Characterization of the Role of N-Linked Glycosylation on the Cell Signaling and Expression of the Human Thromboxane A2 Receptor Alpha and Beta Isoforms

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Accepted for publication April 14, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

The alpha and beta isoforms of the thromboxane A2 receptor (TP) mediate the actions of the prostanoid thromboxane A2 and its mimetics in humans. The amino terminal region of the TPs contains two consensus N-linked glycosylation sites at asparagine (N) residues N4 and N16. In this study, we explored the significance of N-linked glycosylation on the signaling and surface expression of the human TP isoforms. Inhibition of N-linked glycosylation reduced selective radioligand binding by either TP in both human erythroleukemia cells and in transfected human embryonic kidney 293 cells. Moreover, site-directed mutagenesis of the putative glycosylation sites of TPa revealed that radioligand binding also was reduced greatly for both the single (TPaN4-Q4, TPaN16-Q16) and double (TPaN4,N16-Q4,Q16) mutants, yielding levels of 8% binding relative to the wild-type TPα for the double mutants. Reductions in ligand binding were caused by decreased maximal binding and not by changes in affinity (Kd) or in specificity of the receptors for [3H]SQ29,548 or other ligands. Subcellular fractionation confirmed that, in relation to total TP expression, membrane expression was not altered in TPaN4-Q4 or TPaN16-Q16 but was reduced to levels of 55% of total expression in TPaN4,N16-Q4,Q16. Inhibition of glycosylation reduced, but did not abolish, agonist (U46619) mediated intracellular Ca2+ mobilization by TPα or TPβ and cAMP production by TPα. Thus, N-linked glycosylation of the human TP isoforms is important for ligand binding, efficient second messenger signaling and efficient membrane expression.

TXA2 induces many cellular responses, including platelet shape change and aggregation and constriction of bronchial and vascular smooth muscle cells (Negishi et al., 1993). These actions are mediated through interaction with the shared endoperoxide prostaglandin (PG)H2/TP (Kinsella et al., 1997), a member of the seven-transmembrane domain G-protein coupled receptor family. The human TP is encoded by a single gene on chromosome 19p13.3 (Nusing et al., 1997), a member of the seven-transmembrane domain G-protein-coupled receptor family. The human TP is encoded by a single gene on chromosome 19p13.3 (Nusing et al., 1997), a member of the seven-transmembrane domain G-protein-coupled receptor family. The human TP is encoded by a single gene on chromosome 19p13.3 (Nusing et al., 1997), a member of the seven-transmembrane domain G-protein-coupled receptor family. The human TP is encoded by a single gene on chromosome 19p13.3 (Nusing et al., 1997), a member of the seven-transmembrane domain G-protein-coupled receptor family.

Received for publication November 14, 1997.

1 This research was supported by grants from The Wellcome Trust, The Irish Heart Foundation, The Health Research Board of Ireland and University College Dublin, Presidents Research Award (BTK).

ABBREVIATIONS: Ca2+ in intracellular calcium; 8-epi PGF2α, 8-epi Prostaglandin F2α; EGTA, ethyleneglycoltetraacetic acid; Endo H, endoglycosidase H; FBS, fetal bovine serum; FURA2, 1-[2-5-carboxyoxazol(-2-yl)-6-aminobenzofuran-5-oxyl]-2-[2'-amino-5'-methylphenoxy]-ethanone-N,N',N'-tetraacetic Acid, Pentaacetoxymethyl Ester; GPCR, G-protein coupled receptor; HEDG, Heps buffered EGTA, dithiothreitol, glycerol, salt solution; HEL, human embryonic kidney; HEPES, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; HBSS, Heps buffered-solution; IP3, inositol 1,4,5 triphosphate; HBSSHB, Hanks' buffered salt solution (HBSS) containing 10 mM HEPES, pH 7.67 and 0.1% bovine serum albumin; PG, prostaglandin; PLC, phospholipase C; SDS, sodium dodecyl sulfate; TP, thromboxane A2 receptor; TXA2, thromboxane A2. Thromboxane A2 receptor is abbreviated to TP and its splice variants are designated by the Greek letters α and β, as recommended by the IUPHAR classification on prostanoid receptors.
whereas the number of TPβ sites increased (Yukawa et al., 1997). Furthermore, protein kinase C activation by the phorbol myristic acid inhibited intracellular Ca\(^{2+}\) mobilization in response to another TP agonist, I-BOP, in cells expressing TPα but not TPβ.

Many GPCRs contain one or two potential N-linked glycosylation sites (defined by Asparagine-X-Serine/Threonine, where X represents any amino acid; Marshall, 1972). Of the fully characterized GPCRs, it is evident that N-linked glycosylation of GPCRs may confer varying functional roles depending on the receptor. For example, site-directed mutagenesis studies on the consensus glycosylation sites of the hamster beta\(_2\)-adrenergic receptor (beta\(_2\) AR) amino (N) terminus, Asn\(^4\) and Asn\(^16\), revealed that although glycosylation does not affect ligand binding, it is important for normal coupling to the G\(_\alpha\)/adenyl cyclase system and for the correct subcellular localization of the receptor on the cell membrane (Rands et al., 1990). On the other hand, inhibition of N-linked glycosylation of the alpha\(_1\)-adrenergic receptor has no apparent effect on ligand binding or on membrane insertion of receptor (Sawutz et al., 1987). The retinal rhodopsin receptor has two highly conserved N-linked glycosylation sites. A naturally occurring mutation in one of the N-linked glycosylation consensus sites, at Thr\(^17\), is responsible for a form of the autosomal dominant disorder retinitis pigmentosus (Sung et al., 1991). N-linked glycosylation also has been reported as functionally important in the thrombin receptor. Treatment of fibroblasts with tunicamycin, a specific inhibitor of N-linked glycosylation, inhibited thrombin binding to its receptor (Frost et al., 1991) and inhibited thrombin-induced Ca\(^{2+}\) mobilization in human T-lymphoblastoid cells in a dose-dependent manner (Tordai et al., 1995).

