# Biased suppression of TP homodimerization and signaling through disruption of a TM GxxxGxxxL helical interaction motif

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**Abstract Thromboxane $A_2$ ($TXA_2$) contributes to cardiovascular disease (CVD) by activating platelets and vascular constriction and proliferation. Despite their preclinical efficacy, pharmacological antagonists of the $TXA_2$ receptor (TP), a G protein-coupled receptor, have not been clinically successful, raising interest in novel approaches to modifying TP function. We determined that disruption of a GxxxGxxxL helical interaction motif in the human TP's (α isoform) fifth transmembrane (TM) domain suppressed TP agonist-induced Gq signaling and TPα homodimerization, but not its cell surface expression, ligand affinity, or Gq association. Heterodimerization of TPα with the functionally opposing prostacyclin receptor (IP) shifts TPα to signal via the IP-Gs cascade contributing to prostacyclin's restraint of $TXA_2$ function. Interestingly, disruption of the TPα-TM5 GxxxGxxxL motif did not modify either IP-TPα heterodimerization or its Gs-cAMP signaling. Our study indicates that distinct regions of the TPα receptor direct its homo- and heterodimerization and that homodimerization is necessary for normal TPα-Gq activation. Targeting the TPα-TM5 GxxxGxxxL domain may allow development of biased TPα homodimer antagonists that avoid suppression of IP-TPα heterodimer function. Such novel therapeutics may prove superior in CVD compared with nonselective suppression of all TP functions with $TXA_2$ biosynthesis inhibitors or TP antagonists.**—Frey, A. J., S. Ibrahim, S. Gleim, J. Hwa, and E. M. Smyth. **Biased suppression of TP homodimerization and signaling through disruption of a TM GxxxGxxxL helical interaction motif. *J. Lipid Res.* 2013.** 54: **1678–1690.**

**Supplementary key words** G protein-coupled receptor • dimerization • transmembrane • transmembrane domain • biased antagonism • thromboxane $A_2$ receptor

Thromboxane $A_2$ ($TXA_2$) is generated by thromboxane synthase metabolism of prostaglandin $H_2$, the immediate product of cyclooxygenase (COX) action on arachidonic acid (1–3). Platelet COX-1, the only COX isoform expressed in mature platelets, is the dominant source of $TXA_2$ synthesis under normal conditions (4). Other cells, including macrophages and monocytes, contribute to $TXA_2$ generation via both COX-1 and COX-2 with the latter isozyme being particularly relevant during inflammation (2, 5). $TXA_2$ acts as a local autocrine or paracrine mediator to mediate a range of physiological and pathophysiological responses that include platelet activation, vasoconstriction, and smooth muscle cell proliferation (3, 6–10). These processes are of particular relevance to cardiovascular disease (CVD) in which $TXA_2$ generation is markedly elevated and expression of its receptor, the $TXA_2$ receptor (TP), is increased (11–13). In humans, inhibition of platelet COX-1 with low-dose aspirin is widely used for prevention of heart attack and stroke (14–17), while in mouse models of atherogenesis and injury-induced vascular proliferation or remodeling, disease severity was blunted by antagonism or deletion of the TP (8, 18, 19). Interestingly, in hyperlipidemic mice the TP antagonist was more effective in reducing atherogenesis than COX inhibition (20). This may reflect antagonism of COX-independent TP ligands, such as the isoprostanes, free radical-derived metabolites of arachidonic acid that can activate the TP in vivo (21). These, and other studies, have placed significant emphasis on the TP as a therapeutic target in CVD (8, 20, 22). Despite their potential, however, pharmacological antagonists of the TP have been

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Abbreviations: BRET, bioluminescence resonance energy transfer; COX, cyclooxygenase; CVD, cardiovascular disease; GpA, glycophorin A; GPCR, G protein-coupled receptor; HA, hemagglutinin; InosP, inositol phosphate; IP, prostacyclin receptor; mBu, milli BRET units; $PGI_2$, prostacyclin; rLuc, Renilla luciferase; TM, transmembrane; TP, thromboxane $A_2$ receptor; $TP_{WT}$, wild-type thromboxane $A_2$ receptor, $TXA_2$, thromboxane $A_2$; WT, wild type; YFP, yellow fluorescent protein.

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clinically disappointing compared with low-dose aspirin, in large part because none replicate aspirin's irreversible inhibitory effect on platelets (22–25). Most recently, the TP receptor antagonist terutroban showed comparable, but not superior, efficacy with aspirin in preventing recurrent ischemic stroke in clinical trials (26).

The TP is a cell surface G protein-coupled receptor (GPCR) that is expressed in a wide variety of tissues and cells including platelets, smooth muscle cells, endothelial cells, lungs, kidneys, heart, thymus, and spleen (27–29). In humans, but not in other species, there are two splice variants, the TPα and TPβ, which, despite reported differences in their upstream promoter use, posttranslational modifications, interacting proteins, and agonist-induced regulation, do not display significant physiological or pathophysiological divergence (22). A number of tissues express both variants (30), although TPα is the only isoform expressed in platelets (31). Research from our group and others has defined the TP's functional and regulatory pathways (31–36). Signaling via the TP can be transduced through multiple G proteins with Gq and G12/13, which stimulate respectively the phospholipase-C pathway of inositol phosphate (InosP)/intracellular calcium elevation and RhoA, appearing most relevant its biological actions (37, 38). Agonist activation of the TP leads to its internalization and degradation, although sustained agonist activation can increase TP stability and surface expression driving TP responsiveness (32).

Across the GPCR superfamily, there is substantial evidence for receptor dimerization (39, 40) and a significant contribution therein to receptor trafficking and ligand recognition, signaling, and regulation (41–44). We reported that the TP forms dimeric or oligomeric receptor complexes (45–48). In addition to homodimerization, TPα can heterodimerize with TPβ leading to enhanced isoprostane responsiveness (47). Further, we observed equal propensity for TPα to heterodimerize with the receptor for prostacyclin ($PGI_2$) (48). A predominantly COX-2-derived mediator, $PGI_2$ acts via its receptor, the prostacyclin receptor (IP), to activate the Gs-adenylyl cyclase signaling pathway causing vasodilation and inhibition of platelet activation (49). In mice, the restraint placed by the $PGI_2$-IP system on $TXA_2$-TP function limits the proliferative and platelet response to vascular injury (18) demonstrating the in vivo relevance of this interplay. Further, the elevated cardiovascular hazard in patients treated with COX-2 inhibitors can be explained by selective suppression of COX-2-derived $PGI_2$ without alteration of COX-1-derived $TXA_2$ levels (50). We determined that heterodimerization of the TP with the IP contributes to the $PGI_2$-$TXA_2$ interplay; dimerization with the IP dramatically shifts TP function from a lipid raft-excluded Gq-coupled receptor to a raft-associated Gs-coupled receptor that yields a robust Gs-cAMP response, concomitant with reduced Gq-InosP signaling to TP agonists (45, 48). Loss of this shift in TP function in individuals heterozygous for a signaling-deficient IP mutant, $IP^{R212C}$, may contribute to their accelerated CVD (51).

