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ANTI-PEPTIDE ANTIBODIES AGAINST THE HUMAN BLOOD PLATELET THROMBOXANE A_2/PROSTAGLANDIN H_2 RECEPTOR

PRODUCTION, PURIFICATION AND CHARACTERIZATION*

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Abstract—Two anti-peptide antibodies have been raised against the human blood platelet thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) receptor. Based on the published sequence of the placental TXA_2/PGH_2 receptor, two decapeptide segments were selected as potential antigens: one in the first extracellular loop corresponding to residue 89 through 98, and the other in the C-terminal region of the intracellular domain corresponding to residue 314 through 323. Rabbits were immunized with each peptide, and the antisera were subjected to a two-step purification procedure. The IgG fraction was purified using a DEAE Affi-Gel Blue column, and the peptide-specific IgG was further purified by affinity chromatography employing each peptide as the immobilized ligand. The combined purification factor for both procedures was approximately 60-fold. By ELISA, both antibodies displayed immunoreactivity toward their synthetic antigens, solubilized platelet membranes and affinity-purified TXA_2/PGH_2 receptor protein. Furthermore, Western blot analysis revealed that: (1) each antibody reacted with the purified platelet TXA_2/PGH_2 receptor protein (55 kDa); and (2) each antibody recognized a single band (55 kDa) in solubilized platelet membranes. These findings establish antibody specificity for the human platelet TXA_2/PGH_2 receptor protein. Functional analysis demonstrated that neither antibody interfered with ADP- or U46619-induced platelet aggregation or [^{3}H]SQ29,548 binding to the solubilized receptor. These results suggest that the antibody epitopes are separate from the TXA_2/PGH_2 binding domain. In summary, two specific anti-peptide antibodies have been raised against the human platelet TXA_2/PGH_2 receptor. These antibodies should prove to be of value in the further investigation of the platelet TXA_2/PGH_2 receptor.

Synthetic peptides which correspond to certain regions of a protein are routinely utilized to elicit antiserum that reacts with the native protein [1, 2]. One significant advantage of such anti-peptide antibodies is that specific amino acid sequences within a protein can be selectively targeted for antibody production. These antibodies so produced can, in turn, be employed as specific probes to study protein structure and function, as well as to resolve subtle differences between subpopulations within a family of proteins [3–7]. In addition, anti-peptide antibodies have been useful in a number of other applications including studies investigating the expression of specific amino acid sequences in a mature protein as deduced by its nucleic acid coding sequence in cDNA clones [8], defining cellular protein location [9], defining functional receptor binding domains [10], identifying individual protein domains after proteolytic cleavage [11–13], and immunopurifying specific proteins in large quantities [14, 15]. Thus, anti-peptide antibodies have proven to be of significant value in the characterization and isolation of numerous biologically important proteins.

Although the human platelet thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2§) receptor is intimately involved in the pathogenesis of various forms of cardiovascular disease [16, 17], specific antibodies directed against this receptor protein have been previously unavailable. Nevertheless, substantial progress has been made in TXA_2/PGH_2 receptor characterization through the utilization of synthetic receptor agonists, e.g. 15 (*S*-hydroxy-11,9-epoxymethano-prosta-5*Z*, 13*E*-dienoic acid (U46619) [18, 19], U44069 [18, 20] and I-BOP [21] or antagonists, e.g. 13-APA [22], BM13.177 [23], I-PTA-OH [24], [1*S*-[1α,2β(5*Z*),3β,4α]]-7-[3-

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§ Abbreviations: BSA, bovine serum albumin; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; MBS, *meta*-maleimidobenzoic acid *N*-hydroxysuccinimide ester; PEI, polyethyleneimine; PGH_2, prostaglandin H_2; PRP, platelet-rich plasma; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SQ29,548, [1*S*-[1α,2β(5*Z*),3β,4α]]-7-[3-[[2[-(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; TFA, trifluoroacetic acid; TXA_2, thromboxane A_2; U46619, 15(*S*)-hydroxy-11,9-epoxymethano-prosta-5*Z*,13*E*-dienoic acid; FITC, fluorescein isothiocyanate.

