Expression and tissue distribution of the mRNA's encoding the human thromboxane A$_2$ receptor (TP) α and β isoforms.

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Abbreviations: TXA$_2$, thromboxane A$_2$; thromboxane A$_2$ 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; VSM, vascular smooth muscle, SMC, smooth muscle cell; depc, diethyl pyrocarbonate; RT-PCR, reverse-transcriptase polymerase chain reaction; HEL, human erythroleukemia cells; HUVEC, human umbilical vein endothelial cell; FBS, foetal bovine serum.

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Summary

The human (h) TXA_2 receptor (TP), a G protein-coupled receptor, exists as two isoforms, TP_α and TP_β, which arise by alternative mRNA splicing and differ exclusively in their carboxyl terminal cytoplasmic regions. In this study, a reverse transcriptase - polymerase chain reaction (RT-PCR) based strategy was developed to examine the expression of the TPs in tissues of physiologic relevance to TXA_2. Although most of the 17 different cell / tissue types examined expressed both TP isoforms, the liver hepatoblastoma HepG2 cell line was found to exclusively express TP_α mRNA. In most cell types, TP_α mRNA predominated over TP_β mRNA. Moreover, although the levels of TP_α mRNA expression were similar in most of the cell / tissue types examined, extensive differences in the levels of TP_β mRNA were observed. Consequently, the relative expression of TP_α : TP_β mRNA varied considerably due to extensive differences in TP_β mRNA expression. Most strikingly, primary HUVEC's were found to express: (i) low levels of TP_β and (ii) approximately 6- fold greater levels of TP_α than TP_β. These data were confirmed in the spontaneously transformed HUVEC derived ECV304 cell line. Expression of TP mRNAs in the various tissue / cells correlated with protein expression, as assessed by radioligand binding using the selective TP antagonist [3H] SQ29,548.

Introduction

The prostanoid thromboxane A_2 (TXA_2), mainly synthesised in platelets, induces a variety of cellular responses including platelet shape change and aggregation, constriction of bronchial and vascular smooth muscle (VSM) cells, stimulation of mitogenesis and / or hypertrophic responses in VSM (1 - 5). TXA_2 mediates its actions through interaction with the shared endoperoxide prostaglandin (PG)H_2 / TXA_2 receptor (termed TP; 6), a member of the rhodopsin like G protein coupled receptor (GPCR) family. Imbalances in the levels of TXA_2 or in its specific TXA_2 synthase or its receptor (TP) have been implicated as mediators in a number of disease states including myocardial infarction (7 - 9), atherosclerosis (10), angina (9, 7), pregnancy induced hypertension (11), ureteral obstruction (12), lupus nephritis (13), dysmenorrhea and uterine ischemia (14), endometrial cancer (15) and bronchial asthma (16). In addition, U46619, a TP agonist has been reported to induce apoptotic cell death of immature thymocytes (17) implying that TXA_2 may play a role in modulation of immune responses (18).

Early pharmacological and biochemical evidence indicated both inter and intra-species variations of TPs (19 - 22). Moreover, the selective TP antagonist GR32191 could distinguish between two forms of the receptor in human platelets; one linked to phospholipase C activation, resulting in platelet aggregation and granule secretion and a second linked to calcium mobilization, mediating platelet shape change (2). The cDNA for the human TP was initially cloned from placenta and the platelet like MEG-01 cell line (23) and since then cDNAs for TP receptors from a number of species have been cloned including TPs from mouse (24), rat (25,26) and bovine (27) sources. All TPs are predicted to share similar 7 transmembrane, α helical domain arrangements typical of members of the GPCR superfamily. Despite the evidence for receptor heterogeneity, genomic cloning of the human TP gene confirmed the existence of a single gene, located on chromosome 19p13.3 (28). The gene consists of 3 exons and 2 introns and has 2 putative promoters; DNA sequence analysis indicates polymorphism, including polymorphism within the putative promoter 2 (29). In 1994, Raychowdhury *et al.*, (30) isolated a cDNA encoding a second isoform of the TP from a human umbilical vein endothelial cell (HUVEC) cDNA library. The endothelial receptor, termed TP_β, and the platelet/placental TP, termed TP_α, are encoded by the same gene but arise by a novel differential splicing event. The two receptors are identical for the first 328 amino acids but differ in their C-terminal terminal cytoplasmic tails. Polymerase chain reaction (PCR) experiments indicated that HUVECs express TP_β only (30) whereas platelets were reported to express both TP_α and TP_β isoforms (31).

Both receptor isoforms have been shown to be regulated both *in vitro* and *in vivo* by phosphorylation (32,33). Direct functional coupling of TP_α to Gq and G11 in response to the TXA_2 mimetic U46619 and the isoprostane 8-epi prostaglandin $F_{2\alpha}$ has been demonstrated *in vivo* (34). Moreover, in stably transfected Chinese hamster ovary cells, Hirata *et al.,* (31) reported that the individual receptors may activate different intracellular signalling pathways. Whilst showing similar phospholipase C activation, each receptor oppositely regulate adenylyl cyclase activity, with TP_α activating it and TP_β inhibiting it. None the less, despite these studies, the physiologic significance of the existence of 2 receptors for TXA_2 is presently unclear but it is possible that each receptor isoform may be involved in mediating specific functions among the diversity of those attributed to the autocoid TXA_2. To date, no detailed study has been undertaken to analyse the relative expression of the TP_α or TP_β isoforms in cells or tissues of relevance to TXA_2 biology. Whereas Northern blot analyses have confirmed the existence of TP mRNA in human MEG-01 cells, placental and lung tissue (23), in human erythroleukemic (HEL) cells (35,29) and in mouse thymus, lung, spleen, ileum brain and kidney (36), the probes utilised in these studies did not discriminate between

expression of the α or β isoforms. Similarly, at the protein level, TP selective radioligands currently available, such as [^{3}H]SQ29,549 (30,37) or [^{3}H]S-145 (31) do not discriminate between either receptor isoform and sufficiently sensitive receptor specific antibodies that will allow for detection of low amounts of TP expressed on cell membranes are not currently available. As a necessary prelude to studies which may elucidate the possible differential physiologic roles of the TPs, it is necessary to establish the expression and tissue distribution of the individual TP isoforms. Hence, in this study, a semi quantitative reverse-transcriptase PCR (RT-PCR) approach has been developed that allows for the identification of and the assessment of relative expression and abundance of the mRNAs for the TP$_\alpha$ and TP$_\beta$ isoforms from different tissue and cell types. Where possible, TP receptor expression was validated by radioligand binding studies using the highly selective TP antagonist SQ29,548.   This study will provide a starting foundation for elucidating the roles of TP isoforms in physiologic and pathophysiologic states.

Materials and Methods

Materials. Ultraspec[TM] total RNA isolation system was purchased from Biotecx Laboratories (Houston, TX). All oligonucleotides were synthesised by Genosys Biotechnologies (Cambridgeshire, UK). Mouse moloney leukemia virus (MMLV) reverse transcriptase (RT), ribonuclease inhibitor RNasin®, Klenow fragment, RQ DNase I and deoxynucleotides were purchased from Promega. Random hexamers were purchased from Life Technologies Inc. Taq DNA polymerase, T4 DNA ligase, calf intestinal alkaline phosphatase and restriction endonucleases were purchased from Boehringer Mannheim. Sequenase Version 2.0 DNA sequencing kit was purchased from U.S. Biochemical Corp. Foetal bovine serum (FBS) was purchased from PAA laboratories Ltd., (Kingston Upon Thames, UK.). Dulbecco's minimal essential medium, RPMI 1640 and minimal essential medium were purchased from HyClone Europe (Northumberland, UK). Endothelial growth medium (EGM) and supplements were purchased from BioWhittaker Inc., (Walkerville, MD, U.S.A.). All human tissue RNA samples were purchased from Clontech. The *Escherichia coli* (*E.coli*) cloning vector pBluescript II SK(-) was purchased from Stratagene.

Culture of Primary and Immortalised Cells:

Primary (1°) human umbilical vein endothelial (HUVEC) cells and first trimester trophoblast cell lines, namely ED-27, ED-31, ED-77 and Tm-1, derived from 4 individual donors were obtained from Professor Desmond Fitzgerald, Royal College of Surgeons, Dublin, Ireland. The following immortalised human cell lines were purchased from the American Type Culture Collection (ATCC): Human erythroleukemia (HEL) 92.1.7 cells; hepatoblastoma (Hep) G2 cells; ECV304 cells. The foetal and adult aortic vascular smooth muscle (VSM) cell lines VLTR and ALTR and the uterine smooth muscle cell (SMC) line ULTR, described by Perez-Reyes *et al.*, (38), were obtained from J. K. McDougall, Dept. Pathology, University of Washington, Seattle, USA.

