Evidence for novel binding sites on the platelet glycoprotein IIb and IIIa subunits and immobilized fibrinogen

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The present study was designed to examine the interaction of the purified platelet glycoprotein IIb–IIIa complex (GP IIb–IIIa or integrin $\alpha_{IIb}\beta_3$) and the individual subunits of the complex with immobilized fibrinogen. Although ^{125}I -GP IIb–IIIa binding to fibrinogen immobilized on Sepharose was specific, this interaction exhibited properties distinct from those of reversible fibrinogen binding to platelets: ^{125}I -GP IIb–IIIa binding appeared irreversible, but non-covalent, Ca^{2+} -independent, and was inhibited only weakly, or not at all, by the anti-(GP IIb–IIIa) monoclonal antibodies 10E5 and 7E3 and synthetic peptides from known platelet-binding domains of fibrinogen. Reversibly dissociated GP IIb or GP IIIa subunits inhibited

^{125}I -GP IIb–IIIa binding to immobilized fibrinogen and bound directly to the fibrinogen. However, these subunits did not bind to peptides derived from known platelet-binding domains within the fibrinogen α - and γ -chains, although the GP IIb–IIIa complex did. These results show that the complexed form of full-length GP IIb and GP IIIa is required for binding to these synthetic peptides, but not necessarily for binding to immobilized fibrinogen. Thus GP IIb–IIIa can bind to immobilized fibrinogen by a distinct mechanism that appears to involve novel binding sites on each subunit of the GP IIb–IIIa complex and on fibrinogen.

INTRODUCTION

The glycoprotein IIb–IIIa complex (GP IIb–IIIa) is a Ca^{2+} -dependent heterodimer [1–3] on the platelet surface that binds to fibrinogen and other adhesive proteins, thereby mediating platelet aggregation [4–6] and adhesion to the subendothelium [7,8]. The heterodimer consists of GP IIb ($M_r = 136\,000$ nonreduced) and GP IIIa ($M_r = 95\,000$ nonreduced). [GP IIb is also referred to as the α -subunit, and GP IIIa is referred to as the β -subunit. GP IIb is composed of two disulphide-linked subunits: a heavy chain of 120 kDa, formerly called GP IIb $_{\alpha}$, and a light chain of ~ 23 kDa formerly called GP IIb $_{\beta}$. GP IIIa is a single polypeptide of ~ 95 kDa.] Glycoprotein IIb–IIIa (or integrin $\alpha_{IIb}\beta_3$) is a member of a large family of adhesion receptors termed integrins, which are found on diverse cell types (for reviews, see [9,10]).

One especially well characterized aspect of fibrinogen's interaction with GP IIb–IIIa is the reversible phase of soluble fibrinogen binding to activated platelets, which is believed to initiate platelet aggregation. Several binding sites likely to mediate this phase have been identified on fibrinogen and on GP IIb–IIIa. On fibrinogen, these sites include a 10–12-amino acid sequence at the C-terminus of the fibrinogen γ -chain [11], termed the L10 or H12 peptide respectively [12] (residues 402– or 400–411) and the Arg-Gly-Asp (RGD) sequence in positions 95–97 and 572–574 of the fibrinogen α -chain [13–15]. Sites on GP IIb–IIIa that may recognize these fibrinogen peptides include residues 296–306 on GP IIb [16] and 109–171 [17], 211–222 [18], or both, on GP IIIa.

Under some circumstances, the interaction of platelets with

fibrinogen displays properties that are less well understood. Examples include the apparently irreversible, but non-covalent, binding of ^{125}I -fibrinogen to activated platelets after extended incubations [19,20], the partially irreversible binding of platelets to fibrin immobilized in a clot [21], and the adhesion and subsequently irreversible spreading of resting platelets on immobilized fibrinogen through a GP IIb–IIIa-dependent mechanism [22]. Structural differences can be detected in fibrin(ogen) immobilized in a clot [23,24], bound to the platelet surface [25], or adhering to an artificial surface [26] (e.g. as neoepitope exposure), which may be responsible for some of these observations.

To gain additional insight into the interaction of GP IIb–IIIa with immobilized fibrinogen, we investigated the ability of GP IIb–IIIa and its reversibly dissociated subunits to bind to fibrinogen immobilized on Sepharose. Both the GP IIb–IIIa complex and each subunit of the complex were found to bind to immobilized fibrinogen. However, the properties of this binding suggest that it is mediated by sites on GP IIb–IIIa and fibrinogen that are distinct from those that mediate reversible fibrinogen binding to platelets.

EXPERIMENTAL

Fibrinogen purification

Fibrinogen was purified from human plasma by the glycine precipitation procedure of Kazal et al. [27], modified as previously described [28]. Purified fibrinogen was stored in a buffer of 50 mM Tris, 0.1 M NaCl and 0.02% NaN_3 , pH 7.4, at -80°C .