In the prostaglandin receptors, N-linked glycosylation also apparently plays divergent functional roles. For the EP\(_\alpha\) subtype of the PGE\(_2\) receptor, site-directed mutagenesis studies on two potential N-linked glycosylation sites at Asn\(^16\) and Asn\(^193\) indicated an essential role for glycosylation in determining both affinity and specificity for PGE\(_2\) binding (Huang and Tai, 1995). The human PGI\(_2\) receptor also was glycosylated (Smyth et al., 1996). The human TP isoforms have two potential N-linked glycosylation sites in their NH\(_2\)-terminal region at Asn\(^4\) and Asn\(^16\). These sites are conserved in the mouse, rat and bovine TP receptors (Namba et al., 1991) and 44 kDa (Raychowdhury et al., 1994). N-linked glycosylation also has been reported as functionally important in the thrombin receptor. Treatment of fibroblasts with tunicamycin, a specific inhibitor of N-linked glycosylation, inhibited thrombin binding to its receptor (Frost et al., 1991) and inhibited thrombin-induced Ca\(^{2+}\) mobilization in human T-lymphoblastoid cells in a dose-dependent manner (Tordai et al., 1995).

Site-directed mutagenesis led to the elucidation of the importance of particular residues within the TPα, particularly in identifying those residues involved in ligand binding and/or receptor-effector coupling (Funk et al., 1993; D’Angelo et al., 1996, Chiang et al., 1996).

In this study, we sought to determine the importance of N-linked glycosylation on the signaling and surface expression of the human TP isoforms. We used both tunicamycin and endo H treatment of cells combined with site-directed mutagenesis of the Asn\(^4\) and Asn\(^16\) residues of the TPα to explore the significance of N-linked glycosylation on TP function. Our results indicated that N-linked glycosylation of the human TP isoforms is important for ligand binding and for efficient G protein coupling, and that N-linked glycosylation, in at least one site (Asn\(^4\) or Asn\(^16\)), is required for membrane expression.

Materials and Methods

Materials. The following chemicals were obtained from Cayman Chemical Company (Ann Arbor, MI): 5-Heptenoic acid, 7-(6-(3-hydroxy-1-octenyl)-2-oxacyclic) [2,2.1] hept-5-yl)] 1H-[1a,4a,5β,2α,6O(E,5αS)]-9,11-dideoxy-9α,11α-methanoepoxy prostaglandin F\(_2\alpha\) (U66569); 5-Heptanoic acid, 7-[3-[Z-(phenylamino) carbonyl] hydrazine] methyl \(-7\)- oxacyclic [2,2.1] hept -2-yl],[1S-(1a,2a,Z),3a,4a]l]-SQ29,548, thrombin, G418, 1-[(5-(carboxyoxazol-2-yl)-6-aminobenzofuran-2-oxyl)-2’(2’-amino-5’-methylphenoxy)-ethane-N,N,N’-tetraacetic acid, pentacetoxyethyl ester] (FURA2/AM), D-myo-inositol 1,4,5 triphosphate, 3-deoxy-hexa sodium salt (stable analog of IP\(_3\)) and ionomycin.

[G]H]SQ29,548 (50.4 Ci/mmol) and \(^{125}\)I-labeled goat anti-rabbit IgG (7.61 \(\mu\)Ci/\(\mu\)g; NEX 167) were obtained from DuPont NEN (Boston, MA). GaoGo11 (C19) specific antibody was obtained from Santa Cruz Laboratories (Santa Cruz, CA). \([\text{H}]\text{HAMP} (15–30 \text{Ci/mM})\) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). Dualon-UV nylon membranes were obtained from Stratagene (LaJolla, CA). Tunicamycin was purchased from Sigma Chemical Inc. (St. Louis, MO). Ultraspec RNA isolation system from Biotecx (Houston, TX). Endo-\(\beta\)-N-acetylgalactosaminidase H, recombinant enzyme, was obtained from Boehringer Mannheim (Basel, Switzerland).

Plasmid construction and site-directed mutagenesis. The plasmids pCMV5 and pCMVTRX, containing the full-length cDNA (nucleotides \(-4\) to \(+1035\)) for the human platelet/placental TP\(_\alpha\), as an EcoRI-Hind111 insert in pCMV5, have been described previously (Kinsella et al., 1994).

The plasmid pBlueScript11 KS:TPβ was obtained from Dr. Anthony Ware, Harvard Medical School. The latter plasmid contains a 1.5 kB EcoRI insert encoding the full-length coding sequence (nucleotides \(+1224\)) for TPβ plus additional 5’ (140 base pair) and 3’ (67 base pair) untranslated sequences (Raychowdhury et al., 1995, 1995). The plasmid pCMV:TPβ was constructed by subcloning the full-length cDNA coding sequence for TPβ (nucleotides \(+1224\)) into the EcoRI-Hind111 sites of pCMV5. To facilitate the construction of stable cell lines, the full length cDNAs encoding TPα or TPβ were subcloned further into the plasmid pCDNA3 conferring G418 resistance: pCDNA3:TPα contains a Hind111–BamH1 insert (nucleotides 1–1032) encoding TPα, whereas pCDNA3:TPβ contains a Hind111 flanked insert (nucleotides 1–1224) encoding TPβ.

The plasmid pGEMTXR was constructed by subcloning the full-length cDNA for TPα from pCMVTRX into the EcoRI–Hind111 sites of the plasmid pGEM4 (Promega).

To facilitate site-directed mutagenesis of the asparagine (N) to glutamine (Q) residues at amino acids 4 (N4–Q4) or 16 (N16–Q16) of TPα, the plasmid pG3TXRNKp was constructed by subcloning the EcoRI–Kpn1 subfragment (encoding nucleotides \(-4\) to \(+248\)) from pCMVTRX into pGEM3. PCR-assisted, site-directed mutagenesis at N4 or N16, respectively, was carried out with pG3TXRNKp as template and the following mutator oligonucleotides:

Mutator oligonucleotide 1. Mutation N4-Q4. 5’ AAC AGG GCC CCA GGG AAC TGC CTT GGG GCC ACA TA G 3’ (Complementary to coding region).

Mutator oligonucleotide 2. Mutation N16-Q16. 5’ TCCCTG GGG CCC TGG TTC CCG CCC ACA CAG ATT ACC CTT GAG 3’ where the nucleotides carrying the mutated sequences are underlined.

Site-directed mutagenesis was carried out essentially as previously described (Kinsella et al., 1991) with the plasmid pG3TXRNKp.
as template to generate the plasmids pG3TXR^N4,Q4^ NKp and pG3TXR^N16,Q16^ NKp containing single amino acid mutations (N-Q) at codons 4 and 16, respectively. The latter plasmids were then used to create the double-mutant plasmid pG3TXR^N4,Q4,Q16,Q16^ NKp by direct ligation of subfragments. All mutations were verified by double-stranded DNA sequencing with Sequenase Version 2.0 (United States Biochemical, Cleveland, OH).

