The two most extensively characterized thromboxane/prostaglandin endoperoxide (TP) receptors, from human platelets and rat vascular smooth muscle, exhibit thromboxane agonist [15-(1α,2β(5Z),3α-(1E,3S),4α)]-7[3-hydroxy-4-(p-iodophenoxy)-1-buteryl-7-oxabicycloheptenoic acid (I-BOP) binding affinities that differ by an order of magnitude, rat TP having the higher affinity. We utilized this difference in I-BOP affinity to identify structural determinants of TP receptor heterogeneity. No significant difference was found in the rank order of affinities for a series of thromboxane receptor ligands to bind to cloned human TPα versus rat TP, indicating that these represent species homologs, not distinct TP subtypes. Structural determinants for observed differences in I-BOP binding $K_d$ were localized by creating chimeric human/rat TP followed by mutational substitution of specific critical amino acids. Initially, seven chimeric receptors with splice sites in transmembranes 1, 2, 4, or 7 were constructed and expressed in HEK293 cells for analysis of ligand binding properties. Substitution of any part except the carboxyl tail of the human TP into the rat TP resulted in a receptor with I-BOP binding affinity intermediate between the two. Analysis of chimeras in which only the extracellular amino terminus and a portion of transmembrane 1 were switched localized the determinant of high affinity binding to the region between amino acids 3 and 40. Using this chimera, amino acids in the human portion (extracellular amino terminus and part of transmembrane 1) were replaced with analogous amino acids from rat TP to regain high affinity I-BOP binding. Only when amino acid Val$^{37}$ and either Val$^{36}$ or Ala$^{40}$ were reverted to their respective rat TP counterparts (Ala$^{36}$, Leu$^{37}$, and Gly$^{40}$, respectively) was high affinity I-BOP binding recovered. The mechanism for the increased I-BOP affinity may be the lengthening of the amino acid side chain at position 37, thus extending this group further into the putative I-BOP binding pocket, with compensatory shortening of side chains in spatially adjacent amino acids.

Thromboxane $A_2$ is one of the most potent platelet-aggregating and vasoconstricting agents known. High affinity interactions of thromboxane $A_2$ or prostaglandin $H_2$ (1, 2) and lower affinity interactions of prostaglandin $F_2\alpha$, and $E_2$ (3) at membrane thromboxane/prostaglandin endoperoxide (TP) receptors transduce these effects in platelets and vascular smooth muscle. To date, two human TP subtypes as well as mouse and rat TP have been cloned (4–8). The two human subtypes, designated TPα and TPβ, are the alternately spliced products of a single gene, diverge only in the intracellular carboxyl terminus, and display identical ligand binding characteristics but different patterns of coupling to G-protein effectors (9).

The cloned rat and mouse TP are 93% identical at the amino acid level, while, compared with the human TPα, the rat TP is 73% identical. Several laboratories have compared the ligand binding characteristics of human platelet and rat vascular TP and have found that the rat receptor exhibits unique pharmacology exemplified by a binding affinity for the agonist 125I-I-BOP, which is 10-fold greater than human TP (3, 10, 11, 12). A comparative study of transfected human TPα and rat TP has confirmed these findings (7).

There is a great deal of interest in identifying the structural determinants of thromboxane receptor ligand binding due to the potential for development and refinement of subtype-specific agonists and antagonists. To date, two studies have employed mutagenesis to examine the effects of single amino acid substitutions on ligand binding. Funk et al. (13) modified several amino acids within the seventh transmembrane-spanning domain of human TPα and characterized changes in antagonist binding. However, since the amino acids in transmembrane domain 7 are absolutely conserved in all known TP receptors, these studies do not help to define differences between the naturally occurring receptors. In the second study, our laboratory examined the functional consequences of substitution mutagenesis of cysteine residues within human TPα and identified three cysteines that affected ligand binding (14). Cysteines 105 and 184, in the first and second extracellular loops, respectively, were absolutely required for binding and were assumed to form an intramolecular disulfide bond. Cysteine 102, in the first extracellular loop, was found to contribute to optimal binding, although the nature of its interaction with ligand was not defined.