Abbreviations used: GP IIb–IIIa, glycoprotein IIb–IIIa complex; C_{12}E_8 , hexa(ethylene glycol) mono-n-decyl ether; H12, the dodecapeptide from the C-terminus of the fibrinogen γ -chain with the sequence His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val; L10, the decapeptide from the C-terminus of the fibrinogen γ -chain, i.e., Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val; IPTG, isopropyl β -D-thiogalactopyranoside; Me_2SO , dimethyl sulphoxide.

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Glycoprotein IIb–IIIa complex purification

Glycoprotein IIb–IIIa was purified from outdated human platelets by the method of Fitzgerald et al. [29] with modifications as previously described [30]. The purified GP IIb–IIIa complex was stored at -80°C in a buffer containing 20 mM Tris, 150 mM NaCl, 1 mM CaCl_2 , 0.1% Triton X-100 and 0.05% NaN_3 , pH 7.4 (Buffer A).

Reversible dissociation of glycoprotein IIb–IIIa

To form reversibly dissociated subunits, i.e. subunits that retained the ability to reform the heterodimer complex, GP IIb–IIIa was dissociated by the method of Steiner et al. [30]. Unlabelled or ^{125}I -labelled GP IIb–IIIa was diluted in half with a buffer comprising 20 mM Tris, 100 mM NaCl, 0.05% Triton X-100, 0.05% NaN_3 and 1 mM EGTA (pH 9.0 at room temperature), incubated in a glass test tube at 37°C for 5–6 min, and immediately cooled to 4°C . If the glycoprotein was to be used as a mixture of dissociated subunits, CaCl_2 (5 mM final concn.) was added to the 4°C solution, followed by titration to pH 7.3.

H.p.l.c. separation of reversibly dissociated glycoprotein IIb and IIIa subunits

To obtain h.p.l.c.-separated GP IIb and GP IIIa subunits, the mixture of reversibly dissociated GP IIb and GP IIIa was cooled to 4°C and HCl was added to return the pH to 7.2–7.3. The subunits were immediately loaded on to a 300 mm $\times$ 7.5 mm Bio-Sil TSK-400 h.p.l.c. column (Bio-Rad, Richmond, CA, U.S.A.) attached to a Beckman model-110A h.p.l.c. pump at room temperature [31]. The column was pre-equilibrated for several weeks with a buffer comprising 20 mM Tris, 100 mM NaCl, 0.05% Triton X-100 and 0.05% NaN_3 , pH 7.2, such that the A_{280} of the column input and output were identical, and run at 0.5 ml/min. Fractions were collected every 6 min (3 ml). Each fraction was immediately cooled to 4°C and 2 mM CaCl_2 was added. To locate and pool subunits, aliquots of each fraction were examined on silver-stained [32] 7.5% polyacrylamide minigels (Bio-Rad). Pooled fractions of h.p.l.c.-separated GP IIb and GP IIIa were concentrated 8–10-fold by ultrafiltration (Amicon Concentrator with a YM 30 membrane, Amicon Corp., Danvers, MA, U.S.A.). For control samples, buffer alone was concentrated to the same extent. In some experiments, h.p.l.c.-separated GP IIb and GP IIIa were labelled with ^{125}I while attached to DEAE-Sephacel, as described below. Because the condition of the h.p.l.c. column changed with time, different degrees of separation of GP IIb and GP IIIa were achieved in different experiments.

Irreversible dissociation of glycoprotein IIb–IIIa

^{125}I -GP IIb–IIIa (labelled as described below) was diluted in half with a buffer composed of 20 mM Tris, 100 mM NaCl, 0.05% Triton X-100, 0.05% NaN_3 and 2.4 mM EDTA (pH 8.4 at 37°C), incubated for 110 min at 37°C and cooled on ice to 4°C . CaCl_2 (7 mM) was re-added, and the pH was adjusted to 7.2. Glycoprotein IIb and GP IIIa dissociated under these conditions do not retain the ability to reform heterodimer complexes under any known conditions [30]. As a control, the ^{125}I -GP IIb–IIIa complex was diluted into a buffer that had the same final concentration of components as above (i.e., 2.4 mM EDTA and 7 mM CaCl_2).

