Antibodies to GPIIb$_\alpha$ (300–312) Inhibit Fg Binding, Clot Retraction, and Platelet Adhesion to Multiple Ligands (43674)

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Abstract. **We previously reported that a peptide with the sequence Gly-Ala-Pro-Leu (GAPL) found as residues 309—312 in glycoprotein IIb$_\alpha$ (GPIIb) comprises at least part of a Fg binding site on GPIIb (1). Subsequent studies demonstrated that a peptide corresponding to residues 300–312 of GPIIb$_\alpha$ can bind to Fg and Vn, and is a potent inhibitor of platelet aggregation and the adhesion of activated platelets to at least four adhesive ligands: Fg, Fn, Vn, and vWf (2). Here, the production and initial characterization of polyclonal antibodies against this peptide are described. ELISAs and dot-blot assays reveal the specificity of the antibodies for the peptide immunogen. In immunoblots, the antibodies recognize GPIIb under reducing conditions. The binding of Fg to immobilized GPIIb/IIIa, the rate of clot retraction and the adhesion of stimulated platelets to Fg, fibronectin (Fn), vitronectin (Vn), and von Willebrand factor (vWf) were inhibited by these antibodies, but not by a control IgG. These results together with our earlier published data indicate that GPIIb$_\alpha$ (300–312) comprises at least part of a common ligand binding site within the integrin α_{IIb} subunit.**

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The platelet fibrinogen (Fg) receptor (GPIIb/IIIa) is an integrin which plays a critical role in hemostasis by recognizing at least the four adhesive ligands: Fg, fibronectin (Fn), vitronectin (Vn), and von Willebrand factor (vWf) (3–6). The recognition of these large adhesive glycoproteins by activated platelets and subsequent cellular adhesion events are critical for the maintenance of normal vascular integrity. GPIIb/IIIa, like many of the integrins, recognizes the RGD amino acid sequence as at least part of its minimum ligand recognition site (7). Synthetic peptides corresponding to the individual RGDX sequences present in Fg, Fn, Vn, and vWf each can inhibit the binding of these ligands to GPIIb/IIIa (8–12). Consistent with this ability, RGD containing peptides have been shown to interact directly with $\alpha_{IIb}\beta_3$ (α_{IIb} = GPIIb; β_3 = GPIIIa) (13–15).

Fg also possesses a non-RGD containing GPIIb/IIIa recognition sequence (HHLGGAKQAGDV), which is present as the carboxyl terminus of the γ-chain of Fg (16, 17). A synthetic peptide derivative of the γ-chain recognition sequence has been demonstrated to cross-link selectively to residues 294–314 of GPIIb (18). We reported that a peptide with the sequence Gly-Ala-Pro-Leu (GAPL) found as residues 309–312 in GPIIb appears to comprise at least part of a Fg binding site on GPIIb (1). In collaboration with Amrani, Kirschbaum and Matsueda, we recently demonstrated that the adhesion of resting or stimulated platelets to Fg is mediated by the γ-chain (19), and GAPL-containing peptides inhibit the binding of the Fg γ-chain to platelets (20, 21). Further studies demonstrated that a peptide corresponding to residues 300–312 of GPIIb$_\alpha$ (G13) binds to purified Fg and Vn, inhibits Fg binding to purified $\alpha_{IIb}\beta_3$, platelet aggregation, and the adhesion of activated platelets to at least four adhesive ligands (2). Here, the production and initial characterization of polyclonal antibodies against this peptide (G13) are described.

Materials and Methods

Materials. Human α-thrombin was kindly donated by Dr. J. W. Fenton II, New York State Depart-

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ment of Health (Albany, NY). Purified human peak 1 fibrinogen was a generous gift of Dr. D. L. Amrani, Department of Medicine, University of Wisconsin (Madison, WI). Polyclonal rabbit anti-GPIIb/IIIa was generously provided by Dr. Lisa K. Jennings, Department of Medicine, University of Tennessee (Memphis, TN). ADP, L-epinephrine, KLH (keyhole Limpet hemocyanin), 4-chloro-1-napthol, carbodiimide, Sepharose-4B, 6-amino hexanoic acid Sepharose 4B, goat anti-rabbit IgG conjugated FITC (fluorescein isothiocyanate), and BSA (bovine serum albumin) were from Sigma Chemical Co. (St. Louis, MO). Sodium chromate (^{51}Cr) was from NEN, Dupont (Wilmington, DE). Protein G-agarose was purchased from Boehringer Mannheim (USA division). Goat anti-rabbit conjugated to horseradish peroxidase was purchased from Biorad (Richmond, CA). t-Boc amino acids and PAM resins for synthetic solid phase peptide synthesis were from Bachem Inc. (Torrance, CA) and Peninsula Labs (Belmont, CA). All other reagents were analytical or reagent grade.

