Mutagenic Analysis of Platelet Thromboxane Receptor Cysteines

ROLES IN LIGAND BINDING AND RECEPTOR-EFFECTOR COUPLING*

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The human platelet thromboxane A₂ receptor is a member of the G-protein-coupled superfamily of receptors. Previous pharmacologic studies examining the effects of biochemical reduction, oxidation, or sulfhydryl alkylation on thromboxane receptors have suggested a role for cysteines in determining receptor binding characteristics. To characterize the roles of individual cysteines, we employed site-directed mutagenesis to substitute serines for cysteines at seven positions throughout the human K562 thromboxane receptor and analyzed mutant receptor radioligand ([1-3-H]-7-[3-(3-hydroxy-4-(p-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]heptane-2-yl]-5-heptenoic acid) binding and calcium signaling. Replacing cysteines in the amino terminus (amino acid position 11), and transmembrane domains two and six (positions 68 and 257) had little effect on thromboxane receptor binding or signaling. Introduction of serines for cysteines in the first (position 105) or the second (position 183) extracellular loop eliminated thromboxane receptor binding, consistent with the existence of a critical disulfide bond between these positions. Mutation of a second cysteine in extracellular loop one (position 102) resulted in a receptor with decreased binding affinity and low binding capacity that transduced only a low amplitude calcium signal, suggesting the involvement of a free sulfhydryl group at this location in receptor-ligand interactions. Finally, mutation of the cysteine at position 223, located in intracellular loop three, resulted in a receptor with normal ligand binding characteristics, but which did not transduce a calcium signal. Some additional amino acid substitutions in this region of the receptor (Cys-223 → Ala, Thr-221 → Met) resulted in receptors that had normal binding but transduced low amplitude calcium signals, while other mutations in the same region (His-224 → Arg and His-227 → Arg) exhibited normal binding and calcium signaling characteristics. These findings demonstrate that cysteines in extracellular loops one and two contribute to proper ligand binding to thromboxane receptors and show the importance of discrete amino acid sequences in the third intracellular loop, especially cysteine 223, in thromboxane receptor-effector coupling.

Physiologic consequences of thromboxane agonism such as platelet shape change/aggregation and vascular smooth muscle contraction are mediated via a high affinity interaction of thromboxane A₂ or prostaglandin H₂ with specific target cell membrane receptors (1, 2). Thromboxane receptor-mediated activation of phospholipase C hydrolyzes phosphatidylinositol bisphosphate and forms inositol 1,4,5-triphosphate, which stimulates release of calcium from intracellular stores (3, 4). Recently, a mutant human platelet thromboxane receptor with a single amino acid substitution in the first intracellular loop (Arg-60 → Leu) was identified as causing an inherited hemorrhagic disorder characterized by defective thromboxane receptor coupling to phospholipase C (5). The existence of this mutant thromboxane receptor and its associated disease illustrates the importance of proper thromboxane receptor function to normal hemostasis and emphasizes the need to identify structural determinants of thromboxane receptor ligand binding and receptor-effector coupling.

A comparison of the deduced amino acid sequences of various members of the superfamily of G-protein-coupled receptors reveals several highly conserved cysteine residues, suggesting that cysteines help to determine receptor function as related to receptor structure. Cysteines can modulate secondary and tertiary receptor structure due to their unique ability to form intramolecular disulfide bonds. The human platelet (K562) receptor contains eight cysteine residues (6), and identifying a role for these amino acids in thromboxane receptor ligand binding formed the rationale for a prior study that investigated the effects of disulfide and sulfhydryl reactive agents on thromboxane receptors (7). In that study the effects of thromboxane receptor binding on disulfide bond reduction with dithiothreitol, oxidation with dithionitrobenzoic acid, and alkylation with N-ethylmaleimide were assessed. Dithiothreitol treatment decreased ligand binding capacity, an effect that was reversed by dithionitrobenzoic acid, suggesting that (one or more) disulfide bonds were crucial for proper binding of thromboxane to its receptor. Alkylation of free sulfhydryl groups with N-ethylmaleimide also reduced [15-(1α,2β)-7-[3-(3-hydroxy-4-(p-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]heptane-2-yl]-5-heptenoic acid ([125I]BOP) binding capacity, further suggesting that a cysteine(s) was involved in facilitating binding of thromboxane to its receptor independent of disulfide bond formation.