Labelling glycoprotein IIb–IIIa or the dissociated subunits with ^{125}I

Glycoprotein IIb–IIIa was labelled with ^{125}I in one of two methods designed to avoid labelled Triton X-100. In one method, GP IIb–IIIa or the h.p.l.c.-purified subunits were diluted with Buffer A lacking NaCl to decrease the NaCl concentration to 50 mM. The glycoprotein was attached to a 0.4 ml column of DEAE-Sephacel. The column was washed with a buffer composed of 20 mM Tris, 50 mM NaCl, the detergent hexa(ethylene glycol) mono-n-decyl ether (C_{12}E_6 ; 0.5 mg/ml) 1 mM CaCl_2 , and 0.02% NaN_3 (Buffer C) to remove the Triton X-100. The glycoprotein was labelled while attached to the column by applying 490 μl of Buffer C containing chloramine T (0.5 mg/ml) with 10 μl of ^{125}I (1 mCi). The column was washed with 0.5 ml of Buffer C containing sodium metabisulphite (0.49 mg/ml) to stop the iodination reaction. The free ^{125}I was removed by washing the column with 10 ml of Buffer A with 50 mM NaCl. The glycoprotein was eluted with Buffer A containing 500 mM NaCl and stored at -80°C . In the second method, GP IIb–IIIa in Buffer A was incubated with ^{125}I (1–2 mCi) and two Iodo-Beads as described [28], diluted 10-fold with Buffer A without Triton X-100 (Buffer B) and separated from free ^{125}I and unbound Triton X-100 by gel filtration through a PD 10 column (Pharmacia) equilibrated with Buffer B. Fractions containing ^{125}I -GP IIb–IIIa were pooled, and 0.1% Triton X-100 was re-added. The specific activity of the glycoprotein was determined by the protein precipitation microassay of Peterson [33], using BSA as the standard, and by ^{125}I radioactivity measurements. The ^{125}I -GP IIb–IIIa complex that was labelled by either procedure remained as a complex with a 'native' conformation, as demonstrated by the resistance of the heavy-chain GP IIb to hydrolysis by thrombin [34,35].

Coupling proteins or peptides to Sepharose

Proteins [fibrinogen, β -galactosidase (Worthington) or ovalbumin, transferrin, and immunoglobulin (from Sigma), glycine, at a concentration of 1 mg/ml of Sepharose, or Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK) (Biosearch, San Rafael, CA, U.S.A.; pre-extracted with ethyl acetate) at 6 mg/ml of Sepharose] were coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Glycine was used to block sites that had not reacted. The coupled Sepharose was resuspended in an equal volume of 0.02 M HEPES/0.1% Triton X-100/0.1 M NaCl, pH 7.35, and stored at -4°C . The peptide Cys-Tyr-Gly-His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val (CYGHHLLGGAKQAGDV) (Biosearch) was coupled by a stable sulphur–carbon bond via the cysteine residue to activated thiol-Sepharose 4B (Sigma) premodified with 1,6-bis(maleimido)hexane (Pierce) [36].

Binding assay

Assays to measure the binding of ^{125}I -GP IIb–IIIa complex or subunits to proteins or peptides coupled to Sepharose were carried out as follows. ^{125}I -labelled glycoprotein (10–20 μl) was mixed with or without excess unlabelled protein or buffer in 0.5 ml Eppendorf test tubes (Brinkman Instruments Co., Westbury, NY, U.S.A.), in order to determine non-specific and total binding respectively or to examine the effects of various proteins on ^{125}I -glycoprotein binding. Specific binding is defined as total minus non-specific ^{125}I -glycoprotein binding. Crystallized BSA (Figure 1 below) was from Sigma. Binding was initiated by addition of the protein–Sepharose (20 μl). The final volume was 250 μl unless otherwise indicated. Incubations were generally performed for 3 h at 4°C , except as indicated in the Figure

legends, with constant mixing on a Nutator (Clay Adams, Parsippany, NJ, U.S.A.). The final buffer composition for a typical binding experiment was approx. 15 mM Hepes, 2.5 mM CaCl_2 , 0.1 mM NaCl, 0.02 % NaN_3 , 0.1 % Triton X-100 and 3 mM Tris, pH 7.35–7.4. In some instances 25 mM Tris was used instead of Hepes, without apparent effect on the results. Incubations involving reversibly or irreversibly dissociated but unseparated ^{125}I -GP IIb and ^{125}I -GP IIIa also contained EGTA or EDTA from the added ^{125}I -GP IIb-IIIa, as described above. Thus, in the experiment with reversibly dissociated but unseparated subunits, the final Ca^{2+} concentration was ~ 2.7 mM and that of EGTA was 0.02 mM. With the irreversibly dissociated subunits, the final Ca^{2+} concentration in the binding assay was ~ 2.8 mM and the EDTA concentration was ~ 0.1 mM. In experiments with h.p.l.c.-separated subunits, the EGTA was most likely removed during the h.p.l.c. step and the final Ca^{2+} concentration in the binding assay was ~ 2.6 mM. Protein-Sepharose was sedimented at 8370 *g* for 1 min in a Beckman Microfuge B (Beckman Instruments, Palo Alto, CA, U.S.A.), resuspended briefly in 250 μl of an ice-cold buffer consisting of 0.02 M Hepes, 3 mM CaCl_2 , 0.1 % Triton X-100, 0.1 M NaCl and 0.02 % NaN_3 , pH 7.35, and quickly re-sedimented. The tips of the tubes containing the Sepharose pellets with bound ^{125}I -labelled protein were cut off, and the radioactivity of each was quantified in a γ -radiation counter.