[[2[- (phenylamino)carbonyl]hydrazino]methyl] - 7 - oxabicyclo[2.2.1]hept - 2 - yl] - 5 - heptenoic acid (SQ29,548) [25] and S-145 [26]. Indeed, this receptor was purified recently to homogeneity by two separate affinity ligands [27, 28], and cDNA for the human placental receptor was cloned, sequenced and expressed in cos-7 cells [29]. These results, in turn, revealed the primary structure of the protein that predicts the organization and the topography of the receptor [29].

In the present experiments we have extended this work by raising anti-peptide antibodies against the human platelet TXA_2/PGH_2 receptor. Based on the deduced protein sequence of the placental receptor protein [29], rabbits were immunized to generate polyclonal antibodies against two decapeptide segments of the receptor: one in the first extracellular loop, and the other in the C-terminal region of the intracellular domain. The antibodies were subsequently purified by peptide affinity chromatography and shown to be highly specific for the platelet TXA_2/PGH_2 receptor, as well as for separate binding sites on the receptor protein itself. Moreover, neither antibody interacted with the agonist or antagonist binding domains, nor did they interfere with TXA_2/PGH_2-mediated platelet aggregation. Based on these conditions, these antibodies should therefore prove to be of significant value as novel and alternative molecular probes for the identification, localization, purification and characterization of TXA_2/PGH_2 receptors in platelets and other tissues.

MATERIALS AND METHODS

Materials. Outdated platelet concentrates were obtained from Life Source Blood Services, Chicago, IL. The keyhole limpet hemocyanin (KLH), L-α-lysophosphatidylcholine, *meta*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS), complete and incomplete Freund's adjuvant, *o*-phenylenediamine and U46619 were purchased from the Sigma Chemical Co., St. Louis, MO. DEAE Affi-Gel Blue, Affi-Gel 501, goat anti-rabbit IgG (H + L) conjugated to horseradish peroxidase (HRP), and 4-chloro-1-naphthol (HRP color development reagent) were obtained from Bio-Rad Laboratories, Richmond, CA. Immulon 2 microtiter plates were from Dynatech Laboratories, Inc., Chantilly, VA. Biotinylated goat anti-rabbit IgG (H + L) and the Vectastain ABC kit were purchased from Vector Laboratories, Burlingame, CA. SQ29,548 was provided by Squibb Institute for Medical Research, Princeton, NJ. [^{3}H]U46619 and [^{3}H]SQ29,548 were obtained from Dupont, Boston, MA.

Peptide synthesis and purification. Peptides P_1 (CAVLRRLQPRL) and P_2 (CHAALFEWHAV), designated by the single-letter amino acid code corresponding to their sequences, were prepared by solid phase synthesis using an ABI model 431 peptide synthesizer and Fastoc programs. A cysteine residue was introduced to the N-terminal positions to facilitate peptide coupling to the KLH carrier protein and to Affi-Gel 501. Additionally, since both peptide sequences represent interior regions of the receptor protein, they were synthesized as the peptide amide in order to mimic the charge environment of the native protein.

The crude lyophilized peptides were dissolved in 0.1% trifluoroacetic acid (TFA) and 1% β-mercaptoethanol, and were then purified by preparative HPLC using a C-8 aquapore column eluted with a linear gradient of 0.1% TFA and 63% acetonitrile. The purity of each peptide was determined by analytical HPLC to be >95%. Mass spectrometry confirmed that the molecular masses of the peptides were equal to their theoretical values.

Coupling of peptides to KLH carrier protein. Each peptide was conjugated to KLH using MBS [30, 31]. Briefly, KLH (1 mg/mL) was first dialyzed against 10 mM potassium phosphate, pH 7.2, and then incubated with MBS for 30 min at room temperature (the KLH:MBS ratio was 1:40, and the final pH of the mixture was 6.6). The activated KLH was then desalted using a sephadex G-50 column equilibrated with 50 mM sodium phosphate, pH 6.0. Finally, the activated KLH was added to the peptide solution (0.8 mg KLH/mg of peptide) after adjusting the pH to 7.4, the solution was stirred for 3 hr at room temperature. The coupling efficiency of this reaction was determined by sized exclusion HPLC to be approximately 60–80%.

