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The role of N-linked glycosylation in determining the surface expression, G protein interaction and effector coupling of the alpha (α) isoform of the human Thromboxane A₂ receptor.

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Running Title: Glycosylation of the thromboxane receptor.

Key Words: thromboxane receptor, N-linked glycosylation, surface expression, G protein interaction, effector signalling.

Abbreviations: C-tail, carboxyl-terminal tail; [Ca²⁺]; intracellular calcium; ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; HA, hemagglutinin; HEK, human embryonic kidney; IP₃, inositol 1, 4, 5-trisphosphate; PAGE, polyacrylamide gel electrophoresis; PG, prostaglandin; PK, protein kinase; PL, phospholipase; PM, plasma membrane; TP, thromboxane receptor; TXA₂, thromboxane A₂.
Summary
In humans, thromboxane (TX) A2 signals through two TXA2 receptor (TP) isoforms, termed TPα and TPβ, that diverge exclusively within their carboxyl terminal cytoplasmic domains. The amino terminal extracellular region of the TPs contains two highly conserved Asn (N)-linked glycosylation sites at Asn4 and Asn16. Whilst it has been established that impairment of N-glycosylation of TPα significantly affects ligand binding/intracellular signalling, previous studies did not ascertain whether N-linked glycosylation was critical for ligand binding per se or whether it was required for the intracellular trafficking and the functional expression of TPα on the plasma membrane (PM). In the current study, we investigated the role of N-linked glycosylation in determining the functional expression of TPα, by assessment of its ligand binding, G-protein coupling and intracellular signalling properties, correlating it with the level of antigenic TPα protein expressed on the PM and/or retained intracellularly. From our data, we conclude that N-glycosylation of either Asn4 or Asn16 is required and sufficient for expression of functionally active TPα on the PM while the fully non-glycosylated TPαN4,N16-Q4,Q16 is almost completely retained within the endoplasmic reticulum and remains functionally inactive, failing to associate with its coupling G protein Gαq and, in turn, failing to mediate phospholipase Cβ activation.
Introduction

Thromboxane (TX) A\textsubscript{2}, the primary cyclooxygenase product of arachidonic acid in platelets, is a potent stimulator of platelet shape change, aggregation, secretion and a constrictor of bronchial and vascular smooth muscle (1). Synthesis of TXA\textsubscript{2} is increased in a variety of cardiovascular diseases including myocardial infarction, stroke, bronchial asthma and pregnancy-induced hypertension, all of which are believed to coincide with imbalances either in the levels of TXA\textsubscript{2}, its synthase or its receptor (2). TXA\textsubscript{2} mediates its actions through interaction with the shared endoperoxide prostaglandin (PG)H\textsubscript{2}/TXA\textsubscript{2} receptor termed TP (3), a member of the G protein coupled receptor (GPCR) superfamily. Two alternatively spliced variants of the human TP receptor, termed TP\textalpha and TP\textbeta, exist (4,5). TP\textalpha and TP\textbeta are identical for the N-terminal 328 amino acids, but differ in their carboxy-terminal domains (4,5).

As members of the GPCR superfamily, TP\textalpha and TP\textbeta functionally couple to G\alpha\textsubscript{q}, members to activate phospholipase (PL) C\beta, resulting in increased intracellular concentrations of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and mobilization of intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}; 4,5,1,6). It has also been reported that TP\textalpha and TP\textbeta may differentially activate other secondary intracellular signalling pathways; specifically, while both TP\textalpha and TP\textbeta show similar PLC\beta activation in transfected Chinese hamster ovary cells, they oppositely regulate adenylyl cyclase (AC) activity (7). More recent reports show that both TP\textalpha and TP\textbeta are subject to differential heterologous desensitization. For instance TP\textalpha, but not TP\textbeta, is subject to cross-desensitization by prostacyclin, mediated through direct protein kinase (PK) A phosphorylation of TP\textalpha at Ser\textsuperscript{329}; these latter findings led to the proposal that TP\textalpha may be the isoform physiologically relevant to TP:prostacyclin receptor (IP)-mediated vascular hemostasis (8). Furthermore TP\textalpha, but not TP\textbeta, is subject to prostaglandin (PG) D\textsubscript{2} receptor (DP)-mediated cross desensitization (9). On the other hand, TP\textbeta, but not TP\textalpha, is subject to both tonic and agonist-induced internalization (10,11).

Asparagine (N)-linked glycosylation is a major co-translational modification which occurs in the highly specialised environment of the endoplasmic reticulum and the golgi apparatus, and involves the initial transient interaction of specific lectin like chaperones, such as calnexin and its soluble homologue calreticulin (12), as well with co-chaperones which aid in protein folding and trafficking (13,14). In general, N-linked glycosylation is believed to play a major role in protein folding, maintaining the protein’s correct conformation, aiding in intracellular trafficking, protein stability and in the cell surface expression of many glycoproteins (14-18). TP receptors from a number of species contain two highly conserved N-linked glycosylation sites at Asn\textsuperscript{4} and Asn\textsuperscript{16} within their amino terminal domain (4,5,19-21). Impairment of N-linked glycosylation of the human TP\textalpha isoform, either through treatment with the antibiotic tunicamycin (22,23) or through site-directed mutagenesis, resulted in significantly reduced ligand binding, G protein coupling and intracellular...
signalling (24, 25). However, these latter studies did not directly ascertain whether N-linked glycosylation was critical for ligand binding \textit{per se} or whether it was actually required for the intracellular trafficking and surface expression of functionally active TPs on the plasma membrane. Thus, the aim of the current study was to investigate the role of N-linked glycosylation in determining the level of functional TP expression, as determined by assessment of the ligand binding and intracellular signalling properties, correlating it with the level of antigenic TP protein expression on the plasma membrane. Thus, to this end, the ligand binding, intracellular signalling and antigenic expression of hemagglutinin (HA)-epitope tagged forms of the wild type TP\(\alpha\) was compared to that of its N-linked glycosylation defective mutants HA:TP\(\alpha^{N4-Q4}\), HA:TP\(\alpha^{N16-Q16}\) and HA:TP\(\alpha^{N4,16-Q4,16}\). From our data, we conclude that whilst N-linked glycosylation at either Asn\(^4\) or Asn\(^{16}\) is required for functional TP\(\alpha\) expression, in the case of the HA:TP\(\alpha^{N4,16-Q4,16}\) these effects are largely due to an accumulation of the non-glycosylated, inactive receptors within the endoplasmic reticulum (ER) and, consequently, result in a significant reduction in TP surface expression and intracellular signalling.
Materials and Methods

Materials.

