0026-895X/93/050934-06$03.00/0

MOLECULAR PHARMACOLOGY, 44:934-939

Point Mutation in the Seventh Hydrophobic Domain of the Human Thromboxane A_2 Receptor Allows Discrimination between Agonist and Antagonist Binding Sites

COLIN D. FUNK, LUCINDA FURCI, NIAMH MORAN, and GARRET A. FITZGERALD

Department of Pharmacology, Division of Clinical Pharmacology, Vanderbilt University, Nashville, Tennessee 37232 (C.D.F., L.F.), and Center for Cardiovascular Science, Department of Medicine and Experimental Therapeutics, University College Dublin, Mater Hospital, Dublin 7, Ireland (N.M., G.A.F.)

Received February 10, 1993; Accepted August 26, 1993

SUMMARY

Thromboxane A_2, a potent platelet agonist and vasoconstrictor, exerts its actions via specific G protein-coupled receptors. cDNAs encoding the full length thromboxane receptor have been isolated from human placenta mRNA by reverse transcriptase-polymerase chain reaction. An expression construct, under control of the cytomegalovirus promoter, was introduced into human embryonic kidney 293 cells. Membranes from transfected cells bound the thromboxane antagonist SQ29,548 and the agonist [15-(1α,2β(5z)-3α(1*E*,3*S*)-4α)]-7-[3-(3-hydroxy-4-(*p*-iodophenoxy)-1-butenyl)-7-oxabicyclo[2,2,1]hept-2-yl]-5-heptenoic acid) with high affinities, and significantly more receptors were expressed in these cells, compared with platelet preparations. The putative seventh transmembrane segment is highly related in all cloned members of the eicosanoid receptor family and forms a critical portion of the ligand binding pocket for G protein-coupled receptors. Several point mutations in this segment were generated. Binding of SQ29,548 was virtually abolished in cells transfected with all the variant receptor constructs. However, one receptor variant (TxR-W299L), in which a tryptophan at position 299 was substituted for a leucine residue, allowed a definite discrimination between agonist and antagonist binding sites in competition and saturation binding experiments. An antibody directed toward the third intracellular loop of the thromboxane receptor was able to immunoprecipitate native thromboxane receptor in solubilized membranes from human erythroleukemia cells and transfected cells.

TxA_2 is a potent vasoconstrictor and platelet agonist that has been implicated in clinical syndromes of platelet-dependent vascular occlusion, such as unstable angina and stroke (1, 2). Pharmacological studies identified specific binding sites for Tx-like ligands in a variety of tissues, and Hirata *et al.* (3) have reported a cDNA for a TxR obtained from a human placental library. Based on the identity of this clone with a partial length clone obtained from a megakaryocyte cell line, those authors argued for the likely identity of platelet and vascular TxRs (3). In contrast, pharmacological studies raised the possibility of receptor heterogeneity between and possibly within tissues (4–6). Recently, this issue has been given additional importance by the provision of pharmacological data that suggest that the free radical-catalyzed isoprostane 8-epi-prostaglandin $F_{2\alpha}$ exerts its effects on platelets and vascular smooth muscle via specific isoforms of the TxR (7).

This work was supported by National Institutes of Health Grant HL30400. C.D.F. is the recipient of Research Career Development Award HL02710.

The present studies were designed to obtain additional information on the molecular characteristics of TxRs. Specifically, we were interested in using the PCR to clone the receptor with a view toward characterizing the domains of relevance to interaction of the receptor with various ligands, to develop quantitative assays for receptor mRNA based on PCR, and to obtain the reagents necessary to address the issue of receptor heterogeneity.

Materials and Methods

PCR cloning and sequence analysis. Oligonucleotides (gel filtration grade purified) used for PCR were obtained from Midland Certified Reagent Co. (Midland, TX). Total RNA was prepared from human placenta by the acid guanidinium thiocyanate/phenol/chloroform extraction method (8). cDNA synthesis was performed in a 100-μl reaction containing 4 μg of RNA, 0.8 mM deoxynucleoside triphosphates, 80 units of RNAsin (Promega), 200 pmol of random primer (Pharmacia), and 800 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), in 10 mM Tris·HCl, pH 8.3, 50 mM

ABBREVIATIONS: Tx, thromboxane; TxR, thromboxane receptor; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s); HEL, human erythroleukemia; HEK, human embryonic kidney; I-BOP, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate.

KCl, 1.5 mM $MgCl_2$, 0.01% gelatin buffer, for 90 min at 37°, followed by phenol/chloroform extraction and ethanol precipitation. An aliquot (1/10) of the cDNA mixture was subjected to PCR amplification using either *Taq* polymerase (1.25 units; Perkin Elmer Cetus) or Vent polymerase (1 unit; New England Biolabs), with the buffer supplied and recommended by the manufacturer, in a 50-μl reaction volume with 25 pmol of each primer for 35 or 40 cycles, using the following conditions: denaturation, 94–98° for 30–45 sec; annealing, 58–62° for 45 sec to 1 min; extension, 72° for 1–1.5 min. Amplified products were isolated from agarose gels by GeneClean purification (Bio 101) and were cloned directly into the pCR1000 vector (Invitrogen). DNA sequence analysis was performed at 70° with *Taq* polymerase (Gene ATAQ system; Pharmacia) on single-stranded DNA templates cloned into M13 mp18 or mp19 vectors by the Sanger dideoxy chain termination method (9).