Peptide Synthesis. Peptides were synthesized in our laboratory using a PSS-80 automated peptide synthesizer (Applied Protein Technologies, Cambridge, MA), cleaved from the PAM resins with trifluoromethane sulfonic acid, purified, amino acid compositions and sequences confirmed as previously described (1). Prior to use, 10 m*M* stock solutions of individual peptides were made in 0.01 *M* acetic acid and stored at −20°C.

Antibodies. Rabbits were immunized with G13 peptide derivatized to Keyhole Limpet hemocyanin (Sigma) following a standard immunization protocol (22). Antiserum raised against G13 (from six animals) was preadsorbed against nonderivatized Sepharose 4B, precipitated with 40% $(NH_4)_2SO_4$ and IgGs isolated by affinity chromatography on a protein G-agarose column (10 ml) according to the manufacturer's instructions with minor modifications. Briefly, 2 ml of immune salt-precipitated serum was applied to the protein G column which had been equilibrated in phosphate-buffered saline (PBS) {8.77 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4/liter, pH 7.0}. Following incubation with the resin for 60 min, the column was washed until baseline was reached and bound IgG was eluted with 0.2 *M* Glycine, pH 2.8. The eluted protein was collected in 20 ml of PBS on ice, the pH adjusted and the antibodies immediately dialyzed against ice cold H_2O for 48 hr with frequent changes of water. IgG was concentrated by lyophilization and reconstituted in PBS.

Anti-G13 IgG fractions were made monospecific by affinity chromatography with a GDGRHDLLV-GAPL-Sepharose 4B column. Briefly, 5 ml of 6-aminohexanoic acid-Sepharose 4B beads were derivatized with 20 mg of G13 peptide using the water soluble chemical crosslinker carbodiimide (0.1 *M* final). After closely monitoring the pH (maintained at 4.5–6.0) for 1 hr, the peptide resin mixture incubated overnight on an inverted shaker. Unreacted groups were blocked by using 0.1 *M* Tris-HCl, pH 8.0. Purified IgG was allowed to incubate with the peptide column for 1 hr prior to washing and elution of bound antibody by 4.0 *M* $MgCl_2$. IgG fractions were repeatedly passed over G13 matrix until all G13 reactive IgGs were depleted. Eluted monospecific anti-G13 antibody fractions were pooled and concentrated by ammonium sulfate precipitation (40%) and dialyzed against PBS, 0.02% NaN_3, pH 7.0 and stored at −20°C. The sodium azide was removed by dialysis prior to use. Antibody data presented was obtained using a pool of IgGs from the entire collection of antibodies isolated upon immunization. IgGs were isolated from preimmune sera obtained from the same rabbits prior to immunization.

ELISA. G13 peptide at a final concentration of 50 μg/well was incubated in 96-well flatbottom, Pro-Bind ELISA microtiter plates (Falcon) overnight at 4°C. Following drying, washing and blocking the wells with 1% BSA, twofold serial dilutions of purified anti-G13 (1^0 Ab) at a beginning concentration of 20 μg/well or purified preimmune IgG (1° Ab) from the same rabbit were added and allowed to incubate at 37°C for 2 hr. Following washing as described (1), wells were incubated with a 1:1000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate (2° Ab) for 1 hr at ambient temperature. After washing, ELISAs were developed and read at 490 nm with the aid of an automated plate reader (1). All values are reported as total binding. Specific binding was assayed by determining the extent of binding of the antibodies to the plastic wells in the absence of peptide antigen; that is, the amount of binding of the 1° antibodies to wells coated with BSA. Also the amount of binding observed when PBST (PBS with 0.05% Tween 20) was substituted for anti-G13 was assessed.

Nitrocellulose Dot-Blot Assay. A nitrocellulose dot-blot competition assay was used to demonstrate further specificity of binding of G13 to anti-G13 in the fluid phase. Briefly, G13-BSA conjugates were prepared as G13-KLH complexes were made for immunization (22) but substituting BSA for KLH. Peptide-BSA conjugates were adjusted to 1 mg/ml carrier before use. Three μg of conjugate were applied to the center of each nitrocellulose square, allowed to dry and post-coated with 5% BSA, 0.05% Tween 20 in Tris buffered saline for 1 hr at 37°C. In some experiments, prior to the addition of monospecific anti-G13 antibodies to the blots, the antibodies were incubated with 3.33 μ*M* peptide/0.3 ml of PBS at room temperature for 1 hr. After preincubation, antibodies alone or antibody/peptide mixtures or preimmune IgG were transferred to nitrocellulose squares and allowed to incu-

bate for 1 hr. Blots were washed three times with blot buffer (TBS, 0.05% Tween-20 and 1.0% BSA) and subsequently incubated with a 1:1000 goat anti-rabbit IgG horseradish peroxidase conjugate for 1 hr. After washing, blots were developed using H_2O_2 and 4-chloro-1-napthol and comparisons were made between direct binding assays and competition experiments with G13 in the fluid phase.

