Mapping of a Ligand-binding Site for the Human Thromboxane A₂ Receptor Protein*

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The human thromboxane A₂ (TP) receptor, a member of the G protein-coupled receptor superfamily, consists of seven transmembrane segments. Attempts to elucidate the specific segment(s) that define the receptor ligand-binding pocket have produced less than definitive and sometimes conflicting results. On this basis, the present work identified an amino acid sequence of the TP receptor that is directly involved in ligand binding. Mapping of this domain was confirmed by two separate approaches: photoaffinity labeling and site-specific antibodies. The newly synthesized, biotinylated photoaffinity probe, SQBAzide, was first shown to specifically label TP receptor protein. Sequential digestion of this protein with CNBr/trypsin revealed photolabeling of a 2.9-kDa peptide. Using anti-peptide antibodies directed against different regions of the receptor protein, it was established that this peptide represents the predicted cleavage product for CNBr/trypsin and corresponds to amino acids Arg¹⁷⁴-Met²⁰⁵ of the receptor protein. Furthermore, antibody screening revealed that inhibition of the amino acid region Cys¹⁸³-Asp¹⁹³ was critical for radioligand binding and platelet aggregation, whereas inhibition of Gly¹⁷²-Cys¹⁸³ was not. Collectively these findings provide evidence that ligands interact with amino acids contained within the C-terminal portion of the third extracellular domain (ED3) of the receptor protein. This information should be of significant value in the study of TP receptor structure and signaling.

Clinical evidence suggests that inhibition of platelet thromboxane A₂ (TXA₂)₁ production provides a therapeutic basis for the treatment and/or prevention of certain thrombosis states (1–8). Indeed the ability of aspirin to inhibit TXA₂ synthesis is the primary rationale for the widespread use of this agent in recurrent myocardial infarction and more recently in thromboembolic stroke (9, 10). However, despite the clear importance of TXA₂ in these disease processes, the molecular interaction of TXA₂ with its receptor protein remains unknown.

To date, the information available concerning the TP receptor ligand-binding domain(s) has been mostly limited to mutational analyses using receptor chimeras or expressed receptor protein containing site-specific mutations as well as ligand interactions with modified receptor peptides or molecular modeling. Collectively results from these studies have implicated transmembrane domains I, III, IV, V, VI, and VII as well as extracellular domains II and III as potential regions for ligand coordination sites (11–18). Since all of the cited receptor regions would not be expected to participate in or form the ligand-binding domain, it would seem that the interpretation of some of these results may be limited by the potential for gross alterations in receptor tertiary structure. Based on this consideration, the present study used two different approaches to identify critical ligand coordination sites in the TP receptor protein. The first of these approaches utilized a novel and recently characterized bifunctional TP receptor antagonist, SQBAzide, to irreversibly label and track ligand-binding sites; the second approach utilized site-specific antibodies to probe different regions of the TP receptor protein. Our results indicate that the C-terminal portion of ED3 may form a critical ligand-binding domain for the TP receptor protein.

EXPERIMENTAL PROCEDURES

Materials—CHAPS, thrombin, A23187, rabbit preimmune IgG, streptavidin, fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin, trypsin, and CNBr were purchased from Sigma. U46619 and SQ29,548 were obtained from Cayman Chemicals (Ann Arbor, MI). [³H]SQ29,548 and Na⁺/K⁺-ATPase were purchased from PerkinElmer Life Sciences. The continuous elution electrophoresis apparatus and Whole Gel Eluter were provided by Bio-Rad Laboratories. Outdated human platelet pheresis units (5–10 days postdraw) were acquired from Heartland Blood Services (Aurora, IL). Site-specific peptides were synthesized and purified to >95% purity by Multiple Peptide Systems (San Diego, CA). Dynex Technologies (Chantilly, VA) produced the Immulon 2HB microtiter plates used in enzyme-linked immunosorbent assays (ELISAs). Flow cytometric experiments were conducted using a Becton Dickinson FACStar analyzer and Calibrite beads (San Jose, CA).