Immunization. White New Zealand Pasteurella multocida-free rabbits were used for immunization, and a pre-immune bleeding was performed to establish control antibody titers. Equal volumes of the peptide–KLH conjugate solutions and complete Freund's adjuvant were emulsified, and 1 mL of the mixture containing approximately 100 μg of peptide was injected intradermally into each anesthetized (Ketamine: 20 mg/kg) animal. The boosts were administered in incomplete Freund's adjuvant with the first boost given after 3 weeks and successive boosts at 5-week intervals thereafter. Blood (45 mL) was collected from the ear veins 2 weeks after each boost and allowed to clot. The serum was separated by centrifugation (10,000 *g*, 10 min), incubated at 60° for 1 hr to inactivate the complement, and stored at −20° in the presence of 0.02% NaN_3.

Purification of IgG by DEAE chromatography. A DEAE Affi-Gel Blue column was used to separate the IgG fraction of the rabbit serum. Specifically, the serum was first dialyzed overnight against 0.02 M Tris–HCl, 0.028 M NaCl, 0.02% NaN_3, pH 8.0, and then loaded onto the DEAE Affi-Gel Blue column. Unbound IgG was eluted with the above buffer and concentrated by ultrafiltration. The protein concentration of the eluate was determined by UV absorbance at 280 nm.

Antibody purification by affinity chromatography. The affinity column matrix was prepared by covalently coupling Affi-Gel 501 organomercurial agarose, which readily forms covalent mercaptide bonds with free sulfhydryl groups, to the N-terminal cysteine of each peptide. Briefly, 1 mL of gel was mixed with 10 mg of each peptide for 2 hr. After washing excess unbound peptide with 0.1 M sodium acetate (pH 4.0) and 0.1 M sodium bicarbonate (pH 8.1), the column was equilibrated with PBS (0.039 M NaH_2PO_4, 0.061 M Na_2HPO_4, 0.14 M NaCl, 0.02% NaN_3, pH 7.4).

The IgG fraction was loaded onto the column and

incubated overnight at 4°. The column was then washed extensively with PBS until no protein appeared in the eluate. The affinity-purified IgGs were eluted from the column with 0.1 M glycine–HCl, pH 2.5, neutralized immediately with 0.1 vol. of 1 M Tris–HCl buffer, pH 8.0, and dialyzed against phosphate-buffered saline. The protein concentration was measured by UV absorbance at 280 nm. At each purification step, P_1Ab, P_2Ab binding activity was evaluated by ELISA using each peptide to coat microtiter wells.

ELISA. Immulon 2 microtiter plates were coated with either synthetic peptides, solubilized platelet membranes, or purified human platelet TXA_2/PGH_2 receptor. Solubilized platelet membranes and affinity-purified TXA_2/PGH_2 receptor were prepared as previously described [28]. Following incubation for 1 hr at room temperature, the plates were washed three times with a modified Tyrode's buffer containing 0.1% bovine serum albumin (BSA), 5 mM dextrose, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.4, and then blocked by incubation for 1 hr with 5% BSA dissolved in the same buffer. Serial doubled dilutions of antisera were applied to the wells and incubated for an additional 1 hr at room temperature. The wells were washed three times with the modified Tyrode's buffer, and bound antibodies were detected by incubation for 1 hr with a goat anti-rabbit IgG (H + L) conjugated to HRP. After extensive washing, the color reaction was developed by the addition of 50 μL of 0.4 mg/mL *o*-phenylenediamine, 0.012% H_2O_2 in 80 mM citrate phosphate, pH 5. An equal volume of 2 N H_2SO_4 was then added, and the presence of peptide-specific antibodies was measured by absorbance at 490 nm.

Immunoblot assay. Solubilized platelet membranes and purified TXA_2/PGH_2 receptor were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 8.5% polyacrylamide gels as previously described [32]. The proteins were electrophoretically transferred onto nitrocellulose membranes according to the method of Towbin *et al.* [33]. After transfer, the nitrocellulose membranes were blocked with 3% gelatin in 30 mM Tris, 120 mM NaCl, pH 7.4 (TBS), and then incubated for 1 hr at room temperature with the indicated primary antibodies. Bound antibody was detected by the Vectastain method using biotinylated goat anti-rabbit IgG (H + L) as the secondary antibody. Color bands were developed by subsequent additions of a mixture of avidin and biotinylated HRP followed by 0.5 mg/mL of 4-chloro-1-naphthol.