Platelet Immunoblotting. Immunoblots of solubilized platelet membrane proteins were performed as previously described (23) with two minor exceptions: (i) soluble membrane proteins from platelet lysates were electrophoresed on mini-slab gels (Idea Scientific); and (ii) SDS-PAGE was carried out on 10% (wt/vol) polyacrylamide gels. The immunoblots were processed as usual. Immunoreactive bands were visualized using H_2O_2 and 4-chloro-1-napthol.

Cytofluorimetry. Whole blood obtained from aspirin-free human donors was drawn into acid citrate dextrose, ACD (39 m*M* citric acid, 75 m*M* sodium citrate, 135 m*M* glucose, pH 4.5). Washed platelets were prepared as described (1). Platelets (0.075 ml) at a final concentration of 2.0×10^8/ml were suspended in CGS and incubated with 25 μl of clarified IgG (200 μg/ml) or the control preimmune IgG for 1 hr at room temperature. The platelets were washed and incubated with a 1:100 dilution (manufacturer's suggestion) of goat anti-rabbit IgG conjugated to fluorescein isothiocyanate. Following staining in the dark for 1 hr, the platelets were analyzed using a FACS. In some experiments, platelets were stimulated with ADP (20 μ*M*) prior to the addition of anti-G13 antibodies to assess whether the binding of these antibodies to the platelet surface is activation dependent.

Fg Binding. The binding of fibrinogen to purified GPIIb/IIIa was examined using a solid-phase assay system (24). Antibodies were assayed as potential inhibitors of fibrinogen binding exactly as previously described (24). Anti-G13 antibodies and control serum were allowed to incubate with adsorbed GPIIb/IIIa for 60 min prior to addition of biotinylated fibrinogen. Antibodies were assayed by Dr. Israel Charo and Lisa Nannizzi at Cor Therapeutics (San Francisco, CA). In these experiments, nonspecific binding is measured by determining the binding of biotinylated Fg to BSA-coated wells, and also by determining the binding of Fg to immobilized GPIIb/IIIa in the presence of RGDS (250 μ*M*) or EDTA (5 m*M*), and was determined to be less than 10% (24). Dose response curves were performed and all determinations were done in quadruplicate. Standard deviations in these assays were less than 20%, and often less than 10%, of the mean (24).

Clot Retraction. Clot retraction assays were performed as described (25, 26): 300 μl of PRP and 200 μl of PBS in an aggregometer cuvette were treated with 0.15 units of α-thrombin, followed by stirring for 5 sec in a Chrono-log aggregometer. Clot length (mm) was measured at time intervals as the clot retracted. Antibodies were allowed to incubate with the platelet suspension for 15 min with stirring prior to addition of α-thrombin. Platelets with dissociated GPIIb/IIIa failed to support clot retraction as earlier reported (25).

Platelet Adhesion. Platelet adhesion assays were performed exactly as described (1, 2, 27). Chromium labeled platelets were stimulated sequentially with epinephrine and then with ADP as reported (1, 2). For assays of platelet adhesion to vWf, platelets were stimulated with α-thrombin as described (2). Prior to the addition of activated platelets to ligand coated wells, antibodies were incubated for 30 min with the platelet suspension. Adhesion of stimulated platelets to the various ligands was saturable and could be inhibited with either GRGDSP, LGGAKQAGDV, or 5 m*M* EDTA. Two types of controls were used as indicators of nonspecific binding in these assays. Activated platelets were allowed to adhere to wells coated with BSA alone. As the second indicator of nonspecific binding, aliquots of platelets were incubated at 37°C at pH 7.8 in the presence of 5 m*M* EDTA for 15 min to irreversibly inactivate GPIIb/IIIa complexes, thereby minimizing adhesion to Fg mediated by its receptor (28). Typically 5%–8% of the platelets present in the initial suspension adhered to the protein-coated wells.

Platelet Aggregation. Platelets for the aggregation studies were prepared as described (29). Platelet aggregation was measured at 37°C in a single channel aggregometer (Chrono-log Corp., Havertown, PA) using suspensions of gel-filtered platelets, purified peak 1 Fg, and ADP as the agonist. The aggregation studies with gel-filtered platelets and exogenous Fg were essentially as described (29) with the following modification: antibodies were substituted for Fg peptides and allowed to incubate with the platelets and/or the Fg for various time intervals prior to the addition of ADP.

Aggregation studies with washed platelets and α-thrombin were performed as reported (2).

Results

Polyclonal antibodies against G13 were raised in rabbits. IgG from immune sera was purified by affinity chromatography using a protein-G column. Isolated IgG fractions were made monospecific by affinity chromatography on a G13-peptide derivatized Sepharose 4B matrix. IgGs were isolated from preimmune sera obtained from the same rabbit prior to immunization with the G13.