The plasmids pCMVTXR^N4,Q4^, pCMVTXR^N16,Q16^ and pCMVTXR^N16,Q16^ were then created from the latter plasmids by replacing the EcoR1-Kpn1 fragment from pCMVTXR containing the wild-type TPa sequence with the corresponding EcoR1-Kpn1 fragments from the plasmids pG3TXR^N4,Q4^ NKp, pG3TXR^N16,Q16^ NKp and pG3TXR^N16,Q16^ NKp, respectively.

The plasmid pCMV:G HEL 92.1.3 cells and HEK 293 cells were obtained from the American Type Culture Collection (Manassas, VA). HEL cells were routinely grown in RPMI 1640 medium, 10% FBS. HEK 293 cells were grown in minimal essential medium with 10% FBS. HEK 293 cells were grown in minimal essential medium containing 10% FBS. HEK 293 cells were transfected with 10 μg of pCMV:G

**Cell culture and transfections.** HEL 92.1.3 cells and HEK 293 cells were obtained from the American Type Culture Collection (Manassas, VA). HEL cells were routinely grown in RPMI 1640 medium, 10% FBS. HEK 293 cells were grown in minimal essential medium containing 10% FBS. For transfection studies, HEK 293 cells were plated in 100-mm culture dishes, approximately 48 h before transfection. HEK 293 cells were grown in minimal essential medium containing 10% FBS. HEK 293 cells were harvested by centrifugation at 500 × g for 5 min, washed three times in Dulbecco’s phosphate-buffered saline and were resuspended in modified Ca^2+/-Mg^2+-free HBSSHB buffer, containing 10 mM HEPES, pH 7.67, 0.1% bovine serum albumin. Alternatively, to fractionate the cells into their soluble (S100) or membrane (P100) components, washed cells were resuspended and homogenized in HED buffer (20 mM HEPES, pH 7.67, 1 mM EGTA, 0.5 mM dithiothreitol) supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μM iodoacetamide. The homogenates were centrifuged at 100,000 × g for 30 min at 4°C, and the membrane fractions (P100) were resuspended in HEDG buffer (20 mM HEPES, pH 7.67, 1 mM EGTA, 0.5 mM dithiothreitol, 100 mM NaCl, 10% glycerol) supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μM iodoacetamide. Protein determinations were carried out according to the Bradford assay (Kinsella et al., 1997). For ligand binding studies, protein concentrations in the membrane, fractions or whole cell fractions, were diluted to 1 mg/ml in HEDG or HBSSHB buffer, respectively. Radioligand binding assays were carried out in the presence of the TP antagonist [H]SQ29,548 (50.4 Ci/mmol) at 30°C for 30 min in 100-μl reactions (containing 100 μg protein/assayy, unless otherwise specified) in the presence of 0 to 40 nM [H]SQ29,548 for Scatchard analyses or in the presence of 20 nM [H]SQ29,548 for saturation radioligand binding experiments. For competition binding studies, radioligand binding of [H]SQ29,548 (20 nM, 50.4 Ci/mmol) was carried out in the presence of the following competitor ligands: SQ29,548 (10^-6 to 10^-8 M); U46619 (10^-6 to 10^-9 M). In all cases, nonspecific binding was determined in the presence of excess nonlabeled SQ29,548 (10 μM). Reactions were terminated by the addition of 4 ml of ice-cold 10 mM Tris-HCl, pH 7.4, followed by filtration through Whatman GF/C glass filters, and subsequent washing of the filters three times with 10 mM Tris-HCl, pH 7.4, followed by liquid scintillation counting of the filters in 5 ml of scintillation fluid. Radioligand binding data was analyzed with the INPLOT 4 computer program (GraphPad Software Inc., San Diego, CA) to determine the Kd and B_max values.

**Endo H studies.** HEKα1 and HEKβ3 stable cells were plated at a density of 2 × 10^5 cells per 100-mm cell culture dish in 8 ml minimal essential medium, 10% FBS. After 24 h, an aliquot (4 μl; 4 μM) of Endo H was added directly to each dish or, as controls, enzyme that had been heat inactivated by boiling for 10 min. Cells were incubated for an additional 24 h at 37°C. Thereafter, cells were harvested by scraping, and membranes were prepared and assayed by radioligand ([H]SQ29,548 binding (20 nM, 50.4 Ci/mmol) as described previously. Data are presented as picomole radioligand bound per milligram of cell protein ± S.E., (i.e. pmol/mg ± S.E) and are the mean values of four to six experiments.

**Northern Blot analysis.** Total RNA was isolated from transfected HEK 293 cells with the Ultraspec RNA isolation procedure as recommended by the manufacturers. RNA (10 μg/lane) was analyzed by electrophoresis on 1.1 agarose/formaldehyde/formamide gels followed by transfer and cross-linking to Duralon-UV nylon membranes essentially as described by Sambrook et al. (1989). Northern Blots were screened with radiolabeled RNA riboprobes complementary to the human TP receptor mRNA 3’ coding region (nucleotides 474–1032); the probes were prepared by the in vitro transcription of the NotI linearized plasmid pGEMTXR with T7 RNA polymerase in the presence of (α-[^32]P)CTP (800 Ci/mmol, 10 mCi/ml), essentially as described (Melton et al., 1984). Filters were hybridized at 70°C in 5× SSC, 50% formamide, 5× Denhardt’s solution, 0.2% SDS, 100 μg/ml denatured sonicated salmon sperm DNA for 12 to 16 h. Filters were washed in 0.1× SSC, 0.1% SDS at 70°C for 20 min; incubated with RNase A (1 μg/ml) in 2× SSC at room temperature for 10 min and washed again at 70°C in 0.1× SSC, 0.1% SDS for 20 min.

**Measurement of intracellular Ca^{2+} mobilization.** Ca^{2+}, measurements in either transfected HEK 293 cells or in HEL cells were made by monitoring the intensity of FURA2 fluorescence. For the transfected cells, 48 h after transfection the HEK 293 cells were washed twice in phosphate-buffered saline, resuspended in HBSSHB buffer at 10^7 cells/ml and incubated in the dark with 5 μM FURA2/AM for 45 min at 37°C. Subsequently the cells were collected by centrifugation (900 × g, 5 min), were washed once in an equal volume of HBSSHB, and were finally resuspended in HBSSHB buffer at 10^7 cells/ml and kept at room temperature in the dark until use. For each measurement of Ca^{2+} mobilization, aliquots of HEK 293 cells were diluted to 0.825 × 10^6 cells/ml in HBSSHB buffer containing 1 mM CaCl_2.