When the deduced amino acid sequence of the human platelet thromboxane receptor is compared to that of related eicosanoid receptors, conserved cysteines are found to be present in the extracellular amino terminus, the first and second extra-

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The abbreviations and trivial names used are: [125I]BOP, [15-(1α,2β)-7-[3-(3-hydroxy-4-(p-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]heptane-2-yl]-5-heptenoic acid; U46619, 15S-hydroxy-11α,9α(epoxymethano)-prosta-5,13E-dienoic acid; SQ29,548, [15-(1α,2β)-7-[3-(3-hydroxy-4-(p-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]heptane-2-yl]-5-heptenoic acid; U46619, 15S-hydroxy-11α,9α(epoxymethano)-prosta-5,13E-dienoic acid; SQ29,548, [15-(1α,2β)-7-[3-(3-hydroxy-4-(p-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]heptane-2-yl]-5-heptenoic acid; U46619, 15S-hydroxy-11α,9α(epoxymethano)-prosta-5,13E-dienoic acid; SQ29,548, [15-(1α,2β)-7-[3-(3-hydroxy-4-(p-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]heptane-2-yl]-5-heptenoic acid; U46619, 15S-hydroxy-11α,9α(epoxymethano)-prosta-5,13E-dienoic acid; SQ29,548, [15-(1α,2β)-7-[3-(3-hydroxy-4-(p-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]heptane-2-yl]-5-heptenoic acid; U46619, 15S-hydroxy-11α,9α(epoxymethano)-prosta-5,13E-dienoic acid; SQ29,548, [15-(1α,2β)-7-[3-(3-hydroxy-4-(p-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]heptane-2-yl]-5-heptenoic acid; U46619, 15S-hydroxy-11α,9α(epoxymethano)-prosta-5,13E-dienoic acid; SQ29,548, [15-(1α,2β)-7-[3-(3-hydroxy-4-(p-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]heptane-2-yl]-5-heptenoic acid; U46619, 15S-hydroxy-11α,9α(epoxymethano)-prosta-5,13E-dienoic acid; SQ29,548.
cellular loops, and the third intracellular loop, supporting the notion that cysteines at these positions may be especially important in this receptor family. Therefore, we utilized site-directed mutagenesis and stable expression of thromboxane receptor mutants with cysteine amino acid substitutions to identify the roles of individual human platelet thromboxane receptor cysteines in receptor-ligand interactions and receptor coupling to cell signal effectors. Four of the seven thromboxane receptor cysteine mutations examined were dysfunctional; three demonstrated an impaired ability to bind ligand, and one exhibited normal ligand binding characteristics, but crippled cell signaling.

**EXPERIMENTAL PROCEDURES**

Materials—Tissue culture supplies (media, serum, trypsin, G418) and molecular biology reagents were purchased from Life Technologies, Inc. The Altered Sites in vitro mutagenesis system was purchased from Promega, Madison, WI. DNA sequencing was performed using Sequenase version 2.0 (U.S. Biochemical Corp.). All radiochemicals were purchased from DuPont NEN. HEK 293 cells were obtained from ATCC, Rockville, MD. Hybond N+ nylon membranes were purchased from Amersham Corp. The eukaryotic expression vector pcDNA3 was from Invitrogen, San Diego, CA. Fura-2 AM was purchased from Sigma, MO, and stored at -20°C. Mouse thromboxane receptor 

**Fig. 1. Position of mutated cysteines within overall molecular structure of the human thromboxane receptor.** Blocked area represents plasma membrane with seven putative membrane-spanning α-helices.

or replaced and maintained in Dulbecco's modified Eagle's medium, 10% donor calf serum supplemented with 0.3 mg/ml G418 to select for stable transfectants.

RNA Analysis—Total RNA was extracted from stably transfected HEK 293 cells using Chomczynski's method (10) and size-fractionated by electrophoresis in 1% agarose gels containing 3% formaldehyde before blotting onto Hybond N+ membranes by vacuum transfer. A thromboxane receptor polymerase chain reaction fragment (11) was 32P-labeled and hybridized to the blot overnight at 42°C in 50% formamide, 6 × SSPE (1× SSPE is 0.15 m NaCl, 0.015 m Na3 citrate, pH 7.0), 0.5% SDS, 100 μg/ml denatured herring sperm DNA. Membranes were washed at 60°C in 0.1 × SSC (1× SSC is 0.15 m NaCl, 0.01 m NaHPO4, 0.001 m EDTA, pH 7.4), 0.5% SDS for 1 h and exposed overnight on x-ray film at -70°C with intensifying screens.