Site-directed Antibody Production—Sequence-specific antibodies were raised by previously described procedures (19) against the following receptor peptide segments: ED2 (ED2Ab: His¹⁹⁶-Val²⁰⁵), the N-terminal segment of ED3 (ED3Ab: Gly¹⁷²-Cys¹⁸³), the C-terminal segment of ED3 (ED3Ab: Cys¹⁸³-Asp¹⁹³), and the N-terminal segment of ED4 (ED4Ab: Thr²⁰⁶-Met²⁰⁷) (Fig. 1A). Each of these antibodies was reactive against their cognate peptide (shown by ELISA; data not shown). In separate studies, immunoblots against solubilized platelet membranes revealed immunoreactivity at 55 kDa that was blocked by preincubation of the antibodies with their cognate peptides (100 µM;
Fig. 1. TP receptor protein recognition by site-specific antibodies. A, location of peptides used for site-specific antibody production within the putative structure of the TP receptor. The model depicts TP receptor topology based upon hydrophobicity plots and indicates the positions of peptides used to raise antisera (filled circles). The dark filled circle represents an overlapping peptide sequence. B, immunoblot of solubilized platelet membranes probed with various site-specific antibodies in the presence (+) or absence (−) of cognate peptides (100 μM) and detected by enhanced chemiluminescence. PI represents premunne control. C, reactivities of anti-ED2 (■), anti-ED3a (□), anti-ED3b (▲), and anti-ED4 (●) IgG against solubilized platelet membranes as detected by ELISAs. Each point represents the mean ± S.E. of triplicate values from three separate experiments in which preimmune base-line values have been subtracted. D, flow cytometric analysis of site-specific antibody binding to intact platelets for ED2Ab (I), ED3aAb (II), ED3bAb (III), and ED4Ab (IV) as compared with preimmune control. Values obtained represent the mean fluorescence intensity for 10,000 events.

Additional experiments demonstrated that the affinity-purified antibodies also recognized solubilized TP receptors coated to ELISA plates (Fig. 1C) as well as TP receptors present on intact platelets (determined by flow cytometry; Fig. 1D). Thus, these antibodies are capable of interacting with their peptide targets in the denatured receptor conformation, in the native receptor conformation, and in the membrane-imbedded conformation.

ELISA—ELISAs were performed to test peptide purified antibody reactivity against conjugate peptide as well as native receptor protein. Immulon 2HB microtiter plates were coated with either 12.5 μg/well synthetic peptide or 125 μg/well solubilized platelet membranes for 18–24 h at room temperature. Following the incubation, the plates were washed three times with 200 μl/well modified Tyrode’s buffer (0.1% bovine serum albumin, 5 mM dextrose, 1 mM CaCl₂, 5 mM HEPES, pH 7.4), and then nonspecific sites were blocked by incubation for 1 h with 5% bovine serum albumin (200 μl/well) in the same buffer. Plates were again washed three times with the modified Tyrode’s buffer prior to applying serial dilutions of various peptide purified site-specific antibodies to the wells in triplicate. Antibodies were allowed to incubate for 1 h followed by three more washes. Antibodies bound to the immobilized peptide or protein were detected by incubation (1 h) with goat anti-rabbit IgG (heavy + light) conjugated to horseradish peroxidase. The wells were then washed a final time before the addition of the horseradish peroxidase substrate solution (50 μl of 0.4 mg/ml o-phenylenediamine, 0.012% H₂O₂ in 80 mM citrate phosphate, pH 5.0). After a 10 min incubation in the dark, the reaction was quenched with 2 × H₂SO₄ (200 μl/well). The presence of specific antibodies was measured by the absorbance at 490 nm.

Flow Cytometry—Resuspended platelets (20) at a concentration of 1 × 10⁶ platelets/ml were incubated with various peptide purified site-specific antibodies (1:100 (v/v)) for 1 h. Samples were washed twice prior to the addition of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (1:80 (v/v)). Samples were incubated for an additional hour prior to analysis. A Becton Dickinson FACStar analyzer was used to perform single-color analysis on the samples. The fluorescence channel was calibrated with 2-μm Calibrite beads and adjusted to reflect logarithmic output. A lower limit threshold was set for data acquisition thereby eliminating background scatter.