Anti-G13 antibodies were detected by indirect ELISA. Using this assay, even the most dilute solutions of the antibodies tested (1:32×) recognized immobilized peptide immunogen (Fig. 1). At this dilution

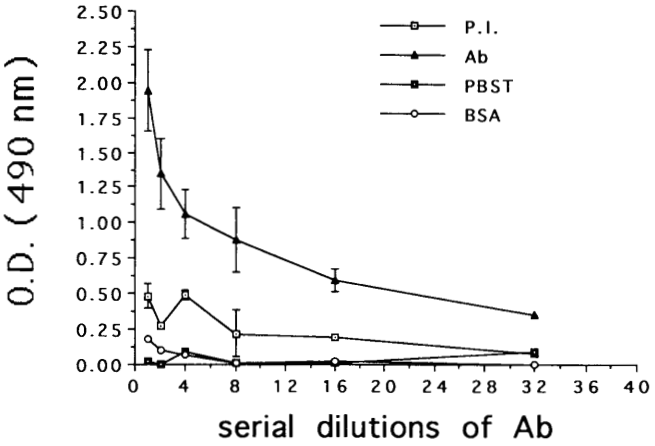

Figure 1. Detection of anti-G13 antibodies by indirect ELISA. 96-well ELISA microtiter plates were coated overnight with G13 peptide at a final concentration of 50 μg/well. Following post coating and washing as described in the materials and methods, 2-fold serial dilutions of purified anti-G13 IgG at a beginning concentration of 20 μg/well were added and allowed to incubate as described. Following washing, wells were incubated with a 1:1000 goat anti-rabbit IgG horseradish peroxidase conjugate, incubated, washed, and ELISAs subsequently developed and absorbance read at 490 nm. Anti-G13 IgG binding to G13 immunogen (Ab); preimmune IgG binding to G13 (P.I.); substitution of wash buffer for antibody (PBST); anti-G13 IgG binding to BSA post coating solution (BSA). Error bars represent standard deviations of the data.

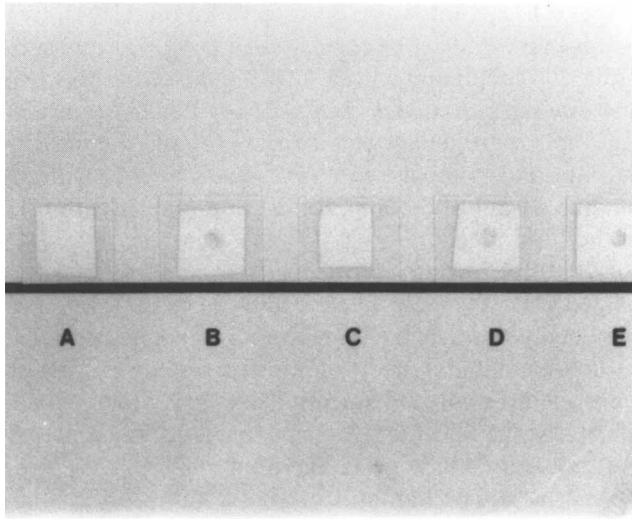

Figure 2. A nitrocellulose dot blot assay demonstrates anti-G13 antibodies are specific for immunogen (G13). Dot blot assays were performed as described in Materials and Methods. Blot B represents binding of immune sera to immobilized G13/BSA. The controls demonstrate that the binding of G13 to Fg is specific. Blot A represents the absence of binding of preimmune antibodies to immobilized G13/BSA; blot C demonstrates the ability of soluble excess G13 to block the binding of anti-G13 antibodies to immobilized G13/BSA; blots D and E reveal the inability of peptides LGGAKQAGDV and GDDYADVFIGAPLF to block the binding of anti-G13 antibodies to immobilized G13/BSA conjugates.

there was a fourfold difference in absorbance between purified immune and preimmune IgG. The controls demonstrate that the binding of antibody to G13 is specific. This is evident since anti-G13 did not recognize and bind to BSA. The second antibodies did not bind to immobilized peptide or BSA since there was no absorbance in the absence of primary antibody, or peptide immunogen.

A dot-blot competition assay directly demonstrated the specificity of the antibodies. In this assay excess G13 peptide in the fluid phase blocked the binding of anti-G13 antibody to immobilized G13/BSA conjugates following the incubation of soluble G13 with antibody prior to addition to immobilized immunogen (Fig. 2). Two control peptides were ineffective when assayed for competition.

To determine whether our antibodies also recognized similar epitope(s) present in GPIIb/IIIa, we performed immunoblots with isolated platelet membrane proteins. The antibodies were able to recognize GPIIb under reducing conditions in these immunoblots (Fig. 3). By Western blot analysis, anti-G13 (Fig. 3, lane B) but not preimmune IgG (lane C) recognized the same upper protein band recognized by the control polyclonal affinity purified anti-GPIIb/IIIa antibody (lane A). The second antibody did not recognize transferred membrane proteins (lane D).

The ability of the antibodies to recognize native protein was assayed by FACS analysis. Flow cytometry demonstrated that the anti-G13 antibodies, but not preimmune IgG bound to the platelet surface (data not shown). Anti-G13 antibodies produced by one of the rabbits bound to the platelet surface in an activation dependent manner (data not shown).