All cells were maintained at 5% CO_2 in an incubator at 37^oC and were cultured using standard conditions, essentially as recommended by the suppliers. Briefly, the megakaryocytic HEL cell line were routinely cultured in RPMI 1640, 10% FBS. Immortalised VSM cell lines, VLTR, ALTR; the uterine SMC cells, ULTR; the 1° trophoblast cell lines ED-27, ED-31, ED-77 and Tm-1, were routinely grown in Dulbecco's minimal essential medium, 10% FBS. The liver-derived cell line, HepG2 cells were cultured in Eagle's minimal essential medium, 10% FBS. The spontaneously transformed HUVEC-derived cell line, ECV304 was cultured in medium 199, 10% FBS whereas the 1° HUVEC cells was grown in EGM supplemented with 10 ng ml^{-1} human epidermal growth factor, 0.5 μg ml^{-1} hydrocortisone, 50 μg ml^{-1} gentamicin sulphate, 50 μg ml^{-1} amphotericin B, 12 μg ml^{-1} bovine brain extract, 2% FBS.

RNA Preparation.

Total RNA from foetal and adult brain tissue, thymus, small intestine and liver were purchased from Clontech. Total RNA was isolated from placental tissue and all cell lines using the Ultraspec® RNA isolation procedure, essentially as described by the supplier. RNA integrity was assessed by electrophoresis on 1.1% agarose/formaldehyde/formamide gels (39). To ensure that the RNA samples prepared using the Ultraspec® method were free from any potential contaminating DNA, aliquots (25μg) of total RNA were treated with 6.25U RQ DNase I in the presence of 40U RNasin® ribonuclease inhibitor, 50mM Tris-HCl, pH 7.5., 10mM $MgCl_2$ for 15 minutes at 37^oC in a total volume of 50μl. Reactions were terminated by the addition of 2.5μl 0.5M EDTA, pH 8.0.

Phenol:chloroform extraction and precipitation of the RNA were performed by standard methodology (39). The lyophilised RNA pellet was resuspended in 20μl diethyl pyrocarbonate (depc) treated H_2O.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

DNase I treated total RNA from immortalised cell lines and total RNA extracted from human tissue were converted to first strand (I^o) cDNA with MMLV RT. Briefly, 1.4μg of total RNA was denatured for 10 minutes at 70^oC in the presence of random hexamers (100μM), then chilled on ice. Reaction buffer, deoxynucleotides, RNasin® and MMLV RT were then added to give a final reaction mix of 50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM $MgCl_2$, 10mM DTT, 40U RNasin, 0.8mM deoxyribonucleotides, 400U MMLV RT in a total volume of 25μl. I^o cDNA synthesis was performed at 37^oC for 40 min, then at 42^oC for 40 min. Reactions were heat inactivated at 80^oC for 10 min. In each RT experiment, the following negative control reactions were routinely carried out: a) 1° cDNA reactions carried out in the absence of MMLV-RT; b) all RT reagents excluding template RNA. Aliquots (3.5μl) of each 1° cDNA were then used as templates in subsequent PCR reactions (25μl) using primers pairs selective for TP_α or TP_β.

The general organization of Exon 2 - Intron 2 - Exon 3 of the human TP gene corresponding to the region encoding the unique C-terminal cytoplasmic tails of TP_α and TP_β are shown in Figure 1. Oligonucleotide primers were designed to specifically amplify TP_α and TP_β mRNA sequences in PCR reactions based on the published cDNA sequences of the placental TP_α (28) and the endothelial TP_β (30). In all cases, the common Primer A with the sequence 5'GAGATGATGGCTCAGCTCCT3' corresponding to nucleotides 724 -743 of Exon 2 was utilised as the 5' or Sense primer; whereas Primer C (5'CCAGCCCCTGAATCCTCA3' ; nt 1123-1106 of TP_α) was used as specific Antisense primer for TP_α and Primer B (5' AGACTCCGTCTGGGCCG3'; nt 1651 - 1643 plus 983 - 976, which correspomds to nt 992-976 of TP_β mRNA) was used as specific antisense primer for TP_β. An additional Primer D (5'TGGGCCACAGAGTGAGACTC3'; nt 1665-1646) was designed to co-amplify both TP_α and TP_β sequences. These primers (Primers A-D) were designed to span across Intron 2 (4.3 kB) such that only PCR products derived from 1° cDNA would be amplified, thereby eliminating genomic artefacts. As an internal control for each experiment, oligonucleotides were also designed to direct PCR amplification of DNA from the constitutively active gene, glyceraldehyde-3-phosphate dehydrogenase (GA3PDH), namely G*f* 5'TGAAGGTCGGAGTCAACG 3' (nt 71-89) and G*r* 5'CATGTGGGCCATGAGGTC3' (nt 1053-1035).

The random primed first strand (1°) cDNA was used as a template for the PCR amplification of TP_α, TP_β, TP_α plus TP_β and GA3PDH specific sequences. In brief, aliquots (3.5 μl) of 1° cDNA were used as templates in each PCR reaction (25 μl) in the presence of 10 mM Tris-Cl, pH 8.3., 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs, 6.7 % glycerol, 1 μM Sense primer, 1 μM Antisense primer, 1 U Taq DNA polymerase. PCR amplifications were carried out using the following repeat cycle conditions for 40 cycles: (denaturation at 95 ^oC, 1 min; primer annealing at 50 ^oC, 30 sec; elongation at 72 ^oC for 3 min) followed by a single final extension reaction (95 ^oC, 1 min; 50 ^oC, 30 sec; 72 ^oC for 10 min). In the case of primer pairs A/D, PCR reaction components were initially assembled without any Taq DNA polymerase and were pre-heated to 95 ^oC for 5 min, then cooled on ice for 1 min. Thereafter, Taq DNA polymerase was added and PCR amplifications were carried out using the following repeat cycle conditions for 40 cycles: (95 ^oC, 1 min; 54 ^oC, 30 sec; 72 ^oC, 3 min) followed by a single final extension reaction (95 ^oC, 1 min; 54 ^oC, 30 sec; 72 ^oC, 10 min). In each PCR experiment the following negative control

reactions were routinely carried out for each primer pair: a) PCR reactions carried out in the absence of 1° cDNA template; b) template DNA resulting from 1° cDNA reactions carried out in the absence of MMLV-RT; c) template DNA resulting from 1° cDNA reactions carried out in the absence of template RNA.

Following amplifications, products of the PCR reactions (7μl) were analysed by agarose gel electrophoresis (39); the gels were then subjected to densitometry by scanning on a UVP GDS800 gel documentation system. The levels of TP_α and TP_β mRNA expression were determined by measurement of PCR product band intensities on the densitometric scans; in each case, expression levels, in arbitrary units, are represented as a ratio relative to GA3PDH expression (i.e TP_α /GA3PDH ± standard error of the mean (S.E.M.); TP_β/GA3PDH ± S.E.M., in arbitrary units) and the relative levels of TP_α to TP_β are represented as a ratio of these values in each case, i.e TP_α/TP_β ± S.E.M., arbitrary units. For each cell or tissue type screened, at least three independent experiments were carried out. Results are expressed as mean ± S.E.M. where n ≥ 3. For each independent cell / tissue type screened, the ratio of TP_α / GA3PDH and TP_β / GA3PDH were analysed using the unpaired Student t-test. P values of less than or equal to (≤) 0.05 were considered to indicate a statistically significant difference and, where values were found to be significant, are indicated in the Figures.

Southern Blotting and Phosphor Image Analysis.

Southern Blot analysis of PCR generated fragments was carried out using standard methodology essentially as described by Sambrook *et al*., 1989.

Briefly, following agarose gel electrophoresis, PCR products were transferred to Duralon -UV nylon membrane (Stratagene) by upward capillary transfer (39). Aliquots (10 pmoles) of the radiolabelled oligonucleotides Probe X (5' CTGTCCCGCACCACGGAG 3', corresponding to nucleotides 844-861 of the TP_α and TP_β mRNA) or the Probe *Gp* (5' CAGAAGACTGTGGATGGC 3', corresponding to nucleotides 552-569 of the GA3PDH mRNA) were each 5'end labelled with 16 units of T4 polynucleotide kinase for 45 min at 37^oC in a final reaction volume of 25μl in the presence of 70mM Tris-Cl, pH 7.6, 10mM $MgCl_2$, 5mM DTT and 20 pmoles [γ -^{32}P] ATP (6000Ci/mmol; 10mCi/ml). Carrier nucleic acid (55μg yeast tRNA) was added and phosphorylated oligonucleotide primers were co-precipitated with 0.1 volumes of 3M sodium acetate, pH 5.2 and 2.5 volumes of absolute ethanol at -20°C for 30 min. The resultant pellet was washed in 70% ethanol and dried *in vacuo*. The lyophilised pellet was then re-suspended in 20 μl dH$_2$O and used as a probe for hybridization in the presence of 6x SSC, 0.5% SDS and 100μg/ml denatured, sonicated salmon sperm DNA overnight at 44°C. Membranes were washed as previously described (39). Radioactive images were captured by autoradiography on Fuji New RX Film and quantified by Phosphor Image analysis using a GS 250 Molecular Imager (BioRad).

Cloning and sequencing of TP_α and TP_β PCR generated fragments

In order to confirm the absolute identity of the TP_α and TP_β specific PCR products, generated using primer pairs A/C and A/B, respectively, each product was subcloned into the vector pBluescript II SK(-) and then subjected to DNA sequence analysis. To facilitate subsequent subcloning, PCR amplifications were carried out in the presence of oligonucleotide primers that had been 5' phosphorylated using T4 polynucleotide kinase prior to the PCR reactions.