Photoaffinity Labeling—CHAPS-solubilized platelet membranes were incubated with SQBAzide (2 μM) for 15 min in the dark, and the samples were then subjected to photolysis with ultraviolet light for 1 min at a distance of 5 cm using a 100-watt Olympus mercury lamp. Photolysis was terminated by the addition of dithiothreitol (4 mM) to the samples followed by a 10× concentration of the product by centrifugal filtration through 0.1-μm low binding Durapore membranes (Millipore, Bedford, MA). Proteins within the concentrated samples were separated by SDS-PAGE, and the entire gel was transferred to a polyvinylidene difluoride membrane. The receptor protein was then blocked for 1 h with 5% nonfat dried milk and probed for irreversibly bound SQBAzide by incubating the membrane for 2 h with 5% nonfat dried milk containing 37.5 nM 125I-streptavidin (which binds the biotin functional group of SQBAzide). After washing with 0.1% Tween in 5% bovine serum albumin, 5 mM dextrose, 1 mM CaCl₂, 5 mM HEPES, pH 7.4, and then nonspecific sites were blocked by incubation for 1 h with 5% bovine serum albumin (200 μl/well) in the same buffer. Plates were again washed three times with the modified Tyrode’s buffer prior to applying serial dilutions of various peptide purified site-specific antibodies to the wells in triplicate. Antibodies were allowed to incubate for 1 h followed by three more washes. Antibodies bound to the immobilized peptide or protein were detected by incubation (1 h) with goat anti-rabbit IgG (heavy + light) conjugated to horseradish peroxidase. The wells were then washed a final time before the addition of the horseradish peroxidase substrate solution (50 μl of 0.4 mg/ml o-phenylenediamine, 0.012% H₂O₂ in 80 mM citrate phosphate, pH 5.0). After a 10 min incubation in the dark, the reaction was quenched with 2 × H₂SO₄ (200 μl/well). The presence of specific antibodies was measured by the absorbance at 490 nm.

Chemical digestion of TP receptors was performed with CNBr according to the method described by Gross (21). In this procedure, receptor protein was suspended in 70% formic acid resulting with digestion carried out at room temperature while tilting the solution for 24–36 h in the dark. Digestion was terminated by diluting the sample solution to 7% formic acid with deionized water immediately followed by lyophilization.

SDS-Continuous Elution Electrophoresis (CEE)—Receptor proteins...
were partially purified by the technique of SDS-CEE as described previously (22). In this procedure, using a continuous elution electrophoresis apparatus, a typical SDS-polyacrylamide gel was cast in a cylindrical electrophoresis tube bathed in a tank of running buffer. Solubilized platelet membranes in sample buffer were then applied to the top of the tube gel. The apparatus was operated at 2 watts constant power until the dye front migrated through the tube gel. At this time, an outlet connection at the bottom of the tube gel was connected to a peristaltic pump (rate = 0.1 ml/min), and 1-mL fractions containing proteins of decreasing electrophoretic mobilities were isolated over a 16-h period. A modification of this technique was used to fractionate receptor protein fragments following chemical digestion (23). To isolate these low molecular weight proteins, 16.5% Tricine gels were cast in the tube gel system using buffers as first described by Schagger and von Jagow (24). Protein digests were then electrophoresed at 4 watts and fractionated as described above.

**Immunoprecipitation**—Immunoprecipitation was performed with either ED3aAb or rabbit preimmune IgG. Specifically 400 µl of antibody (1 mg/ml) was incubated with 400 µl of receptor digestion fragments overnight followed by addition of protein A-Sepharose beads (200 µl) for 4 h at 4°C. Samples were washed three times with 400 µl of 10 mM CHAPS in phosphate-buffered saline. After the final wash, the supernatant was removed and the remaining beads were boiled for 5 min and then microcentrifuged for 2 min. The supernatant served as the immunoprecipitated product.

**Tryptic Digestion**—Protein fragments were dialyzed and lyophilized to dryness prior to resuspension in 100 mM ammonium bicarbonate, pH 8.0. The samples were subsequently incubated with trypsin at a 1:50 dilution of enzyme:substrate for 4 h at 37°C.

**Competition Binding**—CHAPS-solubilized platelet membranes (100 µl; 2 mg/ml protein) were incubated with [3H]SQ29,548 (2 nM) for total binding samples. [3H]SQ29,548 (2 nM) plus unlabeled SQ29,548 (2 µM) were co-incubated in the nonspecific binding samples, and [3H]SQ29,548 (2 nM) plus various amounts of site-specific antibodies or rabbit preimmune IgG (1 nM–1 µM) were co-incubated in the competition binding samples. After a 30-min incubation at room temperature, the protein samples were immobilized on Whatman GF/B glass fiber filters (presoaked in 0.3% polyethyleneimine for 1 h) by vacuum filtration and immediately washed twice with 5 ml of buffer (25 mM Tris base, 5 mM MgCl2, pH 7.4; 4°C). The filters were assayed for radioactivity by liquid scintillation spectroscopy using a Beckman LS 6800.