The importance of transmembrane (TM) helical interactions to protein structure and function is evident across multiple diverse integral membrane protein families (52, 53). Consequently, there is significant interest targeting TM domains to modulate the function of membrane-spanning proteins, including GPCRs (39, 41, 54–57). Among TM domains, the GxxxG motif, in which two glycines are separated by any three other residues, is strongly over-represented (58), highly conserved across species (59, 60), and can direct homologous or heterologous helical interactions (52, 53, 61). Neighboring residues, especially the large aliphatic residues isoleucine, valine, and leucine, appear critical to GxxxG-mediated helix-helix interactions (59, 60). In a number of proteins (62–65), including at least two GPCRs (42, 66), placement of a leucine three residues after the second glycine to create a GxxxGxxxL motif, directs for protein-protein interaction and function. We identified a GxxxGxxxL motif within the fifth transmembrane of the TPα and examined its relevance for TP function. We determined that mutation of the three critical amino acids $G_{205}$, $G_{209}$, and $L_{213}$ dramatically reduced TPα signaling via the Gq-InosP cascade without altering the receptor's cell surface expression, ligand recognition, or Gq association. TPα homodimerization was, however, significantly impaired, suggesting the normal homodimerization of the TPα is necessary for signal transduction. Strikingly, neither TPα-IP heterodimerization nor signaling of the heterodimer to Gs-cAMP in response to TP agonists was impacted by mutation of the TP-TM5 GxxxGxxxL motif indicating the specificity of this motif for TPα homodimerization. We suggest that targeting the TP-TM5 GxxxGxxxL motif may allow selective suppression of the TPα homodimer signal without altering TPα-IP heterodimer function.

## METHODS

### Constructs

Hemagglutinin (HA)-tagged human IP and TPα cloned into the mammalian expression vector pcDNA3 (Invitrogen, CA) were as described previously (48). QuikChange site-directed mutagenesis (Stragagene, CA) was used to replace $G_{205}$ and $G_{209}$ with leucines, a small-to-large replacement that disrupts helix-helix interaction (62, 64, 65). We replaced $L_{213}$ with a tyrosine based on the studies the GxxxGxxxL motif in the β2-adrenergic receptor (42). The resulting mutant was termed $TP_{L205,L209,Y213}$. HA-tagged IP, TPα, and $TP_{L205,L209,Y213}$ were fused at their C termini to either Renilla luciferase (rLuc) or yellow fluorescent protein (YFP), previously described (67). Briefly, the stop codon was removed by PCR and each stopless construct cloned into prLuc-N3(h) (Perkin Elmer, MA) and pEYFP-N1 (Clontech, CA) plasmids in frame with the fusion protein start site. All sequences were verified by DNA sequencing.

### Cell culture and transfection

Cell lines were from the American Type Tissue Culture Collection (Rockville, MD). HEK 293 cells were maintained as described previously (48); Meg-01 cells were grown in RPMI-1640 (Invitrogen) containing 10% fetal bovine serum and 1% penicillin-streptomycin. Transient transfections were performed using FuGENE 6 (Roche Applied Science, IN) for HEK 293 cells (2 μg total DNA), as previously described (48) or for Meg-01s (3 μg total DNA) by nucleofection using an Amaxa Nucleofector™ II and Nucleofector™ Kit C (Lonza, NJ) per the manufacturer's instructions.

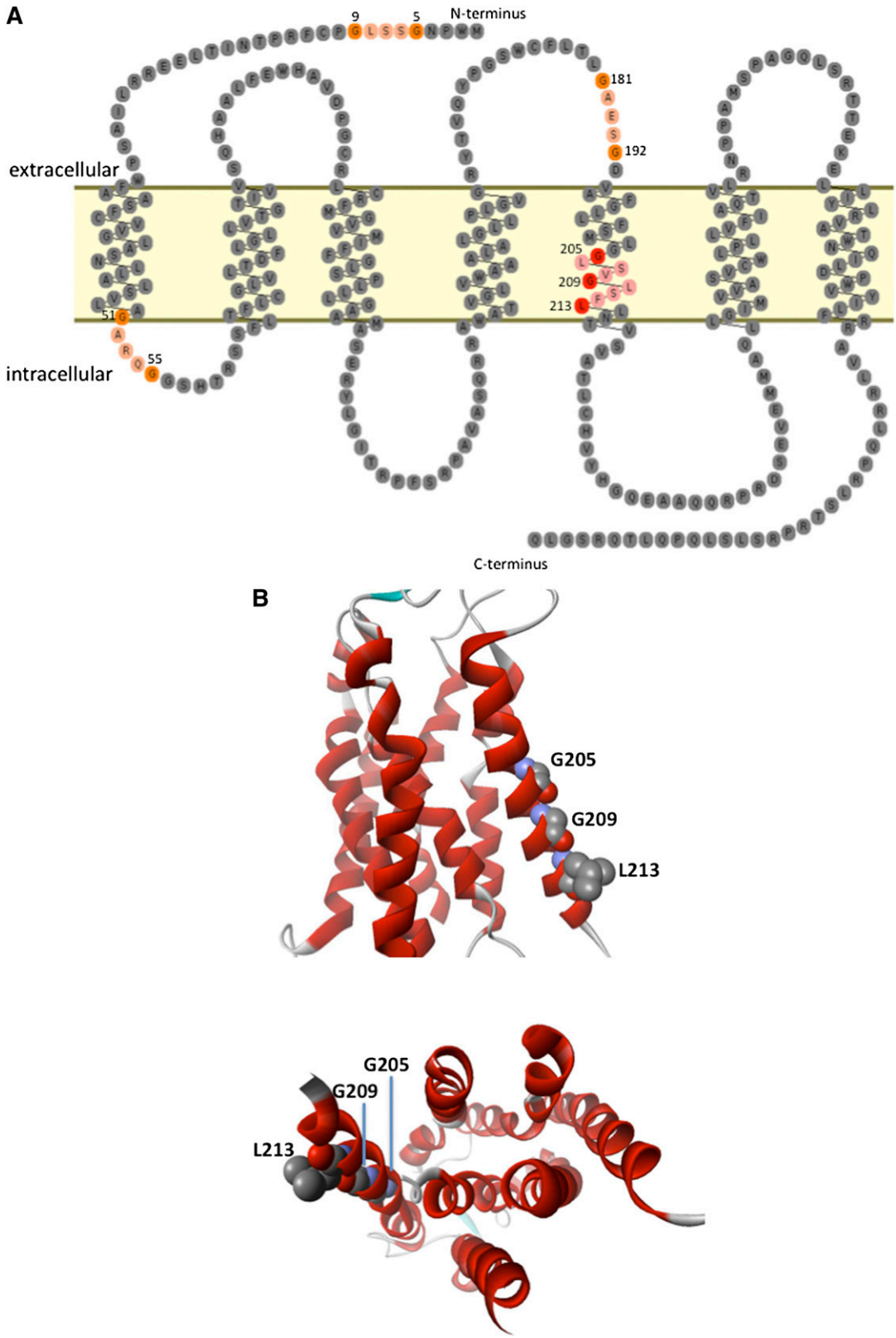


**Fig. 1.** A: Snake plot of the human TPα. GxxxG motifs in the N terminal, first intracellular and second extracellular domains are indicated in orange. The TM5 GxxxGxxxL motif under investigation in our study is highlighted in red. B: Homology modeling (SWISS-MODEL) of the human TPα based on a 2.8 Å crystallographic bovine rhodopsin template. Relative positions of $G_{205}$, $G_{209}$, and $L_{213}$ are highlighted. Each appeared to face the lipid bilayer aligned on one side of TM5.

DNA levels were equalized in all transfections using empty pcDNA3 vector. Assays were performed 48 h after transfection.