Briefly, 500pmoles of the required oligonucleotide primers (A, C or B) were 5' phosphorylated with 16 units of T4 polynucleotide kinase in the presence of 50μM ATP, 10mM $MgCl_2$, 70mM Tris-Cl., pH 7.6 in a final volume of 100μl. Reactions were incubated at 37°C for 90 min and were heat inactivated at 65°C for 10 min. Carrier nucleic acid (5.5μg yeast tRNA) was added and phosphorylated oligonucleotide primers were co-precipitated with 0.1 volumes of 3M sodium acetate., pH 5.2 and 2.5 volumes of absolute ethanol at -20 ^{o}C for 30 min. The resultant pellet was washed in 70% ethanol and dried *in vacuo*. The lyophilised pellets were resuspended in 20μl distilled H_2O. Aliquots (1.25μl) of each phosphorylated primer was used to direct PCR amplification of either TP_α or the TP_β from 1° cDNA template as previously described. Following amplification, any recessed DNA ends were filled in by the addition of 10 units of DNA polymerase I Klenow fragment to each of the resulting 25μl PCR reactions and were further incubated at 30°C for 15 min. Thereafter, the Klenow treated PCR fragments were subjected to 1.2% agarose gel electrophoresis and the TP_α (400bp) and the TP_β (269bp) fragments were gel purified using the GeneClean II$^®$ kit. Phenol-chloroform extraction and precipitation of the gel purified PCR products was performed in the presence of 0.1 volumes of 3M sodium acetate, pH 5.2 and 2.5 volumes of absolute ethanol at -20°C. The washed pellet was resuspended in distilled H_2O.   PCR fragments were then subcloned by blunt end ligation into the Sma I site of pBluescript II SK (-) using standard methodology (39). Recombinant plasmids containing either the TP_α or the TP_β specific PCR products were sequenced using the Sequenase Version 2.0 DNA sequencing kit.

Saturation Radioligand binding assay

Cells were harvested by scraping, centrifuged at 500 x g at 4°C for 5 min and then washed three times in phosphate buffered saline (PBS). Protein concentrations were determined using the Bradford assay (40). Cells were then harvested and were resuspended in HBSSHB (modified Ca^{2+}/Mg^{2+}-free Hanks' buffered salt solution, containing 10mM HEPES pH 7.67, 0.1% bovine serum albumin) at a final concentration of 1mg/ml. Saturation radioligand binding experiments with the TP antagonist [^{3}H]SQ29,548 (20 nM, 50.4 Ci/mmol) were carried out at 30°C for 30 min in 100μl reactions using approximately 100μg cell protein per assay. Non-specific binding was determined in the presence of excess nonlabeled SQ29,548 (10μM). Reactions were terminated by the addition of ice-cold 10mM Tris, pH 7.4 (4 ml) followed by filtration through Whatmann GF/C glass filters, and subsequent washing of the filters 3 times with 4 ml 10mM Tris, pH 7.4, followed by liquid scintillation counting of the filters in 5ml of scintillation cocktail. Results were expressed as the fmol [^{3}H]SQ29,548 incorporated per mg cell protein ± S.E.M, where $n \geq 4$.

Results.

In this study, we have developed a semi quantitative reverse transcriptase (RT)-PCR based approach to screen for and determine the relative expression of TP_α and TP_β mRNA sequences in a variety of human cell and tissue types of relevance to TXA_2 biology. Where possible, in order to confirm actual TP protein expression, measurements of mRNA expression levels were complimented by radioligand binding studies using the selective radiolabelled TP antagonist [3H]SQ29,548.

Figure 1 outlines the general organisation of the human TP gene in the region where the differential splicing event giving rise to the two receptor isoforms occurs and the oligonucleotide primer pairs which were used in the RT-PCR reactions to amplify either TP_α (Primers A/C; nucleotides 724 - 1123; 400bp) or TP_β (Primers A/B; nucleotides 724 - 992 of TP_β mRNA; 269bp) specific sequences. As a confirmatory PCR reaction, an additional primer pair was used to co-amplify both TP_α and TP_β (Primers A/D; nucleotides 724 - 1665 of TP_α; 942bp and nucleotides 724 - 1006 of TP_β; 283 bp) specific sequences. In all cases, the 5' and 3' primers of the TPs were designed to flank the large 4.3kB Intron 2 thereby ensuring that the PCR products derived from 1° cDNA could readily be distinguished from those which might be generated from trace genomic DNA contamination of the RNA preparations. As an internal control for a constitutively transcribed gene, oligonucleotide primers (Primers G*f* / G*r*; nucleotides 71 - 1053; 983 bp) were also used to amplify GA3PDH mRNA sequences in each experiment.

A typical electrophoretogram of RT-PCR generated products from the platelet like human erythroleukemic (HEL) megakaryocyte cell line is presented in Figure 2A. Clearly, TP_α (Primer A/C, lane 2), TP_β (Primer A/B, lane 1), TP_α plus TP_β (Primer A/D, lane 9) as well as GA3PDH (Primer G*f*/G*r*; lane 3) DNA fragments of the predicted sizes were specifically amplified in each case. The amplified fragments were subsequently cloned into pBluescript II SK(-) vector and were confirmed to be correct by DNA sequence analysis (data not shown). Whereas HEL cells express both TP_α and TP_β mRNA sequences at ratio's of 0.87 ± 0.06 and 0.40 ± 0.008 relative to GA3PDH, respectively, these cells express 2.32 ± 0.34 ($p \le 0.05$) fold more TP_α than TP_β mRNA (Figure 2B). Consistent with previous data (32), TP expression in HEL cells was confirmed by radioligand binding studies and were found to express 36.7 ± 6.52 fmol [3H]SQ29,548 / mg cell protein (Figure 2C).

Routinely, in order to validate data obtained from densitometric scans of ethidium bromide stained gels, PCR fragments were also screened by Southern Blot analysis using an internal ^{32}P radiolabelled oligonucleotide Probe X (Nucleotides 844 - 861 of TP_α and TP_β mRNA sequences) as specific TP cDNA probe and radiolabelled oligonucleotide Probe G*p* (Nucleotides 552 - 569 of GA3PDH mRNA) as specific GA3PDH cDNA probe. Southern blots containing the PCR generated cDNA fragments (primer pairs A vs C to ampify Tp_α; primers A vs B to amplify TP_β cDNA; Gf vs Gr to amplify GA3PDH cDNA) were screened by hybridization, using standard methodology. Thereafter, the Southern Blots were subjected to autoradiography and densitometric scanning by Phosphor Image Analysis using a BioRad GS 250 Molecular Imager. Results of a typical autoradiogram are presented in Figure 2D. In each case, data generated by Phosphor Image analyses of the Southern Blots were consistent with the data generated from the ethidium bromide stained gels.

TP expression in uterine and vascular smooth muscle.

The immortalised smooth muscle cells lines derived from adult aortic (ALTR), foetal aortic (VLTR) and uterine (ULTR) smooth muscle cells (SMC's) have been previously described (38). These cells were used as model cell

lines and using the RT-PCR approach, were each found to co-express both TP_α and TP_β isoforms. Specifically, foetal and adult SMC's express comparable levels of TP_α mRNA with ratio's of TP_α / GA3PDH were 0.90 ± 0.06 and 0.81 ± 0.08, respectively (Figure 3A). In contrast, there is greater expression of TP_β in foetal VSM compared to adult VSM, with ratio's of TP_β / GA3PDH mRNA's found to be 0.81 ± 0.14 and 0.36 ± 0.06, respectively. Hence, the adult aortic SMCs express 2.37 ± 0.43 fold more TP_α than TP_β (Figure 3B, $p \leq 0.05$) whereas foetal SMC express 1.16 ± 0.16 fold greater levels of TP_α than TP_β.

Uterine SMCs express over 3-fold greater levels of TP_α relative to TP_β (Figure 3B, $p \leq 0.025$). In contrast, uterine tissue, which comprises uterine SMC, fibroblasts and a number of other cell types, expresses similar, high levels of both TP isoforms (ratio TP_α and TP_β mRNA's relative to GA3PDH were 1.35 ± 0.36 and 1.22 ± 0.36, respectively).

Expression of TP in the immortalised foetal, adult and uterine smooth muscle cells were confirmed by radioligand binding studies (Figure 3C), however, as the radioligand [³H]SQ29,548 is not receptor isoform selective, no direct correlation between the level of isoform-specific TP protein and mRNA expression could be made.

Analysis of TP expression in HUVEC's.

Primary human umbilical vein endothelial cells (1° HUVECs) were confirmed to express TP_β mRNA, albeit at low levels (ratio of TP_β / GA3PDH was 0.07 ± 0.005; Figure 4A & 4B). In contrast however to previous reports (Raychowdhury *et al.,* 1994), 1° HUVEC's were also found to express TP_α mRNA (Figure 4A); in fact, these cells were found to express 5.8 fold greater levels of TP_α relative to TP_β ($p \leq 0.01$; Figure 4B & C). These data were also confirmed by Southern Blot followed by Phosphor Image analysis where the ratio of TP_α / TP_β mRNA expression was found to be 6.09 (Figure 4D). Consistent with these findings, the spontaneously transformed HUVEC-derived cell line, ECV-304, was also found to express both TP isoforms, with greater levels of both TP_α and TP_β mRNA expression relative to 1° HUVECs (Figure 4B & 4C). TP protein expression in the immortalised ECV-304 cells was also confirmed by radioligand binding studies (13.9 ± 3.51 fmol / mg protein) .
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Expression of TP in trophoblasts.