**Aggregation in Resuspended Platelets**—Resuspended platelets used in the aggregation studies were prepared from platelet-rich plasma obtained from the University of Illinois Hospital Blood Bank (Chicago, IL) according to the method of Kattelman (20). Briefly platelet-rich plasma was treated with aspirin (3 mM) to inhibit endogenous platelet activity by liquid scintillation spectroscopy using a Beckman LS 6800.

**RESULTS**

SQBAzide (25) represents a unique probe that combines a photolabile azide moiety with a biotin functional group (Fig. 2A). To assess the ability of SQBAzide to specifically and irreversibly label TP receptor protein, competition photoaffinity labeling studies were first performed. Labeling of solubilized platelet membranes with SQBAzide followed by SDS-PAGE and autoradiography with 125I-streptavidin yielded a major labeled fragment that corresponds to one of these predicted fragments, i.e. an 8.1-kDa peptide (Fig. 2C and Table I). The presence of this 8.1-kDa fragment suggests that photolabeling of the receptor protein occurs within a peptide sequence spanning amino acids Ala127 through Met202, which represents a region between ID2 and the leading edge of transmembrane domain V (TM5).

The sequence identity of this labeled 8.1-kDa fragment was next investigated by studies using a new library of rabbit antibodies (see “Experimental Procedures”) directed against different peptide regions of the TP receptor protein. It can be seen that when this putative amino acid sequence (Ala127–Met202) was probed with antibodies against ED2 (ED2Ab: His199–Val198), ED3 (ED3Ab: Gly172–Cys183; ED3bAb: Cys182–Asp193), and ED4 (ED4Ab: Thr206–Met276) no immunoreactivity was observed in the region of ED2 or ED4 (Fig. 3A). On the other hand, positive immunoreactivity was observed for both ED3aAb and ED3bAb (Fig. 3A), which targets the sequence between Gly172 and Asp193 comprising ED3. These findings therefore indicate that the 8.1-kDa photolabeled fragment contains an amino acid sequence (Gly172–Asp193) known to be present in ED3 and that the presence of this sequence in the CNBr digest is consistent with the known CNBr cleavage sites.

The sequence identity of the 8.1-kDa labeled fragment was further confirmed by immunoprecipitation studies. Specifically
samples containing the 8.1 ± 1-kDa CNBr digest were pooled, concentrated, and immunoprecipitated with ED3aAb, which recognizes the N-terminal half of ED3 containing amino acids Gly\(^{172}\)–Cys\(^{183}\). The immunoprecipitated protein was then probed by dot blot analysis using \(^{125}\)I-streptavidin for assessment of SQBAzide photolabeling. It can be seen (Fig. 3B) that relative to preimmune IgG, ED3aAb immunoprecipitated the SQBAzide-photolabeled 8.1-kDa CNBr fragment. Thus, the predicted chemical CNBr cleavage sequence for the 8.1-kDa fragment (Ala\(^{172}\)–Met\(^{202}\)) was confirmed by immunoblotting, and photolabeling of this predicted sequence was confirmed by immunoprecipitation.

However, while the above studies establish that the Gly\(^{172}\)–Cys\(^{183}\) sequence constitutes a portion of the 8.1-kDa labeled fragment, they do not determine whether photolabeling occurs within the Gly\(^{172}\)–Cys\(^{183}\) sequence itself. This possibility was investigated by a series of experiments in which the concentrated 8.1-kDa sample was subjected to further digestion with trypsin. Specifically the concentrated 8.1-kDa CNBr digest was divided into two samples. One sample was treated with trypsin vehicle, and the other sample was digested with trypsin as described under “Experimental Procedures.” Each sample was then subjected to SDS-CEE. To determine the efficiency of trypsic digestion, fragments in each sample migrating to 8.1 ± 1 kDa were collected, pooled, and concentrated. Furthermore, since trypsic digestion of the CNBr 8.1-kDa fragment is predicted to yield a 2.9-kDa peptide (Arg\(^{174}\)–Met\(^{202}\)) containing ED3 (previously shown to be immunoreactive against ED3aAb and ED3bAb; Fig. 3A), fractions migrating to 2.9 ± 1 kDa were also collected, pooled, and concentrated. The SDS-CEE fractions corresponding to the following molecular masses were collected: the 8.1-kDa fraction (no trypsin), the 2.9-kDa fraction (no trypsin), the 8.1-kDa fraction (plus trypsin), and the 2.9-kDa fraction (plus trypsin). These fractions were then immunoprecipitated with ED3aAb. The immunoprecipitated protein was probed by dot blot analysis using \(^{125}\)I-streptavidin for assessment of SQBAzide photolabeling and normalized for non-specific immunoprecipitation by using preimmune serum.