SDS/PAGE and densitometry

^{125}I -Labelled glycoproteins were electrophoresed on SDS/7.5 % polyacrylamide gels under reducing conditions by the method of Laemmli [37] as described in [35]. The ^{125}I -labelled glycoproteins were detected by autoradiography of dried gels at -80°C , using Kodak X-OMAT AR film with du Pont Cronex intensifying screens. The intensity of bands on X-ray film was determined by densitometry on a Hoefer instrument.

Platelet aggregation

Washed human platelets were prepared on the day of use from drug-free donors as described [38] and the platelets were resuspended in a Hepes/Tyrod's buffer (5.5 mM glucose, 0.14 M NaCl, 11.9 mM NaHCO_3 , 1.4 mM CaCl_2 , 0.5 mM MgCl_2 , 2.7 mM KCl and 10 mM Hepes, pH 7.4) to a concentration of $3.3 \times 10^8/\text{ml}$. Stirred platelets (450 μl) were pretreated for 1 min at 37°C with β -galactosidase (Cooper Biomedical, Philadelphia, PA, U.S.A.), isopropyl β -D-thiogalactopyranoside (IPTG) (Sigma) or Hepes/Tyrod's Buffer (50 μl) before addition of A23187 (0.12 μM) or thrombin (0.05 unit/ml). Aggregation was measured in a Lumi aggregometer (Chrono-Log Corp., Havertown, PA, U.S.A.) by the turbidometric method originally described by Born [39].

Calculations

In calculations to determine the molar amounts of glycoprotein added or bound, we assumed M_r values of 240000 for GP IIb-IIIa, 140000 for GP IIb and 100000 for GP IIIa [2].

RESULTS

Many of the experiments to characterize ^{125}I -GP IIb-IIIa complex binding to immobilized fibrinogen were performed at 4°C because these conditions were necessary to maintain the glyco-

protein subunits in a dissociated state for latter experiments. A time-course study carried out over 29 h at 4°C indicated that the binding of ^{125}I -GP IIb-IIIa (0.7 nM) to fibrinogen-Sepharose approached maximal levels within 3 h (results not shown); this time of incubation was therefore used in most experiments. The binding of ^{125}I -GP IIb-IIIa was specific and saturable since it was maximally inhibited by ~ 100 fold excess unlabelled GP IIb-IIIa or by soluble fibrinogen, but much less so by other proteins such as transferrin, orosomucoid, or BSA (Figure 1). Only a much higher concentration of BSA, representing a 4000-fold molar excess over ^{125}I -GP IIb-IIIa, was found to partially suppress total, non-specific and specific binding. Unexpectedly, β -galactosidase, which was initially used as a control protein, was found to strongly inhibit ^{125}I -GP IIb-IIIa binding (Figure 1). In a further test of specificity, ^{125}I -GP IIb-IIIa (~ 0.1 nM) was added to several different proteins immobilized on Sepharose. If the specific binding of ^{125}I -GP IIb-IIIa to fibrinogen-Sepharose is set at 100 % (0.73 fmol), binding to immobilized β -galactosidase was similar (98.9 %), whereas binding to beads lacking protein (reactive sites blocked with glycine) was only 8.7 %. ^{125}I -GP IIb-IIIa binding to other immobilized proteins was also low: egg albumin, 28.2 %; transferrin, 20.6 %; and IgG, 19.9 %.

Since β -galactosidase bound to ^{125}I -GP IIb-IIIa, inhibited ^{125}I -GP IIb-IIIa binding to fibrinogen (Figure 1) and contains the Arg-Gly-Asp-Phe (RGDF) sequence [40] (see the Discussion section), it was tested for its ability to inhibit platelet aggregation. When aggregation of washed platelets was induced by the Ca^{2+} ionophore A23187 or thrombin, β -galactosidase inhibited aggregation in a concentration-dependent manner ($\text{IC}_{50} \sim 0.5 \mu\text{M}$). This inhibition was not reversed by up to 16 mM IPTG, a potent inhibitor of the enzymic activity of β -galactosidase [41] (results not shown), suggesting that β -galactosidase inhibits aggregation by a mechanism independent of its lactase activity, most likely by blocking fibrinogen binding to GP IIb-IIIa on the platelet surface.

The reversibility of ^{125}I -GP IIb-IIIa binding to the immobilized fibrinogen or β -galactosidase was tested by incubating ^{125}I -GP IIb-IIIa (3 nM) with the immobilized proteins for 2 h, followed by the addition of > 100 -fold excess of unlabelled GP IIb-IIIa or control buffer. Even after 1.5 h, ^{125}I -GP IIb-IIIa binding to immobilized fibrinogen or β -galactosidase most often showed no

Figure 1 Selective inhibition of ^{125}I -GP IIb-IIIa binding to immobilized fibrinogen by excess unlabelled proteins

Fibrinogen immobilized on Sepharose (equivalent of $\sim 0.1 \mu\text{M}$ fibrinogen) was incubated with ^{125}I -GP IIb-IIIa (~ 0.5 nM) in the presence of various concentrations of unlabelled proteins as indicated. Each point represents the average of two or more samples. Standard errors are indicated.