Construction of expression vectors and mutagenesis. *Eco*RI/*Hind*III (1.1-kb) cDNA fragments from M13 vectors containing the TxR coding sequence were inserted into the pCMV5 expression vector (10). Mutagenesis was performed on the 1.1-kb insert cloned in M13 vectors by the method of Taylor and Eckstein (11), using a kit from Amersham. All expression constructs contained the same 5′ (27 bp from the pCR1000 vector and 4 bp of the original receptor cDNA 5′ noncoding sequence before the ATG start codon) and 3′ (3 bp of the original receptor cDNA 3′ noncoding sequence and 28 bp from the pCR1000 vector) noncoding sequences and TxR coding regions, with the exception of various point mutations in the region encoding putative transmembrane region 7. Similar expression levels were seen with closely related pCIS2 (12) or pcDNA-1 (Invitrogen) expression vectors, which also contain the cytomegalovirus promoter (data not shown).

Cell culture and transfection. HEK 293 cells were obtained from the American Type Culture Collection and were cultured in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics. Transfection experiments were carried out as described (12). Briefly, HEK 293 cells were plated at a density of 1.0–1.5 × 10^6 cells, the day before transfection, in P-100 culture dishes. The next day cells were transfected with 10 μg of pCMV5-TxR DNA and 1 μg of pADVA (plasmid containing the genes encoding adenovirus VA RNA) by the calcium phosphate co-precipitation method (12).

Membrane preparations and binding experiments. Approximately 48 hr after transfection, cells were washed once with phosphate-buffered saline and were scraped off in ice-cold homogenization buffer (15 mM HEPES, pH 7.4, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride). After brief homogenization with a Dounce homogenizer, the suspension was passed through a 25-gauge needle three times on ice. The homogenate was centrifuged at 100,000 × *g* for 30 min and the pellet was homogenized as described above and recentrifuged. The membrane fraction was taken up in binding buffer (modified Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution, 10 mM HEPES, pH 7.4, 0.1% fatty acid-free bovine serum albumin, for SQ29,548 binding or 25 mM HEPES, pH 7.4, for I-BOP binding) to a protein concentration of 1 mg/ml. Protein was assayed by the method of Bradford (13). Membranes were used immediately or, in some cases, were frozen at −80° and used later, with minimal losses in binding activity. Washed human platelets were obtained as described (14) and membranes were prepared in the same manner as transfected cells.

Saturation binding experiments with the Tx antagonist [^{3}H] SQ29,548 (0.5–15 nM, 30 Ci/mmol; DuPont/NEN) were carried out for 40 min at room temperature, as described previously (5). Nonspecific binding was determined by adding 10 μM unlabeled ligand. The reaction was terminated by dilution with 4 ml of ice-cold 10 mM Tris, pH 7.4, the mixture was immediately filtered through Whatman GF/A glass filters (Fisher), and the filters were washed an additional three times. After addition of 8 ml of scintillation fluid, the filters were assayed by liquid scintillation counting. Specific binding to membranes of the expressed wild-type TxR ranged between 89 and 96%.

Competition with ^{125}I-BOP (2000 Ci/mmol, 50–60 pM, ≈25,000 cpm; Cayman Chemical Co.) binding to membranes by unlabeled I-BOP (50 pM to 500 nM), SQ29548 (1 nM to 10 μM), and U46619 (1 nM to 5 μM) was performed for 30 min at 37°, in 200 μl, in silanized glass tubes. Incubations were terminated by dilution with 4 ml of ice-cold binding buffer (25 mM HEPES, pH 7.4), repeated three times, and filtration over Whatman GF/C glass fiber filters. Nonspecific binding was determined in the presence of 2 μM I-BOP and was typically 5–10% of total binding (seven experiments). Filters were counted in a Beckman 5500B γ counter at 70% counting efficiency.

Antibody preparation and immunoprecipitation of TxR binding activity. A polyclonal antipeptide antibody (Ab-643) was raised in rabbits by Immuno-Dynamics Inc. The peptide YHGQEA-AQQRPRDSE, directed against the putative third cytoplasmic loop of the receptor, was conjugated to keyhole limpet hemothiocyanin for intradermal injections. Nonimmune serum (Ab-642) served as control antibody.

For immunoprecipitation experiments, membranes were prepared as described above. After the first centrifugation, however, membranes were solubilized in a buffer containing 10 mM CHAPS detergent, 10% glycerol, 50 mM Tris, pH 7.4, and 5 mM EGTA. After centrifugation at 100,000 × *g* to remove insoluble matter, the supernatant was incubated for 1 hr at 4° with Protein A-Sepharose (Pharmacia)-antibody complex (prepared by 1-hr preincubation with shaking at 4°, followed by washing three times with phosphate-buffered saline containing 0.1% Tween 20). The immune complexes were pelleted and washed three times with phosphate-buffered saline/Tween 20, followed by two washes with binding buffer. The washed complexes were resuspended in binding buffer and were added to assay tubes for binding experiments as described above. Additionally, samples were prepared for SDS-polyacrylamide gel electrophoresis. Samples were blotted to nitrocellulose, incubated with primary antibody followed by ^{125}I-Protein A, and subjected to autoradiography.