**Binding of [125I]BOP to Thromboxane A₂ Receptors—**HEK 293 cells expressing wild type or mutant thromboxane receptors were resuspended in 25 μl HEPEs (pH 6.5), 125 μm NaCl, 0.9 mg/ml glucose for whole cell binding essentially as described previously (6). Briefly, 100,000 cpm [125I]-BOP (∼20 fmol) plus competing ligand were concomitantly incubated with 10^6 cells in a final volume of 0.2 ml for 30 min at 30°C. Bound ligand was separated from free by vacuum filtration over Whatman GF/A filters. Nondisplaceable binding was determined by addition of 10 μM SQ29,548 or 1 μM non-radioactive I-BOP and was 10–20% of total binding. HEK 293 cells transfected with pcDNA3 exhibited no significant non-displaceable [125I]BOP binding (6).

**Calcium Measurements—**Agonist-stimulated increases in intracellular free Ca²⁺ ([Ca²⁺]) were quantitated by monitoring fluorescence of fura-2-loaded cells as described previously (6, 12).

**Statistical Methods—**[125I]BOP binding competition experiments were computer-fitted to nonlinear models (LIGAND program, Ref. 13) for simultaneous analysis of multiple experiments using a Macintosh IIci. The following parameters were derived: Kd (dissociation constant), Bmax (maximal binding capacity), IC50 (concentration for 50% inhibition of binding), and nonspecific binding. In competition binding involving different analogs, IC50 values were generated from IC50 values using the Cheng-Prusoff equation (14). The specific activity of [125I]BOP was assumed to be 2,200 Ci/mmol. U46619 concentration-response curves in fura-2 studies were linearized by log-logit transformation to derive the concentration producing half-maximal response (EC50). Data are reported as mean ± S.E. unless otherwise stated. Multiple data sets were compared using one-way analysis of variance. Significant differences between individual means were determined using a two-tailed group comparison test. 

**RESULTS**

**Construction of Thromboxane Receptor Cysteine—**Serine Substitution Mutants—The human platelet thromboxane receptor has eight cysteines (Fig. 1): one located extracellularly in the amino terminus (Cys-11), one each in the first (Cys-35) and second (Cys-68) transmembrane domains, two in the first extracellular loop (Cys-102 and Cys-105), one in the second extracellular loop (Cys-183), one in the third intracellular loop (Cys-223), and one in the sixth transmembrane domain (Cys-257). Comparison of deduced amino acid sequences of the hu-
The effect of substituting serines for cysteines on thromboxane receptor-ligand interactions was assessed in equilibrium binding experiments using the thromboxane agonist [125I]BOP and competition with structurally dissimilar thromboxane agonists and antagonists (Table I and Fig. 3). Substitution of serine for cysteine at amino acid positions 11, 68, 223, and 257 resulted in receptors with agonist affinity and binding capacity similar to wild type thromboxane receptor, showing that cysteines at these positions do not determine agonist binding in thromboxane receptors. However, Cys-11→Ser and Cys-257→Ser exhibited small, yet statistically significant, decreases in affinity for the antagonist compound SQ29548. A similar result was observed for U46619 binding to Cys-232→Ser. Although the noted changes are small, they suggest the possibility that cysteines in these positions may in some small way contribute to thromboxane receptor binding.

Three of the mutant receptors (Cys-102, Cys-105, and Cys-183→Ser) were found to have greatly diminished or no capacity to bind [125I]BOP, although Northern blot analysis revealed that the mutant cell lines expressed full-length (1.6 kilobase pairs) receptor-specific transcripts (Fig. 4). Thus, diminished [125I]BOP binding to these cells was not due to failure to transcribe the transfected mutant thromboxane receptor cDNAs. Cells expressing Cys-105→Ser and Cys-183→Ser exhibited no specific binding (Table I). Cysteines at these respective positions are widely conserved throughout the superfamily of G-protein-coupled receptors and are felt to constitute a structurally necessary disulfide bond between extracellular loops one and two (15), and it appears that elimination of this disulfide bond, by removing either of the two involved cysteines, destroys thromboxane binding to its receptor.

The other mutant receptor which exhibited attenuated ligand binding was Cys-102→Ser. Although the receptor cDNA was transcribed at similar or higher levels than the other mutant receptors (Fig. 4), Scatchard analysis of [125I]BOP binding indicated that few receptors capable of binding ligand were expressed as the binding capacity was diminished by approximately 72% compared to wild type (Table I). To the extent that Cys-102→Ser did bind [125I]BOP, the affinities for I-BOP and SQ29548 were decreased by 2- and 5-fold, respectively. Together, these findings indicate that Cys-102 plays a role in both antagonist and agonist binding.