Figure 2 Binding of reversibly dissociated ^{125}I -labelled GP IIB and GP IIIa to immobilized fibrinogen

^{125}I -GP IIB-IIIa was dissociated under 'reversible' conditions by treatment with EGTA, pH 8.0, for 5 min at 37 °C (see the Experimental section). The temperature of the ^{125}I -glycoprotein was then decreased to 4 °C, excess Ca^{2+} was re-added, and the pH returned to 7.2. As a control, ^{125}I -GP IIB-IIIa was also diluted directly into the final buffer used above except at 4 °C initially so as to maintain the heterodimer complex. The ^{125}I -labelled glycoprotein preparations were then incubated with fibrinogen-Sepharose for 3 h at 4 °C in the presence or absence of 0.21 μM unlabelled GP IIB-IIIa (final vol. 250 μl). Specific binding (total minus non-specific binding) is shown and is the average of triplicate samples. Solid circles represent the binding of ^{125}I -GP IIB-IIIa complex and open circles the binding of dissociated ^{125}I -GP IIB and ^{125}I -GP IIIa. Standard error bars are shown and calculated based on rules for propagation of error as described in [54]. Note that the concentration of ^{125}I -glycoprotein added (on the abscissa) refers to the concentration of each individual glycoprotein species.

evidence of reversal, indicating a very strong interaction between ^{125}I -GP IIB-IIIa and the immobilized proteins. This interaction was most likely non-covalent, however, since ^{125}I -GP IIB-IIIa could be dissociated from the immobilized fibrinogen with SDS.

^{125}I -glycoprotein IIB-IIIa binding to the immobilized fibrinogen could not be inhibited by many agents known to inhibit fibrinogen binding to platelets [11,42–45] or to purified GP IIB-IIIa incorporated into phospholipid vesicles [28]. Fibrinogen normally binds to GP IIB-IIIa on activated platelets with a K_d of $\sim 150 \text{ nM}$ [42]. The RGDS peptide from the fibrinogen α -chain and the L10 peptide from the fibrinogen γ -chain block fibrinogen (0.1–0.2 μM) binding to platelets with K_i values of 16 μM and 50 μM respectively, and the effect of both peptides together is additive [46]. However, when immobilized fibrinogen (representing 0.1 μM fibrinogen) was incubated with 0.2 nM ^{125}I -GP IIB-IIIa in the presence of these peptides, significant inhibition was not observed [$100 \pm 2.0\%$ (SEM) ^{125}I -GP IIB-IIIa binding in the absence of peptides, $111 \pm 2.6\%$ with 0.5 mM RGDS, $91.1 \pm 4.1\%$ with 0.5 mM L10 peptide and $122.3 \pm 6.9\%$ with 0.5 mM of each peptide together]. Significant inhibition was also not observed regardless of the incubation time (1 or 3 h) or temperature (4 or 22 °C) or if immobilized fibrinogen was present at a 10-fold lower concentration. The RGDS peptide was also ineffective in inhibiting ^{125}I -GP IIB-IIIa binding to immobilized β -galactosidase. In addition, the anti-(GP IIB-IIIa) monoclonal antibodies 10E5 [44] and 7E3 [45] in concentrations ranging from ~ 30 to 3000-fold over that of ^{125}I -GP IIB-IIIa were ineffective in inhibiting ^{125}I -GP IIB-IIIa binding relative to control antibodies. However, 10E5 and 7E3 specifically immunoprecipitated ^{125}I -GP IIB-IIIa under similar conditions, suggesting that the lack of immunoinhibition may be due to a distinct mechanism of ^{125}I -GP IIB-IIIa binding to the immobilized fibrinogen. The calcium chelator EDTA (3.3 mM

with 1.7 mM CaCl_2) also did not significantly inhibit ^{125}I -GP IIB-IIIa binding to the immobilized fibrinogen either at 4 °C or at room temperature (results not shown). These experiments were performed under conditions that would be expected to maintain GP IIB-IIIa as a complex [30]. Thus ^{125}I -GP IIB-IIIa binding to fibrinogen immobilized on Sepharose appears to occur in a Ca^{2+} -, RGDS-, and L10 peptide-independent manner. The sites on immobilized fibrinogen that mediate ^{125}I -GP IIB-IIIa binding may be distinct from, or involve more than, the RGDS- and the L10-peptide-containing sites. Conversely, distinct sites on GP IIB-IIIa other than those that bind to RGDS and the decapeptide must also be involved.