Because ligand binding affinity is probably determined by multiple contiguous or widely separated amino acid residues, complete identification of the ligand binding pocket is not likely to be accomplished by substitution mutagenesis of single amino acids. A better approach may be to exchange regions between related receptors, and then measure gain or loss of binding.
affinity related to the particular exchanged domain. In the current study, this approach was employed to identify regions in TP receptors conferring species-specific differences in 125I-BOP binding affinity. Analysis of chimeras was followed by site-directed mutagenesis of single and combined nonconserved amino acids in the region of interest. Our results indicate that multiple regions of TP receptors, including the first transmembrane-spanning domain, are necessary for high affinity 125I-BOP binding. Within the first transmembrane domain, a combination of Leu37 with either Ala36 or Gly40 is necessary to produce a high affinity receptor.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were obtained from Life Technologies, Inc. T4 polynucleotide (Perkin-Elmer) was employed in polymerase chain reaction construction of mutant receptors. Site-directed mutagenesis was performed using the Altered Sites kit from Promega. All radionucleotides were purchased from DuPont NEN, DNA Sequenc II kits were from U.S. Biocals. 125I-BOP and 125I-PIA-OH were synthesized as described previously (15) using precursors generously provided by Dr. Perry Halushka (Charleston, SC). SQ29548, t-SAP, and nonradioactive I-BOP were purchased from Cayman. All tissue culture reagents and Lipofectamine were from Life Technologies, Inc. Oligodeoxynucleotides were synthesized and purified at the University of Cincinnati Core DNA Facility. All other reagents were of the highest purity available from Sigma or Fisher.

Nomenclature and Construction of Chimeric and Mutant TP Receptors—Human K562 TPα (16) and rat TP (7) cDNAs were used to construct the chimeric receptors in this study. TP chimeras were engineered by combining rat and human TP cDNAs at common existing restriction sites or at silent restriction sites created by mutagenic substitution of one or two nucleotides as described in Table I. All splice sites used were in one of the transmembrane-spanning domains. Therefore, the chimeric receptor nomenclature employed reflects the transmembrane domain of the splice site. The initial designation, R or H, is the species of origin (rat or human) of the amino terminus of the receptor, followed by a numeral describing the transmembrane domain wherein the two receptors were joined. The splice site is indicated by a slash, and a letter designating the species of origin of the carboxyl portion of the receptor follows.

The mutagenesis protocol for insertion of silent restriction sites is described in Table I, and the approximate location of the sites is depicted in Fig. 1. Oligonucleotides encoding specific mutations included 10 nucleotides flanking each side of the mutation and were employed for mutagenesis exactly as described previously (14). All mutations were confirmed by DNA sequencing, and all chimeric receptors were constructed by three-way ligation into the expression vector pcDNA3. Restriction enzyme and splice site positions are indicated. As necessary, silent restriction sites were introduced as indicated to facilitate construction of TP chimeras.

RESULTS

Ligand Binding Characteristics of Stably Expressed Rat and Human TP Receptors—It has been recognized for some time that there are species-specific differences in ligand binding to thromboxane receptors (19). Of particular interest is the observation that rat TP exhibit an approximately 10-fold higher affinity for the TP agonist I-BOP than human TPα (3, 7). Fig. 1 compares the amino acid sequence of these two TP receptors. Wild type rat TP bound 125I-BOP with a 7-fold higher affinity than did human TPα expressed in the same cell line using the same expression vector (Table II and Fig. 2A). Thus, differences in ligand binding are unlikely to be attributable to variations in cellular milieu. The Ks values for several stable thromboxane or endoperoxide analogs possessing either agonist or antagonist activity at thromboxane receptors are compared for transfected rat and human TP in Table II. Of the nine compounds tested, significant differences in affinity were observed in five, including the antagonist SQ29548 (Fig. 2B). Interestingly, and despite the structural similarity of the two compounds, rat TP exhibited higher affinity for I-BOP but lower affinity for SQ29548 (Table II). The rank order of binding affinity for these nine compounds in human and rat TP was highly correlated (correlation coefficient = 0.916, p < 0.001).