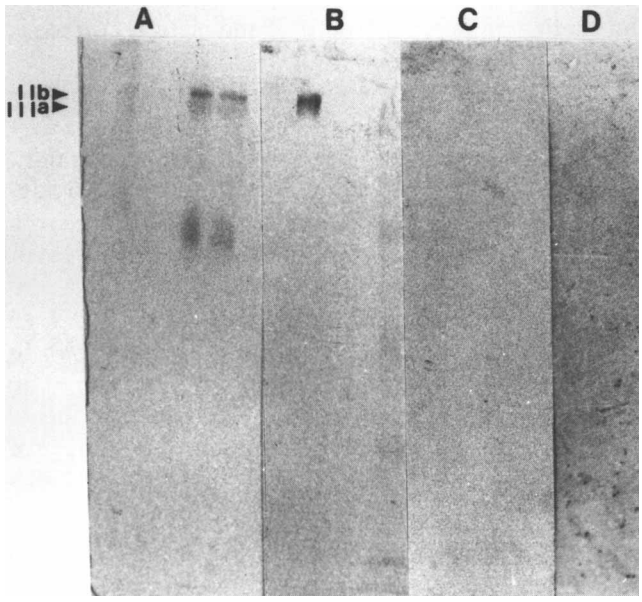

Figure 3. Immunoblot of solubilized platelet membrane proteins separated on a 10% SDS-PAGE mini-gel under reducing conditions. Transferred proteins were probed with various antibodies as described in Materials and Methods. Lane A, Polyclonal anti-IIb/IIIa antibodies; lane B, anti-G13 IgG; lane C, preimmune IgG; lane D, goat anti-rabbit horseradish peroxidase secondary antibody.

Next, the antibodies were assayed in four functional tests which all are dependent on the binding of fibrinogen to its receptor. First the anti-G13 antibodies were tested for the ability to inhibit the binding of Fg to GPIIb/IIIa. The antibodies were assayed in a solid state Fg binding assay which measures the binding of Fg to purified immobilized GPIIb/IIIa (24). Anti-G13, but not control IgG, demonstrated a partial inhibition of Fg binding in this assay (Fig. 4). The inhibition of Fg binding by antibody was dose dependent with 100 μg/ml giving 40% inhibition.

Second, the antibodies were tested as inhibitors of clot retraction (25) since this assay is also dependent on platelet-fibrin(ogen) interaction. It is evident from the data in Figure 5 that anti-G13, but not control IgG, inhibited the rate of clot retraction. The transient inhibition of clot retraction was still evident 45 min after the addition of thrombin. As previously reported, platelets with dissociated GPIIb/IIIa failed to support clot retraction (25).

Third, the antibodies were tested as inhibitors of platelet adhesion to Fg (a platelet functional assay that is dependent on Fg binding to its receptor). The data in Figure 6 reveal that anti-G13 at a final concentration of 200 μg/ml gave ~65% inhibition of the adhesion of epinephrine and ADP-activated platelets to Fg compared to the preimmune IgG. As a measure of nonspecific binding in the platelet adhesion assays, activated platelets were allowed to adhere to BSA coated wells. Less than 10% of the number of platelets which bound to Fg attached to this substrate. Also as a second indication of little nonspecific binding, very few stimulated platelets with dissociated GPIIb/IIIa complexes bound to immobilized Fg.

Since G13 also inhibits the adhesion of platelets to Fn, Vn, and vWf, we determined whether antibodies to G13 could inhibit the adhesion of epinephrine/ADP

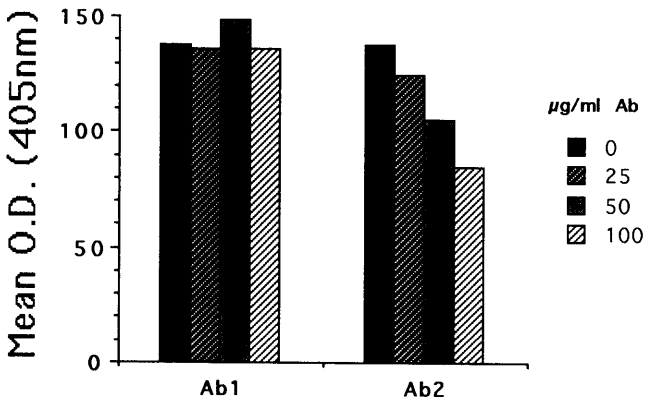

Figure 4. Antibodies to GPIIb peptide 300–312 inhibit fibrinogen binding to immobilized GPIIb/IIIa. Antibodies were tested for their effects on Fg binding using a solid phase Fg binding receptor assay (24) as described in Materials and Methods. Polyclonal monospecific anti-G13 antibodies (Ab 2) demonstrated a dose dependent inhibition while control preimmune IgG (Ab 1) was without effect.