Measurement of platelet aggregation. Platelet-rich plasma (PRP) was isolated from CPD (citrate-phosphate-dextrose)-anticoagulated human blood [34], and washed platelets were prepared as previously described [19]. Aggregation was stimulated with submaximal concentrations (approximately 75% aggregation) of U46619 or ADP in either PRP or washed platelets, and aggregation was measured by the turbidimetric method [35] using a model 400 Lumi-aggregometer (Chronolog Corp., Havertown, PA). The effect of purified antibodies on platelet aggregation was determined by incubating the antibodies with isolated PRP for 5 min prior to the addition of agonists.

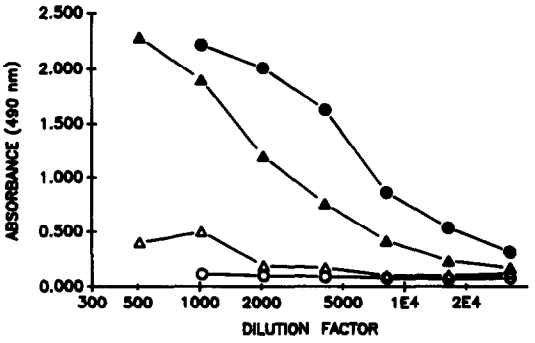


Fig. 1. Reactivities of anti-P_1 and anti-P_2 sera against the synthetic immunogens. Microtiter wells were coated with 12.5 μg of either P_1 or P_2 peptide. Various dilutions of anti-P_1 serum (●), anti-P_2 serum (▲), pre-immune P_1 serum (○) or pre-immune P_2 serum (△) were added to the wells. Immuno-reactivities were detected by ELISA, as described in Materials and Methods. Each point is the mean of triplicate values obtained from two separate experiments. Standard error of the mean was typically less than 10%. Serum dilutions ranged from 500- to 30,000-fold; E4 in the abscissa equals 10,000-fold.

Preparation of permeabilized platelets. Washed platelets were permeabilized with L-α-lysophosphatidylcholine, and the degree of antibody permeabilization was determined by measuring platelet uptake of fluorescein isothiocyanate (FITC)-conjugated, goat anti-rabbit IgG (H + L). Specifically, washed platelets were incubated for 15 min with a 1:100 dilution of FITC-conjugated, goat anti-rabbit IgG (H + L) in the presence or absence of L-α-lysophosphatidylcholine (5 $\mu g/10^8$ platelets final concentration). The platelets were then pelleted through silicone oil [36], and cell-associated fluorescence was determined by microspectrofluorometry as previously described [36], at excitation and emission wavelengths of 487 and 531 nm, respectively.

In the aggregation studies, permeabilized platelets were incubated with the highest concentration (50 μg/mL) of pre-immune IgG that did not produce non-specific inhibition of aggregation. This same concentration (50 μg/mL) of P_1Ab and P_2Ab was then evaluated for inhibition of U46619-induced aggregation.

Competitive binding assays. The binding of [^{3}H]-U46619 to washed platelets was performed as previously described [19] with minor modifications. Specifically, the platelet suspension was preincubated with the peptide-specific IgG (50 μg/mL) for 5 min before adding [^{3}H]U46619 (10 nM). The platelet suspension was then filtered through Whatman GF/C filters, followed by washing (2 × 5 mL) with the modified Tyrode's buffer. Specific binding (70%) was defined as the decrease in binding activity observed upon simultaneous addition of a 10 μM concentration of unlabeled U46619.

Evaluation of [^{3}H]SQ29,548 binding to solubilized platelet TXA_2/PGH_2 receptors was performed by filtration through polyethyleneimine (PEI)-treated

Table 1. Evaluation of the cross-reactivity between P_1Ab and P_2Ab by ELISA

	Absorbance at 490 nm	
	Wells coated	
Wells incubated	P_1	P_2
P_1Ab (1/2000)	1.14	0.07
P_2Ab (1/2000)	0.09	1.00

Data are means of two values.