### Bioluminescence resonance energy transfer assay

Dimerization of rLuc and YFP fused receptors was examined by measuring bioluminescence resonance energy transfer (BRET) from a donor (rLuc-fused) receptor to an acceptor (YFP-fused) receptor following addition of substrate for rLuc (coelenterazine H; Molecular Probes, Life Technologies, NY). In BRET saturation experiments cells were transfected with a fixed amount of rLuc receptor (0.25 μg) together with increasing amounts of YFP receptor (0.125–1.75 μg). In BRET competition assays increasing amounts of HA-tagged competitor receptor were cotransfected together with a fixed ratio (1:7) of receptor-rLuc + receptor-YFP. BRET measurements were performed essentially as described previously (45). Briefly, cells were harvested (phenol red-free Hank's Balanced Salt Solution containing 0.02% EDTA), redistributed in 96-well plates (black, clear; 100,000 cells/well) and maintained at 37°C. Total YFP (Ex485 nm, Em555 nm) was first collected using a luminescence multi-plate reader (VICTOR3, Perkin Elmer) and calculated as fold over basal. Using the same plate, donor (485 nm) and acceptor (555 nm) emissions were gathered sequentially from each well, following addition of coelenterazine H (5 μM in $Ca^{2+}$-free phosphate-buffered saline rested for 30 min at room temperature before use). Milli BRET units (mBu) were calculated as the ratio of Em555 over Em485 nm corrected for cells expressing the rLuc receptor alone, and multiplied by 1,000.

### Cell surface expression of the TP

HEK 293 and Meg-01 cells were transfected with HA-tagged wild-type TP ($TP_{WT}$) or $TP_{L205,L209,Y213}$. Cells were harvested into ice-cold FACS buffer (DPBS containing 1% BSA and 0.1% sodium azide). Cell suspensions were stained with anti-HA mouse IgG1 (monoclonal 16B12) conjugated to Alexa Fluor® 488 (Invitrogen, CA) for 30 min prior to washing. Median fluorescence intensity (MFI) was collected using a flow cytometer as a measure of cell surface expression.

### Measurement of second messenger generation

Measurement of intracellular IP1 or cAMP was performed using the IP-One Tb kit (Cisbio Bioassays, MA) or LANCE cAMP 384 kit (Perkin Elmer, MA), respectively, according to the manufacturer's instructions. Cells were stimulated with or without the TP agonist U46619 (Cayman Chemicals, MI) for 1 h.

### Radioligand binding

HEK 293 cells, transfected with HA-tagged $TP_{WT}$ or $TP_{L205,L209,Y213}$ in poly-L-lysine-coated 12-well plates, were washed with radioligand binding buffer (HBSS with 2% BSA). $^{3}$H-SQ 29548 was held constant at 0.25 nM and competing ligands were added 5 min prior to the radioligand. After 60 min at 37°C, cells were washed with ice-cold binding buffer to remove unbound ligand, lysed with 1 M NaOH for 30 min at 37°C, and radioactivity counted by scintillation counting.

### Immunoprecipitation and immunoblotting

HA-tagged $TP_{WT}$ or $TP_{L205,L209,Y213}$ were immunoprecipitated from transfected HEK 293 cells using the Pierce HA Tag IP/Co-IP kit (Thermo, IL), according the manufacturer's instructions. Eluted proteins were resolved via NuPAGE electrophoresis (Invitrogen, CA) under reducing conditions. HA-tagged $TP_{WT}$ or $TP_{L205,L209,Y213}$ were visualized with anti-TP (Cayman Chemicals, MI; 1:100) while immunoprecipitated Gqα was visualized with anti-Gq/11α (Millipore, CA; 1:1000). Antigen-antibody complexes were revealed using horseradish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch, PA; 1:10,000) and visualized by enhanced chemiluminescence (ECL Plus, GE Healthcare/Amersham, NJ). Quantification of proteins was by densitometry.

### Receptor modeling

The human hTPα sequence was aligned with solved crystal structures, bovine rhodopsin (OPSD, UniProt P02699) and the human β2-adrenergic receptor (ADRB2, UniProt P0755) in ClustalW. Both the PAM250 and BLOSUM algorithms indicated closer alignment of hTPα to align more closely with OPSD (similarity score 30.16) than with ADRB2 (33.48). Each bundle of seven transmembrane α-helices was therefore based on a 2.8 Å crystallographic bovine rhodopsin template (1HZX) (68) to using the internet-based protein-modeling server, SWISS-MODEL (GlaxoSmithKline, Geneva, Switzerland), and energy minimized using the Gromos96 force field in DeepView. Extracellular and cytoplasmic loop regions were manually constructed, built according to JPred consensus, and energy minimized using the NAMD molecular dynamics simulator.

### Statistical analysis

Data were analyzed using GraphPad Prism software. Comparisons were made using a one-sample *t*-test or by ANOVA suitable post hoc multiple comparison testing as appropriate.

## RESULTS

### A GxxxGxxxL motif is located in the fifth transmembrane of the TPα

Analysis of the TPα amino acid sequence revealed a GxxxGxxxL motif in TM5, $G_{205}LSVG_{209}LSFL_{213}$ (**Fig. 1A**).

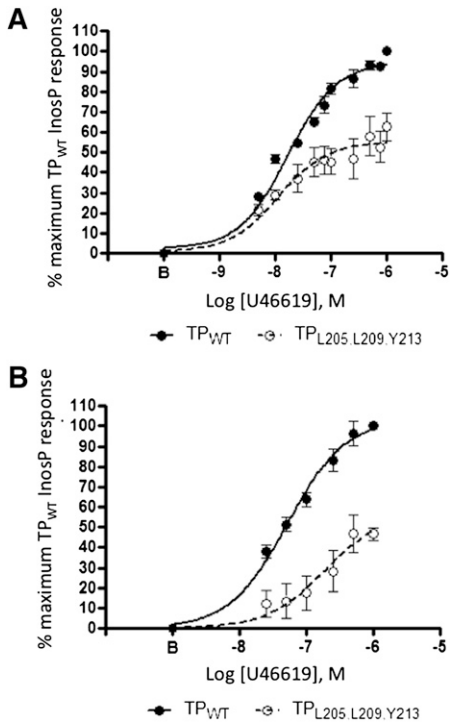


**Fig. 2.** InosP signaling through WT and mutant TP. A: Maximal InosP generation was reduced by 25 ± 5% ($P < 0.0001$) in $TP_{L205,L209,Y213}$ (open circles) compared with $TP_{WT}$ (closed circles) transfected HEK 293 cells. There was no significant change in $EC_{50}$. B: Maximal InosP generation was reduced by 50 ± 7% ($P < 0.01$) with a significant rightward shift in $EC_{50}$ ($P < 0.05$), in $TP_{L205,L209,Y213}$ (open circles) compared with $TP_{WT}$ (closed circles) transfected Meg-01 cells. Data are percent of maximum response (in $TP_{WT}$) and are mean ± SEM, n = 4–6.