RNA preparation and Northern blot analysis. Total RNA was prepared from transfected cells as described above and aliquots were subjected to electrophoresis in a 1% agarose gel containing 0.22 M formaldehyde. After transfer to nitrocellulose, hybridization with the ^{32}P-labeled 1.1-kb *Eco*RI/*Hind*III TxR fragment was performed for 20 hr at 65° in 20% formamide, 10% dextran sulfate, 4× SSPE (1X is 0.15 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA), 5× Denhardt's solution (1X is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 0.5% SDS, 0.1 mg/ml sonicated salmon sperm DNA. Membrane washing was performed at 65° with 0.1× standard saline citrate (0.15 M NaCl/0.015 M trisodium citrate, pH 7) containing 0.1% SDS and the blot was exposed to X-ray film for 8 hr at −70°.

Results

PCR cloning of TxR cDNA. Hirata *et al.* (3) reported the cDNA cloning of the human TxR by library screening with oligonucleotides based on peptide data for the purified receptor. The TxR mRNA appears to be present in very low abundance (3). We were interested in obtaining the cDNA to extend our previous studies on regulation of the TxR (14) and to characterize domains important for ligand binding. Initially, PCR amplification was attempted with *Taq* polymerase and oligo-

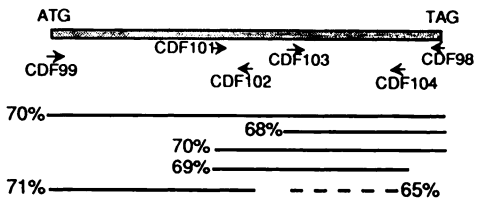

Fig. 1. TxR clones generated by PCR. *Shaded box*, human TxR cDNA; *arrows*, positions of the various oligonucleotides used for PCR amplification. *Solid lines*, clones obtained exclusively with Vent polymerase, with their respective GC contents. *Dotted line*, 0.3-kb cDNA generated by both *Taq* and Vent polymerases. See text for details.

nucleotide primers CDF98 and CDF99 (Fig. 1), to isolate the complete 1.0-kb coding region for the TxR. Numerous attempts with various PCR conditions proved unsuccessful. Attempts to amplify shorter fragments using primer sets CDF98/CDF101 or CDF99/CDF102 (Fig. 1) also failed. Sequence analysis revealed that the TxR coding region is highly GC-rich (70%). Two new primers (CDF103/CDF104) designed from the least GC-rich region enabled us to amplify a 0.3-kb fragment with *Taq* polymerase, which when sequenced was found to be an authentic TxR cDNA fragment. These results suggested that our inability to amplify other TxR fragments might be related to the high GC content and "difficult" secondary structure. Use of high-temperature denaturation (98°) and the thermostable Vent polymerase (New England Biolabs) enabled PCR amplification of all possible fragments (Fig. 1).

Detailed sequence analysis of two subcloned 1.1-kb TxR cDNA clones derived from amplification with the CDF98/CDF99 primer set revealed one difference in each clone (TGG to CGG, nucleotide 895, TxR-W299R; CTC to TTC, nucleotide 871, TxR-L291F), compared with the published sequence (3). The original 0.3-kb TxR cDNA fragment, when sequenced, was also found to contain one point mutation (ATT to ATC, nucleotide 795) and a 1-bp deletion (adenosine at position 815).

Expression of wild-type and mutant TxRs in HEK 293 cells. PCR-derived cDNA clone TxR-W299R was subcloned into the pCMV5 expression vector and was transfected into HEK 293 cells. Binding of the Tx antagonist [^{3}H]SQ29,548 (15) to membranes prepared from these cells was consistently low. As mentioned above, this clone contains a single point mutation. This change would cause a single amino acid substitution in putative transmembrane region 7 (tryptophan to arginine at position 299) of the receptor. Site-directed mutagenesis of TxR-W99R to yield tryptophan at position 299 was performed. Expression of this construct (TxR) in HEK 293 cells resulted in high affinity, saturable binding of SQ29,548, with a K_d of 3.5 nM (Fig. 2). Scatchard analysis revealed a single class of binding sites, with a B_{max} of 3.1 pmol/mg of protein (Fig. 2, *inset*). SQ29,548 binding to platelet membranes, compared in the same experiments, was found to be approximately 15% of binding to HEK 293-transfected cell membranes (Fig. 2).

Because a single point mutation appears to profoundly reduce Tx antagonist binding, we decided to investigate the effects of additional mutations within the highly conserved transmembrane region 7 of eicosanoid receptors (see Fig. 7). Alteration of the wild-type TxR to yield TxR-W299L and TxR-R295Q caused large (≈90%) decreases in specific binding of SQ29,548, to values similar to those obtained for the TxR-W299R mutant (Fig. 3A). Another PCR-derived mutant, TxR-L291F, displayed virtually no specific binding (i.e., indistinguishable from binding performed with mock-transfected cell membranes). Cells transfected with either TxR-R295Q or TxR-W299R expression vectors expressed levels of TxR mRNA comparable to those of the wild-type expressed TxR (Fig. 3B), whereas cells transfected with variants TxR-W299L and TxR-L291F consistently expressed only 25–50% of these mRNA levels (Fig. 3B).