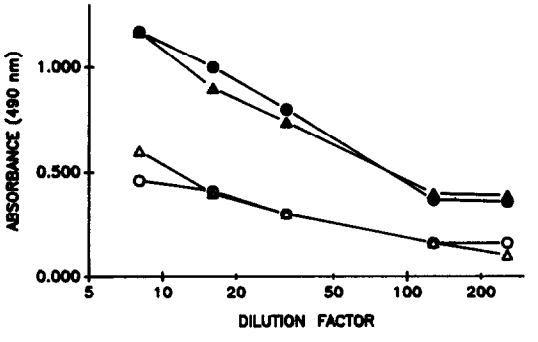


Fig. 2. Reactivities of anti-P_1 and anti-P_2 sera against solubilized platelet membranes. Microtiter wells were coated with solubilized platelet membranes (125 μg protein/well). Various dilutions of anti-P_1 serum (●), anti-P_2 serum (▲), pre-immune P_1 serum (○) or pre-immune P_2 serum (△) were added to the wells. Immuno-reactivities were detected by ELISA, as described in Materials and Methods. Each point is the mean of values obtained from three separate experiments. Standard error of the mean was typically less than 10%.

glass fiber filters. Briefly, solubilized platelet membranes were incubated for 20 min with [^{3}H]-SQ29,548 (3 nM) and peptide specific IgG (150 μg/mL). At the end of the incubation period, the mixture was filtered under vacuum through GF/B filters that had been presoaked in 0.3% PEI [37]. The filters were washed (2 × 5 mL) with 25 mM Tris, 5 mM $MgCl_2$, pH 7.4, at 4°, and the [^{3}H]SQ29,548-receptor complex retained on the filters was measured by liquid scintillation spectrometry. Specific binding (85%) was defined in the presence of a 1 μM concentration of unlabeled SQ29,548.

RESULTS

Each serum was first evaluated by ELISA for immunoreactivity toward the synthetic peptides P_1 and P_2. It can be seen (Fig. 1) that the serum from the P_1- and P_2-immunized rabbits specifically reacted, in a concentration-dependent manner, with their respective peptides. On the other hand, there was no reactivity observed between either peptide and the pre-immune sera. It was also found by ELISA (Table 1) that there was no cross-reactivity between anti-P_1 serum and P_2, or between anti-P_2 serum and P_1 at a serum dilution of 1/2000. These findings therefore indicate that specific antibodies (P_1Ab and P_2Ab) against each peptide had been successfully raised.

Figure 2 illustrates the immunoreactivity profile of P_1- and P_2-anti-sera (at various dilutions) against solubilized platelet membranes. As can be seen, a positive reaction was observed for both sera, indicating the presence of the P_1 and P_2 peptide sequences in solubilized platelet membranes. Since P_1 and P_2 represent segments of the placental TXA_2/PGH_2 receptor, experiments were performed to determine whether the platelet TXA_2/PGH_2 receptor was reactive with the antibodies against P_1 and P_2.

The IgG fractions of the P_1- and P_2-sera were first purified 6-fold by DEAE chromatography and subjected to further characterization. Specifically, the platelet TXA_2/PGH_2 receptor was partially purified by affinity chromatography as previously described [28] and evaluated by ELISA for immunoreactivity against the IgG fractions. Figure 3 demonstrates that both anti-peptide antibodies interacted with the purified receptor preparation within an IgG dilution range of 1/20 to 1/130. These findings are consistent with the notion that P_1Ab and P_2Ab interact with peptide sequences in the TXA_2/PGH_2 receptor protein.

Based on this consideration, the IgG fractions of P_1Ab and P_2Ab were further purified an additional 10-fold by affinity chromatography utilizing P_1 and P_2 as the immobilized ligands. The combination of both DEAE and affinity chromatography therefore yielded a 60-fold purification relative to the activity found in serum.

To further establish P_1Ab and P_2Ab interaction with the TXA_2/PGH_2 receptor, immunoblotting procedures were employed. Specifically, the TXA_2/PGH_2 receptor was first purified to homogeneity [28] and transferred onto nitrocellulose membranes. It can be seen (Fig. 4; lanes 1 and 3) that both P_1Ab

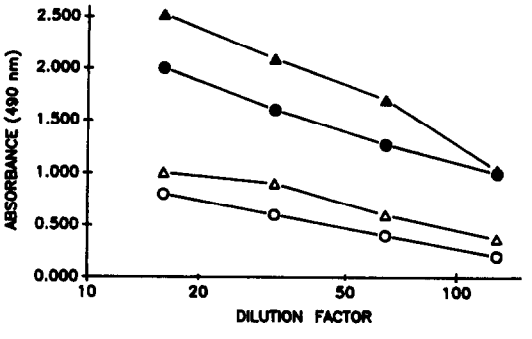


Fig. 3. Reactivities of P_1Ab or P_2Ab IgG fractions against the affinity-purified TXA_2/PGH_2 receptor. Affinity-purified proteins (500 ng) were coated onto microtiter wells. IgGs from P_1Ab or P_1 pre-immune serum (● and ○), and IgGs from P_2Ab or P_2 pre-immune serum (▲ and △) were added at the indicated dilutions. Data are means of triplicate values from one experiment.