Table II illustrates that the efficiency of trypsin digestion was ~60%, i.e., the counts in the 8.1-kDa fragment decreased from 16,758 to 6,480 cpm. It can also be seen that following trypsin digestion the bulk of the counts shifted to the 2.9-kDa fragment. More importantly, however, the decrease in counts in the 8.1-kDa fragment can be completely accounted for by the increase in counts in the 2.9-kDa fragment. This finding establishes that the labeling of the 8.1-kDa fragment is exclusively localized in the 2.9-kDa subfragment. Furthermore, the ability of ED3aAb to immunoprecipitate this 2.9-kDa fragment is consistent with the known cleavage sites for both CNBr and trypsin and provides evidence that SQBAzide photolabels the amino acid sequence Arg\(^{174}\)–Met\(^{202}\) of the TP receptor protein.

Subsequent competition radioligand binding studies confirmed this notion and identified a subregion of the Arg\(^{174}\)–Met\(^{202}\) sequence that is required for ligand interaction. Specifically affinity-purified antibodies against ED2 (ED2Ab), ED3 (ED3aAb and ED3bAb), and ED4 (ED4Ab) were tested for their ability to compete with binding of the TP receptor antagonist \(^{3}H\)SQ29,548 to solubilized TP receptors. It was found (Fig. 4, A and B) that throughout the concentration range of 10\(^{-9}\)–10\(^{-6}\) M, ED2Ab (His\(^{89}\)–Val\(^{98}\)) or ED4Ab (The\(^{268}\)–Met\(^{276}\)) had no measurable effect on \(^{3}H\)SQ29,548 binding. This finding may be expected since neither antibody targets the photolabeled sequence (Arg\(^{174}\)–Met\(^{202}\)). Furthermore, when the N-terminal portion of this sequence was probed with ED3aAb (Gly\(^{172}\)–Cys\(^{183}\)), again no effect on ligand binding was observed (Fig. 4C). This lack of inhibition cannot be due to an inability of ED3aAb to interact with membrane-associated TP receptors since this antibody was shown by flow cytometry to react with intact platelet TP receptors (Fig. 1D). Thus, the N-terminal portion of ED3 does not appear to be critical for ligand interaction. In contrast, the same concentration range of ED3bAb, which targets the C-terminal portion of ED3 (Cys\(^{183}\)–Asp\(^{193}\)),

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**Table I**

| Sequence | Molecular mass (kDa) | Receptor location |
|----------|----------------------|-------------------|
| Trp\(^{2}\)–Met\(^{106}\) | 27.4 (glycosylated) | N terminus, TM1, ID1, TM2, ED2, part of TM3 |
| His\(^{192}\)–Met\(^{126}\) | 1.4 | Most of TM3, part of ID2 |
| Ala\(^{127}\)–Met\(^{202}\) | 8.1 | Most of ID2, TM4, ED3, part of TM5 |
| Leu\(^{203}\)–Met\(^{243}\) | 4.4 | Most of TM5, most of ID3 |
| Ala\(^{245}\)–Met\(^{282}\) | 0.7 | Part of ID3, part of TM6 |
| Val\(^{251}\)–Met\(^{276}\) | 2.7 | Most of TM6, part of ED4 |
| Ser\(^{277}\)–Met\(^{369}\) | 10.5 | Most of ED4, TM7, C terminus |

**Notes:**

* Receptor localization of CNBr-digested fragments based upon sequence hydrophobicity analysis of the putative seven transmembrane-spanning receptor, TP receptor.