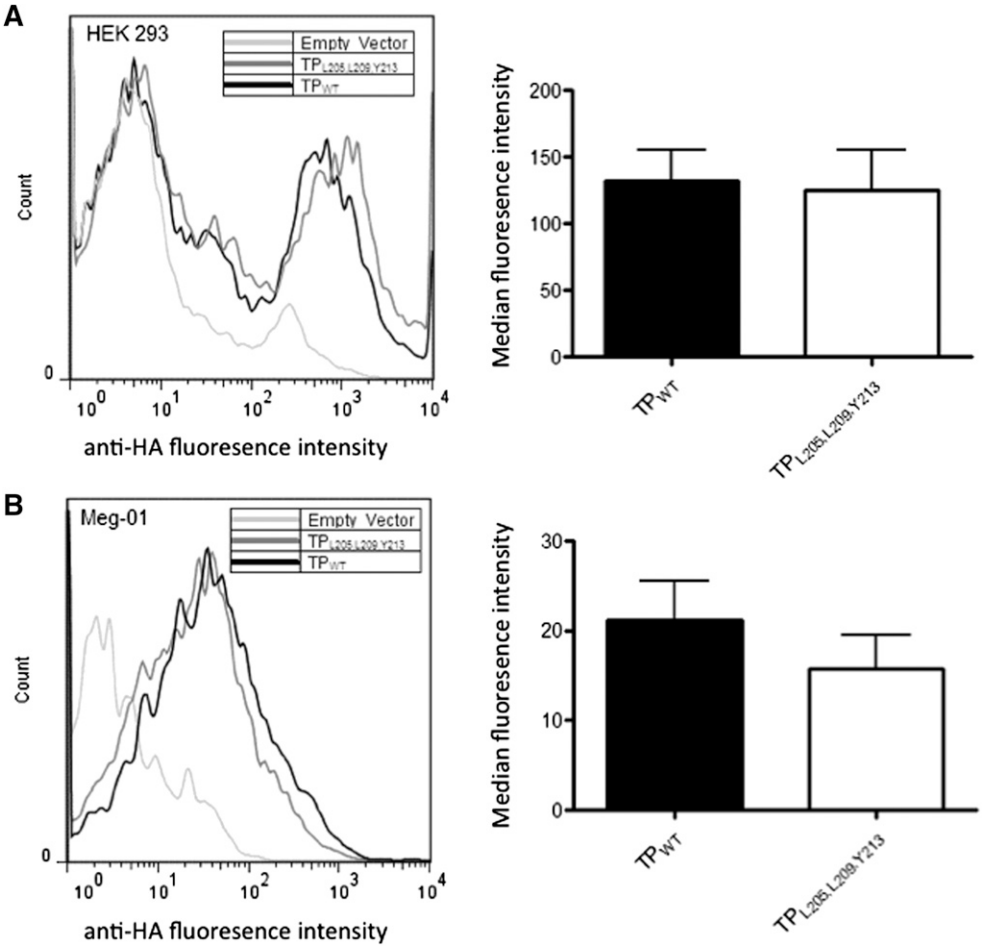


**Fig. 3.** Surface expression of WT and mutant TP. A: HEK 293 cells or (B) Meg-01 cells were transfected with N-terminal HA-tagged $TP_{WT}$ or $TP_{L205,L209,Y213}$ and surface HA quantified by flow cytometry as a measure of surface receptor expression. Left panels show representative histograms taken at one sitting using identical settings; right panels show the median fluorescent intensities (mean ± SEM, n = 7). There was no significant difference in surface expression of $TP_{WT}$ versus $TP_{L205,L209,Y213}$ in either cell model.

Additional GxxxG motifs were identified in N terminus ($G_5SSLG_9$), first intracellular loop ($G_{51}ARQG_{55}$), and second extracellular loop ($G_{188}AESG_{192}$). Given that a TM GxxxGxxxL motif has been implicated in the function of at least two GPCRs (42, 66), we chose to focus further on the $G_{205}LSVG_{209}LSFL_{213}$ domain. Three-dimensional homology modeling of the TP revealed an outward-facing orientation of $G_{205}$, $G_{209}$, and $L_{213}$ (Fig. 1B) in TM5 indicating that this domain is appropriately positioned for protein-protein interaction within the membrane. To define the functional relevance of the TM5 GxxxGxxxL motif in the TP we employed site-directed mutagenesis to replace $G_{205}$ and $G_{209}$ with leucines and $L_{213}$ with a tyrosine to generate $TP_{L205,L209,Y213}$.

### Disruption of the TM5 GGL motif suppressed TP function and but did not alter receptor surface expression

We first measured the ability of the $TP_{WT}$ and $TP_{L205,L209,Y213}$ to transduce a signal via the phospholipase C/InosP pathway in response to the thromboxane mimetic U46619. In transiently transfected HEK 293 cells, the maximal signaling capacity of $TP_{L205,L209,Y213}$ was significantly reduced by ~25% compared with $TP_{WT}$ transfected cells, although there was no significant change in $EC_{50}$ (**Fig. 2A**). Depressed signaling via the $TP_{L205,L209,Y213}$ was also evident in Meg-01 cells (Fig. 2B), which are platelet-like cells that serve as a closer approximation of the TP's normal physiological environment, with an ~50% reduction in InosP generation and a significant rightward $EC_{50}$ shift. Thus, disruption of the TM5 GxxxGxxxL motif markedly suppressed TP response to agonist.

We examined whether this loss of receptor responsiveness reflected simply reduced cell surface expression of the mutant receptor. Cell surface expression of the $TP_{WT}$ or $TP_{L205,L209,Y213}$, both tagged at their N terminus with the HA epitope tag, was examined by flow cytometry in transfected HEK 293 or Meg-01 cells. No significant difference in cell surface receptor levels, as measured by median surface HA fluorescence intensity, was apparent between $TP_{WT}$ and $TP_{L205,L209,Y213}$ transfectants in either cell type (**Fig. 3**). Thus, disruption of the TM5 GxxxGxxxL motif did not substantially modify receptor processing to the surface, indicating that the signaling deficit we observed

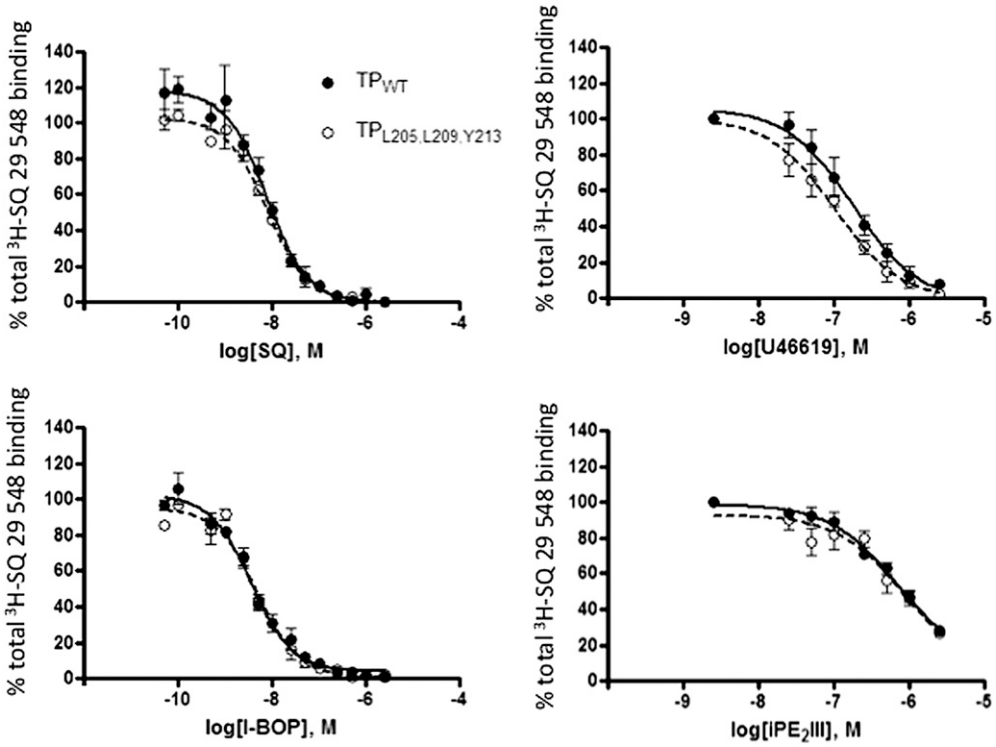


**Fig. 4.** Displacement of $^3$H-SQ 29,548 by various ligands. Displacement of $^3$H-SQ 29,458 (TP antagonist) by SQ 29,548, the TP agonists U46619 or I-BOP or the isoprostane $iPE_2III$ in HEK 293 cells transiently transfected with $TP_{WT}$ (closed circles) or $TP_{L205,L209,Y213}$ (open circles). Data are expressed as percent of total binding (no displacer) and are mean ± SEM (n = 3–8). No significant change in $K_i$ values for displacement between $TP_{WT}$ and $TP_{L205,L209,Y213}$ was seen for any TP ligands used.

could not be explained by quantitative changes in the receptor population on the plasma membrane.