Analysis of Mutant Thromboxane Receptor Cell Signaling—In platelets and cultured platelet-like cells, thromboxane receptor activation results in phospholipase C-mediated increases in intracellular free calcium (3, 4). We have previously demonstrated similar increases in intracellular free calcium in HEK293 cells transiently transfected with platelet thromboxane receptors (6). Therefore, to determine whether the cysteine substitution mutant thromboxane receptors were appropriately coupled to calcium signal effectors, we measured intracellular free calcium transients in fura-2-loaded HEK293 cells stably expressing the wild type and mutant thromboxane receptors.

Cells expressing wild type platelet thromboxane receptors responded to (thromboxane mimetic) U46619 stimulation with a dose-dependent increase in intracellular free calcium having an EC50 value of 56 ± 7 nM (Fig. 5A, Table I). As expected, the two mutant receptors that failed to specifically bind [125I]BOP (Cys-105→Ser and Cys-183→Ser) also showed no calcium response to thromboxane stimulation (Table I). Cys-102→Ser, which had greatly reduced binding capacity, transduced only a small calcium signal at maximal concentrations of U46619 (10 ± 2% of wild type signal n = 3), which was not of sufficient amplitude for accurate determination of the concentration-response characteristics.

Thromboxane Receptor Cysteines

![Thromboxane Receptor Cysteines Diagram](http://www.jbc.org/content/images/throboxane-receptor-cysteines.png)
TABLE I

Results of radioligand binding and calcium signaling in HEK 293 cells expressing wild type and mutant thromboxane receptors

All binding values were calculated from competition binding experiments using nonlinear models in the LIGAND program (13). Binding data are presented as mean ± S.E. for six experiments per compound. Hill coefficients are for I-BOP binding. Calcium EC_{50} values were determined from log-logit transformation of concentration-response relationships (n = 5). *, p < 0.05 compared to wild type.

| Receptor   | B_{max} \times 10^6 sites/cell | K_d | nM | I-BOP | SQ29548 | U46619 | Hill coefficient | EC_{50} U46619 |
|------------|---------------------------------|-----|----|-------|---------|--------|-----------------|---------------|
| Wild type  | 241 ± 43                        | 4 ± 1|    | 28 ± 2| 109 ± 14| 95 ± 0.01| 56 ± 7          |               |
| Cys-11 → Ser | 214 ± 41                        | 4 ± 1| 48 ± 5* | 98 ± 24| 1.0 ± 0.01| 61 ± 19       |               |
| Cys-68 → Ser | 261 ± 20                        | 4 ± 1| 24 ± 3| 122 ± 17| 1.0 ± 0.01| 74 ± 14       |               |
| Cys-102 → Ser | 67 ± 9*                         | 10 ± 1*| 149 ± 37* | 191 ± 34| 0.98 ± 0.01| Small signal  |               |
| Cys-105 → Ser | No binding or calcium signaling | No binding or calcium signaling | 193 ± 10* | 193 ± 10* | 0.98 ± 0.02 | No signal     |               |
| Cys-183 → Ser | No binding or calcium signaling | No binding or calcium signaling | 193 ± 10* | 193 ± 10* | 0.98 ± 0.02 | No signal     |               |
| Cys-223 → Ser | 193 ± 41                        | 5 ± 1| 34 ± 8| 193 ± 10*| 0.98 ± 0.02| 35 ± 4        |               |
| Cys-257 → Ser | 118 ± 4                         | 6 ± 1| 95 ± 12* | 163 ± 32| 0.98 ± 0.01| 35 ± 4        |               |

Fig. 3. Displacement of [125I]BOP from wild type human thromboxane receptor cDNA-transfected HEK 293 cells. Compounds used are agonist [125I]BOP (squares), agonist SQ29548 (circles) and agonist U46619 (triangles). The data are presented as means ± standard deviation of six paired experiments per compound. Calculated values are in Table I.

Fig. 4. Northern analysis of wild type and mutant thromboxane receptor cDNA-transfected HEK 293 cells. Approximately 30 μg of total RNA from indicated mouse prostaglandin EP3 receptor (EP) or mutant thromboxane receptor expressing HEK293 cells was analyzed. Expression of 1.6 kilobase transcript was detected in all cell lines. The positions of 18 and 28 S ribosomal RNAs are indicated. Pictured below are the results of subsequent hybridization to β-actin.