U46619 was obtained from Cayman Chemical Company. G418, 1-[2-(5-Carboxyzazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2’-amino-5-methylphenoxy)-ethane-N,N,N’,N’-tetraacetic Acid, pentacetoxyethyl Ester (FURA2/AM), D-myo-inositol 1,4,5-trisphosphate, 3-deoxyhexasodium salt were obtained from Calbiochem. [3H]IP3 (20-40 Ci/mmol) was obtained from American Radiolabelled Chemicals Inc. Mouse monoclonal antibody HA.11 (MMS-101R) anti-HA-peroxidase antibody (5-7 mg/ml), clone 16B12 was obtained from BABCO (Berkeley, CA). Chemiluminescence Western blotting kit, anti-HA-Peroxidase High Affinity (3F10) was obtained from Roche Molecular Biochemicals. Polyvinylidene difluoride membrane was obtained from Amersham. [3H]SQ29,548 (50.4 Ci/mmol) was obtained from DuPont NEN (Boston, MA). Anti-Gαq (C19) specific antibody was obtained from Santa Cruz Laboratories (Santa Cruz, CA). Protein G sepharose, Rhodamine 6G and tunicamycin were purchased from Sigma Chemical Inc. (St. Louis, MO). Fluorescein isothiocyanate (FITC) labelled goat anti-mouse secondary antibody was purchased from Jackson Immunoresearch Lab. Inc.

Plasmid construction and site-directed mutagenesis.

The plasmids pCMV:TXR, pCMV:TXRN4-Q4, pCMV:TXRN16-Q16 and pCMV:TXRN4,N16-Q4,Q16, encoding TPα, TPαN4-Q4, TPαN16-Q16, TPαN4,N16-Q4,Q16 were previously described (24). To facilitate hemagglutinin (HA) epitope tagging, the respective cDNAs for TPα, TPαN4-Q4, TPαN16-Q16 and TPαN4,N16-Q4,Q16, were subcloned into the HindIII-EcoR1 site of pHM6 (Roche) to generate the plasmids pHM:TPα, pHM:TPαN4-Q4, pHM:TPαN16-Q16 and pHM:TPαN4,N16-Q4,Q16, respectively.

Cell Culture and Transfections

Human Embryonic Kidney (HEK) 293 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in minimal essential medium with Earle’s salts (MEM) containing 10 % foetal bovine serum (FBS) and were maintained at 37 °C in 5 % CO₂.

For transfections, HEK 293 cells were routinely plated in 10-cm culture dishes at a density of 2 X 10⁶ cells/dish in 8 ml media approximately 48 h prior to transfection and thereafter were transfected with 10 μg pADVA (26) and 25 μg pcDNA-, pCMV- or pHM- based vectors using the calcium phosphate/DNA co-precipitation procedure essentially as previously described (27). For transient transfections, cells were harvested 48 h post transfection. To create the HEK.TPα, HEK.TPβ, HEK.TPΔ328, HEK.TPαN4-Q4, HEK.TPαN16-Q16, HEK.TPαN4,N16-Q4,Q16 cell lines stably over-expressing the HA-epitope tagged forms of TPα, TPβ or their respective variants, HEK 293 cells were transfected with 10 μg of Scal-linearised pADVA plus 25 μg of the appropriate PvuI-linearised
pHM:TPα, pHM:TPαN4,Q4, pHM:TPαN16,Q16 or pHM:TPαN4,N16,Q4,Q16 plasmids respectively. Forty-eight hr post transfection, G418 (0.8 mg/ml) selection was applied and after approximately 21 days, G418 resistant colonies were selected and individual pure clonal stable cell lines/isolates were examined for TP expression by evaluation of their radioligand binding properties.

Radioligand binding studies

TP radioligand binding assays were carried out at 30 °C for 30 min in 100 μL reactions in the presence of 0-40 nM [3H]SQ29,548 for Scatchard analyses or in the presence of 20 nM [3H]SQ29,548 for saturation radioligand binding experiments using approximately 100 μg whole cell protein per assay essentially as previously described (27). Alternatively, cells were fractionated into their soluble (S100) or crude membrane/particulate (P100) components prior to carrying out radioligand binding assays as previously described (24). Protein determinations were carried out according to the Bradford assay (28). In general, it was determined that there was approximately 3 fold more protein in the P100 fraction relative to that in the S100 fraction; hence, routinely, 75 μg and 25 μg aliquots of the appropriate P100 and S100 fractions, respectively, was used in the radioligand binding assays of fractionated cells. Radioligand binding data was analysed with the GraphPad Prisim V4.0 computer program (GraphPad Software Inc., San Diego, CA) to determine the Kd and Bmax values.

Measurement of intracellular calcium (\([Ca^{2+}]_i\)) mobilization

Measurements of intracellular calcium mobilization \([Ca^{2+}]_i\), in FURA2/AM preloaded HEK 293 stable cell lines (HEK.TPα, HEK.TPαN4,Q4, HEK.TPαN16,Q16, HEK.TPαN4,N16,Q4,Q16 and as controls, HEK 293), was carried out as previously described (27). Briefly, cells were stimulated at 50 sec with 1 μM U46619 (from a 10 mM stock solution dissolved in ethanol), diluted in modified Ca2+/Mg2+-free Hank’s buffered salt solution, containing 10 mM HEPES, pH7.67, 0.1 % bovine serum albumin (BSA) (HBSSHB) buffer at the appropriate concentration such that addition of 20 μl of the diluted drug/inhibitor to 2 ml of cells resulted in the correct working concentration (8). The HBSSHB buffer alone had no effect on \([Ca^{2+}]_i\), mobilization by the TPs and thus did not affect experimental data. The ratio of the fluorescence at 340 nm and 380 nm is a measure of \([Ca^{2+}]_i\) (27) which assumes a Kd of 225 nM Ca²⁺ for FURA2/AM. The results presented in the figures are representative data from at least three independent experiments and are plotted as changes in intracellular \([Ca^{2+}]_i\), mobilized (Δ[Ca²⁺]i, (nM)) as a function of time (s) upon ligand stimulation. Changes in \([Ca^{2+}]_i\), mobilization were determined by measuring peak rises in intracellular \([Ca^{2+}]_i\), mobilized (Δ[Ca²⁺]i) and are represented as mean changes in Δ[Ca²⁺]i ± S.E.M (nM).

Measurement of IP₃ levels
Intracellular IP$_3$ levels were measured as previously described (8, 29). Briefly, HEK.TPα, HEK.TPα$^{N4,Q4}$, HEK.TPα$^{N16,Q16}$ and HEK. TPα$^{N4,N16-Q4,Q16}$ and as controls, HEK 293 cells were washed twice in ice-cold PBS and 2 X 10$^6$ cells were resuspended in 200 µl HEPES-buffered saline (HBS; 140 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 11 mM glucose, 15 mM HEPES-NaOH, pH 7.4) supplemented with 10 mM LiCl. Cells were pre-equilibrated in this buffer at 37 °C for 10 min and stimulated at 37 °C for 1 min in the presence of U46619 (1 µM) or, in the presence of an equivalent volume (50 µl) of HBS vehicle to determine basal IP$_3$ levels in cells. IP$_3$ was extracted by the addition 50 µl ice-cold 30 % trichloroacetic acid (TCA). After vortexing and centrifugation (3500 rpm, 5 min), 250 µl of each supernatant was removed to glass tubes and 1.0 ml H$_2$O-saturated diethyl ether was added for extraction of TCA and the samples were placed in a methanol-dry ice bath until the aqueous layers froze and the ether layers could be decanted. Ether extraction was repeated three times and the samples were checked for complete extraction of TCA (pH of sample > 5.0). Nitrogen was bubbled through the sample to remove diethyl ether and the pH raised to 7.0 with addition of NaHCO$_3$. Levels of IP$_3$ were determined using a radio-competition binding assay employing [$^3$H]-D-myo-IP$_3$ as the radioactive standard. Analytical samples (100 µl) were added to 100 µl assay buffer (0.1 M Tris-Cl, 4 mM EDTA, pH 9.0, containing 4 mg/ml BSA). [$^3$H]IP$_3$ (100 µl, 20-40 Ci/mmol, 10 mCi/ml diluted 1:147 in H$_2$O) was added and the tubes were vortexed before addition of 100 µl binding protein isolated from bovine adrenal medulla essentially as described by Godfrey et al., (29), followed by further vortexing and centrifuged for 5 min at 14,000 rpm in microcentrifuge. The level of IP$_3$ produced was quantified by radio competition essentially as described by Godfrey et al., (29). Levels of IP$_3$ produced in ligand or in vehicle treated cells were calculated as pmol IP$_3$/10$^6$ cells ± S.E.M and are expressed as fold increases in IP$_3$ levels determined in agonist stimulated cells relative to basal levels, determined in vehicle (HBS) treated cells. The data presented are the mean data of four independent experiments, each performed in duplicate.