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**Fig. 3.** Receptor localization of a photolabeled, CNBr-generated fragment of the TP receptor. A, immunoblot of the photolabeled and purified 8.1-kDa CNBr digestion product of the TP receptor with site-specific antibodies directed against the TP receptor extracellular domains. B, immunoprecipitation of photolabeled TP receptor digestion products by the anti-peptide antibody ED3aAb. The 8.1 ± 1-kDa CNBr-digested, photolabeled TP receptor fragment was isolated by Tricine SDS-CEE and concentrated. Equal amounts of the sample were subjected to immunoprecipitation with ED3aAb or rabbit preimmune IgG. The immunoprecipitate from each sample was then transferred onto polyvinylidene difluoride membranes under vacuum filtration and dot-blotted with \(^{125}\)I-streptavidin. Dots were punched out uniformly and counted on a γ counter. PI, preimmune IgG.
TABLE II  
Immunoprecipitation of photolabeled fragments from CNBr/trypsin digestion  

| CNBr | CNBr + trypsin |
|------|---------------|
| 8 kDa | 3 kDa | 8 kDa |
| 16,758 | 6,480 | 11,359 |

*ND, not detectable.

FIG. 4. Competition binding curves for site-specific antibody IgGs to CHAPS-solubilized platelet TP receptors. Various amounts of the site-specific antibodies (A, ED2Ab; B, ED4Ab; C, ED3Ab; D, ED3bAb) or rabbit preimmune IgG (1 nM–1 μM) were assessed for their ability to displace [3H]SQ29,548 binding to platelet membrane TP receptors. Each point represents the mean ± S.E. of triplicate values obtained from three separate platelet preparations in which preimmune baseline values have been subtracted.

produced a dose-dependent inhibition of binding with the highest concentration resulting in an 80% reduction of binding activity (Fig 4D). Furthermore, evidence that this inhibition was sequence-specific was provided in additional experiments in which ED3bAb was preabsorbed with its cognate peptide. Under these conditions, the ability of the antibody to inhibit binding was reversed (data not shown). Based on these considerations, it appears that the C-terminal (Cys183–Asp193) and not the N-terminal portion (Gly172–Cys183) of ED3 is important for ligand coordination.

Since the above results demonstrate that ED3bAb blocks TP receptor ligand binding, it might be expected that this antibody should also affect TP receptor-mediated platelet aggregation. This was indeed found to be the case. In these experiments, resuspended platelets were treated with ED3bAb or preimmune IgG prior to addition of the TXA2 mimetic U46619 (200 nM) at 24 °C for 30 min prior to the addition of the TP receptor agonist U46619 (200 nM) (A), thrombin (1 unit/ml) (B), or the divalent cation ionophore A23187 (2 μM) (C). Each aggregation curve is representative of multiple traces obtained from three separate blood donors.

had no effect on aggregation induced by either thrombin or the divalent cation ionophore A23187 (Fig. 5, B and C). These results indicate that antibody interaction with the C-terminal segment (Cys183–Asp193) of ED3 specifically blocks platelet aggregation mediated through the TP receptor pathway. ED3bAb therefore represents the first functional antibody against the TP receptor protein. Additional studies with ED2Ab and ED3aAb revealed no effect on U46619-induced aggregation (Fig. 6). However, ED4Ab was found to produce limited inhibition. Since this antibody had no effect on radioligand binding (Fig. 4B), the nature of the inhibition is unclear. Finally, none of these antibodies had any effect on thrombin- or A23187-induced aggregation (data not shown).

DISCUSSION

The present experiments combined two separate approaches to map a ligand-binding site in human TP receptors. The biotinylated photoaffinity probe SQBAzide was shown to specifically label TP receptor protein, and digestion of this protein with CNBr revealed that the bulk of the photolabeling appeared in an 8.1-kDa fragment predicted to represent the receptor segment between Ala127–Met202. The identity of this predicted sequence and the location of labeling within this sequence was next determined by the application of site-specific antibodies directed against different regions of the receptor protein. Consistent with the predicted cleavage sites for CNBr, it was found that the labeled 8.1-kDa CNBr fragment was not recognized by antibodies against sequences in ED2 (His189–Val199) or ED4 (Thr266–Met276) but could be immunoblotted and immunoprecipitated by antibodies raised against contiguous sequences in ED3, i.e. Gly172–Cys183 and Cys183–Asp193. Furthermore, the digestion of this CNBr fragment with trypsin yielded a labeled 2.9-kDa fragment with a predicted sequence of Arg174–Met202. This predicted sequence was in turn confirmed by immunoprecipitation studies using an antibody (ED3aAb) that recognizes the Gly172–Cys183 sequence contained within ED3. Subsequent studies used different antibodies to probe the subregion of the 2.9-kDa fragment that is involved in ligand binding. It was found that only ED3bAb effectively blocked [3H]SQ29,548 binding, suggesting that the sequence Cys183–Asp193 may contain a critical ligand coordination site. The inability of the other antibodies to interfere with binding cannot be due to slight differences in affinity for TP receptors (Fig. 1D) since the radioligand binding studies were conducted over a 3 log unit antibody concentration range.