Primary trophoblast cell lines were generated from four independent placental donors and were termed ED-27, ED-31, ED-77 and Tm-1. Both TP isoforms are expressed at the mRNA level in the ED-27, ED-31, ED-77, and Tm-1 trophoblast cell lines (Figure 5A- 5C). However, variations occur in the relative expression of the TP isoforms; TP_α is expressed at approximately equivalent levels in all four cell lines (Figure 5B). Considerable differences in the expression of TP_β mRNA were found with lowest levels of TP_β being expressed in ED-27 (ratio of TP_β / GA3PDH was 0.23 ± 0.08), TP_β mRNA is most abundantly expressed in ED-77 and Tm-1 (ratio of TP_β / GA3PDH were 0.87 ± 0.08 and 0.87 ± 0.03, respectively). Thus, TP_α predominates in ED-27 and ED-31, with significantly greater levels of TP_α relative to TP_β expressed in ED-27 cells ($p \leq 0.005$). No significant differences in the expression of TP_α relative to TP_β were observed in ED-77 or Tm-1 cells. Radioligand binding studies confirmed protein expression and indicated similar clonal variation in TP expression levels (Figure 5D).

Expression of TP isoforms in brain tissue, small intestine and thymus.

Foetal and adult brain tissue expressed both TP_α and TP_β mRNA with 2-fold greater expression of TP_α relative to TP_β in both cases (ratio TP_α /TP_β were 2.12 ± 0.25; $p \leq 0.05$ and 1.97 ± 0.33, $p \leq 0.05$ for foetal and adult brain tissue, respectively). Hence, there were no significant differences in the level or relative expression of the TP isoforms in foetal brain tissue compared to adult brain tissue.

Human thymus and small intestine tissue were also found to express both TP_α and TP_β mRNAs. The TP_α predominated in both tissues with greater levels of TP_α relative to TP_β mRNA expressed in thymus (ratio TP_α/TP_β was 3.31 ± 0.15, $p \leq 0.01$) and small intestinal (ratio TP_α/TP_β was 1.51 ± 0.12) tissue.

Exclusive expression of TP_α in a hepatoblastoma cell line.

Liver tissue express both TP_α and TP_β mRNAs with significantly ($p \leq 0.05$) greater levels of TP_α expressed relative to TP_β (ratio of TP_α / GA3PDH was 0.78 ± 0.11; ratio of TP_β / GA3PDH was 0.29 ± 0.05; Figure 7B). However, in contrast to the tissue sample, the hepatoblastoma (Hep) G2 clonal cell line express TP_α mRNA exclusively with no detectable expression of TP_β mRNA in these cells (Figure 7A -7C). Furthermore, TP_α is expressed at equivalent levels in both HepG2 cell and total liver (ratio of TP_α / GA3PDH were 0.78 ± 0.11; ratio of TP_α / GA3PDH were 0.80 ± 0.09, in total liver and HepG2 cells, respectively; Figure 7C). TP protein expression in HepG2 cells was confirmed by saturation radioligand binding, with 44.4 ± 16.1 fmoles [^{3}H]SQ,29548 bound/mg protein (Figure 7D).

Discussion

Prostanoids, autocoids synthesised mainly from membrane derived arachidonic acid, mediate diverse physiological and pathophysiological actions throughout the body. Distinct G-protein coupled receptors (GPCRs) have been cloned for each of the primary prostanoids including receptors for TXA_2 (TP, 23), prostaglandin (PG) D_2 (DP,41), PGE_2 (EP_1 - EP_4,42-47), $PGF_{2\alpha}$ (FP, 48) and PGI_2 (IP, 49). Among the large superfamily of GPCRs, the prostanoid receptors are somewhat unique in that receptor isoforms, derived from alternative mRNA splice variants, have now been cloned and identified for the EP_3 (50, 51), the TP (30) and the FP (52) receptors. In each case, the specific isoforms are identical thoughout their seven transmembrane domains but diverge exclusively within their C-terminal cytoplasmic tail regions. The significance of the existence of isoforms for prostanoid receptors is unclear, but in the case of EP_3 isoforms, differences in receptor / G-protein coupling (50, 53 - 55) and receptor desensitization (56) have been found. In the case of the TP_α and TP_β isoforms, studies to date indicate that both receptors exhibit identical ligand binding profiles, identical coupling to phospholipase C but may oppositely regulate adenylyl cyclase activity (31, 37). In terms of receptor localization, PCR analyses indicated that HUVECs express TP_β mRNA only (30) and platelets express mRNAs for both TP_α and TP_β isoforms (31). In view of the diverse actions attributed to TXA_2 and its receptor, in both physiological and pathophysiological situations, it was considered imperative to investigate the distribution of TP_α and TP_β in tissues of relevance to TXA_2 biology, and indeed, pathophysiology. Hence, in this study, we have developed a semi-quantitative RT-PCR approach to examine the relative expression of the individual TP_α and TP_β isoforms. In all, 17 different human cell / tissue types were examined, including 6 whole tissue samples and 5 primary cell lines. In the absence of available tissue or primary cells, a number of representative immortalised cell lines were also examined including platelet-like megakaryocytes, adult and foetal aortic and uterine SMC's, human umbilical vein endothelial cells and hepatoblastoma cell lines which may usefully reflect the relative expression of the TP isoforms in the progenitor tissues / cells.

Initially, vascular tissue and an erythroleukemia-like cell line were chosen for analysis since TXA_2 is known to play a role in platelet aggregation (57), vascular smooth muscle mitogenesis (58) and contraction (59, 7). The mRNAs coding for both TP isoforms were identified in the platelet-like HEL 92.1.7 cell line. In addition, mRNAs for both TP isoforms were identified in vascular and uterine smooth muscle. However, greater levels of TP_β were found in foetal VSM relative to adult VSM, which may imply an age dependant down-regulation of TP_β mRNA expression in adult tissue, with no alteration of TP_α expression.

Uterine tissue, which encompasses a milieu of cell types, including uterine SMC, blood vessels, stromal cells, endometrial glands and fibroblasts (60) was also found to express both TP isoforms. A previous study reported the existence of a 2.8kb TP transcript in proliferative phase human uterus (60). In our study, uterine tissue expresses greater levels of both TP isoforms relative to that expressed by the uterine smooth muscle ULTR cell line which may indicate essential differences between uterine tissue and uterine derived smooth muscle cells. However, there may, of course, be essential differences in TP expression between primary and immortalised smooth muscle cells which will require further investigation.

The endothelial TP_β has been so called since this novel spliced variant of TP was first cloned from HUVECs (30) which were reported to express TP_β exclusively with no detectable expression of TP_α mRNA in these cells. However, in direct contrast to those studies reported by Raychowdhury *et al* (30), in the present study we found that whereas the endothelial TP_β was expressed, albeit at low levels in 1° HUVECs, TP_α mRNA was also

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identified in these cells. Furthermore, TP_α mRNA levels far exceeded those of TP_β. The immortalised HUVEC-derived cell line, ECV-304 was also found to express both TP isoforms, with expression of TP_β being greater in the immortalised cell line. TP_α was also expressed at greater levels in the immortalised cell line when compared to the 1° HUVECs.

Trophoblasts mediate the transport of nutrients and immunoglobulins from the maternal to the foetal circulation and also functions as an endocrine organ (61). Recently, TXA synthase mRNA was identified in trophoblasts (62). In addition, serotonin-induced vasoconstriction in the human fetoplacental circulation appears to be mediated by TXA_2 release and action (63). Increased fetoplacental resistance, a feature of pre-eclampsia, may be due, in part, to TXA_2 as it is known to be a potent vasoconstrictor (59). Because of the role trophoblasts play in fetoplacental circulation, examination of TP isoform expression in trophoblast was undertaken. Both TP isoforms were identified in all trophoblast cell lines; however, differences were observed in the relative expression of the mRNA's for the individual isoforms. Whereas TP_α mRNA is expressed at approximately equivalent levels in all 4 cell lines, up to 4 fold differences in the level of TP_β mRNA were observed. Corresponding differences in TP expression by the clonal trophoblast lines were observed in radioligand binding studies using the non-discriminatory TP antagonist SQ29,548 as a radioligand. The significance of the differential expression of TP_α and TP_β in trophoblasts is currently unknown. However, taking into account the inter-individual variation in endogenous mRNA and protein levels which were observed in trophoblast cell lines derived from normal individuals in this study, it is possible that the individual isoforms may have distinct roles to play in pregnancy-induced hypertensive disorders including pre-eclampsia and pregnancy-induced hypertension.