**Western blot analysis.**

HEK.TPα, HEK.TPα$^{N4,Q4}$, HEK.TPα$^{N16,Q16}$, HEK.TPα$^{N4,N16-Q4,Q16}$ and as controls, HEK 293 cells were harvested by centrifugation at 500 X g at 4 °C, washed twice in ice cold PBS and resuspended in HED buffer, (20 mM HEPES, pH. 7.67, 1 mM EGTA, 0.5 mM dithiothreitol supplemented 1 mM phenylmethylsulfonyl fluoride, 10 µM indomethacin). Cells were then homogenized and centrifuged at 100,000 X g at 4 °C for 1 h. The pellets (P$_{100}$) fraction, representing crude membranes, was resuspended in the HEDG buffer (20 mM HEPES, pH. 7.67, 1 mM EGTA, 0.5 mM dithiothreitol, 100 mM NaCl, 10% glycerol supplemented 1 mM phenylmethylsulfonyl fluoride, 10 µM indomethacin) and protein determinations were carried out according to the Bradford assay (28). Aliquots of P$_{100}$ (75 µg) and S$_{100}$ (25 µg) fractions were resuspended in Sample Buffer (10% β-
mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 30% glycerol, 0.025% bromophenol blue, 50 mM Tris-HCl, pH 6.8); samples were loaded without boiling and resolved on by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), on 10 % gels, followed by Western blot transfer onto PVDF membranes.

**Immunoprecipitations.**

HEK.TPα, HEK.TPαN4-Q4, HEK.TPαN16-Q16, HEK.TPαN4,N16-Q4,Q16 and as controls, HEK 293 cells were co-transfected with 25 μg pCMV:Gαq or with the vector pCMV5. Approximately 48 h post-transfection, cells were stimulated with 1 μM U46619 or, as controls, with the vehicle HEPES-buffered saline buffer (HBS; 140 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 1.2 mM KH₂PO₄, 11 mM glucose, 15 mM HEPES-NaOH, pH 7.4). Thereafter, cells were washed once in ice-cold phosphate-buffered saline (PBS; 3 ml/dish) and immunoprecipitated essentially as described previously (8). Briefly, cells were lysed with 0.6 ml of Radioimmune Precipitation Buffer (RIP buffer; 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 % Nonidet P-40 (v/v), 0.5 % sodium deoxycholate (w/v), 0.1 % SDS (w/v) containing 10 mM sodium fluoride, 25 mM sodium pyrophosphate, 1 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml antipain, and 1 mM sodium orthovanadate) and incubated on ice for 15 min. Cells were then harvested and disrupted by sequentially passing through hypodermic needles of decreasing bore size (gauge 20, 23, and 26), and soluble lysates were harvested by centrifugation at 13,000 x g at room temperature (R.T.) for 5 min. HA epitope tagged TP receptors (HA.TPα, HA.TPαN4-Q4, HA.TPαN16-Q16, HA.TPαN4,N16-Q4,Q16) were immunoprecipitated using the anti-HA 101R antibody (1:300 dilution) at R.T. for 2 h followed by the addition of 10 μg of protein G-Sepharose 4B and further incubated at room temperature for 1 h. Immune complexes were collected by centrifugation at 13,000 X g at R.T. for 5 min and washed three times in 0.5 ml RIP buffer and finally resuspended (75 μg protein/assay; determined by Bradford assay) in 1X Solubilisation Buffer (10 % β mercaptoethanol (v/v), 2 % SDS (w/v), 30 % glycerol (v/v), 0.025 % bromophenol blue (w/v), 50 mM Tris-HCl, pH 6.8; 40 μl). Samples were loaded without boiling onto 10 % polyacrylamide gels, analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and thereafter electroblotted onto poly-vinylidene difluoride membranes, essentially as described previously (30). ImmunobLOTS were either screened with the anti-Gαq (C19) specific antibody and HRP-conjugated secondary antibody or, to detect HA-tagged TP receptors, with the anti-HA-Peroxidase High Affinity 3F10 antibody followed by visualization of the immunoreactive proteins by chemiluminescence detection according to the supplier’s instructions.
**Immunofluorescence Microscopy**

HEK.TPα, HEK.TPα\(^{(N4-Q4)}\), HEK.TPα\(^{(N16-Q16)}\) and HEK. TPα\(^{(N4,N16-Q4,Q16)}\) and, as controls, HEK 293 cells were plated onto coverslips pre-coated for 1 min with poly-L-lysine (0.1 mg/ml) at a density of 0.5 X 10^5 cells in 60 mm cell culture dish. Following 24 h, cells were washed twice with PBS, and fixed with 3.7 % formaldehyde in PBS, pH 7.4 for 15 min at R.T. Cells were then washed three times with PBS, followed by permeabilisation with 0.2 % Triton X-100 in PBS for 10 min on ice. As a negative control, to confirm antibody specificity, non-permeabilised cells were used in parallel with permeabilised cells. Cells were rinsed with Blotto solution (20 mM Tris-HCl, pH 7.2, 0.1 M NaCl, 5% dried skimmed milk powder followed by incubation with the primary anti-HA 101R antibody (1:2500 dilution in Blotto solution) in a total volume of 100 μl for 2 h at R.T. The antibody solution was removed and the cells were then washed three times with Blotto solution. The goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:120 dilution in Blotto) was added to the cells and then incubated at R.T. for 30 min. To detect the endoplasmic reticulum, cells were washed three times with PBS followed by incubation with Rhodamine 6G (1:2000 dilution) at R.T for 30 sec. The cells were then washed once in PBS, followed by gentle rinsing with distilled H_2O. A drop of antifade mounting solution (90% glycerol, 0.1 X PBS, 2.5% DAPCO; sigma) was added to the cells and the cover slips were mounted onto microscope slides. Immunofluorescent staining of cells was observed using an Olympus BX60 fluorescence microscope with either an U-MNIBA filter for the observation of FITC fluorescence staining or an U-MWIG filter for the observation of Rhodamine 6G fluorescence staining. Images were then captured photographically using an ISO 400 colour photographic film with exposure time ranging between 10-30 sec. Co-stained images were captured by first individually photographing the cells using the individual U-MNIBA and U-MWIG filters which were then overlaid before the final co-stained photographic images were taken.