To identify which glycoprotein subunit(s) mediate GP IIB-IIIa binding to the immobilized fibrinogen, it was necessary to determine whether ^{125}I -GP IIB-IIIa that was dissociated under reversible conditions [30] retained fibrinogen-binding activity. As Figure 2 shows, when ^{125}I -GP IIB-IIIa was dissociated under reversible conditions, the mixture of dissociated, but unseparated, subunits bound as well to fibrinogen-Sepharose as did the ^{125}I -GP IIB-IIIa complex. The extent of dissociation of the GP IIB-IIIa complex under these conditions is $\sim 90\%$ [30]. In contrast, when the ^{125}I -GP IIB-IIIa complex was dissociated so that ^{125}I -GP IIB and ^{125}I -GP IIIa were no longer able to reform heterodimer complexes (i.e. under irreversible conditions), the specific binding was greatly reduced (results not shown).

To examine whether the results presented in Figure 2 were affected by an exchange between the dissociated ^{125}I -labelled glycoproteins and the added unlabelled GP IIB-IIIa, the following experiment was performed. ^{125}I -glycoprotein IIB and ^{125}I -GP IIIa formed under reversible conditions were incubated with and without excess unlabelled GP IIB-IIIa under conditions of a typical binding assay, and analysed by sedimentation through sucrose gradients [2]. In both cases, the ^{125}I -GP IIB and ^{125}I -GP IIIa sedimented in positions characteristic of the dissociated subunits, but not of the GP IIB-IIIa complex (results not shown). Thus the possibility of significant levels of exchange occurring between dissociated and complexed glycoprotein subunits under the conditions of these experiments is unlikely.

Because ^{125}I -GP IIB-IIIa binding to the immobilized fibrinogen was not inhibited by synthetic peptides from the putative platelet-binding domains of fibrinogen or fibronectin, we examined the ability of the ^{125}I -labelled GP IIB-IIIa complex to bind directly to similar peptides immobilized on Sepharose, as has been shown for unlabelled platelet GP IIB-IIIa [47,48]. Indeed, we found that ^{125}I -GP IIB-IIIa bound in a specific manner to an RGDS-containing peptide or to a pentadecapeptide that contains H12, the 12-amino-acid sequence of the C-terminus of the fibrinogen γ -chain (Table 1). This binding was specific in that it was inhibited by an excess of related peptides in solution. However, whereas a mixture of unseparated ^{125}I -GP IIB and ^{125}I -GP IIIa subunits that were dissociated under reversible conditions did not bind in a specific manner to peptides containing either the RGD or H12 sequence, the same preparation of dissociated ^{125}I -GP IIB and ^{125}I -GP IIIa subunits did retain the ability to bind to the immobilized fibrinogen (Table 1), in agreement with earlier results.

Two approaches were used to determine which of the dissociated subunits was responsible for the binding to immobilized fibrinogen detected in Figure 2 and Table 1. Dissociated GP IIB and GP IIIa subunits were separated from one another by h.p.l.c. In the first approach, the direct binding of ^{125}I -GP IIB, GP IIIa or the complex was detected by incubating each with immobilized fibrinogen and eluting the bound material with SDS/electrophoresis sample buffer, followed by autoradiography of SDS/polyacrylamide gels (Figure 3). Lanes

Table 1 Reversibly dissociated glycoprotein subunits, in contrast with the complex, do not bind to immobilized adhesive peptides but do bind to immobilized fibrinogen

The same preparation of ^{125}I -GP IIb–IIIa complex or reversibly dissociated ^{125}I -GP IIb and ^{125}I -GP IIIa subunits (4×10^{-13} mol of each species in $300 \mu\text{l}$ or 1.3×10^{-12} M) was incubated with fibrinogen– or GRGDSPK–Sephacrose. Approx. 9×10^{-13} mol (or 3×10^{-12} M) of reversibly dissociated ^{125}I -GP IIb and ^{125}I -GP IIIa subunits were incubated with Sephacrose–CYGHHLLGGAKQAGDV. Binding to the immobilized fibrinogen or peptides was determined in the absence (total binding) or the presence (non-specific binding) of either free fibrinogen ($2.35 \mu\text{M}$), GRGDSP (0.25 mM) or HHLGGAKQAGDV (0.33 mM) respectively. Data represent the mean ($\pm$ S.E.M.) of triplicate determinations. Specific binding is total binding minus non-specific binding. S.E.M. values for specific-binding values were calculated based on rules for propagation of error [54].