### Ligand affinity and Gq association are not modified by mutation of the TM5 GxxxGxxxL motif

We considered whether suppressed agonist-induced signal transduction in $TP_{L205,L209,Y213}$ reflected a change in ligand binding leading to reduced agonist affinity. Intact HEK 293 cells expressing either $TP_{WT}$ or $TP_{L205,L209,Y213}$ were labeled with a single concentration of $^3$H-SQ 29,548 and displacement examined for two TP agonists, U46619 ($K_i$ = 90 nM for $TP_{WT}$ vs. 52 nM for $TP_{L205,L209,Y213}$) and IBOP ($K_i$ = 1.8 nM for $TP_{WT}$ vs. 2.5 nM for $TP_{L205,L209,Y213}$), or by unlabeled SQ 29,548 ($K_i$ = 4 nM for both $TP_{WT}$ and $TP_{L205,L209,Y213}$) as a reference. No significant difference in displacement was evident between the WT and mutant receptors. We also examined an isoprostane, $iPE_2III$ ($K_i$ = 334 nM for $TP_{WT}$ vs. 403 nM for $TP_{L205,L209,Y213}$), a free radical-generated metabolite of arachidonic acid that can activate the TP in vivo (21), and again saw no difference in radioligand displacement (**Fig 4**). Thus, disruption of the TM5 GxxxGxxxL motif did not alter the receptor ligand binding properties.

We considered also whether disruption of the TM5 GxxxGxxxL motif interferes with the association of the TPα to its effector, Gq, leading to suppressed signaling. As for other GPCRs, association of the G protein with the TP in the inactive conformation provides a high affinity state for agonist (69, 70). In displacement analyses, we detected no change in the $K_i$ for either of the TP agonists U46619 or IBOP, arguing against dissociation of the $TP_{L205,L209,Y213}$ from Gq. Further, comparable levels of Gq coimmunoprecipitated

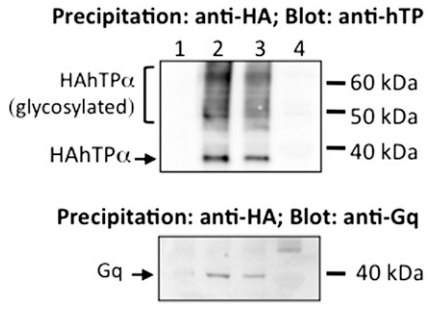


**Fig. 5.** Coimmunoprecipitation of Gq with HA-$TP_{WT}$ or HA-$TP_{L205,L209,Y213}$. Lysates from HEK 293 cells transfected with empty pcDNA3 (lane 1), HA-$TP_{WT}$ (lane 2), or HA-$TP_{L205,L209,Y213}$ (lane 3) were subjected to immunoprecipitation with anti-HA. In lane 4 lysate is from *Escherichia coli* expressing an unrelated HA-tagged control (HA-GST-PI3 kinase-SH2 domain; supplied by the manufacturer). The upper blot was stained with anti-TP antibody. Molecular species corresponding to unglycosylated TP and differentially glycosylated TP are indicated. The lower blot was probed with an anti-Gq antibody. A representative experiment, which was repeated with similar results, is shown. Densitometric quantification of Gq relative to HA-TP showed no difference between $TP_{WT}$ and $TP_{L205,L209,Y213}$ transfected cells.