TXA_2 is thought to play a pathophysiological role in neuronal death during cerebral ischaemia (64). Previous studies have shown that the level of brain TXB_2, the non-enzymatic degradation metabolite of TXA_2, increases 55 fold in cerebral ischaemia, and has thus been implicated in post-ischaemic hypoperfusion and the development of cerebral injury after ischaemia (65). Using ligand based (SQ31,491) affinity chromatography, Borg *et al,* (66) identified two distinct TP receptors of 52kDa and 55kDa in rat brain. Recently, a TXA_2 receptor was cloned from rat astrocytes (67) and was found to be 71.8% homologous to the human TP_α cDNA. Interestingly, no alternatively spliced variant of the TP gene was identified in rat brain tissue (67). In this study, we identified both TP_α and TP_β mRNAs in normal human foetal and adult brain tissue, with no age-related alterations in TP expression levels. TP_α expression predominates in both adult and foetal brain tissue. The significance of this is unclear but may point to TP_α playing a greater role in mediating cerebral ischaemia. Our finding of TP expression at the mRNA level correlates with Blackman *et al.,* (68) who reported the existence of functional TP protein in both rat neonatal oligodendrocytes and human oligodendroglioma cells by radioligand binding analysis and immunocytochemistry.

Northern blot analysis indicated that, in the mouse, TP mRNA is most abundantly expressed in thymic tissue (36). Additional studies indicated that the TP is most highly expressed in $CD4^-8^-$ and $CD4^+8^+$ immature thymocytes, followed by $CD4^+8^-$ and $CD4^-8^+$ cells (17). Previous studies have shown that the TXA_2 agonist, STA_2, induces apoptotic cell death in immature thymocytes and mediates thymocyte maturation and selection (17). Therefore, we examined thymic tissue for the presence of TP_α and TP_β encoding mRNA's. We found that both TP isoforms are expressed at the mRNA level in thymic tissue, with 3-fold greater expression of TP_α relative to TP_β. The relative role of each TP isoform in DNA fragmentation and apoptosis remains to be determined.

Northern blot analysis of various mouse organs revealed the presence of TP mRNA in thymus, spleen, lung, kidney, uterus, heart and brain tissue but not liver, stomach and ileum (36). In contrast, another study reported the existence of TP in endothelial cells, hepatocytes and Kupffer cells of rat liver, with an increase in TP mRNA expression observed with experimentally induced alcoholic liver disease in each of these cell types (69). Moreover, Taniguchi *et al*., (70) reported that TXA_2 antagonists may prove useful in the treatment of imflammatory bowel disorder implicating the importance of TP in intestinal tissue. In view of these findings, we investigated the existence of mRNAs encoding the TP isoforms in human liver and small intestine tissue. Both TP isoforms were expressed in small intestine and liver tissue, with TP_α expression predominating in both cases. Furthermore, RT-PCR analyses for TP mRNAs in the immortalised hepatoblastoma cell line, HepG2, revealed the expression of TP_α exclusively with no detectable expression of TP_β mRNA. The differential expression of TP_α and TP_β mRNA's in these cells clearly indicates that the 2 receptor isoforms are subject to distinct gene regulatory events in HepG2 cells, at least.

In conclusion, the TP is abundantly expressed at both the mRNA and protein level in tissues of relevance to TXA_2 biology, such as erythroleukemia cells, vascular and uterine smooth muscle, uterus and placental tissue, endothelium, epithelium, trophoblasts, thymus, liver and small intestine with TP_α expression predominated in most tissues examined. Whereas relative to GAP3DH mRNA, TP_α is expressed at approximately equal levels in all cell / tissue types analysed (0.80 ± 0.04, n = 47), considerable differences in TP_β mRNA (0.49 ± 0.04, n = 47) expression were observed. The apparent greater physiological requirement for TP_α over TP_β remains to be studied in the future. In addition, the hepatoblastoma cell line, HepG2, exclusively expresses TP_α, implicating a redundant physiological role for TP_β in this cell type. Contrary to previous reports, HUVECs express both the TP_α and TP_β mRNA's with significantly greater levels of TP_α than TP_β expressed. Thus, it is evident from this study that mRNA's for both isoforms are co-expressed in placental/platelet tissue, in endothelial cells and, indeed, in a number of other cell/tissue types. This fact raises doubts as to whether the TP_α should be referred to as the platelet/placental TP and the TP_β referred to as the endothelial receptor (30). Our findings also suggest that the molecular mechanisms responsible for TP_α and TP_β mRNA expression are subject to subtle regulation in different tissues and cell types. Further investigations are required to understand the molecular regulation in TP_α and TP_β mRNA expression.

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A. TP PRIMER POSITIONING

B. PREDICTED SIZE FRAGMENTS

TP	Primers	Size product
TP_α	A vs C	400bp
TP_β	A vs B	269bp
$TP_{\alpha/\beta}$	A vs D	942/283bp

C. GA3PDH PRIMER POSITIONING

GA3PDH	Primers	Size product
GA	Gf vs Gr	983bp
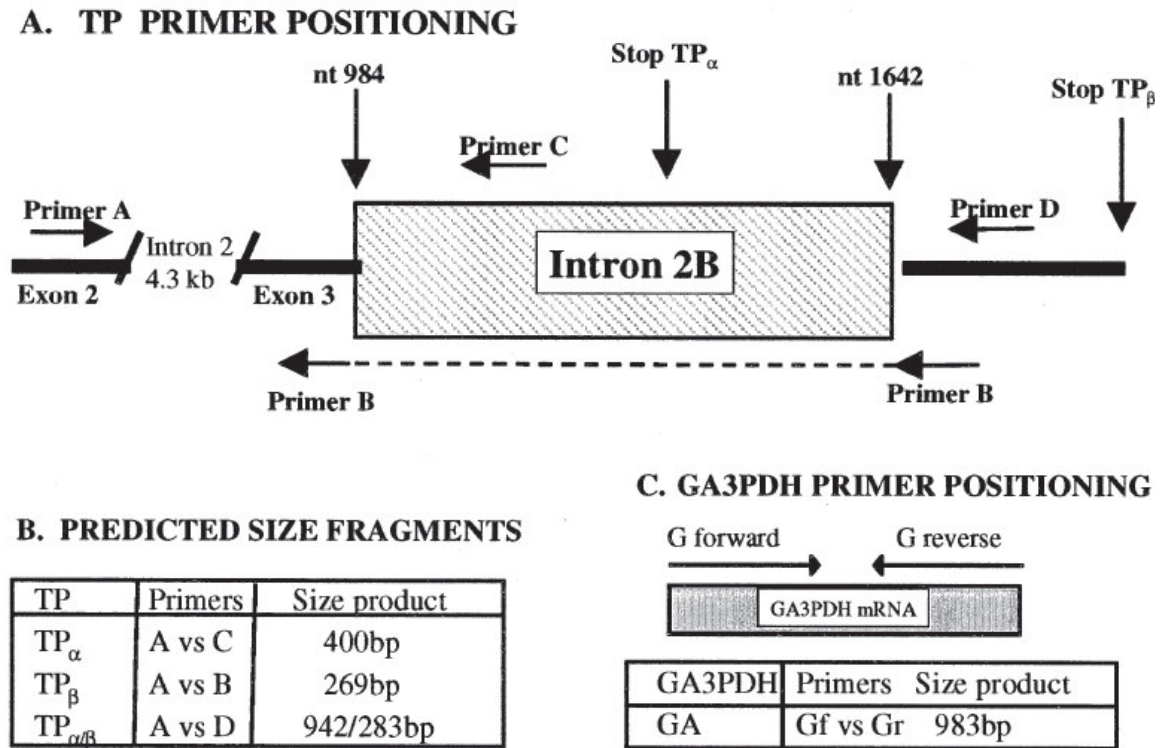
Figure 1. Organization of the h. TP gene.

A. The general organization of Exon 2 - Intron 2 - Exon 3 of the human TP gene corresponding to the region encoding the unique C-terminal cytoplasmic tail of TP_α and TP_β are shown. Exon 3 spans nucleotides 811- 1029, encoding amino acid residues 271 - 343 of TP_α, plus an additional 942 nucleotides (corresponding to nucleotides 1029 -1971) of 3' untranslated sequence (3' UTR). Nucleotides 984 - 1642 of the TP_α coding sequences behave as a potential intron (Intron 2B) in the TP_β mRNA; splicing of nucleotides 983 / 1643 results in an mRNA which has a new open reading frame and encodes a protein of 407 amino acids, of which amino acids 328 - 427 are unique to the TP_β. **B.** The following primer pair combinations were used in the PCR reactions: Primer pairs A/C specifically amplify TP_α; Primer pairs A/B specifically amplify TP_β; Primer pair A/D was used to co-amplify TP_α and TP_β isoforms. **C.** Primer pairs G*f* and G*r* were used to amplify a 982 bp fragment of GA3PDH cDNA sequences.