**Enzyme Linked Immuno-sorbent Assay (ELISA)**

For quantitation of TP receptor antigenic protein expression, HEK.TPα, HEK.TPα\(^{(N4-Q4)}\), HEK.TPα\(^{(N16-Q16)}\) and HEK. TPα\(^{(N4,N16-Q4,Q16)}\) and as controls, HEK 293 cells, were plated at 10,000 cells / 96 well tissue culture dish pre-coated for 1 min with 0.1 mg/ml poly-L-lysine. After 24 h, cells were washed once with PBS and fixed in 3.7 % formaldehyde in 1 X Tris-buffered saline (TBS), pH 7.4, for 5 min at R.T. The cells were washed three times with TBS and non-specific binding was blocked with TBS containing 1 % bovine serum albumin (BSA) for 45 min at room temperature. Cells were then incubated in the presence of the primary antibody (monoclonal HA 101R antibody: 1:1000 in TBS/BSA) for 1 h at R.T. Thereafter, cells were washed three times with TBS and then reblocked in...
TBS/BSA for 15 min at R.T. and then incubated with goat anti-mouse-conjugated alkaline phosphatase (diluted 1:1000 in TBS/BSA) for 1 h at R.T. Cells were washed three times with TBS, and colorimetric alkaline phosphatase substrate (K Blue, TMB substrate (Neogen) was added and the reaction stopped by adding Red Stop TM (Neogen). When adequate colour change was reached, 100 μl samples were taken for colorimetric reading at A<sub>650nm</sub>. As controls, HEK 293 cells were studied concurrently to determine background. All experiments were carried out in triplicate.

**Data analyses**

Radioligand binding data were analysed using GraphPad Prisim V4.0 programme (GraphPad Software Inc., San Diego, CA, U.S.A.) to determine the $K_d$ and $B_{max}$ values. Statistical analyses were carried out using the unpaired Student’s $t$ test using the Statworks Analysis Package. $P$-Values ≤ 0.05 indicated statistically significant differences.
Results:
The role of N-linked glycosylation in determining the surface expression of TPα.

In the current study, we sought to investigate the role of N-linked glycosylation in determining the level of functional TPα expression, as determined by assessment of its ligand binding, G-protein coupling and intracellular signalling properties, correlating it with the level of antigenic TPα protein expressed on the plasma membrane and/or retained intracellularly. Thus, mammalian cell lines stably over-expressing HA-epitope tagged forms of TPα and its respective N-linked glycosylation defective variants TPαN4-Q4, TPαN16-Q16 and TPαN4,N16-Q4,Q16 were established in human embryonic kidney (HEK) 293 cells. Scatchard analysis confirmed that the presence of the HA-epitope tag per se did not affect the ability of TPα to bind its radiolabelled antagonist [³H]SQ29,548 (Table 1; Walsh et al., 1998). Moreover, consistent with previous results (24), there were no observed differences in the affinity (Kd) of either the TPαN4-Q4, TPαN16-Q16 or TPαN4,N16-Q4,Q16 receptors for [³H]SQ29,548 relative to that of the wild type TPα (Table 1). However, in each case, there was a substantial reduction in their maximal ligand binding (Bmax) with TPαN4-Q4 and TPαN16-Q16 exhibiting only 41 % and 70 % maximal surface expression relative to that of TPα, respectively, whilst TPαN4,N16-Q4,Q16 exhibited only 8% maximal surface expression relative to TPα (Table 1).

To ascertain whether the observed reductions in ligand binding by the TPαN4-Q4, TPαN16-Q16 and TPαN4,N16-Q4,Q16 receptors were due to an impaired ability to bind the radioligand or due to decreased receptor expression on the plasma membrane, saturation radioligand binding assays were performed on whole cells to determine relative surface expression of the TPs and on cells fractionated into their respective crude membrane (P100) and soluble (S100) fractions to determine relative total expression and data was correlated with their levels of antigenic TP protein expression as determined by ELISA in either non-permeabilised or permeabilised cells (Figure 1). Consistent with data obtained from Scatchard analyses, there was a strong correlation between the level of surface expression of TPαN4-Q4, TPαN16-Q16 and TPαN4,N16-Q4,Q16 in the non-fractionated cells as determined by saturation radioligand binding (Figure 1A) and ELISA in non-permeabilised cells (Figure 1B) whereby the level of surface expression of TPαN4-Q4, TPαN16-Q16 and TPαN4,N16-Q4,Q16 was reduced to approximately 47%, 65 % and 8.4%, respectively, relative to the wild type TPα (Figure 1A & 1B).

Moreover, the fractionated cells displayed similar corresponding reductions in radioligand binding by their respective membrane (P100) and soluble (S100) fractions for each of the TPαN4-Q4, TPαN16-Q16 and TPαN4,N16-Q4,Q16 receptors relative to TPα (Table 2; Figure 1C) and thereby exhibited similar reductions in the total radioligand binding relative to the wild type TPα (Figure 1C). In terms of membrane (P100) expression, some 82.4% of total radioligand binding occurred in the P100 fraction for the TPα (Table 2) while in the case of the TPαN4-Q4 and TPαN16-Q16 that figure was not substantially
different with some 74% and 80.5% of the total TPα<sup>N4,Q4</sup> and TPα<sup>N16,Q16</sup> ligand binding occurring in their respective P<sub>100</sub> fractions (Table 2). In the case of the TPα<sup>N4,N16,Q4,Q16</sup>, the level of ligand binding in the P<sub>100</sub> fraction relative to its total ligand binding was reduced to 63.5% (Table 2). However, ELISA analysis of permeabilised cells indicated that the relative levels of total antigenic TP expression was not substantially different for the TPα<sup>N4,Q4</sup>, TPα<sup>N16,Q16</sup> and TPα<sup>N4,N16,Q4,Q16</sup> relative to the wild type TPα indicating that impairment of N-linked glycosylation per se did not appreciably affect the overall level of TP protein expression (Figure 1D). Hence, there was a poor correlation between the relative levels of total TP expression as determined from radioligand binding from the fractionated cells and from ELISA of permeabilised cells (compare Figure 1C & 1D).

Consistent with the latter findings, Western blot analysis confirmed that in the case of the wild type TPα, TPα<sup>N4,Q4</sup> and TPα<sup>N16,Q16</sup>, the majority of the antigenic TP protein was expressed in their respective P<sub>100</sub> fractions, appearing as a higher molecular mass protein of 64 – 66 kDa approximately and a lower molecular mass protein of 30 – 46 kDa, with only relatively low level of TP protein expression evident in the corresponding S<sub>100</sub> fractions (Figure 2). Conversely, in the case of the TPα<sup>N4,N16,Q4,Q16</sup> most of the receptor protein expression was evident in the S<sub>100</sub> fraction where it appeared largely as the lower molecular mass protein of 30 – 46 kDa (Figure 2).