Characterization of Recombinant TP Receptors—Wild type human TPα and rat TP were stably expressed in HEK293 cells as described previously (14, 16). Recombinant receptors were transiently transfected in HEK293 cells using calcium phosphate precipitation. Nontransfected HEK293 cells have no thromboxane receptor expression defined by the absence of specific binding of 125I-BOP and the absence of calcium signaling with U46619 (16). Cells were prepared for equilibrium bind-

| Chimera | Enzyme | Restriction site | Mutagenesis | Restriction site | Mutagenesis |
|---------|--------|------------------|-------------|------------------|-------------|
| TP R7/H | XhoII  | bp 902           | None        | bp 893           | None        |
| TP R1/H and TP H1/R | BstXI | bp 129          | None        | bp 129           | None        |
| TP R2/H and TP H2/R | BstEI | bp 237          | T^42_50 → C, creation of BstEI site | bp 234 | None |
| TP R4/H and TP H4/R | NotI | bp 473       | None        | bp 470           | G^470 → C and G^476 → C, creation of NotI site |
FIG. 1. Comparison of amino acid sequences for human TPα and Rat TP. Rat and human TP are presented as defined by the seven-transmembrane-spanning α-helical domain receptor model. Amino acids conserved between human and rat are shown as solid black circles. Nonconserved amino acids are depicted with the human amino acid followed by the rat analogue. Human and rat receptors are 75% identical at the amino acid level. Arrows indicate positions of restriction sites used to construct human/rat TP chimeras.

Table II
Comparison of ligand binding affinities for human TPα and rat TP

| TPα | Rank | Rat TP | Rank | p value |
|-----|------|--------|------|---------|
| Agonists | | | | |
| I-BOP | 4.0 ± 0.5 nM (8) | 2 | 0.6 ± 0.07 nM (8) | 1 | 0.001 |
| U46619 | 157,516 ± 19,266 sites/cell | 5 | 124,499 ± 1,022 sites/cell | 4 | 0.257 |
| U44069 | 60 ± 3.7 nM (6) | 6 | 41 ± 6 nM (6) | 6 | 0.339 |
| CTA2 | 77 ± 7 nM (6) | 7 | 33 ± 14 nM (6) | 6 | 0.001 |

| Antagonists | | | | |
| SQ29548 | 13 ± 2 nM (13) | 3 | 34 ± 4 nM (15) | 4 | 0.004 |
| I-PTA-OH | 300 ± 34 nM (8) | 7 | 365 ± 17 nM (8) | 8 | 0.319 |
| 13-APA | 0.44 ± 0.05 nM (6) | 1 | 0.34 ± 0.1 nM (6) | 1 | 0.531 |
| PTA2 | 50 ± 3.3 μM (6) | 9 | 37 ± 2.6 μM (6) | 9 | 0.014 |
| | 27 ± 1.7 μM (6) | 4 | 14 ± 1 μM (6) | 3 | 0.001 |

* For I-BOP, Kd and Bmax values are given.
rat receptor and transmembrane domain 1 of the human receptor was created. Select mutations then reverted distinct nonhomologous amino acids in the first transmembrane domain to the rat counterparts. It was anticipated that replacement of functionally important amino acids with their rat analogs would, in the context of TP H1/R, restore \( {^{125}}I \)-BOP binding characteristics to those of wild type rat TP. Initially, the four divergent amino-terminal amino acids were substituted \textit{en bloc}, as were the three divergent amino acids within the first transmembrane domain. As depicted in Fig. 4, replacement of the extracellular domain amino acids with their rat counterparts had no effect on \( {^{125}}I \)-BOP binding, whereas replacement of the transmembrane amino acids rescued high affinity I-BOP binding. Since this indicated that amino acid(s) at position 36, 37, and/or 40 was necessary for high I-BOP affinity, each of these amino acids was individually replaced with the rat analog, again in the context of TP H1/R. Surprisingly, none of these individual amino acid substitutions was sufficient to rescue high affinity binding for \( {^{125}}I \)-BOP (Fig. 4).

Therefore, to examine the possibility that two of these residues interacted cooperatively to confer high affinity for \( {^{125}}I \)-BOP, amino acids 36, 37, and 40 were mutated to their rat analogs in all three possible pairs. As depicted in Fig. 4, the combination of Leu\(^\text{37}\) with either Ala\(^{36}\) or Gly\(^{40}\) rescued high affinity I-BOP binding, whereas the combination of Ala\(^{36}\) and Gly\(^{40}\), like individual replacement of any of the three amino acids, did not.