Introduction of serine at position 223 in the third intracellular loop resulted in a receptor that did not transduce a U46619-induced calcium signal at concentrations of U46619 up to 5 μM (Fig. 5B and Table II), even though it had normal ligand binding characteristics (see above). This finding suggested that Cys-223 was critically important for proper thromboxane receptor coupling to G-protein effectors. To determine if defective signaling by the Cys-223 → Ser thromboxane receptor was specifically due to elimination of the cysteine at that position, or instead resulted from introduction of serine, we constructed a second mutation changing Cys-223 to alanine. When transiently expressed in HEK293 cells, Cys-223 → Ala had similar (125I)BOP binding characteristics to wild type and Cys-223 → Ser (Table II). In fura-2 studies, Cys-223 → Ala exhibited an attenuated U46619-induced calcium transient compared to cells transiently expressing wild type receptor (Table II). Together, the results with Cys-223 → Ser and Cys-223 → Ala indicate that a cysteine at amino acid position 223, while not absolutely critical for receptor-effector coupling, is necessary for normal efficiency of thromboxane receptor coupling to calcium signaling.

Functional Effects of Mutating Other Amino Acids in the Thromboxane Receptor Third Intracellular Loop—It is generally accepted that the third intracellular loop of seven transmembrane-spanning receptors is especially important in receptor coupling to G-protein effectors (16–18). Our studies indicated that Cys-223, in the thromboxane receptor third intracellular loop at a region close to the fifth transmembrane domain, played a role in receptor-effector coupling. To determine whether other amino acids in this region of the receptor were similarly important for proper receptor coupling to calcium signaling we constructed additional mutant thromboxane receptors having amino acid substitutions upstream and downstream of Cys-223. The residues examined were Thr-221 (mutated to arginine), His-224 (mutated to arginine), and His-227 (mutated to arginine) (Fig. 6). These mutant receptors were transiently expressed in HEK293 cells and assayed for ligand binding and receptor-mediated calcium signaling as described above. Similar to the Cys-223 → Ser and Cys-223 → Ala substitution mutations, each of these third intracellular loop thromboxane receptor mutations exhibited wild type (125I)BOP binding affinities (Table I). The Thr-221 → Met receptor, similar to the Cys-223 → Ser and Cys-223 → Ala mutants, exhibited an attenuated U46619-induced calcium transient (Table II). In contrast, neither of the histidine substitutions (His-224 → Arg and His-227 → Arg) showed altered U46619-stimulated calcium signaling (Table II). These data demonstrate the importance of specific amino acids at the amino terminus of intracellular loop three in coupling of human platelet thromboxane receptors to calcium signaling effectors.

DISCUSSION

In this study we have used substitution mutagenesis to define the roles for cysteine residues in human platelet thromboxane receptor function. Introduction of serine for cysteine in the amino terminus (Cys-11) or transmembrane domains two or six (Cys-68 and Cys-257) of the thromboxane receptor had no effect on agonist binding or cell signaling. In contrast, substi-
tution of serine for cysteines in the first (Cys-105) or the second (Cys-183) extracellular loops eliminated thromboxane receptor binding, and mutation of a second cysteine in extracellular loop one (Cys-102) resulted in a receptor with decreased affinity for both an agonist and an antagonist and low levels of agonist binding despite high level expression of mRNA transcripts. The most interesting result derived from analysis of a cysteine residue in the third intracellular loop (Cys-223), which, when changed to serine or alanine, resulted in a receptor with ligand binding properties similar to wild type, but with impaired calcium signaling. The importance of this region of the thromboxane receptor's third intracellular loop, and of Cys-223 in particular, was supported by mutational analysis of additional amino acids in the region of Cys-223, one of which (Thr-221 → Met) also resulted in a receptor with diminished cell signaling. We believe that these studies are the most comprehensive to date investigating the structure-function relationships for ligand binding and cell signaling of the human thromboxane receptor.