Parameter	Immobilized fibrinogen		Immobilized GRGDSPK		Immobilized CYGHHLLGGAKQAGDV	
	^{125}I -GP IIb–IIIa complex	^{125}I -Dissociated subunits	^{125}I -GP IIb–IIIa complex	^{125}I -Dissociated subunits	^{125}I -GP IIb–IIIa complex	^{125}I -Dissociated subunits
Total binding (fmol)	14.9 ± 0.1	16.8 ± 1.2	82.5 ± 9.1	28.7 ± 1.8	36.1 ± 1.3	16.1 ± 0.8
Nonspecific binding (fmol)	3.9 ± 0.1	3.7 ± 0.2	46.5 ± 1.2	28.9 ± 1.3	17.8 ± 0.5	16.1 ± 1.4
Specific binding (fmol)	11.0 ± 0.2	13.1 ± 1.2	36.0 ± 9.1	-0.2 ± 2.2	18.3 ± 1.4	0 ± 1.6

Figure 3 Direct binding of ^{125}I -GP IIb–IIIa and h.p.i.c.-separated ^{125}I -GP IIb and ^{125}I -GP IIIa to immobilized fibrinogen

Lanes 1–3, fibrinogen immobilized on Sepharose was incubated with ^{125}I -GP IIb–IIIa complex (26 fmol in $250 \mu\text{l}$ of total incubation volume) in the absence (lane 1) or presence of excess unlabelled GP IIb–IIIa ($1.5 \mu\text{M}$, lane 2) or fibrinogen ($1 \mu\text{M}$, lane 3). Bound ^{125}I -labelled protein was eluted as described in the Experimental section and observed by autoradiography of SDS/7.5% polyacrylamide gels. Lanes 4–5, h.p.i.c.-purified GP IIb (lane 4) and GP IIIa (lane 5) that were labelled with ^{125}I and used in subsequent experiments. Twice the volume of ^{125}I -GP IIb relative to ^{125}I -GP IIIa was added per lane, or $\sim 1.4 \times 10^6$ c.p.m. of each. Lanes 6–9, fibrinogen–Sephacrose was incubated with ^{125}I -GP IIb (18 fmol in $250 \mu\text{l}$) in the absence (lane 6) or the presence of excess unlabelled GP IIb–IIIa ($3.3 \mu\text{M}$, lane 7), fibrinogen ($3.3 \mu\text{M}$, lane 8) or orosomucoid ($3.3 \mu\text{M}$, lane 9), and treated as in lanes 1–3 above. Lanes 10–13, fibrinogen–Sephacrose was incubated with ^{125}I -GP IIIa (21 fmol in $250 \mu\text{l}$) in the absence (lane 10) or presence of excess unlabelled GP IIb–IIIa ($3.3 \mu\text{M}$, lane 11), fibrinogen ($3.3 \mu\text{M}$, lane 12), or orosomucoid ($3.3 \mu\text{M}$, lane 13), and treated as in lanes 1–3 above. Note that although the intensities of the bands can be compared within groups, they cannot necessarily be compared between groups because the Figure was compiled from separate experiments and the autoradiographs were exposed under optimal conditions for each group.

1–3 show the specific binding of ^{125}I -GP IIb–IIIa. Note that GP IIIa appears darker than the heavy chain of GP IIb in lane 1, because of the characteristically increased labelling of GP IIIa with ^{125}I [49]. The h.p.i.c.-separated ^{125}I -GP IIb and ^{125}I -GP IIIa preparations, which appeared $> 95\%$ pure as judged by densitometry, are shown in lanes 4 and 5. When ^{125}I -GP IIb was incubated with immobilized fibrinogen, ^{125}I -GP IIb bound specifically (lane 6), since its binding was inhibited by excess unlabelled GP IIb–IIIa (lane 7) or fibrinogen (lane 8), but not by orosomucoid (lane 9). More ^{125}I -GP IIIa was present than expected in lane 6 and 9, based on the apparent purity of the ^{125}I -GP IIb preparation. However, densitometric analyses of the

ratios of ^{125}I -GP IIb to GP IIIa in lanes 6 and 9 versus lanes 1–3 indicated that $> 80\%$ of the observed specific binding must have been due to dissociated ^{125}I -GP IIb as opposed to a potentially small amount of contaminating GP IIb–IIIa complex. Purified ^{125}I -GP IIIa also bound specifically to the fibrinogen–Sephacrose as demonstrated in lanes 11–13.

In the second approach, the ability of different concentrations of the individual unlabelled subunits to inhibit ^{125}I -GP IIb–IIIa binding to the immobilized fibrinogen was compared with that of unlabelled GP IIb–IIIa. Both subunits inhibited binding at concentrations similar to that of GP IIb–IIIa, suggesting that both subunits have fibrinogen-binding activity (results not shown).

DISCUSSION

The results of the present study demonstrate that purified GP IIb–IIIa and immobilized fibrinogen can bind to one another with characteristics distinct from those of soluble reversible fibrinogen binding to activated platelets. These distinct characteristics include the inability of EDTA, anti-(GP IIb–IIIa) monoclonal antibodies or the RGDS or L10 peptides derived from sequences within the fibrinogen α - and γ -chains to inhibit ^{125}I -GP IIb–IIIa binding to immobilized fibrinogen, the apparently irreversible nature of this binding and the ability of the dissociated GP IIb and GP IIIa subunits to bind to the fibrinogen. These results suggest that the observed binding is mediated at least in part by sites on GP IIb–IIIa and fibrinogen that are separate from those that mediate reversible fibrinogen binding to platelets.