FURA2 fluorescence was recorded in HEK 293 cells (2-mL aliquots) at 37°C with gentle stirring with a Perkin Elmer-Cetus LS50-B spectrofluorometer at excitation wavelengths of 340 nm and 380 nm and emission wavelengths of 510 nm, respectively (Kinsella et al., 1997). For each of the cell types used in this study, a dose-response curve to U46619 was determined and individual values were found to be identical irrespective of the cell type. Thus, each experiment was performed with the dose of agonist corresponding to 2 μM U46619 unless otherwise specified. Intracellular Ca^{2+} mobilization was monitored in response to ionomycin (1 μM) or in response to the stable P_{αi} analog d-myo-inositol 1,4,5-triphosphate, 3-deoxy-hexa sodium salt (0.1 μM) in saponin (10 μg/ml)-permeabilized cells. A rapid, transient rise and fall in Ca^{2+}, levels in response to ligand stimulation was interpreted as receptor-mediated Ca^{2+}, mobilization. The calibration of the signal was performed in each sample by adding 0.2% Triton X-100 to obtain the maximal fluorescence ratio (R_{max}) and then 1 mM EGTA to obtain the minimal fluorescence ratio (R_{min}). The ratio of the fluorescence at 340 nm and 380 nm is a measure of Ca^{2+}, (Kinsella et al., 1997) which assumes a K_d and B_{max} values.

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[3H]SQ29,548 for Scatchard analyses or in the presence of 20 nM [H]SQ29,548 for saturation radioligand binding experiments. For competition binding studies, radioligand binding of [H]SQ29,548 (20 nM, 50.4 Ci/mmol) was carried out in the presence of the following competitor ligands: SQ29,548 (10^-6 to 10^-8 M); U46619 (10^-6 to 10^-9 M). In all cases, nonspecific binding was determined in the presence of excess nonlabeled SQ29,548 (10 μM). Reactions were terminated by the addition of 4 ml of ice-cold 10 mM Tris-HCl, pH 7.4, followed by filtration through Whatman GF/C glass filters, and
are plotted as changes in intracellular Ca\(^{2+}\) mobilized (\(\Delta[Ca^{2+}]\), (nM)) as a function of time (sec) upon ligand stimulation.

**Measurement of cAMP.** Transfected cells were washed three times in ice-cold phosphate-buffered saline; cells (approximately 1–2 \(\times\) 10\(^6\) cells) were resuspended in 200 \(\mu\)l 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) containing 1 \(\mu\)M 3-isobutyl-1-methylxanthine and preincubated at 37°C for 10 min. Thereafter, cells were stimulated in the presence of 1 \(\mu\)M U46619 (from a 5 \(\mu\)M U46619 stock, 50 \(\mu\)l) or in the presence of HBS (50 \(\mu\)l) at 37°C for 10 min. Reactions were terminated by heat inactivation (100°C, 5 min) and the level of cAMP produced was quantified by radioimmunoassay with the cAMP-binding protein from bovine adrenal medulla, essentially as described by Farndale et al., (1992). Protein determinations were carried out with the Bradford assay (Bradford, 1976). Levels of cAMP produced by U46619-stimulated cells relative to basal stimulation, in the presence of HBS, were expressed in picomoles cAMP per milligram protein ± S.E.M. (pmol/mg ± S.E.M.) and as fold stimulation to basal (fold increase ± S.E.M.). The data presented are representative of four independent experiments.

**Data analyses.** Radioligand binding data were analyzed with the INPLOT 4 computer program (GraphPad Software Inc.) to determine the \(K_d\) and \(B_{max}\) values. Statistical analyses were carried out with the unpaired Student’s \(t\) test with the Statworks Analysis Package.

**Results**

**Effect of inhibition of N-linked glycosylation on TP expression.** HEL cells were incubated in the presence of tunicamycin, a direct inhibitor of N-linked glycosylation, to investigate initially whether inhibition of N-linked glycosylation had any effect on TP expression. Tunicamycin has been shown to inhibit the incorporation of oligosaccharides into proteins with little or no effect on de novo protein synthesis itself and to exhibit low general toxicity in the effective concentration range of 1 to 20 \(\mu\)g/ml (Struck and Lennarz, 1977). Thus, a time course assay (0–48 h) was used initially to examine the effect of tunicamycin on TP expression used at nontoxic concentrations of 2 \(\mu\)g/ml. When used at these concentrations, tunicamycin was confirmed to be noncytotoxic to both HEL and HEK 293 cells with the Trypan Blue dye-exclusion assay. TP expression was monitored by radioligand binding assays with the radiolabeled TP antagonist \(^{3}H\)SQ29,548. The platelet-like HEL cells are known to express TPα (Kinsella et al., 1994); with a reverse transcriptase polymerase chain reaction approach, we have confirmed that HEL cells express both the TP\(\alpha\) and TP\(\beta\) isoforms (Miggin and B.T. Kinsella, unpublished). Control HEL cells, not treated with tunicamycin, displayed specific binding of \(^{3}H\)SQ29,548 of 19.9 ± 5.9 fmol/mg cell protein. In the presence of tunicamycin, TP expression was reduced to levels of 45 ± 3.2% relative to the nontreated cells after 12 h incubation (fig. 1), indicative of a role of N-linked glycosylation in TP expression or function.

To investigate further whether this effect was isoform specific, stable cell lines expressing either the TP\(\alpha\) or TP\(\beta\) isoform were constructed in HEK 293 cells. The latter cells display very low levels of TP expression (Kinsella et al., 1997), providing an ideal background in which to create stable cell lines for TPs. Thus, HEK\(\alpha\) cells are stably transfected with TP\(\alpha\) and display an affinity (\(K_d\)) of 9.43 ± 0.92 nM (n = 4) and \(B_{max}\) of 3.02 ± 0.43 pmol/mg cell protein (n = 4) for \(^{3}H\)SQ29,548, respectively. HEK\(\beta\) cells are stably transfected with TP\(\beta\) and display an affinity (\(K_d\)) of 8.44 ± 1.44 nM (n = 6) and \(B_{max}\) of 3.24 ± 0.33 pmol/mg cell protein (n = 6) for \(^{3}H\)SQ29,548, respectively. Incubation of HEK\(\alpha\) and HEK\(\beta\) cells with tunicamycin during a 0 to 48 h period showed a time-dependent decline in TP\(\alpha\) and TP\(\beta\) expression (fig. 2). Levels of TP\(\alpha\) and TP\(\beta\) expression were reduced to 78.3 ± 7.70% and 80.2 ± 5.50%, respectively, after 4 h incubation in the presence of tunicamycin relative to the control cells. On prolonged incubations (24 h or 48 h), HEK\(\beta\) cells were significantly (P = .05) more sensitive to tunicamycin treatment than HEK\(\alpha\) cells (fig. 2).