**Fig. 5.** A, representative analog tracing of U46619-induced increases in intracellular free calcium in wild type human thromboxane receptor cDNA-transfected HEK 293 cells. Each tracing represents a single experiment with addition of U46619 at arrow in concentrations indicated. Inset, concentration-response relationship to U46619-stimulated wild type thromboxane receptor calcium signaling. Each point is mean ± standard error for five separate experiments. EC₅₀ was 56 ± 7 nM with maximum U46619-stimulated increase in calcium of 108 ± 12 nM. B, comparison of U46619-induced increases in intracellular free calcium in wild type and Cys-223 → Ser mutant human thromboxane receptor expressing HEK 293 cells. Each tracing represents a single experiment with addition of 1 μM U46619 at arrow. No U46619-induced rise in intracellular free calcium is seen in HEK293 cells expressing Cys-223 → Ser mutant human thromboxane receptors.

**Table II**

Results of radioligand binding and calcium signaling in HEK cells expressing wild type and mutant thromboxane receptors

Data were calculated as described in Table I legend and are presented as mean ± difference for two binding or mean ± S.E. for four calcium experiments. Maximal calcium signal was measured with 1 μM U46619. Wild type base-line calcium was 100 ± 16 nM and maximum increase in intracellular calcium was 154 ± 21 nM. *, p < 0.05 compared to wild type.

| Receptor  | Bmax (× 10⁴ sites/cell) | Kd (nM) | Hill coefficient | Wild type calcium signal (%) |
|-----------|------------------------|---------|------------------|-------------------------------|
| Wild type | 140 ± 14               | 4 ± 1   | 0.90 ± 0.01      | 100                          |
| Thr-221 → Met | 166 ± 51         | 4 ± 1   | 0.97 ± 0.03      | 52 ± 5*                      |
| Cys-223 → Ser  | 259 ± 4             | 5 ± 1   | 0.98 ± 0.02      | No signal*                   |
| Cys-223 → Ala  | 184 ± 30            | 3 ± 1   | 0.97 ± 0.01      | 44 ± 4*                      |
| His-224 → Arg  | 947 ± 200           | 4 ± 1   | 1.02 ± 0.01      | 104 ± 4                      |
| His-227 → Arg  | 338 ± 62            | 4 ± 1   | 0.99 ± 0.02      | 63 ± 13                      |

**Fig. 6.** Amino acid sequence of transmembrane domain five, the third intracellular loop, and transmembrane domain six of the human platelet thromboxane receptor. Filled blocked area represents plasma membrane. Position of cysteine 223 is noted. Arrows indicate positions of other mutated amino acids (Table II).
A special role for cysteines in determining the functional characteristics of G-protein-coupled receptors was first suggested by chemical studies of β-adrenergic receptors, where disulfide reducing agents resulted in loss of ligand binding sites and reduction in binding affinity (15, 20). Similar results were later described for human platelet thromboxane receptors, where reduction with dithiobiotin or sulphydryl alkylation with N-ethylmaleimide resulted in reduced thromboxane receptor ligand binding, implying that cysteines could determine receptor binding characteristics through disulfide bridging as well as due to the contribution of one or more free sulphydryl groups (7). Therefore, we undertook the present studies to more clearly determine the effect on thromboxane receptor function of altering individual cysteines.

Replacement of cysteine by serine at positions 11, 68, and 257 did not affect the ability of thromboxane receptors to bind thromboxane agonist or to increase intracellular free calcium levels, suggesting that these amino acids do not play an important role in receptor-agonist or receptor-G-protein interactions (although Cys-11 → Ser and Cys-257 → Ser had minimally decreased affinity for the antagonist SQ29548 and may play minor role in antagonist binding). Furthermore, if any of these cysteines had been involved in formation of a disulfide bond, then substitution by serine should have disrupted that bond and impaired receptor function in some regard. It is not surprising that Cys-11, in the extracellular amino terminus of the receptor, does not contribute in a critical manner to thromboxane receptor binding or cell signaling. However, our results with Cys-68 and Cys-256 demonstrate the different functions that analogous cysteine residues can perform in thromboxane receptors compared to G-protein-coupled receptors (21, 22).

Cysteine 68 is located in the second transmembrane-spanning domain of the thromboxane receptor. While Cys-68 → Ser behaved normally in binding and calcium signaling studies, mutation of the analogous cysteine (amino acid position 69) in the M1 muscarinic acetylcholine receptor impaired agonist binding, but not antagonist binding (21). In contrast, mutation of the analogous cysteine (amino acid position 77) of the β2 adrenergic receptor, like the thromboxane receptor, did not alter receptor function in any measurable way (22). Thus, a sulphydryl group in the second transmembrane domain appears to contribute to normal ligand binding of muscarinic, but not adrenergic or thromboxane receptors.