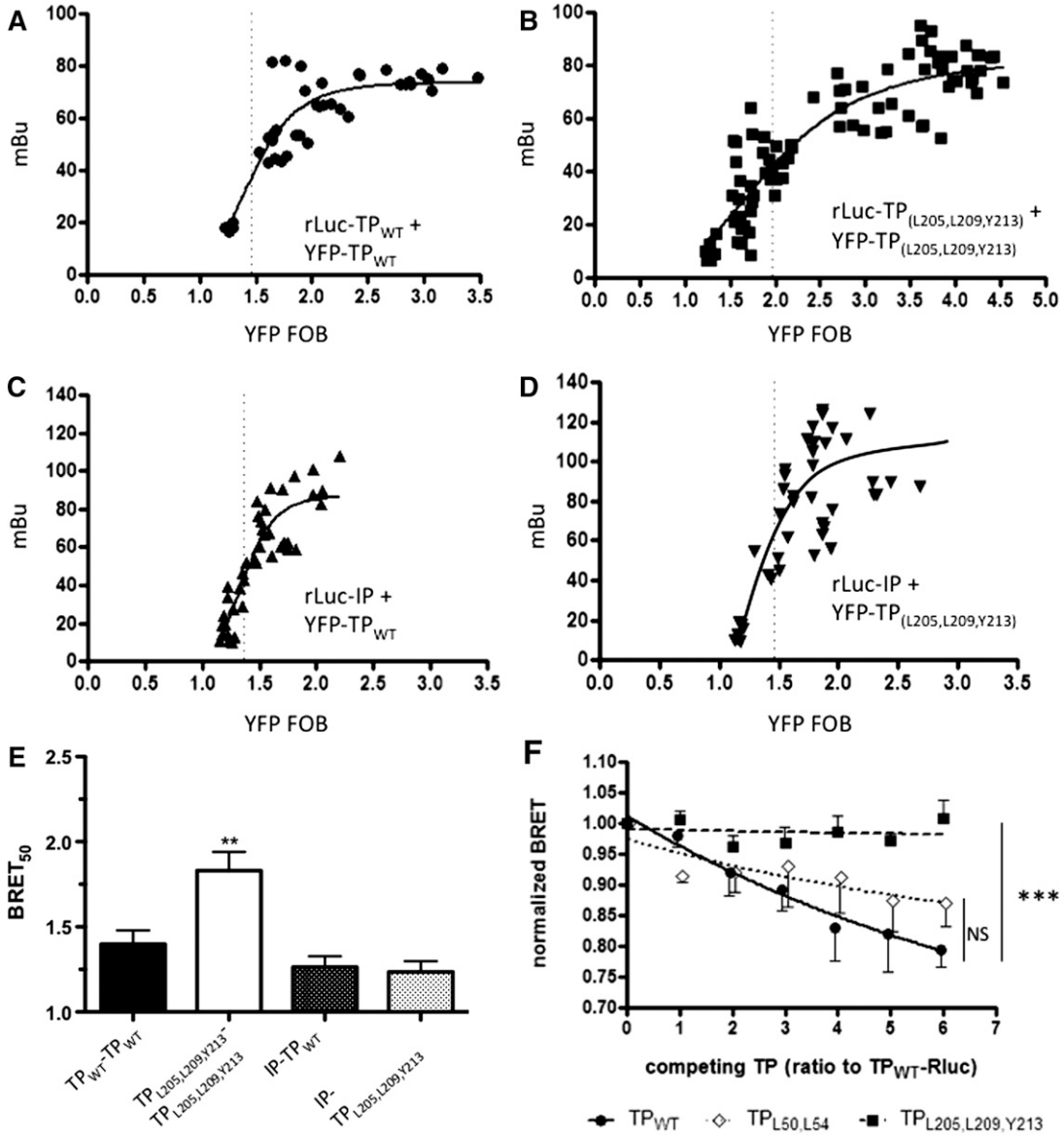


**Fig. 6.** Homo- and heterodimerization of $TP_{WT}$ and $TP_{L205,L209,Y213}$ by BRET. Saturable BRET was observed for rLuc- (donor, 0.25 μg) + YFP- (acceptor, 0.125–0.75 μg) fused pairings of (A) $TP_{WT}$-$TP_{WT}$, (B) $TP_{L205,L209,Y213}$-$TP_{L205,L209,Y213}$ (C) $IP_{WT}$-$TP_{WT}$ or (D) $IP_{WT}$-$TP_{L205,L209,Y213}$ in transiently transfected HEK 293 cells. Representative experiments are shown. Data are mBu plotted against fold over basal total YFP emission (a measure of YFP-fused acceptor receptor expression). Individual $BRET_{50}$ values are indicated by the dotted gray lines. E: $BRET_{50}$ values were calculated from 4–6 individual BRET saturation experiments. Data are mean $BRET_{50}$ values ± SEM from n = 4–6. $^{**}P < 0.005$ relative to all other data sets. F: Competition for BRET in rLuc-$TP_{WT}$ + YFP-$TP_{WT}$ (constant 1:7 ratio) transfected HEK 293 cells by cotransfection with HA-$TP_{WT}$, HA-$TP_{L205,L209,Y213}$ or HA-$TP_{L51,L54}$. Data are normalized to BRET in rLuc-$TP_{WT}$ + YFP-$TP_{WT}$ transfected cells without cotransfection of a competing receptor (set to 1) and are mean ± SEM of n = 3–4. $^{***}P < 0.0001$; NS, not significant.

with either HA-tagged $TP_{WT}$ or HA-tagged $TP_{L205,L209,Y213}$ in HEK 293 transfectants (**Fig 5**). Taken together these analyses indicate that mutation of the TM5 GxxxGxxxL motif in TPα allows normal formation of the high affinity receptor-Gq complex at the cell surface.

### Disruption of the TM5 GxxxGxxxL motif modifies TP homodimerization

We reported previously that, similar to other GPCRs, the TP physically associates to form homodimers (32, 47, 48). The molecular determinants of TPα homodimerization have not been defined; similarly, the precise role homodimerization plays in TPα expression and function also remains unclear. However across GPCR studies, one or more TMs have been frequently implicated in dimer formation and function (54, 55, 60). Given the outward-facing orientation of the TP-TM5 GxxxGxxxL motif, thus positioned for intermolecular protein interaction, we examined whether homodimerization was modified in the $TP_{L205,L209,Y213}$ mutant. $TP_{WT}$ and $TP_{L205,L209,Y213}$ were fused at their C termini to either rLuc (energy donor) or YFP (energy acceptor) and energy transfer quantified as a measure of dimerization. In saturation experiments, expression of the donor-tagged receptor is held steady and expression

of the acceptor-tagged receptor (which is quantified independently as fold over basal YFP emission) is gradually increased. A saturable BRET curve indicates a specific interaction of the two protomers to form a dimer while the concentration of acceptor at which the BRET signal reaches 50%, the $BRET_{50}$, reflects the affinity of individual promoters for each other (71) (Fig 6A–D). We determined that although $TP_{L205,L209,Y213}$ retained the capacity to dimerize, the $BRET_{50}$ for $TP_{L205,L209,Y213}$ homodimerization was significantly right shifted ($BRET_{50}$ = 1.83 ± 0.1, n = 5) compared with that of $TP_{WT}$ homodimerization ($BRET_{50}$ = 1.4 ± 0.08, n = 4), indicating reduced efficiency in formation of the homodimer when the TM5 GxxxGxxxL motif was disrupted (**Fig. 6E**). To confirm impaired homodimerization of the mutant receptor, BRET was measured in HEK 293 cells expressing a fixed ratio of rLuc-$TP_{WT}$ + YFP-$TP_{WT}$ (1:7) and competition by unfused $TP_{WT}$ or $TP_{L205,L209,Y213}$ examined. As expected, $TP_{WT}$ efficiently competed for the interaction of rLuc-$TP_{WT}$ and YFP-$TP_{WT}$ reducing the BRET signal in a concentration-dependent manner. $TP_{L51,L54}$, in which the TP-IC1 GxxxG motif $G_{51}ARQG_{55}$ was mutated, was as efficient as the $TP_{WT}$ in competition for rLuc-$TP_{WT}$-YFP-$TP_{WT}$ interaction while, in contrast, $TP_{L205,L209,Y213}$ did not alter the BRET signal confirming its relative deficiency for dimer formation (Fig 6F). Together these data indicate the importance of TP-TM5 GxxxGxxxL for efficient TPα homodimerization.

### TM5 GGL domain disruption does not modify IP-TPα heterodimerization or function

The studies thus far indicate that the GxxxGxxxL motif in TM5 of the TPα is important for efficient homodimerization and that its disruption suppresses receptor signaling. We have also reported the TP can interact with the IP, a Gs-cAMP coupled receptor, to form a heterodimer (45). When heterodimerized with the IP, the TP's microdomain localization, signal transduction, and regulation is markedly altered with reduced "normal" transduction of Gq-InosP signal in response to TP agonists and a concomitant switch to signal via the Gs-cAMP pathway in an IP-like manner (46). This signaling shift likely contributes to the restraint placed on the TP via the IP and to the increased risk of CVD in individuals heterozygous for signaling-deficient IP mutants (51). We next asked whether disruption of the TP-TM5 GxxxGxxxL motif modifies IP-TPα heterodimerization and what, if any, is the function contribution to the IP-TPα-Gs signaling in response to TP activation. Interestingly, disruption of the TM5 GxxxGxxxL motif did not modify heterodimerization of the TP with the IP; the BRET saturation curves and $BRET_{50}$ for IP-$TP_{L205,L209,Y213}$ (1.24 ± 0.06, n = 6) was indistinguishable from the IP-$TP_{WT}$ ($BRET_{50}$ = 1.26 ± 0.06, n = 6). Concordantly, U46619-induced cAMP generation, the signature "switch" in TP signaling from the Gq pathway to the Gs pathways, was not different between IP-$TP_{WT}$ and IP-$TP_{L206,L209,213}$ in transfected HEK 293 cells or MEG-01 cells (**Fig. 7**). Thus, while the TM5 GxxxGxxxL motif was critical for efficient TPα homodimerization and Gq-signaling, this motif did not contribute to IP-TPα heterodimerization or function. These data support the concept that distinct molecular interactions drive the physical association of the TPα-TPα and IP-TPα dimers and their downstream signaling.

### A TM GxxxGxxxL motif is found in numerous class A GPCRs

Given that a TM GxxxGxxxL motif was functionally relevant in at least two other GPCRs, the β2-adrenoreceptor and the α-factor yeast receptor (42, 66), we searched the SwissProt database (http://prosite.expasy.org/scanprosite/) for human GxxxGxxxL-containing GPCRs. Sixty-nine receptors were identified of which, after removal of olfactory (24 hits), taste (2 hits), and orphan (9 hits) receptors, 22 GPCRs were identified that contain one or more TM GxxxGxxxL motifs (**Table 1**). Interestingly, all but one of these 22 were class A GPCRs suggesting a particular prevalence of this motif among rhodopsin-like GPCRs.