Purely as a matter of thoroughness, the binding affinity for SQ29548 was also determined for each of the TP mutant/chimeras. Consistent with our prior observation that the determinants of I-BOP and SQ29548 binding differ in these receptors, there was again no apparent correlation between SQ29548 and I-BOP binding affinities (data not shown).

DISCUSSION

This study identifies cooperative interactions between pairs of amino acids in the rat TP first transmembrane-spanning domain that contribute to species selectivity in TP agonist binding. Leu\(^\text{37}\), paired with either Ala\(^{36}\) or Gly\(^{40}\) increased affinity for the agonist I-BOP but not the structurally related antagonist SQ29548. Furthermore, additional residues in transmembrane domain 4, 5, or 6 were implicated as also contributing to the high affinity I-BOP binding exhibited by wild-type rat TP. The experimental design employed herein took advantage of species-specific differences in ligand binding.
FIG. 4. Rescue of high affinity I-BOP binding to TP H1/R chimera by mutagenic reversion of nonhomologous amino acids in transmembrane domain 1. The extracellular amino terminus and first transmembrane spanning domain are depicted for (top row) human TP (amino acids shown as closed circles), TP H1/R, and rat TP (amino acids shown as open circles). Below are depicted TP H1/R mutants in which various individual or combinations of human amino acids are replaced by their rat analogs. I-BOP $K_d$ is reported for each receptor. These studies show that only when the human amino acid at position 37 and either amino acid 36 or 40 are reverted to the appropriate rat analogs is I-BOP binding $K_d$ restored to rat TP values.
between rat and human TP. Previous reports (3, 10–12) have demonstrated higher affinity of rat platelet and/or vascular smooth muscle TP receptors for I-BOP compared with the human aggregation-coupled TP receptor. Recently, D’Angelo et al. (7) directly compared the binding affinities of transfected human TPα and rat TP and found that, when transiently expressed in identical cell systems using identical expression vectors, rat and human TP differ in their affinity for I-BOP. The current study provides a mechanism that explains these observations.

A comparative analysis of the binding affinities of nine thromboxane/endoperoxide analogs (four agonists and five antagonists) in HEK293 cells stably expressing the rat or human TP showed significant differences in the absolute binding affinities of five of the nine compounds. The rank order of ligand binding affinity, however, did not significantly differ between the rat and human TP. Thus, based on the current binding studies in transfected cells, on previously demonstrated similarities in cell signaling (3, 7, 15, 16), and on the high percentage of shared amino acids between rat and human TP, these two receptors should most appropriately be considered as species variants of a single pharmacologic subtype.

The similarities in rat TP and human TPα facilitated identification of ligand binding determinants using analysis of chimeric receptors. A general weakness in mutagenic structure-function analysis is differentiating between functional changes conferred by the characteristics of an individual amino acid from more general alterations in protein folding and tertiary structure. Substitution of portions of one receptor with another that is similar was anticipated to alter amino acids without changing the overall structure of the receptor. This approach was employed to analyze binding properties of various rat/human TP chimeras and demonstrated the following: 1) the intracellular carboxyl terminus of TP receptors does not play a regulatory role for I-BOP binding affinity, since chimera TP R7/H exhibited high affinity for I-BOP; 2) multiple receptor domains contribute to the high I-BOP binding affinity exhibited by wild-type rat TP. The latter conclusion derives from comparison of the binding properties of the six mirror-image chimeras constructed by ligating human and rat TP receptors within transmembrane domains 1, 2, and 4. Each of these chimeric receptors displayed an intermediate binding affinity for I-BOP. En bloc replacement of rat TP receptor transmembrane domains 4, 5, and 6 (but not 7; see TP R7/H) with the human counterparts or replacement of the extracellular amino terminus and the first transmembrane domains (TP H1/R) lowered the I-BOP binding dissociation constant from approximately 0.6 nM to 2 nM. Thus, a minimum of at least two separate regions of TP receptors are required for high affinity interactions with I-BOP. Interestingly, the determinants of I-BOP and SQ29648 binding differ, since no correlation was observed in the binding affinities of these two compounds with the wild-type and chimeric TP receptors.