**Effect of inhibition of N-linked glycosylation on intracellular Ca\(^{2+}\) mobilization.** Functional coupling of the TP\(\alpha\) or TP\(\beta\) isoforms to PLC activation was assessed by monitoring mobilization of Ca\(^{2+}\) in FURA2/AM-loaded HEL cells grown in RPMI, 10% FBS (0.5 × 10\(^6\) cells/ml) were incubated in the presence of tunicamycin (2 \(\mu\)g/ml) for 0 to 48 h. At the indicated times, aliquots were removed and cells were assayed for TP expression in the presence of \(^{3}H\)SQ29,548 (50.4Ci/mmol, 20 nM) as described under “Materials and Methods.” Results are expressed as a percentage TP expression relative to the control HEL cells that had not been incubated with tunicamycin ± standard error (% expression ± S.E.); n = 4). TP expression in the control HEL cells was 19.9 ± 5.90 fmol/mg cell protein.

![Fig. 1. Effect of tunicamycin on TP expression in HEL cells.](image-url)
HEKα1 cells and HEKβ3 cells, respectively, in response to the TXA2 mimetic U46619. It has been previously reported that for efficient TPα coupling to Ca^{2+}, mobilization in HEK 293 cells, it was necessary to coexpress a member of the Gαq (Gαq or G11) family (Kinsella et al., 1997). Thus, HEKα1 or HEKβ3 cells were transiently cotransfected with the cDNA for Gα11, where indicated, tunicamycin (2 μg/ml) was added directly to the cells 2 to 44 h after transfection. Thereafter, tunicamycin-treated or nontreated cells were harvested 48 h after transfection, and Ca^{2+}, mobilization, was measured in FURA2/AM-loaded cells by spectrofluorometry in response to U46619 (2 μM). Positive overexpression of Gα11 in HEKα1 and HEKβ3 cells was confirmed by Western Blot analyses (data not shown). Both HEKα1- and HEKβ3-transfected cells displayed a rapid transient rise in Ca^{2+}, mobilization in response to U46619 stimulation (fig. 3 A and B). However, incubation of HEKα1 or HEKβ3 cells with tunicamycin resulted in time-dependent reductions in Ca^{2+}, mobilization, respectively. Treatment of HEKα1 cells with tunicamycin for 48 h reduced Ca^{2+}, mobilization from 96.9 ± 16.3 nM to 15.7 ± 7.09 nM, whereas treatment of HEKβ3 cells with tunicamycin for 48 h reduced Ca^{2+}, mobilization from 94.1 ± 21.1 nM to 11.7 ± 4.99 nM. Tunicamycin treatment of HEKα1 or HEKβ3 cells had no effect on Ca^{2+}, mobilization in response to the IP3-stable analog or in response to ionomycin, indicating that tunicamycin treatment, per se, does not interfere with Ca^{2+}, mobilization (data not shown). Moreover, tunicamycin was confirmed to be noncytotoxic to HEK 293 cells with the Trypan Blue dye-exclusion assay. Thus, the effects of tunicamycin on TPα expression/TPα ligand binding and on Ca^{2+}, mobilization in response to U46619 are evidence that N-linked glycosylation of TPα and TPβ is functionally important.

Site-directed mutagenesis of N-linked glycosylation sites of TPα. To establish definitively whether one or both putative N-linked glycosylation sites are functionally required, site-directed mutagenesis was carried out on the TPα isoform whereby the pivotal asparagine (N) residues were mutated to glutamine (Q) residues at amino acid residues N4, N16 or both to generate the mutated receptors TPα^{N4,Q4}, TPα^{N16,Q16}, TPα^{N4,N16,Q4,Q16}, respectively. HEK 293 cells were transiently transfected with the cDNAs for the wild-type and mutant TPα receptors and the level of TP expression was assessed by saturation radioligand ([3H]SQ29,548) binding. Levels of TP expression in HEKα1 cells were 4.99 ± 0.32 pmol/mg cell protein. However in the mutant receptors, specific [3H]SQ29,548 binding was reduced to 47.6 ± 3.2% (TPα^{N4,Q4}), 58.4 ± 7.3% (TPα^{N16,Q16}) and 8.3 ± 1.0% (TPα^{N4,N16,Q4,Q16}) relative to the wild-type receptor (100%). To address whether this reduction in binding exhibited by the mutant receptors was caused by a change in the affinity (Kd) or Bmax for [3H]SQ29,548, radioligand binding isotherms were carried out and the results were analyzed by Scatchard analysis (table 1 and fig. 4). Consistent with the previous results, although there were no observed differences in the affinity (Kd) of each of the mutant receptors for [3H]SQ29,548, a substantial reduction occurred in maximal binding, with the double mutant receptor (TPα^{N4,N16,Q4,Q16}) only retaining approximately 8% binding relative to the non-mutant receptor (table 1). In general, HEK 293 cells transfected with the control vector (pcMV5) had between 2 and 5% radioligand ([3H]SQ29,548) binding, representing approximately 48 to 120 fmol [3H]SQ29,548/mg protein, relative to that expressed by cells transfected with the wild-type TPα receptor. Thus, in the double mutant, a considerable portion (48–120 fmol [3H]SQ29,548/mg protein) of radioligand binding associated with this receptor may be caused by endogenous receptor expressed in HEK 293 cells.

To establish whether the observed changes in [3H]SQ29,548 binding associated with the mutant receptors could be caused by changes in the relative subcellular distribution of TP, transfected cells were fractionated into their membrane (P100) and cytosolic (S100) components before radioligand binding. Consistent with data for nonfractionated cells (fig. 4, table 1), the fractionated cells displayed similar corresponding reductions in total, membrane and cytosolic expression for each of the mutants relative to the wild-type receptor (fig. 5A). In terms of membrane expression, 84.8 ± 5.1% of total TPα expression localized to the membrane fraction of the cell for the wild-type receptor (fig. 5). Similarly, in the single mutant receptors, lo-