The irreversible (or very slowly reversible), yet apparently non-covalent, binding of ^{125}I -GP IIb–IIIa to immobilized fibrinogen (or β -galactosidase) observed here and in other studies with intact platelets [19–22] could be explained in part by co-operation between multiple binding sites on the immobilized fibrinogen and GP IIb–IIIa. At least some of these binding sites must be separate from those previously described, however, for the following reasons. First, excess unlabelled GP IIb–IIIa and fibrinogen, but not agents such as monoclonal antibody 10E5 or EDTA, inhibited this binding. Second, the dissociated subunits of GP IIb–IIIa bound to immobilized fibrinogen but not to peptides derived from putative binding domains of fibrinogen. Much evidence exists to show that when fibrinogen is converted into fibrin [23,24], immobilized on some artificial surfaces [26], or even bound to platelets [25], its conformation is altered such that previously cryptic epitopes become exposed, and previously

exposed sites may become inaccessible. It is possible that newly exposed binding sites could co-operate with one another or with previously described sites to cause high-affinity interactions with distinct properties, as observed here. These properties are not associated with all types of immobilized fibrinogen, however. Fibrinogen immobilized on certain plastics has been shown to bind activated platelets [50] or GP IIB-IIIa incorporated into liposomes [51] in a peptide- and antibody-sensitive manner.

The separate GP IIB and GP IIIa subunits each appear to have binding sites that recognize immobilized fibrinogen when generated under conditions known to maintain a certain degree of conformational integrity, that is, when generated under conditions previously determined to result in reversibly dissociated subunits [30]. This binding activity is much diminished when the subunits are formed under more harsh conditions. The subunits generated under reversible conditions do not recognize immobilized H12- and RGD-containing peptides, suggesting that putative recognition sites within these subunits either are not efficiently exposed, perhaps because of subunit folding, or do not bind with high enough affinity unless the subunits are complexed. The former possibility seems the most likely, since Gulino et al. recently found that a fragment of GP IIB (amino acid residues 171–464) when expressed in and isolated from *Escherichia coli*, bound to fibrinogen in a Ca^{2+} -dependent manner and binding was inhibited by RGD- and L10-containing peptides [52].

Of additional interest was the finding that β -galactosidase bound to GP IIB-IIIa, inhibited GP IIB-IIIa binding to fibrinogen and inhibited platelet aggregation. Although the mechanism by which β -galactosidase binds to GP IIB-IIIa is unknown, it may in part be related to the RGDF sequence, which is present in each of this tetramer's four identical subunits [40]. If this sequence is functional when β -galactosidase is soluble, it may bind to platelet GP IIB-IIIa and inhibit aggregation, and may bind to soluble ^{125}I -GP IIB-IIIa, thereby causing a steric inhibition of binding to immobilized fibrinogen. The finding that the related RGDS sequence did not inhibit ^{125}I -GP IIB-IIIa binding to either immobilized β -galactosidase or fibrinogen suggests that either the RGD sequences are not operational in the immobilized proteins, are in an altered conformation that results in a very strong interaction with GP IIB-IIIa, and/or that these sequences function in cooperation with other sites in the immobilized proteins.

Whether the mechanism of GP IIB-IIIa binding to immobilized fibrinogen observed here occurs under physiological or pathological conditions or to fibrinogen coated on prosthetic surfaces is currently unknown. However, our observations suggest that alternative mechanisms of fibrin(ogen) binding to GP IIB-IIIa are possible. Potentially relevant situations include the observation that a significant proportion of platelet binding to 'immobilized' fibrin clots is not inhibited by an anti-(GP IIB-IIIa) monoclonal antibody [21]. Furthermore, Savage and Ruggeri observed that platelet binding to immobilized fibrinogen, although mediated by GP IIB-IIIa, does not require platelet activation, and results in an apparently irreversible binding of spread platelets to the fibrinogen [22], properties clearly distinct from the reversible phase of soluble fibrinogen binding to activated platelets. Finally, fibrinogen and fibrin protofibrils in solution bind to activated platelets in an initially reversible and subsequently irreversible manner that is not well understood [19,20,53].

In summary, our results suggest, first, that each subunit of the GP IIB-IIIa complex can bind to immobilized fibrinogen; secondly, that the binding of GP IIB-IIIa by an RGDS- or γ -chain H12-mediated mechanism appears to require the intact GP IIB-IIIa complex as opposed to the dissociated full-length

subunits; and finally, that GP IIB-IIIa and the individual subunits can bind to immobilized fibrinogen through a novel mechanism, independent of the RGDS and γ -chain H12 sites, which may depend on the conformation of fibrinogen. The additional binding sites on GP IIB-IIIa and fibrinogen may contribute to platelet-fibrinogen interactions under specific circumstances.

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