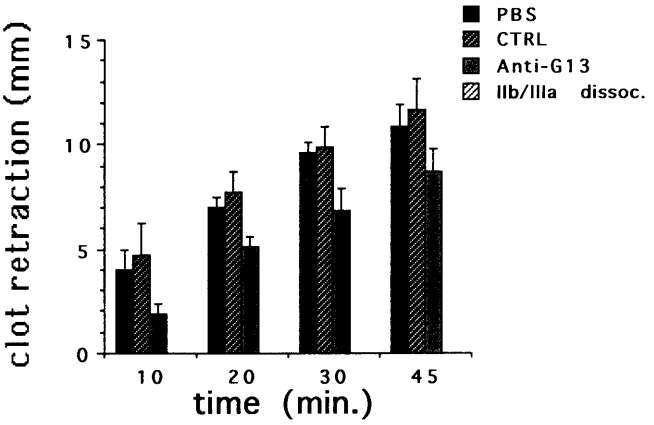

Figure 5. Effect of anti-G13 antibodies on the time course of clot retraction. Clot retraction (mm) is plotted on the y-axis and time in minutes is on the x-axis. The times correspond to 10, 20, 30, and 45 min, respectively, after addition of thrombin (0.3 units/ml). Cuvettes contained: no addition (PBS), preimmune IgG (CTRL) and anti-G13, respectively. Platelets with dissociated GPIIb/IIIa failed to support clot retraction as reported earlier (25). The results are pooled data from three of the immunized rabbits. Error bars represent the range of the data.

or α-thrombin stimulated platelets to all of these ligands as well. The data in Figure 6 demonstrate that anti-G13 antibodies inhibit the adhesion of stimulated platelets to Fg, Fn, Vn, and vWf. At a final concentration of 200 μg/ml the level of inhibition of stimulated platelets to the various ligands was between 60%–70% for each of the particular ligands (n = 3 donors).

Fourth, the antibodies were tested in assays of platelet aggregation. In studies using both washed

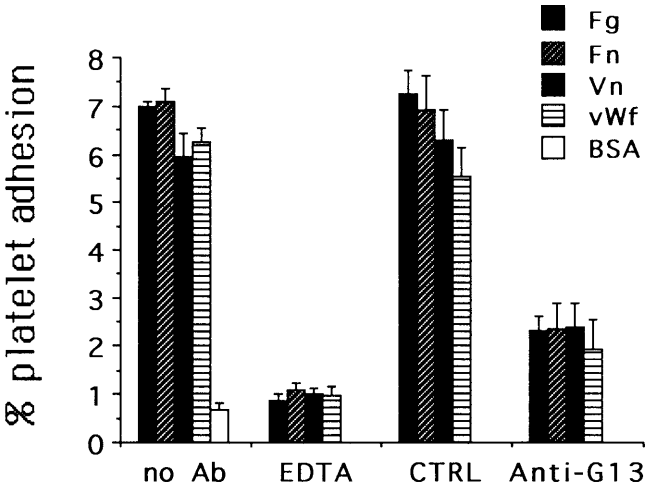

Figure 6. Inhibition of the adhesion of stimulated platelets (see Materials and Methods) to Fg, Fn, Vn, and vWf by anti-G13 antibodies. The ordinate indicates % platelet adhesion. The bars labeled "no Ab" represent the extent of adhesion to the substrate-coated wells in the absence of antibody. The bars labeled "EDTA" correspond to the amount of adhesion to each ligand using platelets with dissociated IIb/IIIa complexes (see Methods). The "CTRL" bars represent the extent of adhesion in the presence of control IgG. Antibodies were tested at a final concentration of 200 μg/ml. The results represent pooled data from immunoglobulins isolated from three of the immunized rabbits. Error bars indicate standard deviation of the data.

platelets stimulated with α-thrombin and gel-filtered platelets stimulated with low levels of ADP in the presence of exogenous Fg, no inhibition of platelet aggregation by anti-G13 antibodies was seen (data not shown).

Discussion

The data described here support the hypothesis that residues 300–312 of GPIIb (G13) are important in adhesive ligand binding to GPIIb/IIIa. Our previous data implicated residues 309–312 in GPIIb as comprising at least part of a Fg binding site on GPIIb (1). These data are consistent with the demonstration that GAPL-containing peptides inhibit the binding of the Fg γ-chain to platelets (21). These results are also consistent with chemical cross-linking data which identified a sequence present in a region of GPIIb (residues 294–314) that was found to be crosslinked by a derivative of a Fg γ-chain peptide (18). Our recent studies demonstrated that G13 binds to immobilized Fg and Vn in a specific fashion, inhibits platelet aggregation, Fg binding to GPIIb/IIIa, the adhesion of epinephrine/ADP stimulated platelets to Fg and most significantly, inhibits the adhesion of stimulated platelets to Fn, Vn, and vWf (2). However, the peptide did not inhibit the GRGDSP-sensitive adhesion of resting platelets to Fn. This lack of inhibition occurs even though the α-subunit of the Fn receptor shares 81% homology with GPIIb in the region of IIb crosslinked by a carboxy-terminal γ-chain peptide with only two amino acid differences relative to G13 [GDGLDDLLVGAPL] (18, 30, 31). This Fn receptor {GPIc-IIa (Ic = α5) has been identified as the receptor which mediates the adhesion of resting platelets to Fn (23). These data demonstrate that G13 cannot inhibit the function of all RGD sensitive integrins. Independently, a peptide corresponding to residues 296–306 of GPIIb (designated B12) was implicated in Fg binding (32). Direct comparisons between the two peptides, 296–306 (generously supplied by Dr. Ed Plow, Scripps Research Clinic) and 300–312, revealed that they have relatively similar potencies as inhibitors of the adhesion of stimulated platelets to Fg (unpublished data). Thus, this entire conserved region of GPIIb (294–314) appears to be important in ligand recognition and together with our earlier published data indicate that GPIIb 300–312 comprises a major ligand binding site within the integrin α_{IIb} subunit.