Fig. 2. Effect of tunicamycin on TPα and TPβ expression in HEK 293 cells. HEKα1 or HEKβ3 cells stably transfected with the cDNA for TPα (Kα) for [3H]SQ29,548 = 9.43 ± 0.92 nM, Bmax = 3.02 ± 0.43 pmol/mg) or TPβ (Kβ for [3H]SQ29,548 = 8.44 ± 1.44 nM, Bmax = 3.24 ± 0.33 pmol/mg, n = 6), respectively, were incubated in the presence of tunicamycin (2 μg/ml) for 0 to 48 h. At the indicated times, cells were harvested and assayed for TPα (●) or TPβ (■) expression in the presence of [3H] SQ29,548 (50.4 Ci/mmol, 20 nM). In each case, results are expressed as percentage TP expression relative to the control cells that had not been incubated with tunicamycin (percentage expression ± standard error of mean = ± S.E.M.; n ≥ 3). Levels of TPα in control HEKα1 cells were 3.29 ± 0.30 pmol/mg cell protein and for TPβ expression in control HEKβ3 cells were 2.85 ± 0.32 pmol/mg cell protein.
TABLE 1
Radioligand binding isotherms analyzed by Scatchard analysis for determination of cause for reduction in binding exhibited by mutant receptors

<table>
<thead>
<tr>
<th>Transfected Cells</th>
<th>$K_d$ (nM ± S.E.)</th>
<th>$B_{max}$ (fmol/mg protein ± S.E.)</th>
<th>Relative $B_{max}$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP$_{a}$</td>
<td>15.1 ± 1.9</td>
<td>2710 ± 416</td>
<td>100</td>
</tr>
<tr>
<td>TP$_{a}$N4–Q4</td>
<td>9.9 ± 0.65</td>
<td>1005 ± 405</td>
<td>43.5</td>
</tr>
<tr>
<td>TP$_{a}$N16–Q16</td>
<td>10.8 ± 1.9</td>
<td>1336 ± 493</td>
<td>60.2</td>
</tr>
<tr>
<td>TP$_{a}$N4,N16–Q4,Q16</td>
<td>11.4 ± 2.6</td>
<td>222 ± 50.2</td>
<td>8.2</td>
</tr>
</tbody>
</table>

HEK 293 cells were transiently transfected with the plasmids coding for the wild-type TP$_{a}$ or mutant TP$_{a}$N4–Q4, TP$_{a}$N16–Q16 and TP$_{a}$N4,N16–Q4,Q16 receptors as indicated. For Scatchard analyses, radioligand binding assays on whole cells (25 μg protein/assay) were carried out in the presence of the TP antagonist $[^3H]$SQ29,548 (50.4 Ci/mmol, 0–40 nM). Radioligand binding data were analyzed with the INPLOT 4 computer program (GraphPad Software Inc.) to determine the $K_d$ and $B_{max}$ values. Data are presented as the mean values of four independent experiments ± standard error (S.E.). Relative $B_{max}$ values are expressed as percentage TP expression relative to levels of the wild-type TP$_{a}$ (% expression).

However, these data do not definitively establish a direct role for N-linked glycosylation on ligand binding by TP$_{a}$. Hence, to address this issue, HEK1 cells and HEK3 stable cell lines were incubated directly in the presence of the enzyme Endo H, specific for N-linked oligosaccharides or, as controls, in the presence of heat-inactivated enzyme. Thereafter, membranes were prepared and were assayed for radioligand $[^3H]$SQ29,548 binding. In the case of TP$_{a}$, Endo H treatment of HEK1 cells reduced radioligand binding to 50.9% (1.59 ± 0.42 pmol/mg, n = 8) relative to the non-treated control cells (100%; 3.12 ± 0.58 pmol/mg, n = 5), whereas the heat-inactivated enzyme did not affect ligand binding (3.25 ± 1.02 pmol/mg, n = 4). Similarly, in the case of TP$_{b}$, Endo H treatment of HEK3 cells reduced radioligand binding to 53.9% (1.83 ± 0.27 pmol/mg, n = 8) relative to the non-treated control cells (100%; 3.39 ± 0.57 pmol/mg, n = 6), whereas the heat-inactivated enzyme only marginally reduced ligand binding (94.1%; 3.19 ± 1.04 pmol/mg, n = 4). Thus, from these experiments, it appears that N-linked glycosylation is required for optimal ligand binding.
Northern Blot analysis. Northern Blot analysis was carried out to address whether the substantial reduction in [3H]SQ29,548 binding by the mutant TPα receptors could be accounted for by reductions in gene expression (fig. 6). RNA was isolated from HEK 293 cells transiently transfected with wild-type or mutant TPα receptors, or with the vector, pCMV5, and Northern Blots were probed with a [32P]-radiolabeled antisense RNA transcript based on the 3' coding region of the TP mRNA. Equal loading of RNA (fig. 6A) and equal intensity of the hybridization signal by cells transfected with either wild-type TPα or mutant TPα (TPαN4–Q4, TPαN16–Q16, TPαN4,N16–Q4,Q16) but not with the vector pCMV5 (fig. 6B) was confirmed by densitometric scanning of the gel and autoradiogram, respectively.

Analyses of intracellular signaling. To address whether the observed differences in radioligand ([3H]SQ29,548) binding by the mutant TPα receptors was confined to a reduced ability to bind the antagonist SQ29,548 exclusively or whether there was also a reduced ability to interact with other TP ligands, competition binding profiles were performed. Competition binding studies of [3H]SQ29,548 were carried out in the presence of U46619 and SQ29,548 on HEK 293 cells transfected with TPα wild-type or mutant receptors. For the competing ligand U46619, ligand binding by the wild-type TPα and each of the mutant receptors (TPαN4–Q4, TPαN16–Q16, TPαN4,N16–Q4,Q16) were not significantly different with typical $K_i$ values between 0.24 and 0.77 μM U46619 (table 2). Similarly, no significant differences were obtained with SQ29,548 as competing ligand.
by the wild-type or mutant TPα receptors with typical $K_i$ values ranging between 0.76 and $1.60 \times 10^{-8}$ M SQ29,548 (table 2). The $K_i$ values reported here compare favorably with those reported previously by Habib et al. (1997) and Muck et al. (1998).