The only other cysteines located within transmembrane domains of the thromboxane receptor are at positions 34 and 257. Despite multiple attempts at mutagenesis of Cys-34 using two different oligonucleotides, this mutant could not be made. In one study of human platelet thromboxane receptors, although mRNA transcript levels were as high or higher than in the other mutant receptor expressing cell lines. The most likely explanation for this observation is that these two cysteines form an intramolecular disulfide bond that is necessary for proper receptor folding, expression in the membrane, ligand binding, or all three. Although it was not possible to measure and localize receptor protein in our studies, and therefore definitively state that the mutant receptor proteins were expressed in levels comparable to the observed levels of mRNA, there is ample evidence supporting the notion that cysteines in these positions are necessary for normal receptor function. A cysteine in the first and second extracellular loops appears to be a common structural feature of nearly all members of the G-protein-coupled receptor family. In the β-adrenergic receptor, mutagenic substitution of the analogous cysteines (amino acid positions 106 and 184) has previously been shown to affect agonist, but not antagonist binding (23), while mutation of the analogous cysteines (amino acid positions 98 and 178) in the M1 muscarinic receptor, like the thromboxane receptor, resulted in a receptor with no binding or signaling (21). In the case of the thromboxane receptor, it has been demonstrated that the oxidation/reduction state of the receptor can affect radioligand binding in a manner similar to that observed in the present study when we mutated these two cysteines (7). Of particular interest are the recent observations that naturally occurring mutations which disrupt disulfide bond formation of rhodopsin and V2 vasopressin receptors cause human diseases characterized biochemically by improperly folded or non-functional receptors (24–26). Taken together, these studies clearly implicate disulfide bonds between cysteines as being critical for normal G-protein-coupled receptor expression and function. It is interesting to note that Cys-102, only three amino acids away from Cys-105, does not appear from our results to be capable of substituting for Cys-105 and forming a disulfide bond with Cys-184: at least not a disulfide bond that results in a functional thromboxane receptor. This indicates that only a single form of disulfide bond between the first and second extracellular loops is adequate for normal thromboxane receptor expression and function.

The other thromboxane receptor mutant that exhibited altered ligand binding was Cys-102 → Ser. In this case, receptor affinity for thromboxane agonist and antagonist was decreased. Additionally, the receptor agonist binding capacity was much lower than one would expect of thromboxane receptors expressed at a level corresponding to the quantity of mRNA transcript we observed by Northern analysis. This result is similar to the previous observation that alkylation of platelet thromboxane receptors with N-ethylmaleimide diminished receptor ligand binding capacity (7). Together, these observations strongly suggest that Cys-102 exists as a free sulfhydryl that contributes to thromboxane receptor ligand binding. A cysteine at this relative position is not conserved in the G-protein-coupled receptor superfamily, nor is it present in the closely related receptors for prostaglandins E2, F2α, or I2, indicating that the requirement for a free sulfhydryl is specific to the thromboxane receptor. The first extracellular loop where Cys-102 is located has previously been implicated as being important in ligand binding to this receptor (27, 28). Halushka’s group used N-bromosuccinimide or diethylpyrocarbonate to biochemically modify histidine residues in the thromboxane receptor and found altered receptor binding affinity. The plate-
let thromboxane receptor contains four histidine residues, two of which are in the first extracellular loop (amino acid positions 89 and 96), while the other two are in the third intracellular loop (amino acid positions 224 and 227). In the present study, the third loop histidines were mutated to arginine with no alteration of I-BOP binding, suggesting that the effects of N-bromosuccinimide and diethlypyrocarbonate are due to modification of histidines in the first extracellular loop. These results, together with our own, indicate that Cys-102 and surrounding amino acids in the first extracellular loop of the platelet thromboxane receptor play critical, but as yet unspecified roles in ligand binding.

Mutation of Cys-223 in the third intracellular loop between transmembrane domains five and six completely abolished thromboxane mimetic-stimulated calcium signaling without appreciably altering the ligand binding properties of the receptor. The third intracellular loop has previously been implicated in coupling of adrenergic receptors to G-proteins (29), and studies using chimeric β2-adrenergic/α2-adrenergic or β2-adrenergic/M1 muscarinic receptors have indicated that specificity of G-protein coupling to these receptors is determined by amino acids at the amino-terminal portion of intracellular loop three (16, 30). Platelet thromboxane receptors are coupled to calcium signaling and phospholipase C via interactions with G-proteins of the Gq and G11 families (31–34). Studies of other phospholipase C-coupled receptors, although relatively few in number, indicate that the third intracellular loop is also important in G-protein coupling to these receptors. Site-directed mutagenesis of amino acids in intracellular loop three of the angiotensin II type 1A receptor has shown that this region is required for proper coupling to phospholipase C (18). This notion is further supported by demonstration that synthetic peptides corresponding to the amino half of angiotensin II 1A receptor intracellular loop three can mimic the effects of agonist-occupied receptor in GTP•S binding assays (35).