## DISCUSSION

Protein-protein interactions are ubiquitous to biological processes and are vital for signaling complex assembly. Compared with soluble protein regions, relatively little is known about the interaction of membrane embedded proteins within lipid bilayers, although there is substantial and increasing interest in therapeutic targeting of TM interactions (61). GPCRs are characterized by their seven transmembrane spanning regions, which are capable of intra- as well as intermolecular interactions that define tertiary and quaternary receptor structure and function. The GxxxG interaction motif, first described in homodimerization of the single TM sialoglycoprotein glycophorin A (GpA), has been identified as a dominant TM motif across diverse protein families (52, 58). In GpA, as in other transmembrane proteins, residues that neighbor the GxxxG domain appear critical and are thought to provide a three-dimensional structure within the helix creating the protein-protein interface. In one particular subclass, termed "glycine zippers," a small residue (glycine, alanine, or serine) is located three positions before or after the GxxxG motif (59). More generally, large residues (isoleucine, valine, or leucine) are commonly found within one or two positions of the GxxxG pair (58), forming a groove (the glycines) and ridges (the large residues) arrangement. In the case of the TPα-TM5 GxxxGxxxL motif we determined a similar arrangement with a groove created by $S_{201}G_{205}G_{209}$ and a ridge created by leucines 203, 206, 210, and 213 (**Fig. 8**). The positioning of $L_{213}$ three residues after the GxxxG pair serves to align the GGL triplet along the same α-helix face (Figs. 1B, 8) and was observed in multiple other class A human GPCRs (Table 1) as well as α integrins (65).

To define its contribution to TPα function, we performed the following triple mutation: $G_{205}\rightarrow L_{205}$, $G_{209}\rightarrow L_{209}$, and $L_{213}\rightarrow Y_{213}$. The choice of glycine-to-leucine and leucine-to-tyrosine was based on studies of other GxxxG motifs

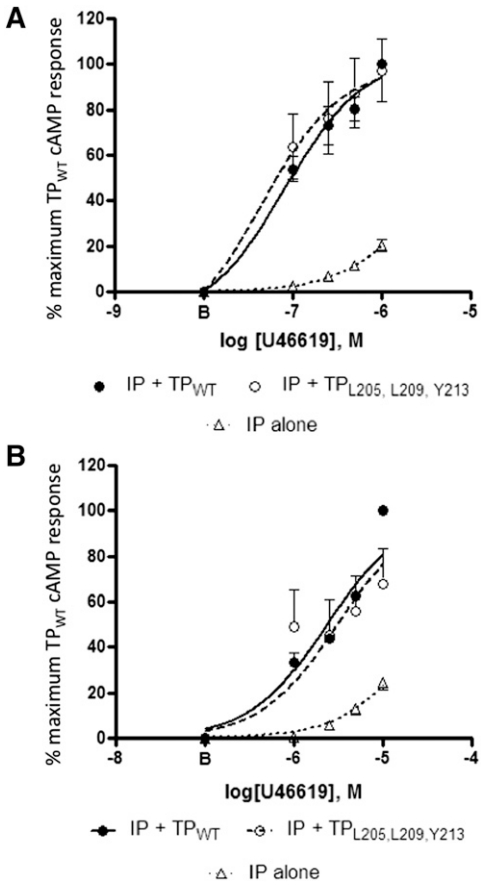


**Fig. 7.** Cyclic AMP signaling through $TP_{WT}$ or $TP_{L205,L209,Y213}$ heterodimerized with the IP. The TP agonist (U46619) simulated a robust cAMP response in (A) HEK 293 cells or (B) Meg-01 cells cotransfected with IP + $TP_{WT}$ (closed circles) or IP + $TP_{L205,L209,Y213}$ (open circles), compared with cells transfected with IP alone (open triangles). No difference in cAMP signaling was observed between IP + $TP_{WT}$ versus IP + $TP_{L205,L209,Y213}$ transfected cells in either model. Data are percent of maximum cAMP response (in IP + $TP_{WT}$ transfectants) and are mean ± SEM of n = 3–4.

(62, 64, 65), and one study of a TM GxxxGxxxL motif in the β2-AR receptor (42), and was designed to disrupt the small-small-big triplet alignment along the outer side of TM5 (see Figs. 1B, 8). Signaling of the $TP_{L205,L209,Y213}$ via the Gq-InosP cascade was markedly reduced in both transfected HEK 293 cells and Meg-01 platelet-like cells. Given the role of GxxxG motifs in helical packing (58) we considered that this loss of function might be due to improper processing of the correctly folded receptor at the cell surface. However, comparable cell surface expression of the WT and TM5 GxxxGxxxL mutant receptor was evident by flow cytometry in both in vitro models, and no alteration in processing of the fully glycosylated receptor was evident by Western blotting (Fig. 5). Further, displacement analysis using a range of TP ligands revealed no difference in the ligand binding properties of $TP_{L205,L209,213}$ compared with $TP_{WT}$, and both the mutant and WT receptor displayed high agonist affinity, consistent with normal G protein association and the comparable levels of Gq that accompanied the WT or mutant receptor in coimmunoprecipitation experiments. Together these analyses clearly indicate no major role for the TM5 GxxxGxxL motif in processing of the TPα to form a high-affinity receptor-Gq complex at the cell surface.

Homodimerization of GPCRs appears universal across the superfamily (40, 44, 72). Given the established contribution of GxxxG motifs to helix-helix interactions, the extensive evidence that TMs are critical for GPCR homodimerization and the outward-facing orientation of the $G_{205}xxxG_{209}xxxL_{213}$ triplet in TPα-TM5, we considered whether this motif contributes to TPα homodimer formation. We found that while saturable BRET was achieved, the $BRET_{50}$ for $TP_{L205,L209,Y213}$ homodimerization was significantly right-shifted compared with $TP_{WT}$. Thus, while $TP_{L205,L209,Y213}$ protomers can dimerize, they do so with a reduced affinity. Importantly, we confirmed independently that $TP_{L205,L209,Y213}$ was unable to compete for $TP_{WT}$-$TP_{WT}$ interaction, confirming the mutant's dimerization deficiency. Thus, similar to the β2-AR (42, 57) and yeast α-factor (66, 73) receptors, a TM motif GxxxGxxxL is necessary for normal efficient TPα homodimerization. Reports vary as to the contribution of homodimerization to receptor function, with substantial evidence that homodimerization is necessary for normal surface expression of the receptor (39, 66, 74–77) and that a dimeric pair coupled to a single G protein forms the basic signaling unit (43, 78, 79). Thus, dimerization-deficient GPCRs often fail to traffic normally to the cell surface, while ER retained GPCRs cause their WT counterparts to stay in the ER in a dominant negative manner (42). Indeed, in the case of β2 adrenergic receptor disruption of the TM6 GxxxGxxxL motif right shifted the $BRET_{50}$ for homodimerization coincident with reduced cell surface receptor expression (42). Our data showing normal processing, cell surface expression, and G protein association of $TP_{L205,L209,Y213}$ despite impaired dimerization suggests that for the TPα the two processes, homodimerization and cell surface expression, are independent. Alternatively, it may be that the level of $TP_{L205,L209Y,213}$ homodimerizes sufficiently to traffic to the cell surface, but that the reduced protomer affinity significantly modifies the efficiency with which signal is transduced. Our data does not reveal how activation of Gq via $TP_{L205,L209,Y213}$ is reduced, but one possibility is the formation of a suboptimal conformation of the $TP_{L205,L209,Y213}$ homodimer, impacting the receptor dimer's ability to undergo the necessary conformational shift to fully activate Gq.