Binding of a high affinity radioiodinated Tx agonist, ^{125}I-BOP (16), was observed in membranes of HEK 293 cells transfected with the wild-type TxR and in human platelet membranes (Fig. 4A). Unlabeled I-BOP competed for radioligand binding with half-maximal inhibition at 11 nM (four

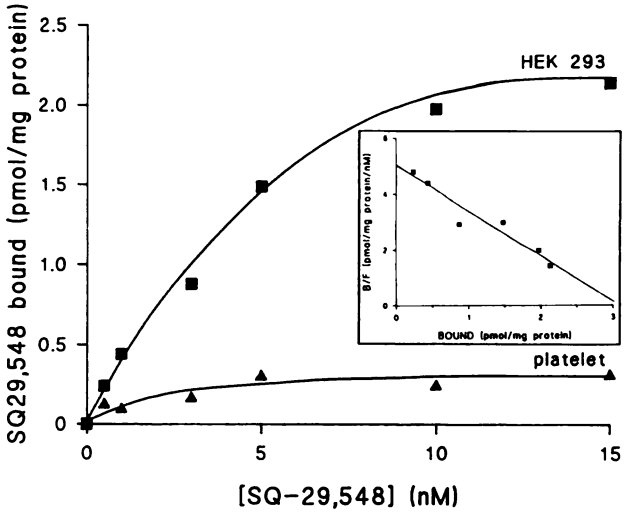

Fig. 2. Saturation binding of the Tx antagonist SQ29,548 to transfected HEK 293 cell and platelet membranes (50 μg of protein for each determination). HEK 293 cells were transfected with a human TxR cDNA expression vector as described in Materials and Methods. Human platelet membranes were prepared similarly. Each *point* represents specific binding and is the average of duplicate determinations from one representative experiment. Similar results were obtained in additional experiments (eight experiments for transfected cells; two experiments for platelets), although absolute values varied, likely due to differences in transfection efficiency. *Inset*, Scatchard analysis of saturation binding data for HEK 293 cell-expressed TxRs.

experiments) and 2.3 nM (two experiments), respectively. TxR mutants L291F, R295Q, and W299R failed to bind I-BOP (four experiments; data not shown). However, membrane fractions derived from the TxR mutant W299L bound approximately the same amount of I-BOP as did platelet preparations (per milligram of protein) and ≈35% of that bound by membranes from wild-type TxR-transfected cells. In separate experiments, saturation binding analysis with I-BOP yielded K_d and B_{max} values of 1.8 nM and 4.0 pmol/mg of protein for the native TxR and 1.9 nM and 0.95 pmol/mg of protein for mutant TxR-W299L, respectively. Competition binding with membranes from native TxR-transfected cells gave the same rank order of potency as determined previously with human platelet membranes (I-BOP > SQ29,548 > U44619) (Table 1; see Ref. 16). However, the rank order was altered (I-BOP > U46619 ≫ SQ29,548) (Fig. 4B; Table 1) for TxR-W299L binding.

An antibody directed toward the putative third cytoplasmic loop of the TxR (Ab-643) was prepared and was able to specifically immunoprecipitate SQ29,548 binding activity from solubilized HEL cell membranes (Fig. 5). Identical experiments with nonimmune serum (Ab-642) resulted in retention of binding activity in the supernatant fraction and no specific binding in the immunoprecipitated fraction (Fig. 5). Similar results were observed using HEK 293 cells transfected with the TxR cDNA (data not shown). Ab-643 recognized a ≈55-kDa band from HEL cells and platelets in immunoblot analysis of the immunoprecipitated proteins (Fig. 6).

Discussion

The GC-rich, full length, coding region of the human TxR cDNA has been isolated by PCR using 98° denaturation and the thermostable Vent polymerase derived from *Thermococcus*