It was unexpected that mutation of Cys-223 to Ser would result in a receptor essentially devoid of calcium signaling activity. Although serine was chosen to substitute for cysteine in our mutagenesis protocols because the hydroxyl of serine and the sulfhydryl of cysteine resemble each other in mass and charge, we wished to test the possibility that insertion of the hydroxyl, rather than removal of the sulfhydryl, accounted for the dramatic loss of receptor-effector coupling. Therefore, we constructed a Cys-223 → Ala mutant receptor. This mutant, like Cys-223 → Ser, had normal ligand binding affinity. Interestingly, however, its ability to transduce a calcium signal was neither as efficient as wild type nor as impaired as Cys-223 → Ser, but was intermediate between the two. This indicates that Cys-223 is important for normal thromboxane receptor coupling to G-protein effectors, but that substitution with a serine at this position has additional deleterious consequences on receptor-effector coupling.

To further explore the roles of individual amino acids in the amino terminus of the thromboxane receptor third intracellular loop, we constructed an additional three substitution mutants, each of which altered a different amino acid near Cys-223. Mutation of an amino acid closer to the fifth transmembrane-spanning segment (Thr-221 → Met), like the Cys-223 → Ala mutation, resulted in a receptor with normal ligand binding properties, but cell signaling properties intermediate between wild type and Cys-223 → Ser. Interestingly, substitution of arginine for His-224 or His-227 generated a receptor that was not significantly different from wild type binding or signal transduction. Thus, specific amino acids in the portion of the third intracellular loop closest to transmembrane-spanning domain five appear to play roles in maintaining normal thromboxane receptor coupling to G-protein effectors.

The exact molecular structure responsible for the third intracellular loop involvement in receptor G-protein interaction is not well understood. Site-directed mutagenesis studies of the hamster β-adrenergic receptor have identified hydrophobic, but not hydrophilic, residues at the amino end of intracellular loop three, which are critical for G-protein coupling (36). Structural analysis indicates this region may form an α-helix with hydrophobic residues along one side, similar to the G-protein activating wap venom peptide mastoparan (37). Interestingly, we have mutated amphipathic (Cys and Thr) residues resulting in impaired signal transduction. However, a conformational preference analysis of the amino acids in the thromboxane receptor third intracellular loop reveals a higher preference for the carboxyl segment of the loop to form an α-helix compared to the region near the fifth transmembrane domain (38).

The first intracellular loop of the thromboxane receptor has also been implicated in receptor-G-protein coupling. A natural mutation has been identified, which changes Arg-60 in the first intracellular loop to leucine resulting in a hemorrhagic disorder (5). Similar to our Cys-223 → Ser mutant, the Arg-60 → Leu mutation resulted in a receptor unable to activate phospholipase C. These studies together show that multiple receptor determinants are necessary for proper thromboxane receptor coupling to effectors.

A second human thromboxane receptor cDNA has recently been cloned from endothelial cells (39). This form of the thromboxane receptor arises from alternative splicing of the thromboxane receptor mRNA and results in a receptor protein identical to the platelet receptor except for an alternate carboxyl tail. The carboxyl tail for the endothelial receptor is 47 amino acids longer than the platelet receptor and contains three cysteines. The role of these cysteines has not been tested, but one may act as a site for palmitoylation forming a forth intracellular loop. In α2a-adrenergic receptors the presence of a palmitoylated cysteine in the carboxyl tail is critical for normal agonist-induced down-regulation (40).

In summary, we have determined the roles of individual cysteine residues in the human platelet thromboxane receptor that help to determine normal receptor-ligand and receptor-effector interactions. We have identified cysteines in the first and second extracellular loops, which, through formation of disulfide bonds between Cys-105 and Cys-184 and via a free sulfhydryl on Cys-102, play roles in agonist and antagonist binding. We have also shown that amino acids, including Cys-223, at the amino end of the third intracellular loop are important for coupling to calcium signaling. These studies represent an initial step toward a full understanding of how thromboxane receptor structure determines its ligand binding and cell signaling characteristics.

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