A. Agarose gel electrophoresis of PCR products derived from HEL 92.1.7

B. Relative expression of TP isoforms

C. Saturation radioligand binding data

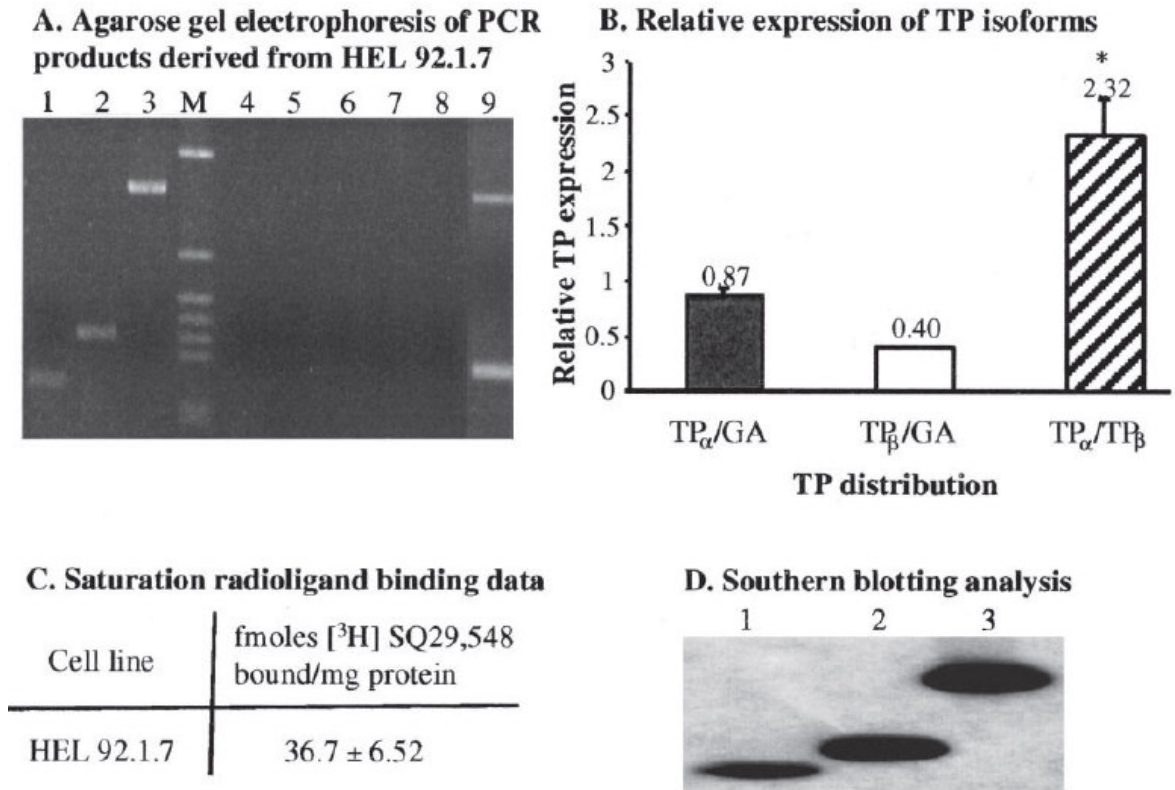| Cell line	fmoles [³H] SQ29,548 bound/mg protein
HEL 92.1.7	36.7 ± 6.52

D. Southern blotting analysis

Figure 2. Analysis of TP expression in HEL cells.

A. Agarose gel electrophoresis of RT-PCR products (7 μl/lane) derived from megakaryocyte HEL 92.1.7 cell 1^O cDNA template: lane 1, TP_β, 269bp product versus primers A/B; lane 2, TP_α, 400bp product versus primers A/C; lane 3, GA3PDH, 983 bp product versus primers Gf/Gr; lanes 4-6, negative control PCR reactions carried out in the absence of template 1^O cDNA in the presence of primers A/B, A/C and Gf/Gr, respectively; lanes 7-8, negative control reactions resulting from 1° cDNA template generated in the absence of MMLV-RT or template RNA, respectively, versus primers A/B; lane 9, TP_α and TP_β, 942 bp and 283 bp products, respectively, derived versus primer A/B. Lane M, pGEM$^®$ DNA markers; only the 179 - 1,198 bp fragments are shown. **B.** In each case, levels of TP_α, TP_β mRNA were each represented as a ratio relative to GA3PDH expression (i.e. TP_α/GA3PDH $\pm$ S.E.M. and TP_β/GA3PDH $\pm$ S.E.M., arbitrary units). Expression of TP_α relative to TP_β are represented as a ratio of these values (i.e. TP_α /TP_β $\pm$ S.E.M, arbitrary units). Both TP_α and TP_β are expressed in HEL 92.1.7 with significantly greater levels of TP_α expressed relative to TP_β (* p$\leq$0.05). **C.** Radioligand binding data are expressed as fmol [³H]SQ29,548 / mg cell protein $\pm$ S.E.M. where n $\geq$4. **D.** Southern blot analysis of TP_β, TP_α and GA3PDH specific RT-PCR products, generated using primers A/B, A/C and Gf/Gr , respectively, screened using the [γ -^{32}P] radiolabelled TP specific Probe X and GA3PDH specific Probe *Gp*.

A. Relative expression of TP isoforms

B. Relative TP isoform expression data

Cell line/tissue	TP_α/TP_β
Foetal VSM	1.16 ± 0.16
Adult VSM *	2.37 ± 0.43
Uterine SMC *	3.24 ± 0.09
Uterus	1.12 ± 0.03

C. Saturation Radioligand binding data

Cell line	fmoles[^{3}H]SQ29,548/mg protein
Foetal VSM	12.1 ± 2.44
Adult VSM	33.1 ± 10.8
Uterine SMC	11.7 ± 3.57

Figure 3. Analysis of TP expression in vascular SMC and uterus.

A. Levels of TP_α, TP_β and GA3PDH mRNA expression in foetal aortic (VLTR), adult aortic (AALTR) and uterine (ULTR) smooth muscle (SM) cells, and uterine tissue, in arbitrary units, were each represented as a ratio relative to GA3PDH expression (i.e. TP_α /GA3PDH $\pm$ S.E.M. and TP_β /GA3PDH $\pm$ S.E.M.). **B.** Expression of TP_α relative to TP_β are represented as a ratio of these values (i.e. TP_α / TP_β $\pm$ S.E.M, arbitrary units). Significantly greater levels of TP_α relative to TP_β mRNA is expressed in adult aortic (* $p \le 0.05$) and uterine (* $p \le 0.025$) SM cells. **C.** Radioligand binding data are expressed as fmol [^{3}H]SQ29,548 / mg cell protein $\pm$ S.E.M. where n ≥ 4.