![Fig. 5. Subcellular distribution of TP receptors in transfected HEK 293 cells.](image)

![Fig. 6. Northern Blot analysis of transfected HEK 293 cells.](image)

<table>
<thead>
<tr>
<th>Cells</th>
<th>$K_i$ Values</th>
<th>$[U46619]$</th>
<th>$[SQ29,548]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPα</td>
<td>$0.77 \pm 0.25$</td>
<td>$1.34 \pm 0.13$</td>
<td></td>
</tr>
<tr>
<td>TPα&lt;sub&gt;N4-Q4&lt;/sub&gt;</td>
<td>$0.41 \pm 0.07$</td>
<td>$0.76 \pm 0.16$</td>
<td></td>
</tr>
<tr>
<td>TPα&lt;sub&gt;N16-Q16&lt;/sub&gt;</td>
<td>$0.57 \pm 0.09$</td>
<td>$1.60 \pm 0.20$</td>
<td></td>
</tr>
<tr>
<td>TPα&lt;sub&gt;N4,N16-Q4,Q16&lt;/sub&gt;</td>
<td>$0.24 \pm 0.15$</td>
<td>$1.56 \pm 0.35$</td>
<td></td>
</tr>
</tbody>
</table>

For competition binding studies, binding of $[^3H]SQ29,548$ (20 nM, 50.4 Ci/mmol) was carried out on HEK 293 cells transiently transfected with the plasmids coding for the wild-type TPα or mutant TPα<sub>N4-Q4</sub>, TPα<sub>N16-Q16</sub> and TPα<sub>N4,N16-Q4,Q16</sub> receptors in the presence of the competitor ligands: U46619 (10$^{-8}$–10$^{-5}$ M) or SQ29,548 (10$^{-8}$–10$^{-6}$ M). Nonspecific binding was determined in the presence of excess non-labeled SQ29,548 (10μM). Radioligand binding data were analyzed using INPLOT 4 computer program (GraphPad Software Inc.) to determine the individual $K_i$ values for U46619 ($\times 10^8$ M ± S.E., n = 4) and SQ29,548 ($\times 10^8$ M ± S.E., n = 4).

To analyze the effect of site-directed mutagenesis of the N-linked glycosylation sites on TPα coupling to PLC, functional coupling of the wild-type and mutant receptors was assessed by monitoring mobilization of Ca$^{2+}$ in HEK 293 cells transiently cotransfected with the cDNAs coding for...
was only 58% (fig. 7). This indicates some retention of functional signal transducing properties by the fully deglycosylated double-mutant receptor.

To evaluate the consequences of site-directed mutagenesis of the N-linked glycosylation sites on TPα coupling to adenyl cyclase, functional coupling of the wild-type and mutant receptors was assessed by measurement of elevations of cAMP concentrations in response to U46619 stimulation. HEK 293 cells, transiently transfected with TPα, produced a 2.65-fold stimulation in cAMP relative to basal levels to produce levels corresponding to 39.8 ± 12.3 pmol cAMP/mg cell protein (table 3). In the single mutant receptors, relative stimulation in cAMP production were 88.1% (TPαN4–Q4 and 75.2% (TPαN16–Q36), whereas, for the double mutant, TPαN4–Q4,N16–Q36, U46619-induced stimulation of cAMP production was reduced to 39.6% relative to that of the wild-type TPα. Thus, the fully deglycosylated TPα also retains the ability to interact with Gαq and partly retains signal transducing properties in this assay similar to that observed in the Ca2+ mobilization assays.

**Discussion**

In this study, we investigated the potential role of asparagine N-linked glycosylation on the subcellular localization, ligand binding properties, G-protein coupling and second messenger signaling of the human TP isoforms. N-linked glycosylation is important in the correct functioning and subcellular distribution of many, but not all, membrane-associated proteins such as the acetylcholine (Giovannielli et al., 1991), transferrin (Ralton et al., 1989) and IgD (Swenson et al., 1993) receptors. When considering this variation in functional responses, it was of interest to determine the significance, if any existed, of alteration in the glycosylation status of both the TPα and TPβ receptor subtypes.

Several genetic bleeding disorders have been described in humans in which the individual’s platelets were unresponsive to TXA2. The molecular basis of one of these dominantly inherited bleeding disorders recently was reported to be caused by a single amino acid substitution (Arg60-Leu60) in the first cytoplasmic loop of the TP (Hirata et al., 1994). The mutant TPα receptor, when expressed in Chinese hamster ovary cells, showed decreased agonist-induced second messenger signaling despite its normal ligand binding affinities. Thus, in this study, a series of experiments were undertaken to investigate possible loss of TP receptor function either because of inhibition of glycosylation in vivo or with tunicamycin or transfection of mammalian cells with site-directed mutants of the TPα receptor in which either one or both consensus N-linked glycosylation sites (Hirata et al., 1991, Raychowdhury et al., 1994, 1995) have been destroyed. The antibiotic tunicamycin is a highly selective inhibitor of N-linked but not O-linked glycosylation, blocking the enzymatic transfer of N-acetylglucosamine 1-phosphate to dolichol-mono-phosphate within 1 h after treatment, thereby preventing the N-linked glycosylation of various proteins (Tkaczez and Lampen, 1975; Elbein, 1987). However, it has little or no effect on de novo protein synthesis itself and exhibits low general toxicity in the effective concentration range of 0.1 to 20 μg/ml (Struck and Lennarz, 1977). Calnexin and calreticulin are molecular chaperones that anchor proteins in the endoplasmic reticulum until they have folded correctly by
recognizing their carbohydrate portions (Wada et al., 1997; Gahmberg and Tolvanen, 1996). Tunicamycin treatment blocks calnexin and calreticulin interactions with their targets. This may result in incorrectly folded polypeptides, increased protein degradation and mislocalization of newly synthesized proteins.

When HEL cells were treated with tunicamycin, we observed a time-dependent reduction in TP receptor expression, measured in radioligand binding assays, with $^{[3]}$H]SQ29,548, to yield a level of binding of 45% relative to control, nontreated cells after 12 h incubation. We have confirmed that HEL cells express the mRNAs coding for both the TPα and TPβ isoforms (S.M. Miggin and B.T. Kinsella, unpublished). To address whether this effect may be isoform specific, stable HEK 293 cells, which exclusively express either the TPα or TPβ isoform, were generated. Treatment of these stable cell lines with tunicamycin resulted in a similar time-dependent decline in TPα and TPβ expression. After a 4-h treatment of HEKα1 and HEKβ3 cells with tunicamycin, TPα and TPβ expression was reduced to 78% and 80%, respectively, relative to the corresponding nontreated control cells. However, on prolonged exposure (24–48 h), the HEKβ3 cells were significantly more sensitive to tunicamycin treatment than HEKα1 cells. The physiologic significance of this is unclear but may reflect differences in TPα and TPβ protein turnover rates or indicate additional functional differences between the two receptor isoforms, such as intracellular trafficking or interaction with endoplasmic reticulum chaperones such as calnexin and calreticulin (Wada et al., 1997). Tunicamycin treatment of the HEKα1 and HEKβ3 cells cotransfected with G11 also resulted in substantial reduction in Ca$^{++}$ mobilization in FURA2/AM-loaded cells in response to the TXA2 mimetic U46619, but had no effect on Ca$^{++}$ mobilization in response to ionomycin or a stable analog of IP3. Thus, inhibition of TPα and TPβ glycosylation with tunicamycin resulted in reduced ligand binding and reduced Ca$^{++}$ mobilization. However, as previously stated, these data do not fully exclude other effects such as alteration in receptor folding or secondary effects on other proteins. The glycosylation of membrane proteins generally is believed to help in protein folding and correct assembly in the endoplasmic reticulum and migration to the plasma membrane (Gahmberg and Tolvanen, 1996).