Since the first transmembrane domain is not generally recognized as a region of critical importance in ligand binding to G-protein-coupled receptors, we focused our efforts toward identifying individual amino acid determinants in this region. While the chimeric analysis assayed for “loss of function” (high affinity I-BOP binding), we utilized the TP H1/R chimera as the substrate for grouped or single amino acid mutagenesis with the goal of “rescuing” high affinity I-BOP binding. The most intriguing findings of this study relate to the requirement for cooperative interactions between Leu37 and Ala36 or Gly40 in the first transmembrane domain to restore high I-BOP binding affinity. A postulated mechanism whereby substitution of Val37 → Leu plus either Val36 → Ala or Ala40 → Gly increases I-BOP binding affinity in the TP H1/R chimera is illustrated in Fig. 5. The shared substitution, that of Val37 → Leu, simply lengthens the side chain of amino acid 37 by a single carbon, extending the isopropyl group further into the putative binding pocket, thereby potentially facilitating interactions with I-BOP. The necessary co-substitutions have opposite effects, diminishing the size of the side chain of an adjoining amino acid (position 36 is continuous with position 37, and position 40 is one additional revolution of the α-helix), thus making room for the larger Leu37 side chain. This space-filling mechanism is supported by several observations: 1) each of the amino acids in positions 36, 37, and 40 of either receptor possesses a noncharged hydrophobic side chain, thus eliminating charge as a factor; 2) each of the two pairs of amino acid substitutions that restores high affinity binding involves the amino acid at position 37 and a spatially adjacent amino acid; 3) The required alteration in either substitution pair is an increase in length of the side chain of amino acid 37 with a compensatory shortening of the side chain of an adjacent amino acid. Unfortunately, these data...
do not identify the portion of the ligand interacting with these receptor domains.

Regional variations in the degree of amino acid conservation between human and rat TP are illustrated in Fig. 1. The seventh transmembrane domain is absolutely conserved between these receptors and has previously been demonstrated to play a critical role in TP ligand binding (13). Interestingly, two of the three nonconserved amino acids in the first transmembrane domain were found to be necessary for species-specific high affinity I-BOP binding exhibited by rat TP. In contrast to these highly conserved regions of the receptor, the intracellular carboxyl terminus is hypervariable and did not affect ligand binding. This degree of variability may occur either because this region is not a critical determinant of receptor structure-function and therefore can accommodate frequent mutations or because this region is responsible for observed species differences in these receptors. To date, two forms of human TP receptors have been identified using molecular techniques, and these receptors differ only in their intracellular carboxyl terminus (5). The ligand binding properties of these two human TP receptors are identical. Furthermore, our laboratory has engineered a mutant human TP receptor lacking an intracellular carboxyl terminus by introducing a termination codon at amino acid position 320 and found that this truncated receptor has normal human TP binding properties with only mild impairment of calcium signal transduction. Thus, several different avenues of investigation support the notion that the hypervariable carboxyl terminus is not directly involved in ligand binding to TP receptors.

The current findings, together with a prior mutagenesis analysis of the seventh transmembrane domain of human TPα (13) and studies of other G-protein coupled receptors that bind small molecules (20–23) suggest that the binding site for thromboxane and endoperoxides resides within the hydrophobic transmembrane-spanning domains. Particular importance has been assigned to transmembrane domains 1 and 7 in determining ligand specificity and binding affinity (current study and Ref. 13). However, some studies have also suggested that residues within the first and second extracellular loop may influence ligand binding to TP receptors, perhaps by determining peptide folding and receptor conformation (14, 24, 25). Furthermore, Halushka and colleagues (26) have recently reported that TPα interactions with different G-protein effectors can modify receptor affinity for I-BOP. Because the binding domain for TP ligands is not well defined and even the orientation of thromboxane with different receptor regions is a matter of speculation, future studies will be needed to address these issues.

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Structural Determinants for Agonist Binding Affinity to Thromboxane/Prostaglandin Endoperoxide (TP) Receptors: ANALYSIS OF CHIMERIC RAT/HUMAN TP RECEPTORS
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