B. Relative TP isoform expression

A. Agarose gel electrophoresis of PCR products derived from 1° HUVEC

C. Relative TP isoform expression data

Cell line/tissue*	TP_α/TP_β
1° HUVEC *	5.81 ± 0.53
ECV 304	1.53 ± 0.12

D. Southern blotting analysis

Figure 4. Analysis of TP expression in 1° HUVEC and ECV-304 cells

A. Agarose gel electrophoresis of RT-PCR products (7 µl/lane) derived from primary HUVEC cell 1° cDNA template: lane 1, TP_β, 269bp product versus primers A/B; lane 2, TP_α, 400bp product versus primers A/C; lane 3, GA3PDH, 982 bp product versus primers G*f*/G*r*; lanes 4-6, negative control PCR reactions carried out in the absence of template 1° cDNA in the presence of primers A/B, A/C and G*f*/G*r*, respectively; lanes 7-8, negative control reactions resulting from 1° cDNA template generated in the absence of MMLV-RT or template RNA, respectively, versus primers A/B. Lane M, pGEM® DNA markers; only the 179 - 1,198 bp fragments are shown. **B.** Levels of TP_α, TP_β and GA3PDH mRNA expression in 1° HUVEC and ECV-304 cell lines, arbitrary units, were each represented as a ratio relative to GA3PDH expression (i.e. TP_α /GA3PDH ± S.E.M. and TP_β /GA3PDH ± S.E.M.). **C.** Expression of TP_α relative to TP_β are represented as a ratio of these values (i.e. TP_α / TP_β ± S.E.M, arbitrary units). The 1° HUVECs express significantly greater levels of TP_α relative to TP_β (* $p \leq 0.05$). **D.** Southern blot analysis of TP_β, TP_α and GA3PDH specific RT-PCR products from 1° HUVEC mRNA, generated using primers A/B, A/C and G*f*/G*r* , respectively, screened using the [γ -^{32}P] radiolabelled TP specific Probe X and GA3PDH specific Probe *Gp*.
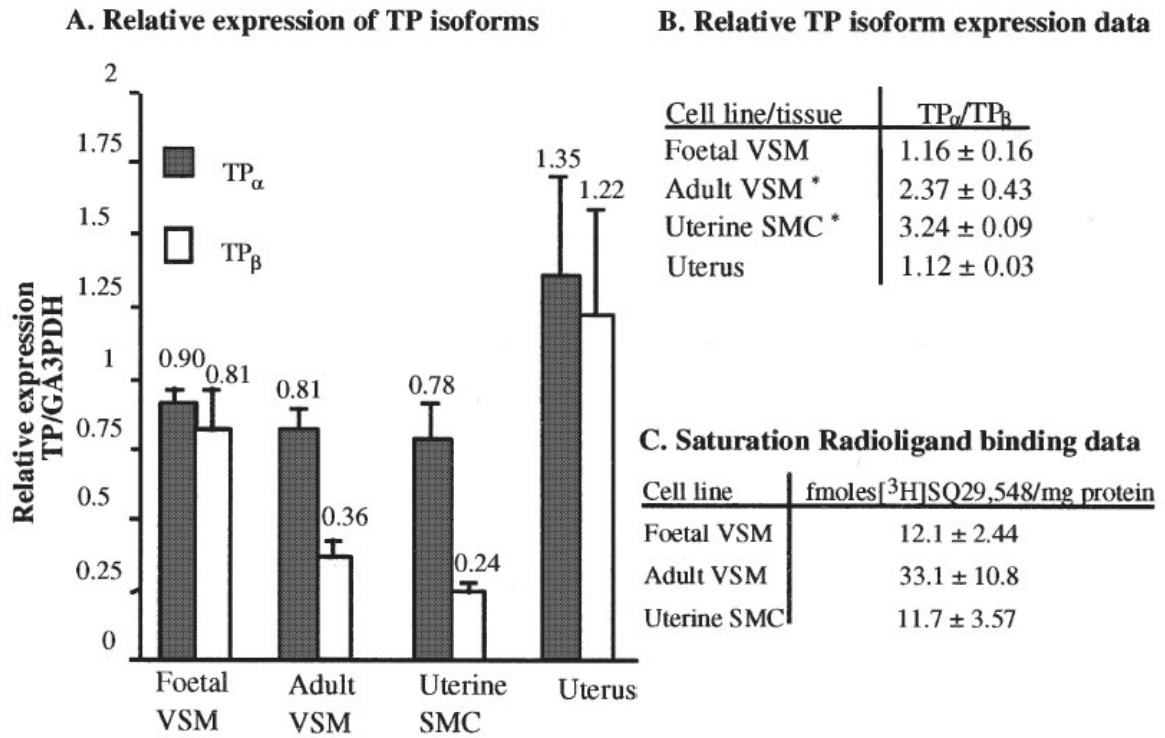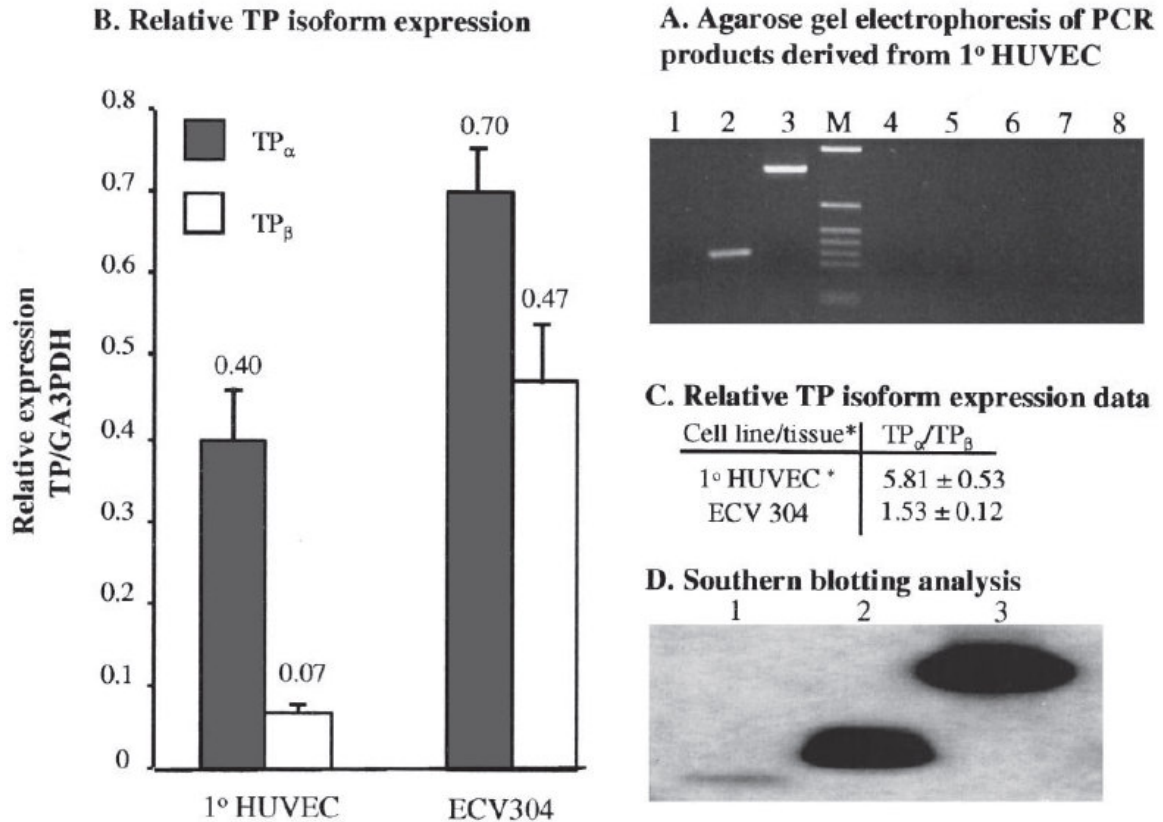
A. Agarose gel electrophoresis of PCR products derived from ED-31

B. Relative TP isoform expression

Cell line

C. TP isoform expression data

Cell line	TP_α/GA3PDH	TP_β/GA3PDH
ED-27	0.73 ± 0.08	0.23 ± 0.01
ED-31	0.85 ± 0.12	0.55 ± 0.12
ED-77	0.86 ± 0.11	0.87 ± 0.08
Tm-1	0.78 ± 0.10	0.87 ± 0.03

D. Saturation radioligand binding data

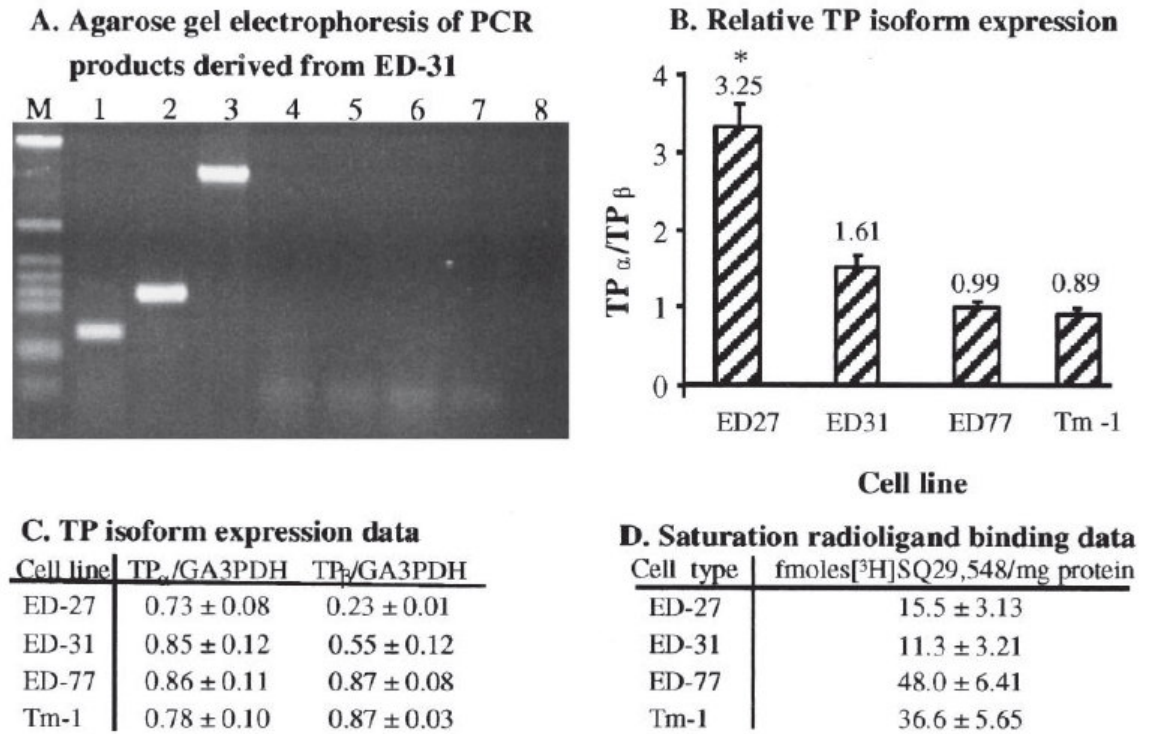| Cell type	fmoles[^{3}H]SQ29,548/mg protein
ED-27	15.5 ± 3.13
ED-31	11.3 ± 3.21
ED-77	48.0 ± 6.41
Tm-1	36.6 ± 5.65