**Immunolocalisation of TPα, TPα<sup>N4,Q4</sup>, TPα<sup>N16,Q16</sup> and TPα<sup>N4,16,Q4,16</sup>**

Thereafter, to further investigate the relative expression and subcellular distribution of the TPα and its N-linked glycosylation variants TPα<sup>N4,Q4</sup>, TPα<sup>N16,Q16</sup> and TPα<sup>N4,N16,Q4,Q16</sup>, immunofluorescence microscopy was carried out on the respective HEK 293 stable cell lines under both permeabilising and non-permeabilising conditions. Similar to that of the wild-type TPα, there was abundant expression of the TPα<sup>N4,Q4</sup> and the TPα<sup>N16,Q16</sup> on the plasma membrane as evidenced by strong immunostaining of cells under non-permeabilising conditions (Figure 3A, 3D & 3G). In permeabilised cells, while there was some evidence of low level expression of the wild-type TPα intracellularly (Figure 3B), there was significantly increased expression of both the TPα<sup>N4,Q4</sup> and the TPα<sup>N16,Q16</sup> as evidenced by increased intracellular immunostaining in permeabilised cells (Figure 3E & 3F). Moreover, on co-staining the respective cell lines with the anti-HA primary and FITC labelled secondary antibodies along with the endoplasmic reticulum (ER) stain Rhodamine 6G, there was evidence of increased retention of both the HA-tagged TPα<sup>N4,Q4</sup> and the TPα<sup>N16,Q16</sup> within the ER (Figure 3F & 3I). In the case of the TPα<sup>N4,N16,Q4,Q16</sup>, only very low levels of this receptor was detected on the plasma membrane under non-permeabilising conditions (Figure 3J) whereas in permeabilised cells there was abundant expression and retention of the TPα<sup>N4,N16,Q4,Q16</sup> within the intracellular compartment (Figure 3K). Moreover, co-staining with the FITC labelled antibody along with Rhodamine 6G confirmed that the majority of this receptor was retained within the ER (Figure 3L).
The role of N-linked glycosylation in G protein interaction and effector signalling.

Thereafter, we sought to investigate the functional requirement for N-linked glycosylation for intracellular signalling by the HA-epitope tagged wild type TPα, TPαN4-Q4, TPαN16-Q16, TPαN4,16-Q4,16 receptors by analysing agonist induced intracellular second messenger generation and receptor: G protein interaction through co-immunoprecipitation studies. Functional coupling of TPα, TPαN4-Q4, TPαN16-Q16, TPαN4,16-Q4,16 receptors to its primary effector PLCβ was assessed by monitoring agonist induced IP3 generation and mobilization of intracellular calcium ([Ca2+]i) in response to the TXA2 mimetic U46619 (Figure 4). Consistent with previous reports (Walsh et al., 2000), stimulation of TPα with U46619 led to a significant increase in intracellular IP3 and to a rapid rise in [Ca2+]i mobilization (Figure 4; Δ[Ca2+]i, mobilization = 160 ± 10.1 nM). While stimulation of both the TPαN4-Q4 and TPαN16-Q16 each resulted in significant agonist-induced increases in IP3 generation and in [Ca2+]i mobilization (Figure 4; Δ[Ca2+]i, mobilization = 101 ± 7.3 nM for TPαN4-Q4; Δ[Ca2+]i, mobilization = 120 ± 7.4 nM for TPαN16-Q16), in each case those levels were significantly reduced accounting for 62% and 75% of those levels generated by the TPα, respectively. Moreover, in the case of the TPαN4,N16-Q4,Q16, both agonist induced IP3 generation and intracellular [Ca2+]i mobilization were significantly reduced (Figure 4; Δ[Ca2+]i, mobilization = 72 ± 3.4 nM for TPαN4,N16-Q4,Q16) and were not substantially greater than those levels generated by the control HEK 293 cells.

To ascertain whether the impaired coupling of TPαN4-Q4, TPαN16-Q16, TPαN4,16-Q4,16 receptors to PLC activation was associated with an impaired ability to interact with the coupling G protein Gαq, HEK 293 cell lines stably over-expressing TPα and its respective N-linked glycosylation variants were transiently co-transfected with pCMV:Gαq or, as controls, with the vector pCMV5. Agonist induced receptor: G protein interactions were assessed by immunoprecipitating the respective HA-tagged TP receptors using the anti-HA 101R antisera followed by screening the immunoprecipitates for co-precipitation of Gαq using the anti-Gαq antibody. Whereas non-stimulated, vehicle (HBS) treated cells showed no TPα: Gαq interaction either in the absence or presence of co-expression of Gαq, stimulation of those cells with U46619 (1 μM) for 5 min prior to immunoprecipitation of HA-tagged TPα resulted in efficient co-immunoprecipitation of Gαq along with TPα (Figure 5Ai). Similarly, stimulation of cells stably expressing TPαN4-Q4 or TPαN16-Q16 led to an efficient agonist-induced co-immunoprecipitation of Gαq along with both TPαN4-Q4 and TPαN16-Q16 in cells over-expressing Gαq, but not in control pCMV5 transfected cells or in vehicle treated cells (Figure 5Aii and 5Aiii, respectively). On the other hand, Gαq was not efficiently co-precipitated with TPαN4,N16-Q4,Q16 irrespective of Gαq expression status or agonist stimulation (Figure 5Aiv). In each case, the presence of HA-tagged TPα, TPαN4-Q4, TPαN16-Q16 and TPαN4,16-Q4,16 in the respective
immunoprecipitates was confirmed by co-screening the blots with anti-HA 3F10 horseradish peroxidase-conjugated antibody (Figure 5B). Moreover, efficient transfection and over-expression of Gaq in the respective cell lines was confirmed by direct immunoblot analysis using the anti-Gαq antibody (Figure 5C).
Discussion.

The carbohydrate moieties of glycoproteins are, in general, believed to be important in facilitating protein folding, intracellular trafficking, cell surface expression and/or secretion and may also protect against proteolysis (15, 16). In general, most glycoproteins are N-glycosylated while the importance and requirements of O-glycosylation where carbohydrates are attached to serine or threonines, remain to be elucidated (14). During N-glycosylation, dolichol glycolipids donate the initial glucose3-mannose3-N-acetylglucosamine oligosaccharides to Asn residues within the lumen of the ER; intracellular lectin like chaperones calnexin and calreticulin bind to the innermost glucose residue of the initial oligosaccharide, anchoring the polypeptide in the ER until it has achieved its correct folding conformation with subsequent glycosylation reactions taking place during the transport of the glycoproteins through the ER and Golgi apparatus en route to the cell surface. Most cell surface and, indeed, secreted proteins are glycosylated or are found associated with other glycoproteins (14) with the exception of certain cell surface proteins with multi-transmembrane spanning domains. Among the 7-transmembrane spanning GPCR superfamily, the role of N-linked glycosylation is somewhat less clear with variable, receptor-dependent effects on ligand binding, signal transduction and cell surface expression (31 - 37). For example, N-glycosylation does not affect ligand binding or coupling to Gs/adenylyl cyclase by the β2 adrenergic receptor (AR) but is required for its presentation on the plasma membrane (38), while inhibition of N-glycosylation of the related α1 AR has no affect in its ligand binding or membrane expression (39). In the case of the thrombin receptor, tuncamycin inhibits thrombin binding to its receptor (40) and inhibits thrombin-induced [Ca\(^{2+}\)] mobilization in human T-lymohoblastoid cells (41).