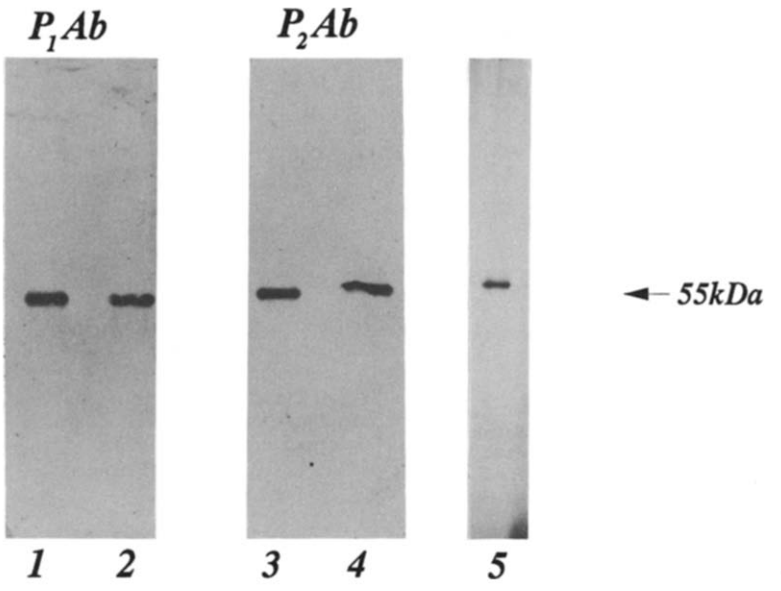


Fig. 4. Immunoblot of purified TXA_2/PGH_2 receptor or solubilized platelet membranes by affinity-purified P_1Ab or P_2Ab (30 μg/mL). Proteins were resolved by SDS–PAGE on 8.5% polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with the indicated antibodies. Lanes 2 and 4 represent solubilized platelet membranes (200 μg protein/lane); lanes 1 and 3 represent purified receptor protein (0.5 μg/lane); and lane 5 represents the silver-stained, purified TXA_2/PGH_2 receptor.

and P_2Ab reacted with the purified receptor protein. Thus, the immunoblotted bands possessed the same electrophoretic mobility (55 kDa) as silver-stained receptor (lane 5). Furthermore, the specificity of P_1Ab and P_2Ab for the TXA_2/PGH_2 receptor (relative to other platelet membrane proteins) was demonstrated by the finding that immunoblotting against solubilized platelet membranes revealed a single band at 55 kDa (Fig. 4; lanes 2 and 4). These results therefore demonstrate that immunization with P_1 and P_2 resulted in the production of specific anti-peptide antibodies against the human platelet TXA_2/PGH_2 receptor protein.

The next experiments were directed at characterizing the functional activity of P_1Ab and P_2Ab. It was found that the purified antibodies neither stimulated platelet aggregation nor inhibited aggregation induced by U46619 or ADP in PRP (Fig. 5) or washed platelets (Fig. 6). The slightly reduced aggregation observed in the presence of P_1Ab or P_2Ab was indistinguishable from that caused by the addition of IgG from pre-immunized rabbits, and consequently was not P_1Ab or P_2Ab specific. Preincubation of PRP with P_1Ab and P_2Ab also did not inhibit platelet shape change induced by U46619 (data not shown).

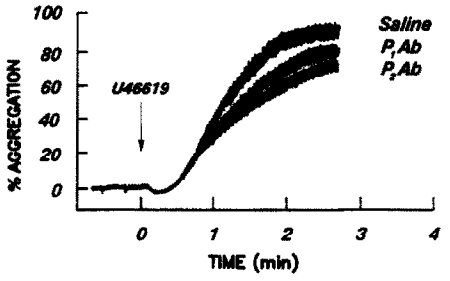
Since the P_1Ab epitope is presumably on the intracellular domain of the receptor, separate experiments measuring the effects of P_1Ab (and P_2Ab) on aggregation in permeabilized platelets were also performed. Platelet permeabilization was established by measuring the uptake of FITC-labeled IgG. Specifically, platelets were incubated with FITC-IgG in the presence or absence of L-lysophosphatidylcholine. The platelets were then centrifuged and their fluorescence was determined. It was found that permeabilization resulted in a 75% increase in platelet-associated fluorescence relative to control, i.e. 34670 ± 3390 cps, N = 3, versus 19890 ± 490 cps, N = 3.