The importance of the region containing the Cys183–Asp193 sequence was confirmed in platelet aggregation studies that revealed that ED3bAb specifically blocked U46619-induced aggregation, while antibodies against other receptor regions (ED2Ab and ED3aAb) were without effect. On the other hand, these studies also showed that ED4Ab produced modest inhibition of U46619-induced aggregation (Fig. 6). However, since...
brane involvement. In this regard, experiments performed on membrane sequence homology with rhodopsin. As a result, the not only sharing structural similarity but also notable trans-

the G protein-coupled receptors identified a family of proteins in the receptor protein (26). The subsequent cloning of several of protein-coupled receptor homolog, rhodopsin, that revealed a receptor and other G protein-coupled receptors has centered on work done to elucidate a ligand-binding domain on the TP receptor. In addition, Chiang et al. (15) reported that mutations of S201A and S255A at TM5 and TM6, respectively, caused altered affinity to the agonist I-BOP but had no effect on binding SQ29,548. Collectively these latter results indicate that alterations in either the TM5, TM6, or TM7 region can diminish ligand binding to TP receptors. Separate studies by Dorn et al. (17) used receptor chimeras to evaluate ligand binding activity. We concluded that residues in TM1 constitute an important portion of the TP receptor binding site. Finally, reports from two different groups suggested that the putative disulfide bond between Cys105 and Cys183/184 in ED2 and ED3, respectively, plays a critical role in receptor-ligand binding. In particular, mutants C105A and C183A from the human placenta TP receptor (15) and mutants C105S and C184S from human K562 TP receptors (16) did not show binding activity of either agonists or antagonists. In addition, both groups reported that Cys102, which is conserved in most seven transmembrane-spanning receptors including the TP receptor (but absent in other prostanoid receptors), also plays an important, yet unspecified role in ligand binding.

Thus, the previous results point to multiple sites in the TP receptor that may seem critical for ligand binding, i.e. trans-

membrane regions I, III, IV, V, VI, and VII as well as cysteine residues in ED2 and ED3. However, since alterations in either hydrophobic segments or the elimination of disulfide bonds can lead to significant effects on protein structure, at least some of these receptor alterations may not be at the critical ligand coordination site(s). Based on this consideration, the existence of a definitive transmembrane ligand-binding domain for the TP receptor has yet to be established.

Regarding other possible ligand-binding sites, evidence also exists to support the notion that ligands can coordinate with extracellular domains. Specifically our previous results using the biotinylated TP receptor antagonist SQB (29) demonstrated that SQB can simultaneously bind to intact platelet TP receptors and the 68-kDa protein avidin. On the other hand, molecular dynamic simulations revealed that the SQB extended conformation is only 20.5 Å, and the relaxed conformation is considerably less. On this basis, we proposed that the ligand coordination site(s) for the TP receptor must reside at or near the external aspect of the plasma membrane. Furthermore, work done on the bradykinin B2 receptor using site-specific antibodies has identified the N-terminal portion of ED3 as crucial to both ligand binding and agonist function (30). More recently a constrained peptide containing amino acids representing ED3 of the TP receptor was shown to change conformation in response to the TP receptor antagonist SQ29,548 (18). These results in combination with the present data suggest that extracellular regions of seven transmembrane receptors may represent important ligand coordination sites. For the TP receptor we have provided evidence that one of these extracellular regions may reside in the C-terminal portion of ED3 that contains amino acids Cys183–Asp184. On the other hand, this finding does not exclude the possibility that other amino acid sequences in the receptor may also participate in ligand coordination. Nevertheless it is believed that identification of this specific amino acid region will serve as an important reference point for future modeling studies describing the three-dimensional structure of the TP receptor binding pocket. This information should, in turn, facilitate our understanding of the molecular mechanism by which TXA2 binds to this biologically important receptor protein.

Fig. 6. Ability of various site-specific antibodies to inhibit TP receptor-mediated platelet aggregation. U46619-induced aggregation was measured as described in the legend for Fig. 5. Bars represent the percent control aggregation ± S.E. obtained from three separate experiments. *, p < 0.05 (ED4Ab versus control); **, p < 0.01 (ED3ab versus control).

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