The combination of ELISAs, dot-blot assays, cytofluorimetry, and Western blot analysis demonstrate that anti-G13 antibodies can recognize both the peptide immunogen and its respective epitope(s) in both the native and denatured protein. Antibodies were determined to be specific since they recognize G13 but not BSA. Furthermore, the binding of the antibodies to the peptide was inhibited by excess soluble G13 but not LGGAKQAGDV (L10), or by a similar peptide to G13 from the homologous region (residues 317–330 [33]) of the Vnα subunit (GDDYADVFIGAPLF). The inability of the Vn receptor peptide to inhibit anti-G13 binding to G13 would suggest that residues in addition to the GAPL sequence at residues 309–312 are important for the binding of the antibodies to their immunogen. Five of the six polyclonal antibodies raised against G13 bound to the platelet surface in a nonactivation-dependent fashion. Only anti-G13 antibodies from one rabbit failed to bind to nonactivated platelets as judged by cytofluorimetry. However, this antibody did bind to ADP stimulated platelets and was indistinguishable from the other anti-G13 IgGs in regard to its inhibitory effect on platelet function. Western blot analysis demonstrated that anti-G13 antibodies recognized reduced GPIIb.

It is interesting that the same peptide conjugate elicited both activation- and nonactivation-dependent antibodies. These results indicate that the peptide immunogen appears to be able to assume different configurations during antigen processing. Since all but one of these antibodies bound to the surface of resting platelets, these results demonstrate that at least part of the 300–312 region of GPIIb is exposed on the surface of unstimulated platelets. Activation dependent binding of one of the IgGs is consistent with a conformation change in GPIIb following platelet activation (42, 47).

Having established that anti-G13 antibodies were specific and could recognize the peptide immunogen and its corresponding epitope in both the native and denatured protein, we next characterized the antibody in four platelet functional tests which are all dependent on Fg binding to its receptor. The G13 antibodies, but not control IgG, inhibited the binding of fibrinogen to immobilized GPIIb/IIIa, the rate of clot retraction, and the adhesion of epinephrine/ADP stimulated platelets to Fg, but had no effect on platelet aggregation. The inhibition of fibrinogen binding by the anti-G13 antibodies was dose dependent while the inhibition of clot retraction was a transient effect on the rate of retraction. This transient inhibition is similar to that of some known inhibitors of clot retraction such as GRGDSP, L10, and certain anti-GPIIb/IIIa monoclonal antibodies (25).

The extent of inhibition of platelet adhesion to Fg was also dependent on the amount of antibody tested with 200 μg/ml of anti-G13 resulting in approximately 65% inhibition compared to the control IgG. Although this concentration of antibody seems to be high, it is consistent with other reports requiring large concentrations of anti-peptide antibodies (24, 34). For example, an immunopurified anti-peptide antibody specific for a peptide sequence found in the signal recognition particle of yeast required a concentration of 1 mg/ml

for full activity (34). These results are probably due in part to the lower affinity of anti-peptide antibodies for the receptor than for the peptides. An alternative explanation for the partial inhibition is that additional site(s) on the GPIIb/IIIa receptor are involved in ligand binding.

Antibodies to $GPIIb_{\alpha}$ 300–312 inhibited the adhesion of stimulated platelets to Fn, Vn, vWf, and Fg. These results are consistent with our recently reported data in which we demonstrated that the soluble peptide (G13) also inhibits the adhesion of stimulated platelets to at least these four ligands in a specific and dose-dependent fashion (2). The antibody data further support the significance of this highly conserved region in adhesive ligand binding to GPIIb/IIIa. These results are significant in that they represent the first report in which antibodies to this region are capable of blocking the adhesion of stimulated platelets to adhesive ligands (Fn, Vn, and vWf) other than Fg.

Interestingly, the anti-G13 antibodies inhibited the adhesion of platelets to Fg but had no effect on platelet aggregation. This apparent discrepancy is similar to that seen with the monoclonal antibody 4A5 (an antibody specific for the carboxyl terminus of the gamma chain of Fg [48]). The monoclonal antibody 4A5 totally inhibits the adhesion of resting and stimulated platelets to immobilized Fg (19) but has no effect on platelet aggregation (unpublished data). These results are not unique to antibodies specific for Fg peptides or GPIIb peptides since it has been reported that polyclonal and monoclonal antibodies that recognize an activation-dependent epitope on GPIIIa (residing in residues 203–236) do not inhibit platelet adhesion to Fg or platelet aggregation (40). The basis for the differential effects observed with these antibodies in different platelet tests is not known.