Figure 7 illustrates that L-α-lysophosphatidylcholine treatment alone neither stimulated nor inhibited platelet aggregation. Furthermore, incubation of permeabilized platelets with P_1Ab or P_2Ab (or the combination of both) did not inhibit aggregation induced by U46619.

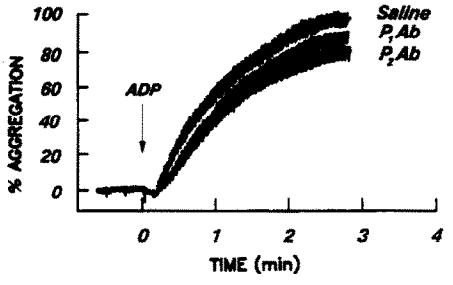
A similar lack of functional activity was observed for agonist or antagonist TXA_2/PGH_2 receptor binding. Thus, preincubation with P_1Ab or P_2Ab did not alter the specific binding of [^{3}H]U46619 (Table 2) to intact platelets or the specific binding of [^{3}H]-SQ29,548 to solubilized platelet membranes.

DISCUSSION

Although previous studies have suggested the existence of different TXA_2/PGH_2 receptor subtypes in platelets and the vasculature, Hirata *et al.* [29] have demonstrated that 43% of the primary structure of the human placental TXA_2/PGH_2 receptor (from residue 194 to the C-terminus) is identical with the sequence of a partial clone from the megakaryocyte TXA_2/PGH_2 receptor (the other 57% of the megakaryocyte sequence is unknown). Based on this consideration, computer analysis was conducted on the published placental TXA_2/PGH_2 receptor sequence to determine the most antigenic peptide fragment for the generation of antibodies against



A



B

Fig. 5. Effects of affinity-purified P_1Ab or P_2Ab on U46619- or ADP-induced platelet aggregation. Platelet aggregation was determined as described in Materials and Methods: U46619 (0.6 μM, panel A); ADP (2 μM, panel B). Antibodies (150 μg/mL) were incubated with PRP for 5 min prior to agonist additions. The aggregation traces are representative of three separate experiments.

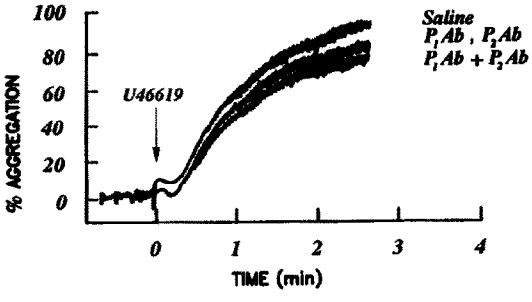


Fig. 6. Effects of affinity-purified P_1Ab, P_2Ab on U46619-induced aggregation in washed platelets. The antibodies (100 μg/mL) were preincubated 5–10 min before the addition of U46619 (180 nM). The aggregation traces are representative of three separate experiments.

the platelet TXA_2/PGH_2 receptor protein [38]. Empirical predictions of antigenicity resulted in the selection of two sequences for the production of antibodies, i.e. a decapeptide sequence

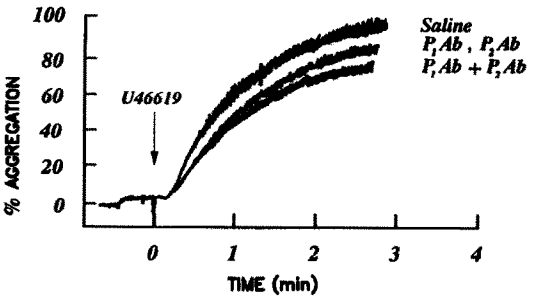


Fig. 7. Effect of affinity-purified P_1Ab, P_2Ab on U46619-induced aggregation in permeabilized platelets. The antibodies (50 μg/mL) were preincubated with L-α-lysophosphatidylcholine (5 $\mu g/10^8$ platelets) for 15 min before the addition of fibrinogen (100 μg/mL platelets) and a submaximal concentration of U46619 (0.5 μM). The aggregation traces are representative of three separate experiments.