N-linked glycosylation is thought to play a somewhat divergent role in the case of the prostanoid subfamily of GPCRs. For the EP\(_{3a}\) subtype of the prostaglandin (PG) E\(_2\) receptor, N-linked glycosylation is essential in determining both its affinity and specificity for PGE\(_2\) binding and in influencing its trafficking to the plasma membrane (42, 43); in contrast, N-linked glycosylation of the EP\(_{3b}\) subtype is required for its expression on the plasma membrane while both ligand binding and intracellular signalling are maintained (44). The human prostacyclin receptor (IP) has also been confirmed to be glycosylated at both Asn\(^{7}\) and Asn\(^{78}\) (45, 46) and depending on the extent of glycosylation, it may influence membrane expression, ligand binding and intracellular of the IP.

TP receptors from a number of species contain two highly conserved N-linked glycosylation sites at Asn\(^{4}\) and Asn\(^{16}\) within their amino terminal domain (4, 5, 19 - 21). Walsh et al., (24) investigated the significance of N-linked glycosylation of TP\(_{\alpha}\) by mutating either one or both Asn\(^{4}\) and Asn\(^{16}\) converting them to Gln (Q) residues. While there was no significant change in the affinity (\(K_d\)) of the mutated receptors for the TP ligand SQ29,548, in each case there were substantial reductions in maximal ligand binding (\(B_{max}\)) relative to wild type TP\(_{\alpha}\) receptor (24). In a related
study, Chiang & Tai, (25) mutated the glycosylated Asn residues to corresponding hydrophobic Leu residues and reported that glycosylation at either Asn4 or Asn16 is absolutely required for ligand binding. However, a major limitation of these latter studies was that they did not directly ascertain whether N-linked glycosylation was critical for ligand binding per se or whether it was actually required for the intracellular trafficking and surface expression of functionally active TPs on the plasma membrane. Thus, employing HA-epitope tagged forms of the wild type TPα and its glycosylation defective TPαN4,Q4, TPαN16,Q16, TPαN4,N16,Q4,Q16 variants, in the current study we sought to employ a combination of ligand binding, intracellular signalling and immunochemical approaches to investigate the role of N-linked glycosylation in determining the level of functional TP expression correlating it with the level of antigenic TP protein expression on the plasma membrane.

Consistent with previous reports (24), Scatchard analysis confirmed that there were no observed differences in the affinity (Kd) of either TPαN4,Q4, TPαN16,Q16 or TPαN4,N16,Q4,Q16 relative to TPα for the radioligand [3H]SQ29,458 while in each case there was a highly significant reduction in their maximal ligand binding whereby the level of TPαN4,Q4, TPαN16,Q16 and TPαN4,N16,Q4,Q16 expression was reduced to 41 %, 70 % and 8 %, respectively, relative to the wild type TPα. There was also a strong correlation between the relative levels of surface expression of TPαN4,Q4, TPαN16,Q16 and TPαN4,N16,Q4,Q16 in non-fractionated whole cells, as determined by saturation radioligand binding, and in non-permeabilised whole cells as determined immunochemically by ELISA. While the fractionated cells displayed similar corresponding reductions in radioligand binding by their respective membrane (P100) and soluble (S100) fractions for each of the TPαN4,Q4, TPαN16,Q16 and TPαN4,N16,Q4,Q16 receptors relative to TPα, ELISA analysis of permeabilised cells indicated that the relative levels of total antigenic TP expression was not substantially different for the TPαN4,Q4, TPαN16,Q16 and TPαN4,N16,Q4,Q16 relative to TPα indicating that impairment of N-linked glycosylation per se did not appreciably affect the overall level of TP protein expression or turnover.

Western blot analysis confirmed that while the TPαN4,Q4, TPαN16,Q16 and TPαN4,N16,Q4,Q16 were expressed at equivalent levels relative to the TPα, the majority of the antigenic TPα, TPαN4,Q4 and TPαN16,Q16 protein was expressed in their respective P100 fractions while most of the TPαN4,N16,Q4,Q16 receptor protein expression was evident in the S100 fraction where it appeared largely as a lower molecular mass non-glycosylated protein of 30 – 46 kDa. Thereafter, immunofluorescence microscopy confirmed that similar to TPα, both TPαN4,Q4 and TPαN16,Q16 were abundantly expressed on the plasma membrane; however, under permeabilising conditions there was evidence of significantly increased retention and co-localisation of both the TPαN4,Q4 and the TPαN16,Q16 within the ER. In the case of TPαN4,N16,Q4,Q16 there was no significant expression of this non-glycosylated receptor on the plasma membrane while it was abundantly expressed and largely retained within the ER. Thus, collectively these data indicate that mutation of the N-linked glycosylation sites at Asn4
and Asn\(^{16}\) leads to retention of the partially glycosylated receptor within the ER and thereby most likely impairs chaperone mediated protein folding and trafficking of functionally active TP receptors to the plasma membrane. Whilst glycosylation at either Asn\(^{4}\) (TP\(\alpha^{N4-Q4}\)) and Asn\(^{16}\) (TP\(\alpha^{N16-Q16}\)) is sufficient to allow for the substantial expression of functional TPs on the plasma membrane, impairment of glycosylation of both Asn\(^{4}\) and Asn\(^{16}\) leads to the almost complete retention of the TP\(\alpha^{N4,N16-Q4,Q16}\) within the ER.

Thereafter, we sought to investigate the functional requirement for N-linked glycosylation for intracellular signalling by analysing agonist-induced PLC\(\beta\) effector coupling, through measurement of intracellular second messenger generation, correlating it with TP receptor: G protein interaction through co-immunoprecipitation studies. While stimulation of both the TP\(\alpha^{N4-Q4}\) and TP\(\alpha^{N16-Q16}\) each yielded significant increases in IP\(_3\) generation and in [Ca\(^{2+}\)] mobilization, in each case those levels were significantly reduced accounting for 62 % and 75 % of those levels generated by the TP\(\alpha\), respectively. In the case of the TP\(\alpha^{N4,N16-Q4,Q16}\), both agonist-induced IP\(_3\) generation and [Ca\(^{2+}\)] mobilization were significantly reduced and were not substantially greater than those levels generated by the control HEK 293 cells.

Thereafter, co-immunoprecipitation studies were employed to ascertain whether the impaired coupling TP\(\alpha^{N4-Q4}\), TP\(\alpha^{N16-Q16}\), TP\(\alpha^{N4,16-Q4,16}\) receptors to PLC\(\beta\) activation was associated with an impaired ability to interact with the coupling G protein G\(\alpha_q\). Agonist induced receptor: G protein interactions were assessed by immunoprecipitating the respective HA-tagged TP receptors followed by screening the immunoprecipitates for co-precipitation of G\(\alpha_q\) where cells co-transfected with the empty vector pCMV5 and vehicle treated cells served as controls in each case. While stimulation of cells stably expressing TP\(\alpha\), TP\(\alpha^{N4-Q4}\) or TP\(\alpha^{N16-Q16}\) led to an efficient, agonist-induced co-immunoprecipitation of G\(\alpha_q\) in cells over-expressing G\(\alpha_q\), but not in control pCMV5 transfected cells or in vehicle treated cells, G\(\alpha_q\) was not efficiently co-precipitated with TP\(\alpha^{N4,N16-Q4,Q16}\) irrespective of G\(\alpha_q\) expression status or agonist stimulation.