We reported previously that in addition to homodimerization, the TPα forms high affinity heterodimers with the IP, which is distinct in sequence, membrane microdomain localization, regulation, and effector signaling (45–48). TP agonists signal through the IP-TPα heterodimer in an IP-like manner cAMP generation, and coincidentally suppress InosP generation (4, 48). Interestingly, in the current study, mutation of the $G_{205}xxxG_{209}xxxL_{213}$ motif did not impact either heterodimerization with the IP or TP agonist-induced cAMP generation through the heterodimer. Thus, it appears that the TPα-TM5 GxxxGxxxL motif contributes selectively to homodimerization and

TABLE 1. Prosite scan of the UniProt/SwissProt protein database (release 2013_01) for the motif G-x(3)-G-x(3)-L

| Receptor | GPCR Family | GxxxGxxxL Sequence | Residues | TM |
|---|---|---|---|---|
| 5-hydroxytryptamine receptor 1A | A | GIIMGTFIL | 348–356 | 6 |
| 5-hydroxytryptamine receptor 1E | A | GLILGAFIL | 294–302 | 6 |
| 5-hydroxytryptamine receptor 5A | A | GILIGVFVL | 288–296 | 6 |
| 12-(S)-hydroxy-5,8,10,14-eicosatetraenoic acid receptor | A | GLECGLGLL | 22–30 | 1 |
| α1A adrenergic receptor | A | GVILGGLIL | 30–38 | 1 |
| | | GIVVGCFVL | 275–283 | 6 |
| α1B adrenergic receptor | A | GLVLGAFIL | 49–57 | 1 |
| | | GIVVGMFIL | 297–305 | 6 |
| β1 adrenergic receptor | A | GIIMGVFTL | 327–335 | 6 |
| β2 adrenergic receptor | A | GIIMGTFTL | 276–284 | 6 |
| β3 adrenergic receptor | A | GLIMGTFTL | 295–303 | 6 |
| Cannabinoid receptor 2 | A | GSLAGADFL | 74–82 | 2 |
| Galanin receptor type 2 | A | GLIWGLSLL | 147–155 | 4 |
| Glucagon receptor | B | GIGWGAPML | 269–277 | 4 |
| Muscarinic acetylcholine receptor M1 | A | GITTGLLSL | 29–37 | 1 |
| Muscarinic acetylcholine receptor M5 | A | GIMIGLAWL | 148–156 | 4 |
| Neuromedin-U receptor 1 | A | GAVWGLAML | 183–191 | 4 |
| Neuromedin-U receptor 2 | A | GIVWGFSVL | 168–176 | 4 |
| Neuropeptide Y receptor type 2 | A | GLAWGISAL | 170–178 | 4 |
| Opsin-5 | A | GFFFGCGSL | 113–121 | 3 |
| Oxoeicosanoid receptor 1 | A | GLWVGILLL | 215–223 | 4 |
| P2Y purinoceptor 4 | A | GLLFGVPCL | 206–214 | 5 |
| Proteinase-activated receptor 4 | A | GHMYGSVLL | 158–166 | 3 |
| Thromboxane A2 receptor | A | GLSVGLSFL | 205–213 | 5 |

Filters were set for species, homo sapiens; description, receptor; and size, >300 and <550.

that distinct receptor regions direct formation of the TPαIP heterodimer.

It has been over a decade since the GPCR dimerization was first reported. Since that time much has been learned about the molecular mechanisms of GPCR dimerization and the biological relevance for receptor function. The most well established model of GPCR dimerization holds that two receptors couple to one G protein (78, 79). In heterodimers, one promoter typically dominates the downstream signal transduced, and hence the biological outcome (79). For example, in heterodimers of the B2 receptor for the vasorelaxant bradykinin and the AT1 receptor for the vasoconstrictor angiotensin II, the latter dominates leading to enhanced AT1-Gq signaling and vasoconstriction (74, 80). It remains unclear whether ligation of one or both protomers is optimal and to what extent G protein activation is symmetrical (the agonist activates the protomer that is directly associated with the G protein) or asymmetrical (the agonist indirectly activates the G protein through the non-G protein associated protomer) (78, 81). In the case of the serotonin type 4 receptor homodimer, evidence supports asymmetrical G protein activation through one ligand binding to its protomer but activating signaling via the companion protomer (78). For the IP-TPα, we established that the IP dominates the heterodimer's signaling through the Gs-cAMP cascade but that agonists for either protomer could activate the complex (48). Our observations that the TM5 GxxxGxxxL mutant did not support normal Gq-InosP signaling in the homodimer but was fully capable of propagating a normal cAMP response to the TP agonist in the IPTPα heterodimer, provides further support for the 2-receptors-1-G-protein model and for asymmetrical G protein activation through one protomer in a dimeric complex (in this case agonism of the TPα led to activation of the IP-associated Gs in the IP-TPα heterodimer).

We reported that the shift in TPα function to Gs signaling when dimerized with the IP likely contributes to the restraint placed by the $PGI_2$-IP system and the $TXA_2$-TP system in vivo (42, 44, 50). It is, therefore, very promising to uncover a molecular region that selectively reduces TP

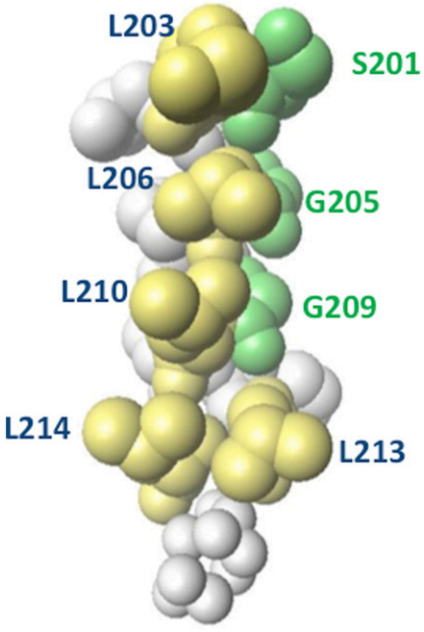


**Fig. 8.** Modeling of the TPα-TM5 highlighting the leucines that neighbor $G_{205}$ and $G_{209}$ and $L_{213}$. By analogy with glycophorin A, the small residues, S201, G205, and G209 align to create a groove (green), while the large residues $L_{203}$, $L_{206}$, $L_{210}$, and $L_{213}$ form an adjacent ridge (yellow).

homodimer function without altering activation and signaling of IP-TPα heterodimer. Efforts to antagonize the TP have proved clinically disappointing (22, 26), perhaps because TP antagonists block activation of the TP in both its TP-TP homodimeric (Gq-coupled) and IP-TP heterodimeric (Gs-coupled) complexes (44). Our work opens novel avenues to biased interference with the TPTP homodimer while sparing the function of the IPTP heterodimer and its beneficial cardiovascular biological effects. Arguably, such an approach should be superior to inhibitors of thromboxane synthase, and even selective inhibition of platelet COX-1 with low-dose aspirin, because the endogenous ligand acting at the IPTP heterodimer would be spared. Recently, computationally designed peptides directed at the GxxxGxxxL motif that mediates interaction of the αIIbβ3 integrins were reported to modify integrin function in platelets (54, 65). We propose that such a peptide targeted at TP-TM5 GxxxGxxxL domain may provide a novel approach to biased TP antagonism. Conceivably, such selective targeting of the TP homodimer would allow us to modify signaling in cell types with high TP-TP expression, such as platelets, while largely preserving the function of cells that have a higher IP-TP dimer population, such as macrophages (51).

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