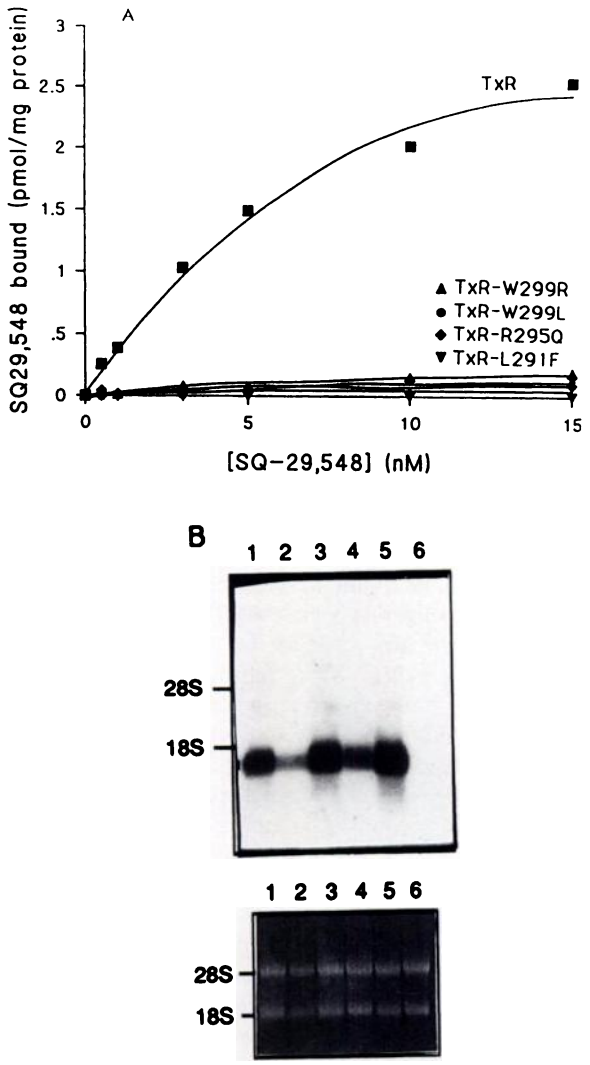

Fig. 3. A, Saturation binding of SQ29,548 to membranes from wild-type and mutant TxR-transfected HEK 293 cells. Each *point* represents specific binding and is the average of duplicate determinations from one experiment. Similar results were obtained in four additional experiments. B, Northern blot analysis of TxR RNA from transfected HEK 293 cells. *Top*, total RNA (*lanes 1* and *3-6*, 7.5 μg; *lane 2*, 3.5 μg) from W299R (*lane 1*), W299L (*lane 2*), R295Q (*lane 3*), L291F (*lane 4*), or wild-type (*lane 5*) TxR-transfected or mock-transfected (*lane 6*) cells was electrophoresed in a 1% agarose/formaldehyde gel, transferred to nitrocellulose, and probed with a 1.1-kb TxR cDNA. The blot was exposed for 8 hr at −70°. *Bottom*, ethidium bromide-stained gel used for Northern analysis, showing integrity of ribosomal bands and equal loading (except 3.5 μg for *lane 2*).

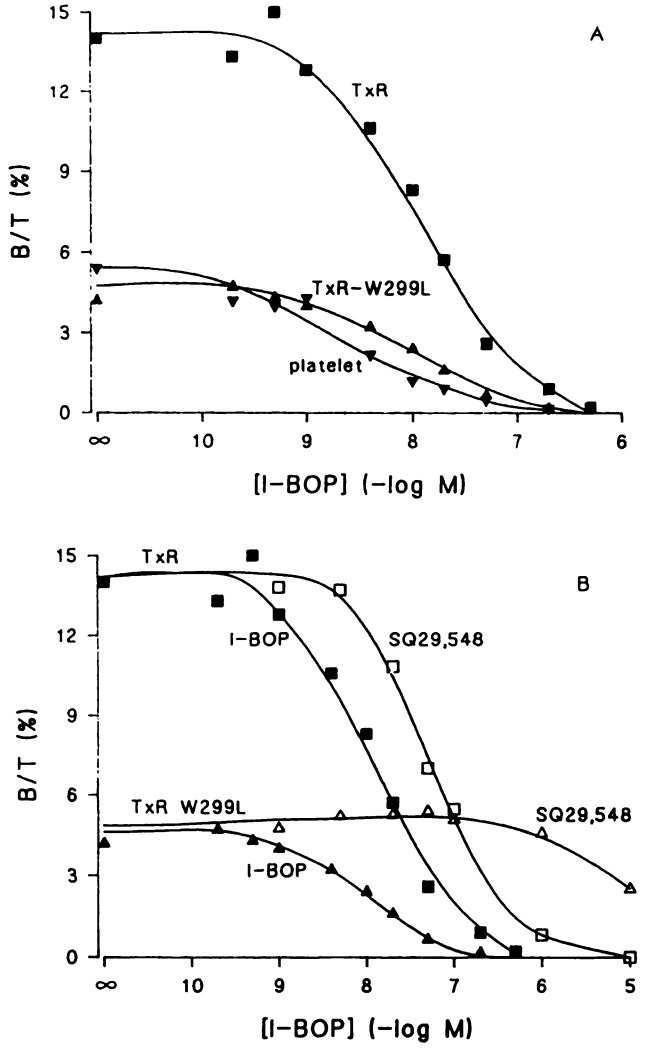

Fig. 4. Competitive inhibition of specific ^{125}I-BOP binding in transfected cell and platelet membranes. ^{125}I-BOP (60 pM) and varying concentrations of agonist (I-BOP) (*closed symbols*) or antagonist (SQ29,548) (*open symbols*) were incubated with platelet membranes (A) (*inverted triangles*), wild-type TxR-transfected membranes (A and B) (*squares*), or TxR-W299L-transfected membranes (A and B) (*triangles*). Forty micrograms of protein were used for each determination. The amount of specific ^{125}I-BOP binding (*B*) is expressed as a fraction of the total (*T*) amount of radioligand added.

littoralis. Attempts by us and by several other investigators[1] to isolate this cDNA by other means proved unsuccessful. Because the TxR mRNA appears to be expressed at very low levels, based on the reported long exposure time (12 days) and use of 20 μg of poly(A)$^+$ RNA during RNA blot analysis (3), the PCR-based assay described here will be useful for investigating regulation of expression of this receptor at the RNA level. We are currently developing a quantitative reverse transcription-PCR assay for this purpose, as we have done for the lipoxygenase and cyclooxygenase mRNAs (17).