Table 2. Effects of P_1Ab and P_2Ab on [^{3}H]U46619 and [^{3}H]SQ29,548 specific binding

	[^{3}H]U46619 binding in whole platelets (dpm/10^8 platelets)	[^{3}H]SQ29,548 binding in solubilized platelet membranes (dpm/50 μg protein)
Control	220 ± 20	1160 ± 25
P_1Ab	250 ± 25	1060 ± 150
P_2Ab	230 ± 5	1000 ± 105

Values are means ± SEM of triplicate values from two separate experiments.

(AVLRRLQPRL) corresponding to residues 314 through 323 in the published sequence, and a decapeptide sequence (HAALFEWHAV) corresponding to residues 89 through 98. Based on the putative transmembrane assignment of the receptor protein [29], AVLRRLQPRL resides in the intracellular domain twenty residues from the C-terminus, and HAALFEWHAV resides in the first extracellular loop. A second computer analysis [39] was next conducted to compare the sequence homology of the two decapeptides with the published sequences for other platelet proteins. This analysis revealed no significant homology between these two peptides and published protein sequences. Consequently, P_1 (CAVLRRLQPRL) and P_2 (CHAALFEWHAV) were selected for antibody production.

Rabbits were immunized with each peptide by standard procedures, and anti-P_1/P_2 sera were evaluated by ELISA for reactivity against the synthetic antigens. It was found that both sera reacted with the immobilized peptides, and exhibited no measurable cross-reactivity between P_1 and P_2. A similar positive reaction was observed by ELISA for each serum against solubilized platelet membranes. These results therefore demonstrated

that specific anti-peptide antibodies (P_1Ab and P_2Ab) had been successfully raised against the synthetic antigens, and that these antibodies reacted with one or more platelet membrane proteins.

To investigate P_1Ab and P_2Ab interaction with the human platelet TXA_2/PGH_2 receptor, the IgG fractions of each serum were prepared and evaluated against partially purified receptor protein. Specifically, solubilized platelet membranes were subjected to ligand-affinity chromatography [40], and the affinity column eluate was evaluated by ELISA against each IgG fraction. The results demonstrated that both fractions reacted positively with a protein(s) contained in the affinity column eluate. These results therefore indicated that at least part of the immunoreactivity observed in solubilized platelet membranes was due to antibody interaction with the receptor itself.

Based on these findings, P_1Ab and P_2Ab were purified by affinity chromatography and evaluated for their ability to interact with purified receptor protein. Immunoblot analysis revealed that both P_1Ab and P_2Ab did, in fact, interact with TXA_2/PGH_2 receptor purified to homogeneity. Studies were then undertaken to establish the specificity of P_1Ab and P_2Ab relative to interaction with other platelet proteins. In these experiments, immunoblotting of solubilized platelet membranes revealed only one distinct band at 55 kDa which represented the TXA_2/PGH_2 receptor [28]. Experiments characterizing the functional activity of P_1Ab and P_2Ab demonstrated that neither antibody stimulated nor inhibited platelet aggregation in PRP, washed platelets and permeabilized platelets. The studies in permeabilized platelets provided evidence that the absence of functional activity was not due to inaccessibility of the antibodies to the intracellular compartment. This lack of inhibitory effects was further confirmed by radioligand binding studies. Specifically, neither antibody was found to inhibit TXA_2/PGH_2 receptor binding of [^{3}H]U46619 in intact platelets or [^{3}H]SQ29,548 in solubilized platelet membranes. Collectively, these data therefore indicate that neither antibody interacts with the receptor ligand binding domain(s) or interferes with the TXA_2/PGH_2 signal transduction pathway.

In summary, the above results describe the generation of the first anti-TXA_2/PGH_2 receptor antibodies. Each of these antibodies has been shown to be highly specific for the receptor protein, to be devoid of functional activity, and to interact with separate epitopes on the receptor, presumably in the extracellular and cytoplasmic regions. The high degree of specificity of these antibodies should be of significant value to large scale receptor purification from platelets and other tissues by immunoaffinity chromatography. Furthermore, the inability of these antibodies to interfere with TXA_2/PGH_2 receptor signal-transduction makes them logical candidates for: (1) purification of the coupled receptor-G-protein complex; and (2) receptor localization and distribution studies in platelets and other organ systems.

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