressing TP, or agonist to cultures expressing the CAMs, and inclusion of ligands during the purification process might also increase stability of the protein. Scale-up of the expression using suspension cultures in a bioreactor using established techniques should allow production of TP, and importantly the A160T CAM at levels suitable for the application of crystallography.

Work in this thesis showed that TP genetic variant A160T was constitutively active. The CAMs by virtue of their higher basal activity are known to be pharmacologically important tools, which aid in distinguishing between antagonists and inverse agonists. The

results using A160T CAM show that SQ 29,548 and Ramatroban are inverse agonists for TP towards IP_3 signaling and platelet activation. Given the crucial role played by TP in maintaining vascular homeostasis, SNPs such as A160T in TP that are also CAMs, can have significant clinical manifestations. In light of the therapeutic relevance, identification of inverse agonists for TP could be beneficial in clinical applications. As indicated in the introduction there are a number of genetic variants in prostanoid receptors whose structure-function and possible role in diseases has not yet been elucidated. With the recent surge in pharmacogenetic studies and emphasis on personalized medicine it will be worthwhile to see the effect of nsSNPs in human patient samples or in knock-in or knock-out mouse models.

Growing evidence suggests that GPCRs now can readily form dimers or higher order complexes. To understand the role of nsSNPs in dimerization and under stress conditions such as hypoxia, functional studies were carried out on TP, A160T and IP. The results clearly indicate that possible dimerization could be affected under hypoxic condition. Furthermore, when co-transfected together in 1:2 ratios of 1x TP or A160T with 2x IP, the number of receptors trafficked to the surface as a single entity gets affected pointing towards improper receptor trafficking. Interestingly, the results also indicate a different signaling crosstalk between the A160T and IP under hypoxic condition. The results will be strengthened if the proposed dimerization model can be validated under hypoxia and normoxia using biophysical approaches using BRET or FRET. With the growing interest in the pharmaceutical industry to retest some old drugs as allosteric modulators, dimerization studies hold enormous potential not only among the prostanoid receptors but across the GPCR superfamily.

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