An efficient, high-level, transient expression of the human

[1] W. L. Smith, P. V. Halushka, and J. A. Ware, personal communication.

TABLE 1

T×A$_2$-prostaglandin H$_2$ agonist and antagonist competition for ^{125}I-BOP binding to wild-type TxR- and TxR-W299L-transfected HEK 293 cells

Values are the mean ± standard error of the indicated number of experiments (in parentheses). Radioligand binding studies were performed as described in Materials and Methods.

	IC_{50}	
	TxR	TxR-W299L
	nM	
Agonists		
I-BOP	11 ± 1 (4)	10 ± 1 (4)
U46619	95 (1)	103 (1)
Antagonist		
SQ29,548	56 ± 5 (3)	10,000[a] (3)

[a] This value is approximate, because concentrations of antagonist greater than 10 μM were not used.

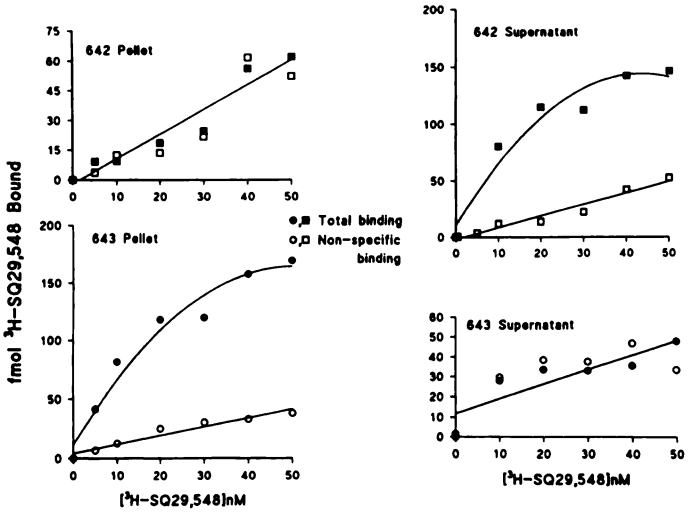

Fig. 5. Immunoprecipitation of TxR antagonist binding from solubilized HEL cell membranes. The pellet and supernatant fractions, after immunoprecipitation from solubilized membrane fractions with Ab-643 (TxR specific) or Ab-642 (control, nonimmune), were incubated with varying amounts of [³H]SQ29,548, and binding was determined as described in Materials and Methods.

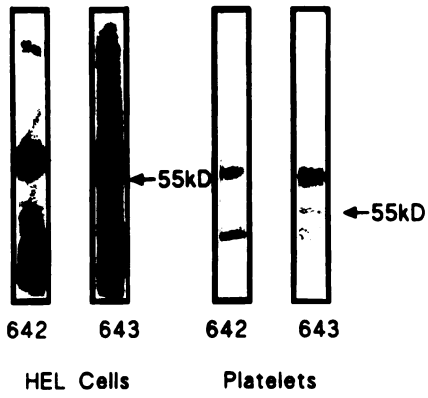

Fig. 6. Immunoblot analysis of TxR expression in HEL cells and platelets. CHAPS-solubilized membranes from HEL cells and platelets were used for immunoprecipitation with Ab-643 (TxR specific) or Ab-642 (control, nonimmune). The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted to nitrocellulose, and probed with the respective antisera. Electrophoresis was for different times for HEL cell and platelet samples.

placenta-derived TxR has been achieved in HEK 293 cells. Assuming a ≈10% transfection efficiency by the calcium phosphate method used here, we estimate that ≈1.5 × 10^6 receptors/cell are expressed. Human platelets have been estimated to possess 1400 sites/platelet, based on SQ29,548 binding data (18). The expressed receptor displayed characteristics similar to those of human platelet and placenta TxR-containing membrane and whole-cell preparations, with respect to various binding parameters with the Tx antagonist SQ29,548 (18, 19). It is not known with certainty whether the placenta and platelet TxRs are identical. We observed differences in competition for ^{125}I-BOP binding between platelet and expressed (placenta) TxRs (IC_{50} values of 2.3 nM and 11 nM, respectively). Additionally, these values are somewhat higher than those previously reported for human platelet and rabbit vascular preparations (16, 20), which may reflect differences in membrane preparations or subtype variations.

The TxR belongs to an unique eicosanoid receptor subclass of the seven-transmembrane region G protein-coupled receptor superfamily (3, 21–24). Receptors in this subclass are highly related within putative transmembrane region 7 (Fig. 7). Four variant transmembrane region 7-containing TxRs were generated during the course of these studies, two from probable PCR-based errors and two from *in vitro* site-directed mutagenesis. Mutant TxR-W299L was virtually incapable of recognizing the TxR antagonist SQ29,548 but was able to bind two different agonists in saturation and competition binding experiments. Affinity for these agonists was not changed with respect to the native receptor. The reduced binding capacity of I-BOP to membranes of TxR-W299L-transfected cells, compared with the native receptor expression, was likely due to lower protein expression levels, because mRNA levels were consistently lower (see Fig. 3B). Previous biochemical studies had demonstrated that differences in pH, likely attributable to variations in histidine charge, could lead to distinctions between Tx agonist and antagonist binding sites in human platelets (25).