The significance of the N-linked glycosylation sites at positions 4 and 16 of TPα was confirmed by site-directed mutagenesis of either one or both asparagine (N) residues of these sites to glutamine (Q) residues in the TPα isoform. After transient transfection of the wild-type or mutant receptors into HEK 293 cells, the level of TP expression was determined by radioligand binding studies. Specific $^{[3]}$H]SQ29,548 binding was reduced to 47% (TPα$^{N4–Q4}$), 58% (TPα$^{N16–Q16}$) and 8.3% (TPα$^{N4,16–Q4,16}$) relative to wild-type TPα receptor. Scatchard analysis indicated that whereas there was no significant change in the affinity ($K_d$) of either mutant receptor for SQ29,548, there were substantial reductions in maximal binding ($B_{max}$) with that of double mutant receptor (TPα$^{N4,16–Q4,16}$) being reduced by 92% relative to the wild type TPα. Furthermore, comparison of $^{[3]}$H]SQ29,548 binding by either U46619 or SQ29,548 failed to demonstrate any difference in $K_d$ values for these ligands between the wild-type or mutant forms of TPα, confirming that glycosylation per se does not have a role in determining TPα ligand affinity or specificity.

The observed reduction in SQ29,548 binding for the mutant TPs could be caused by altered TP expression or by altered subcellular localization of mutated receptors between the membrane and soluble fractions of the cell relative to that of the wild-type TPα. Currently, antibodies to the human TP isoforms are not available to this laboratory, which precludes us from directly determining actual TP protein expression levels or the relative cell surface expression of the wild-type and mutant receptors. However, Northern Blot analysis revealed that TP gene expression was equal among all the TP receptors transiently expressed in HEK 293 cells, which indicates that differential gene expression also may not account for the observed reduction in SQ29,548 binding displayed by the mutant receptors (TPα$^{N4–Q4}$, TPα$^{N16–Q16}$, TPα$^{N4,16–Q4,16}$) relative to the wild-type TPα. Fractionation of transfected cells into their membrane (P100) and soluble (S100) fractions revealed similar corresponding reductions in total, membrane and cytosolic expression for each of the mutants relative to the wild-type receptor. Such a reduction in membrane expression is not, on its own, sufficient to account for the observed reduction in SQ29,548 binding in the double mutant receptor (92% reduction relative to the wild-type TPα) which suggests that glycosylation may be required for ligand binding. Endo H treatment of HEKα1 or HEKβ3 cells, stably expressing either TPα or TPβ, respectively, resulted in approximately 50% reductions in radioligand binding, by either receptor isoform, confirming a direct role for N-linked glycosylation in ligand ([$^{[3]}$H]SQ29,548) binding by the TPs. In the EP3 isoform of the PGE2 receptor, another prostanoid receptor, N-linked glycosylation has been reported to be important not only in determining the affinity, but also the specificity of the EP3 receptor for its ligands (Huang and Tai, 1995). In general, the putative seventh transmembrane domain forms a critical portion of the ligand binding pocket for G-protein-coupled receptors (Baldwin, 1994) and is highly related among the whole prostanoid receptor family (Ushikubi et al., 1995). Previous site-directed mutagenesis studies confirmed that amino acid residues within the putative seventh transmembrane domain of the TPs are critical for ligand binding (Funk et al., 1993). In addition, D’ Angelo et al. (1996) reported that conservation of cysteine (C) residues (C105 and C183) located within the first and second extracellular regions, respectively, are essential for ligand binding; whereas C103, also located within the first extracellular loop, is required for optimal ligand binding affinity, capacity and cell signaling by TPα. Thus, from our current studies, it is evident that conservation of N-linked glycosylation sites at Asn4 and Asn16, located within the extracellular amino-terminal region of TP, may be required for optimal ligand binding.

The TPα functionally couples in vivo to members of the G4 (G4 and G11) family of heterotrimeric G proteins mediating activation of the beta isoforms of PLC and leading to release of Ca$^{++}$ from intracellular stores (Kinsella et al., 1997). For some GPCRs, N-linked glycosylation has been reported to affect G-protein coupling and subsequent second messenger generation (Frost et al., 1991.; Randa et al., 1990). Tunicamycin treatment of HEKα1 or HEKβ3 cells, transiently cotransfected with G11, greatly reduced U46619-induced Ca$^{++}$ mobilization in these cells compared with the un-
treated control cells. In confirmation experiments, U46619-mediated Ca\(^{2+}\) mobilization in HEK 293 cells transiently cotransfected with either the wild-type (TPα) or mutants, and G\(_{\alpha}\) were reduced to 78.5% (TPα\(^{N4–Q4}\)), 88.8% (TPα\(^{N4–Q4, N16–Q16}\)) or 42.2% (TPα\(^{N4–Q4, N16–Q4, Q16}\)) relative to that of the wild-type receptor. Similarly, we also assessed the possible consequences of site-directed mutagenesis of the N-linked glycosylation sites of the TPα on its ability to couple to adenyl cyclase via G\(_{\alpha}\) (Hirata et al., 1996). As observed with mobilization of intracellular Ca\(^{2+}\), adenyl cyclase activity was modestly reduced in cells expressing the single mutants (88.1% for TPα\(^{N4–Q4}\) or 75.2% for TPα\(^{N16–Q16}\)) and reduced to 39.6% in cells expressing the double mutant (TPα\(^{N4–Q4, N16–Q16}\)) compared to the wild-type TPα. Thus, whereas the fully deglycosylated TPα mutant has a 92% reduction in relative expression, as determined by saturation radioligand binding studies, the nonglycosylated receptor (TPα\(^{N4–Q4, N16–Q4, Q16}\)) does retain some functional activity and signal transducing properties, as was indicated by both the Ca\(^{2+}\) mobilization studies and cAMP assays. This may indicate that the overall structure, folding or topography of the fully nonglycosylated receptor maintains some ligand binding properties but retains a substantial ability to couple to G\(_{\alpha}\) and G\(_{\alpha}\) with concomitant activation of the respective signal transduction pathways.

References


D’Angelo DD, Dubank JJ, Davis MG and Dorn GW II (1996) Mutagenic analysis of cosylated TPα receptor isoform (TPα\(^{N4–Q4, N16–Q16}\)). Respective signal transduction pathways.

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