Thus, collectively these data further confirm the functional requirement for N-linked glycosylation for intracellular signalling and indicate that the defective second messenger generation by the TP\(\alpha^{N4,N16-Q4,Q16}\) is due to an impaired interaction with its coupling G protein. The latter impaired coupling is most likely due to the almost complete retention of the non-glycosylated, conformationally inactive TP\(\alpha^{N4,N16-Q4,Q16}\) within the ER, making it inaccessible to interact with its coupling G protein G\(\alpha_q\) and hence to its primary effector PLC\(\beta\). On the other hand, glycosylation of either Asn\(^4\) or Asn\(^{16}\) alone is sufficient to mediate substantial expression of functionally active TP receptor on the plasma membrane and thereby is sufficient to support functional agonist-induced interaction of TP\(\alpha^{N4-Q4}\) and TP\(\alpha^{N16-Q16}\) with G\(\alpha_q\) and, in turn, PLC\(\beta\) effector coupling. The strict requirement for N-linked
glycosylation of the human TPα for intracellular trafficking and functional expression is in striking contrast to that of the human V2 vasopressin receptor (33), for example and adds further credence to the divergent, unpredictable functional role of N-linked glycosylation among specific members of the 7-transmembrane spanning GPCR superfamily.

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References:


36] X. Liu, X., D. Davis, D.L. Segaloff, Disruption of potential sites for N-linked glycosylation does not impair hormone binding to the lutropin/choriogonadotropin receptor if Asn-173 is left intact, J. Biol. Chem. 268 (1993) 1513-1516.


FIGURES:

Figure 1. Determination of relative surface expression and the relative total expression of the wild type and glycosylation defective TPα receptors as determined by radioligand binding assays and ELISAs.

Radioligand binding (Panel A & C) and ELISA (Panel B & D) determinations were carried out in HEK 293 cells stably over-expressing HA-tagged TPα (wild type), TPα$^\text{N4-Q4}$ (N4-Q4), TPα$^\text{N16-Q16}$ (N16-Q16) or TPα$^\text{N4,N16-Q4,Q16}$ (N4,N16-Q4,Q16) to determine surface (Panel A & B) and total (Panel C & D) TP expression, respectively. Panel A: to estimate surface TP expression, radioligand binding assays were performed using 20 nM $[^3\text{H}]$SQ29,548 (50.4 Ci/mmol) and 75 μg whole cell protein per assay; levels of TPα$^\text{N4-Q4}$, TPα$^\text{N16-Q16}$ and TPα$^\text{N4,N16-Q4,Q16}$ expression (pmol $[^3\text{H}]$SQ29,548/mg cell protein) were calculated as a mean percentage relative surface expression (% ± S.E.M, n = 3-4) where the level of wild type TPα expression was represented as 100%. Actual levels of TP surface expression (pmol/mg cell protein) were: TPα, 3.6 ± 1.2 pmol/mg cell protein; TPα$^\text{N4-Q4}$, 1.2 ± 0.7 pmol/mg cell protein; TPα$^\text{N16-Q16}$, 2.5 ± 0.9 pmol/mg cell protein; TPα$^\text{N4,N16-Q4,Q16}$, 0.6 ± 0.32 pmol/mg cell protein. Panel C: to estimate total TP expression, the radioligand binding data (20 nM $[^3\text{H}]$SQ29,548; 50.4 Ci/mmol) from the crude membrane ($P_{100}$; 75 μg protein per assay) and soluble fractions ($S_{100}$; 25 μg protein per assay) were combined to represent total TP expression; levels of TPα$^\text{N4-Q4}$, TPα$^\text{N16-Q16}$ and TPα$^\text{N4,N16-Q4,Q16}$ expression (pmol $[^3\text{H}]$SQ29,548/mg cell protein) were calculated as a mean percentage relative total expression (% ± S.E.M, n = 3-4) where the level of wild type TPα total expression was represented as 100%. Panels B & D. For ELISAs, either whole (Panel B) or permeabilised (Panel C) cells were screened for HA-epitope expression using the anti-HA 101R antibody. Levels of HA-epitope tagged TPα$^\text{N4-Q4}$, TPα$^\text{N16-Q16}$ and TPα$^\text{N4,N16-Q4,Q16}$ expression were expressed as a mean percentage relative surface (Panel B) or total (Panel C) expression (% ± S.E.M, n = 4) where the respective levels of wild type TPα surface or total expression was represented as 100%. The asterisks indicate that mutation of the N-linked glycosylation sites significantly affected TP expression, where *, ** and *** indicates $p \leq 0.05$, $p \leq 0.02$ and $p \leq 0.001$, respectively.
Figure 2. Analysis of TP distribution by Western Blot Analysis

HEK 293 cells (lane 1), HEK.TP\(\alpha\) (lane 2), HEK.TP\(\alpha^{N4-Q4}\) (lane 3), HEK.TP\(\alpha^{N16-Q16}\) (lane 4), HEK.TP\(\alpha^{N4,N16-Q4,Q16}\) (lane 5) were fractionated into their crude membrane (Panel A; 75 \(\mu\)g protein analysed per lane) and soluble (Panel B; 25 \(\mu\)g protein analysed per lane) fractions and analysed by SDS-PAGE/western blot analysis using anti-HA 101R and goat anti-mouse IgG-HRP conjugate as primary and secondary antibody, respectively. Levels of TP expression in the various cell lines were as follows; HEK:TP\(\alpha\), 3.2 \(\pm\) 2.4 pmol \([^{3}H]\)SQ29,548/mg whole cell protein; HEK.TP\(\alpha^{N4-Q4}\), 1.1 \(\pm\) 0.6 pmol \([^{3}H]\)SQ29,548/mg whole cell protein; HEK.TP\(\alpha^{N16-Q16}\), 2.1 \(\pm\) 0.7 pmol \([^{3}H]\)SQ29,548/mg whole cell protein; HEK.TP\(\alpha^{N4,N16-Q4,Q16}\), 0.4 \(\pm\) 0.02 pmol \([^{3}H]\)SQ29,548/mg whole cell protein, and HEK 293, 0.19 \(\pm\) 0.02 pmol \([^{3}H]\)SQ29,548/mg whole cell protein. Data presented are representative immunoblots from four individual experiments. The positions of the molecular weight markers (kDa) are indicated to the left and right of panels A and B, respectively.
Figure 3. Subcellular localisation of TPs by indirect immunofluorescence microscopy.
HEK 293 cell lines stably over-expressing the HA:TPα (Panel A-C), HA:TPαN4,Q4 (Panels D-F), HA:TPαN16,Q16 (Panels G-I) and HA:TPαN4,N16,Q4,Q16 (Panels J-L) were subject to indirect immunofluorescence microscopy under non-permeabilising (Panels A, D, G & J) and permeabilising (Panels B, E, H & K) conditions using the anti-HA 101R as primary antibody (1:2500 dilution) and FITC-labelled goat anti-mouse IgG (1:120 dilution) secondary antibody, employing an Olympus BX60 fluorescent microscope using the U-MNIBA filter. Alternatively, in Panels C, F, I & L, permeabilised cells were co-stained with anti-HA primary / FITC-labelled goat anti-mouse IgG (1:120 dilution) secondary antibody and with Rhodamine 6G (1:2000 dilution); images captured with the U-MNIBA filter and the U-MWIG filter, at magnification of 100X, were overlaid prior to photography and overlaid images are depicted in the respective Panels C, F, I & L.
Figure 4. Analysis of U46619-mediated intracellular signalling.