Much evidence to date suggests that the membrane-spanning regions of G protein-coupled receptors are involved in ligand binding, forming a ligand pocket (26). The retinal chromophore of the visual pigments attaches to a lysine residue within transmembrane region 7 by a Schiff base mechanism (26). Previous studies with α_2- and β_2-adrenergic receptors have shown that the differences in the subtype-specific binding are determined by the seventh hydrophobic domain (27). Extension of these studies revealed that a single point mutation (Phe-412 to Asn-412) could account substantially for the subtype specificities (28).

The three other transmembrane region 7 TxR variants bound insignificant amounts of Tx agonists or antagonist. Because we were unable to document protein expression for these variants, it remains a possibility that structural alterations induced by the mutations lead to aberrant processing to the appropriate membrane sites, although mRNA levels are comparable to those of the native expressed TxR. Therefore, we can only speculate on how these alterations may affect binding. For instance, Arg-295, conserved among all eicosanoid receptors, has been proposed to be the counter-ligand for the prostanoid carboxyl moiety (21). Change of this residue to glutamine could disrupt the charge-charge interaction to abolish binding. Switch of leucine at position 291 to phenylalanine yields the residue found in the corresponding position of the human prostaglandin EP_1 receptor and would not be expected to markedly alter the hydrophobicity profile of the seventh membrane-spanning region. Perhaps the bulkier side chain of phenylalanine disrupts

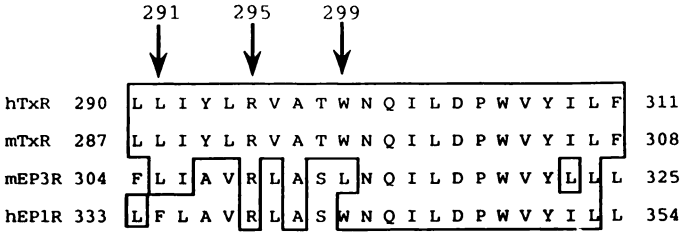

Fig. 7. Comparison of amino acid sequences from transmembrane region 7 of eicosanoid receptors. *Arrows*, the three positions that were altered. *hTxR*, human TxR (3); *mTxR*, mouse TxR (22); *mEP3R*, mouse prostaglandin E receptor, subtype 3 (21); *hEP1R*, human prostaglandin E receptor, subtype 1 (24). Boxed area indicates amino acids identical to hTxR.

the structure of the ligand binding pocket consisting of the arginine and other conserved amino acids.

Ab-643, raised against the putative third cytoplasmic loop of the TxR, was able to specifically immunoprecipitate Tx ligand binding activity from HEL cells (a platelet/megakaryocytic cell line) (Fig. 5) and TxR-transfected HEK cells. Although this antibody recognized a specific ≈55-kDa band from HEL cell and platelet immunoprecipitated proteins in immunoblot analysis (Fig. 6), it did not function well for the transfected HEK 293 cell samples, due to the presence of a nonspecific band in this region.

In conclusion, we have cloned the human TxR cDNA by PCR and have expressed it and mutated forms in a heterologous system. A point mutation in the seventh hydrophobic domain (tryptophan at position 299 to leucine) allows preferential agonist binding and loss of SQ29,548 binding ability.

Acknowledgments

The authors wish to thank Ginger Griffis and Usha Kurre for excellent technical assistance.