Figure 5. Analysis of TP expression in 1° Trophoblasts

A. Agarose gel electrophoresis of RT-PCR products (7 μl/lane) derived from primary ED-31 cell 1^O cDNA template: lane 1, TP_β, 269bp product versus primers A/B; lane 2, TP_α, 400bp product versus primers A/C; lane 3, GA3PDH, 982 bp product versus primers G*f*/G*r*; lanes 4-6, negative control PCR reactions carried out in the absence of template 1^O cDNA in the presence of primers A/B, A/C and G*f*/G*r*, respectively; lanes 7-8, negative control reactions resulting from 1° cDNA template generated in the absence of MMLV-RT or template RNA, respectively, versus primers A/B. Lane M, pGEM$^®$ DNA markers; only the 126 - 1,198 bp fragments are shown. **B.** Levels of TP_α, TP_β and GA3PDH mRNA expression in four individual clonal trophoblast cell lines, namely ED-27, ED-31, ED-77 and Tm-1, in arbitrary units, were each represented as a ratio relative to GA3PDH expression (i.e. TP_α /GA3PDH ± S.E.M. and TP_β /GA3PDH ± S.E.M.). **C.** Expression of TP_α relative to TP_β are represented as a ratio of these values (i.e. TP_α/ TP_β ± S.E.M, arbitrary units). Significantly greater levels of TP_α relative to TP_β expressed in ED-27 cells (* $p \leq 0.005$). **D.** Radioligand binding data are expressed as fmol [^{3}H]SQ29,548 / mg cell protein ± S.E.M. where n ≥ 4.
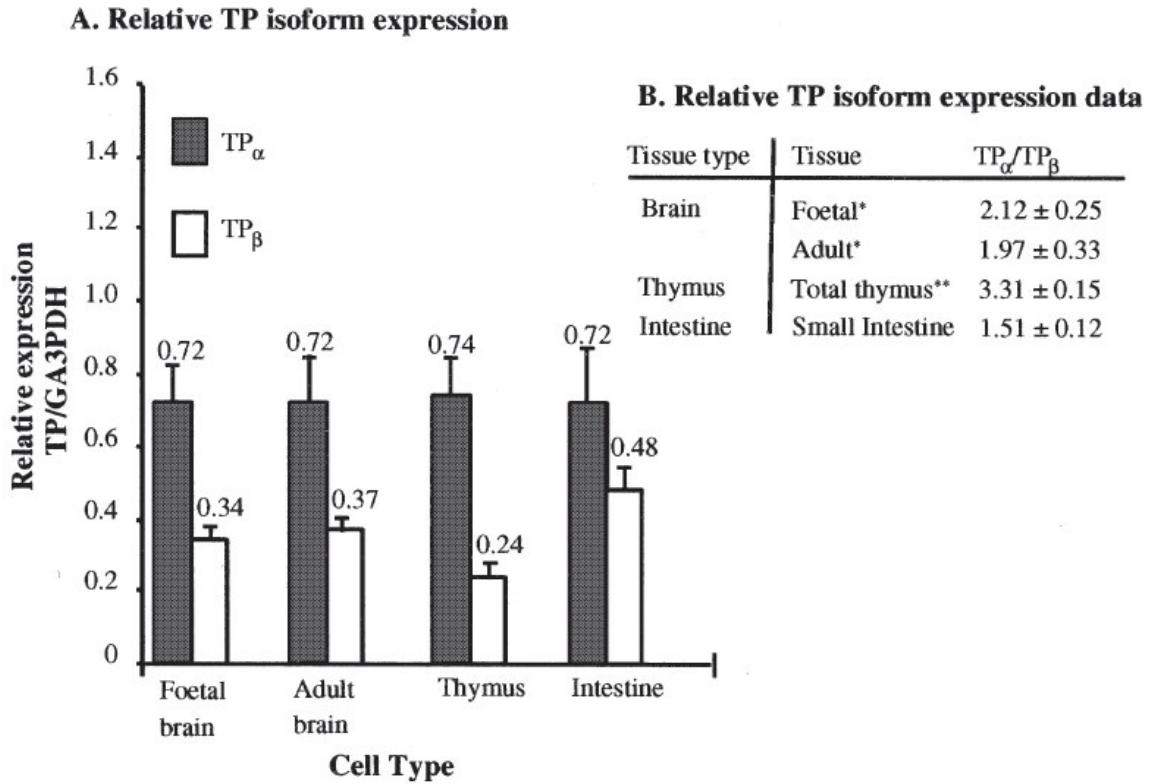
A. Relative TP isoform expression

B. Relative TP isoform expression data

Tissue type	Tissue	TP_α/TP_β
Brain	Foetal*	2.12 ± 0.25
	Adult*	1.97 ± 0.33
Thymus	Total thymus**	3.31 ± 0.15
Intestine	Small Intestine	1.51 ± 0.12

Figure 6. Analysis of TP expression in foetal and adult brain, thymus and small intestine.

A. Levels of TP_α, TP_β and GA3PDH mRNA expression in foetal brain, adult brain, thymus and intestine, in arbitrary units, were each represented as a ratio relative to GA3PDH expression (i.e. TP_α /GA3PDH $\pm$ S.E.M. and TP_β /GA3PDH $\pm$ S.E.M.). **B.** Expression of TP_α relative to TP_β are represented as a ratio of these values (i.e. TP_α / TP_β $\pm$ S.E.M, arbitrary units). Significantly greater levels of TP_α relative to TP_β are expressed in foetal brain tissue (* $p \leq 0.05$), thymus (*$p \leq 0.05$) and small intestine.

B. Relative TP isoform expression

A. Agarose gel electrophoresis of PCR products derived from Hep G2

C. Relative expression of TP isoforms

Cell line/Tissue	TP_α/TP_β
Liver [*]	2.71 ± 0.17
Hep G2	--------

D. Saturation radioligand binding data

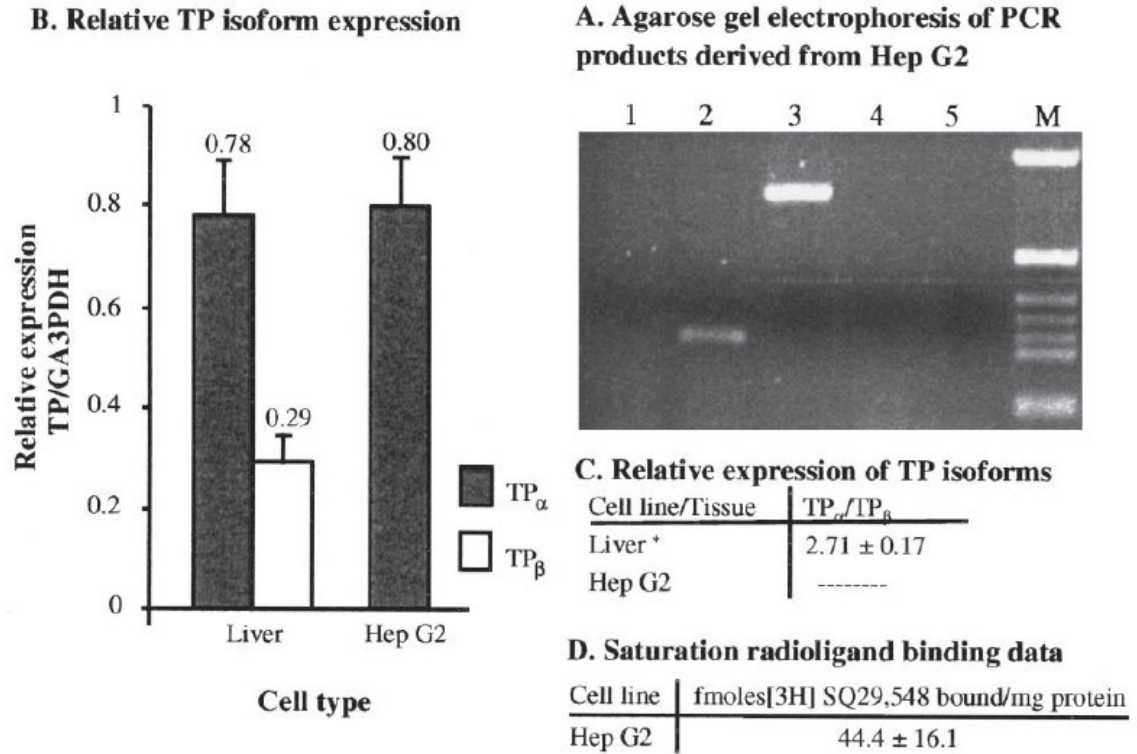| Cell line	fmoles[3H] SQ29,548 bound/mg protein
Hep G2	44.4 ± 16.1

Figure 7. Analysis of TP expression in liver tissue and HepG2 cells

A. Agarose gel electrophoresis of RT-PCR products (7 µl/lane) derived from HepG2 cell 1^O cDNA template: lane 1, TP_β, primers A/B; lane 2, TP_α, 400bp product versus primers A/C; lane 3, GA3PDH, 982 bp product versus primers G*f*/G*r*; lanes 4-5, negative control PCR reactions carried out in the absence of template 1^O cDNA in the presence of primers A/B and A/C. Lane M, pGEM® DNA markers; only the 126 - 1,198 bp fragments are shown. Both the TP_α and the GA3PDH products were amplified (lanes 2 & 3, respectively). However, TP_β specific product (268bp, lane 1) was not detected. **B.** Levels of TP_α, TP_β and GA3PDH mRNA expression in liver and the HepG2 cell line, in arbitrary units, were each represented as a ratio relative to GA3PDH expression (i.e. TP_α /GA3PDH ± S.E.M. and TP_β /GA3PDH ± S.E.M.). **C.** Expression of TP_α relative to TP_β are represented as a ratio of these values (i.e. TP_α / TP_β ± S.E.M, arbitrary units). Both TP_α and TP_β are expressed in liver tissue with significantly greater levels of TP_α expressed relative to TP_β (* $p \leq 0.05$). **D.** Radioligand binding data are expressed as fmol [³H]SQ29,548 / mg cell protein ± S.E.M. where n $\geq$4.