Panels A & B: HEK.TPα (TPα), HEK.TPαN4-Q4 (TPαN4-Q4), HEK.TPαN16-Q16 (TPαN16-Q16), HEK.TPαN4,N16-Q4,Q16 (TPαN4,16-Q4,16) or, as controls, HEK 293 cells, each transiently transfected with pCMV:Gαq, were stimulated with 1 μM U46619 and agonist-mediated IP3 generation (Panel A) and intracellular calcium ([Ca2+]i) mobilization (Panel B) was determined, as outlined in the Materials and Methods. Levels of IP3 produced in ligand-stimulated cells relative to that produced by the vehicle HBS-treated cells (basal IP3) were expressed as fold stimulation of basal (fold increase in IP3 ± S.E.M, n = 4). Changes in U46619-induced [Ca2+]i mobilization were monitored in FURA2/AM preloaded cells; data presented are representative profiles from at least four independent experiments and are plotted as changes in intracellular Ca2+ mobilization (∆[Ca2+]i, nM) as a function of time (second, s). Actual mean changes in [Ca2+]i mobilization (nM ± S.E.M) were as follows: TPα, ∆[Ca2+]i = 160 ± 10.1 nM; TPαN4-Q4, ∆[Ca2+]i = 101 ± 7.3 nM; TPαN16-Q16, ∆[Ca2+]i = 120 ± 9.2 nM; TPαN4,16-Q4,16, ∆[Ca2+]i = 72 ± 3.4 nM; HEK 293 cells, ∆[Ca2+]i = 20 ± 2.1 nM. The asterisks indicate that U46619-mediated IP3 generation was significantly reduced compared to wild type TPα cells stimulated with U46619, where *, ** and *** indicates p ≤ 0.05, p ≤ 0.01 and p ≤ 0.001, respectively.
Figure 5. Co-immunoprecipitation of Gαq with TPα and its N-linked glycosylation defective variants.

Panel A: HEK.TPα (i), HEK.TPαN4-Q4 (ii), HEK.TPαN16-Q16 (iii), HEK.TPαN4,N16-Q4,Q16 (iv) or HEK 293 (v) cells, were transiently co-transfected with either pCMV5 (-Gαq) or pCMV: Gαq (+ Gαq; 25 μg), and were stimulated at 37 °C for 5 min with 1 μM U46619 (+ U46619) or with the vehicle HBS (- U46619). Thereafter, cells were harvested and subjected to immunoprecipitation with the anti-HA 101R antibody; immunoprecipitates were analysed by SDS-PAGE/Western blotting and were screened for the presence of Gαq employing the anti-Gαq antisera (C-19, Santa Cruz).

Panel B: Alternatively, HEK.TPα (lane 1), HEK.TPαN4-Q4 (lane 2), HEK.TPαN16-Q16 (lane 3), HEK.TPαN4,N16-Q4,Q16 (lane 4) or HEK 293 (lane 5) cells, were subject to immunoprecipitation with the anti-HA 101R antibody; immunoprecipitates were analysed by SDS-PAGE/Western blotting and were screened for the presence of the respective HA-epitope tagged receptors employing the anti-HA 3F10 peroxidase conjugated Ig. Panel C: Typical western blot (75 μg total cellular protein analysed) confirming over-expression of Gαq in HEK.TPαN4,N16-Q4,Q16 cells transiently co-transfected with pCMV:Gαq (+ Gαq) or, as a control, with pCMV5 (-Gαq). The relative positions of the molecular weight markers (kDa) are indicated to the left of Panel A and to the right of Panels B & C, respectively.
Table 1. **Scatchard Analysis.**

<table>
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<tr>
<th>Receptor Type</th>
<th>$K_d$ (nM ± S.E.M)</th>
<th>$B_{max}$ (pmol/mg protein ± S.E.M)</th>
<th>Relative $B_{max}$ %</th>
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<td>TPα</td>
<td>13.5 ± 1.1</td>
<td>3.40 ± 5.1</td>
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<tr>
<td>TPα$^{N4-Q4}$</td>
<td>8.1 ± 5.2</td>
<td>1.39 ± 3.8</td>
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<tr>
<td>TPα$^{N16-Q16}$</td>
<td>11.2 ± 0.5</td>
<td>2.40 ± 3.5</td>
<td>70</td>
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<tr>
<td>TPα$^{N4,N16-Q4,Q16}$</td>
<td>7.3 ± 1.7</td>
<td>0.27 ± 3.9</td>
<td>8</td>
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Scatchard analysis on HA-tagged forms of TPα, TPα$^{N4-Q4}$, TPα$^{N16-Q16}$, TPα$^{N4,N16-Q4,Q16}$ were carried out in the presence of the TP antagonist $[^3]$H]SQ29,548 (50.4Ci/mmol, 0-40 nM) using 75 µg whole cell protein per individual assay. Radioligand binding data were analysed with the Graphpad computer program (GraphPad Software Inc.) to determine the $K_d$ and $B_{max}$ values. Data presented are the mean values of four independent experiments ± standard error mean (S.E.M; $n = 4$). Relative $B_{max}$ values are expressed as percentage TP expression relative to levels of the wild-type HA:TPα (expression). HEK 293 control cells expressed 154 ± 4.1 fmol $[^3]$HSQ29,58 / mg protein ± S.E.M.
Table 2. *Saturation Radioligand Binding in Fractionated Cells.*

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>Expression in $P_{100}$ (pmol/mg protein ± S.E.M)</th>
<th>Expression in $S_{100}$ (pmol/mg protein ± S.E.M)</th>
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<td>$T!P!\alpha$</td>
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<td>0.41 ± 0.09</td>
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<tr>
<td>$T!P!\alpha^{N4,16-Q4,16}$</td>
<td>0.42 ± 0.06</td>
<td>0.23 ± 0.03</td>
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</tbody>
</table>

HEK 293 cells stably over-expressing HA-tagged $T\!P\!\alpha$, $T\!P\!\alpha^{N4-Q4}$, $T\!P\!\alpha^{N16-Q16}$ or $T\!P\!\alpha^{N4,N16-Q4,Q16}$ were fractionated into their crude membrane ($P_{100}$) and soluble ($S_{100}$) fractions and it was determined that there was approximately 3 fold more protein in the $P_{100}$ relative to the $S_{100}$ fractions. Thereafter, saturation radioligand binding assays were performed using 20 nM $[^{3}H]$SQ29,548 (50.4 Ci/mmol) and 75 μg $P_{100}$ or 25 μg $S_{100}$ fractions, respectively. Data presented are the mean values of four independent experiments ± standard error mean (S.E.M; $n = 4$).