References

1. Patrono, C., G. Ciabattoni, and G. Davi. Thromboxane biosynthesis in cardiovascular disease. *Stroke* **21** (Suppl. IV):IV130-IV133 (1990).
2. Fitzgerald, D. J., and G. A. FitzGerald. Eicosanoids in myocardial ischaemia and injury, in *Advances in Eicosanoid Research* (P. V. Halushka and D. E. Mais, eds.). MTP Press, Lancaster, UK, 128–158 (1988).
3. Hirata, M., Y. Hayashi, F. Ushikubi, Y. Yokota, R. Kageyama, S. Nakanishi, and S. Narumiya. Cloning and expression of a cDNA for a human thromboxane A_2 receptor. *Nature (Lond.)* **349**:617–620 (1991).
4. Takahara, K., R. Murray, G. A. FitzGerald, and D. J. Fitzgerald. The response to thromboxane A_2 analogues in human platelets. *J. Biol. Chem.* **265**:6836–6844 (1990).
5. Furci, L., D. J. Fitzgerald, and G. A. FitzGerald. Heterogeneity of prostaglandin H_2/thromboxane A_2 receptors: distinct subtypes mediate vascular smooth muscle contraction and platelet aggregation. *J. Pharmacol. Exp. Ther.* **258**:74–81 (1991).
6. Dorn, G. W. Distinct platelet thromboxane A_2/prostaglandin H_2 receptor subtypes. *J. Clin. Invest.* **84**:1883–1891 (1989).
7. Morrow, J. D., T. A. Minton, and L. J. Roberts. The F_2-isoprostane, 8-epi-prostaglandin $F_{2\alpha}$, a potent agonist of the vascular thromboxane/endoperoxide receptor, is a platelet thromboxane/endoperoxide receptor antagonist. *Prostaglandins* **44**:155–163 (1992).
8. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159 (1987).
9. Sanger, F., S. Nicklen, and A. R. Coulson. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467 (1977).
10. Andersson, S., D. L. Davis, H. Dahlback, H. Jörnvall, and D. W. Russell. Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* **264**:8222–8229 (1988).
11. Taylor, J. W., J. Ott, and F. Eckstein. The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. *Nucleic Acids Res.* **13**:8764–8785 (1985).
12. Gorman, C. M., D. R. Gies, and G. McCray. Transient production of proteins using an adenovirus transformed cell line. *DNA Protein Eng. Techniques* **2**:3–10 (1990).
13. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
14. Murray, R., and G. A. FitzGerald. Regulation of thromboxane receptor activation in human platelets. *Proc. Natl. Acad. Sci. USA* **86**:124–128 (1989).
15. Ogletree, M. L., D. N. Harris, R. Greenberg, M. F. Haslanger, and M. Nakane. Pharmacological actions of SQ 29,548, a novel selective thromboxane antagonist. *J. Pharmacol. Exp. Ther.* **234**:435–441 (1985).
16. Morinelli, T. A., J. Oatis, A. K. Okwu, D. E. Mais, P. R. Mayeux, A. Masuda, D. R. Knapp, and P. V. Halushka. Characterization of an ^{125}I-labeled thromboxane A_2/prostaglandin H_2 receptor agonist. *J. Pharmacol. Exp. Ther.* **25**:557–562 (1989).
17. Funk, C. D., and G. A. FitzGerald. Eicosanoid forming enzyme mRNA in human tissues: analysis by quantitative polymerase chain reaction. *J. Biol. Chem.* **266**:12508–12513 (1991).
18. Hedberg, A., S. E. Hall, M. L. Ogletree, D. N. Harris, and E. C.-K. Liu. Characterization of [5,6-^{3}H]SQ 29,548 as a high affinity radioligand, binding to thromboxane A_2/prostaglandin H_2-receptors in human platelets. *J. Pharmacol. Exp. Ther.* **245**:786–792 (1988).
19. Hedberg, A., P. F. Mento, E. C.-K. Liu, A. M. Hollander, and B. M. Wilkes. Evidence for functional thromboxane A_2-prostaglandin H_2 receptors in human placenta. *Am. J. Physiol.* **256**:E256-E263 (1989).
20. Sessa, W. C., P. V. Halushka, A. Okwu, and A. Nasjletti. Characterization of the vascular thromboxane A_2/prostaglandin endoperoxide receptor in rabbit aorta. *Circ. Res.* **67**:1562–1569 (1990).
21. Sugimoto, Y., T. Namba, A. Honda, Y. Hayashi, M. Negishi, A. Ichikawa, and S. Narumiya. Cloning and expression of a cDNA for mouse prostaglandin E receptor EP_3 subtype. *J. Biol. Chem.* **267**:6463–6466 (1992).
22. Namba, T., Y. Sugimoto, M. Hirata, Y. Hayashi, A. Honda, A. Watabe, M. Negishi, A. Ichikawa, and S. Narumiya. Mouse thromboxane A_2 receptor: cDNA cloning, expression and Northern blot analysis. *Biochem. Biophys. Res. Commun.* **184**:1197–1203 (1992).
23. Funk, C. D. Molecular biology in the eicosanoid field. *Prog. Nucleic Acid Res. Mol. Biol.* **45**:67–98 (1993).
24. Funk, C. D., L. Furci, G. A. FitzGerald, R. Grygorczyk, C. Rochette, M. A. Bayne, M. Abramovitz, M. Adam, and K. M. Metters. Cloning and expression of a cDNA for the human prostaglandin E receptor EP_1 subtype. *J. Biol. Chem.*, in press.
25. Mayeux, P. R., T. A. Morinelli, T. C. Williams, E. S. Hazard, D. E. Mais, J. E. Oatis, D. A. Baron, and P. V. Halushka. Differential effect of pH on thromboxane A_2/prostaglandin H_2 receptor agonist and antagonist binding in human platelets. *J. Biol. Chem.* **266**:13752–13758 (1991).
26. Savarese, T. M., and C. M. Fraser. *In vitro* mutagenesis and the search for structure-function relationships among G protein-coupled receptors. *Biochem. J.* **283**:1–19 (1992).
27. Kobilka, B. K., T. S. Kobilka, K. Daniel, J. W. Regan, M. J. Caron, and R. J. Lefkowitz. Chimeric α_2-β_2-adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science (Washington D. C.)* **240**:1310–1316 (1988).
28. Suryanarayana, S., D. A. Daunt, M. Von Zastrow, and B. K. Kobilka. A point mutation in the seventh hydrophobic domain of the α_2 adrenergic receptor increases its affinity for a family of β receptor antagonists. *J. Biol. Chem.* **266**:15488–15492 (1991).

Send reprint requests to: Colin D. Funk, Department of Pharmacology, Vanderbilt University, Nashville, TN 37232.