Anti-GPIIb antibodies have also been studied in other laboratories (32, 35–38). Some of these antibodies are inhibitors of fibrinogen binding and/or platelet aggregation. Antibodies to $GPIIb_{\alpha}$ 296–306 reacted exclusively with GPIIb, blocked fibrinogen binding to the peptide and to purified GPIIb (32). Our data are consistent with these findings, and they are an extension of these results in that they are the first to demonstrate the inhibition of clot retraction, and more importantly the inhibition of platelet adhesion to at least four ligands. Furthermore, our antibody results reinforce our previous peptide data which demonstrated that the region encompassing residues 300–312 of $GPIIb_{\alpha}$ can serve as a common ligand binding site for at least four adhesive ligands (2).

Additional sequences in GPIIb have been implicated in ligand binding. The epitope of the anti-GPIIb monoclonal antibody PMI-1 (whose binding is RGD- and/or EDTA-dependent) was mapped to α842–858 (37). Finally, the epitope for the monoclonal anti-GPIIb antibody (M6) was located to α657–667 (38). This antibody inhibits platelet aggregation and the exposure of this epitope is EDTA, or thrombin dependent. These data led to the hypothesis that this sequence in GPIIb may play a role in ligand binding (38).

Studies with monoclonal and polyclonal antibodies against GPIIIa and various peptide sequences from IIIa have also contributed to our understanding of the apparent complexity of ligand binding to this receptor. Polyclonal antibodies to peptides corresponding to residues 204–229 inhibit fibrinogen binding to purified GPIIb/IIIa and platelet aggregation (24). In a different study, a polyclonal antibody to a peptide corresponding to GPIIIa 217–231 was used to show direct interaction between this peptide and Fg (39). Using polyclonal and monoclonal antibodies specific for synthetic peptides spanning GPIIIa 203–236, an activation-dependent epitope on GPIIIa was identified (40). These antibodies bind to purified GPIIb/IIIa, immunoprecipitate GPIIIa from platelet lysates and bind to denatured GPIIIa on immunoblots but do not inhibit platelet adhesion to Fg or platelet aggregation (40). In another study, the precise localizations of the epitopes for six monoclonal antibodies for the N-terminal region of GPIIIa were determined (41). The fact that some of these antibodies inhibited platelet aggregation and their epitopes were found to be in proximity to the early chymotryptic cleavage site of GPIIIa in whole platelets implicated these N-terminal multiple sequences as putative fibrinogen-binding sites (41). Also, a monoclonal antibody (AC7) against residues 109–128 of GPIIIa interacted only with stimulated platelets and inhibited platelet aggregation and Fg binding (42).

The complexity obtained with the antibody data is supported by results from synthetic peptide studies. These studies have implicated a number of regions as putative ligand binding domains of GPIIIa. Residues 109–171 of GPIIIa were implicated as a ligand recognition site in GPIIIa were implicated as a ligand recognition site in GPIIIa by cross-linking studies with a RGD-containing peptide (15, 43). These results were supported by studies of GPIIb/IIIa from two patients who express the CAM variant of Glanzmann's thrombasthenia. The GPIIIa molecules of these two patients have a single amino acid substitution (Asp^{119} to Tyr^{119}) which results in a loss of ligand binding ability (44). Peptides corresponding to residues 104–229 and 217–231 of GPIIIa have recently been demonstrated to inhibit the binding of Fg, Fn, Vn, and vWf to purified GPIIb/IIIa and bind directly to Fg, Fn, and vWf (24, 39).

Interestingly, several earlier studies demonstrated direct interaction of RGD peptides to both subunits of GPIIb/IIIa (14, 15). Furthermore in cross-linking studies with both RGD and γ-chain peptides, cross-linking could be inhibited by either peptide (14, 15, 18). Evi-

dence obtained with a related member of the integrin family of receptors, $\alpha_v\beta_3$, suggests a RGD binding site composed of noncontiguous sequences of the α-subunit and a region from the β-subunit of the Vn receptor (45, 46).

The results of all these studies suggest a complex picture for the formation of a ligand binding site(s). Further work is required to resolve the apparent complexity of the makeup of the ligand binding site(s) on GPIIb/IIIa. However, the data suggest the existence of noncontiguous ligand-binding sites in both subunits of GPIIb/IIIa. Indeed a model is evolving in which a binding pocket, made of several noncontiguous sequences present in both subunits of GPIIb/IIIa, may form as a result of a conformational change in the Fg receptor following platelet activation (2, 42, 45–47). The binding pocket which includes $GPIIb_\alpha$ 300–312 can recognize at least four ligands.

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