Ca^{2+}-dependent Binding of a Synthetic Arg-Gly-Asp (RGD) Peptide to a Single Site on the Purified Platelet Glycoprotein IIb-IIIa Complex*

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The platelet glycoprotein IIb-IIIa complex (GP IIb-IIIa) is a member of the integrin receptor family that recognizes adhesive proteins containing the Arg-Gly-Asp (RGD) sequence. In the present study the binding characteristics of the synthetic hexapeptide Tyr-Asn-Arg-Gly-Asp-Ser (YNRGDS, a sequence present in the fibrinogen \( \gamma \)-chain at position 570–575) to purified GP IIb-IIIa were determined by equilibrium dialysis. The binding of \( ^{125}I \)-YNRGDS to GP IIb-IIIa was specific, saturable, and reversible. The apparent dissociation constant was \( 1.0 \pm 0.2 \) \( \mu \)mol, and the maximal binding capacity was 0.92 ± 0.02 mol \( ^{125}I \)-YNRGDS/mol of GP IIb-IIIa, indicating that GP IIb-IIIa contains a single binding site for RGD peptides. The binding of \( ^{125}I \)-YNRGDS to purified GP IIb-IIIa showed many of the characteristics of fibrinogen binding to activated platelets: the binding was inhibited by fibrinogen, by the monoclonal antibody AsA1, and by the dodecapeptide from the C terminus of the fibrinogen \( \gamma \)-chain. In addition, the binding of \( ^{125}I \)-YNRGDS to GP IIb-IIIa was divalent cation-dependent. Our data suggest that two divalent cation binding sites must be occupied for YNRGDS to bind: one site is specific for calcium and is saturated at 1 \( \mu \)mol free Ca^{2+}, whereas the other site is less specific and reaches saturation at millimolar concentrations of either Ca^{2+} or Mg^{2+}. The results of the present study support the hypothesis that the RGD domains within the adhesive proteins are responsible for their binding to GP IIb-IIIa.

Adhesion of platelets to extracellular matrices and platelet aggregation are crucial events in hemostasis and thrombosis. The glycoprotein IIb-IIIa complex (GP IIb-IIIa) is a member of the integrin receptor family that recognizes adhesive proteins containing the Arg-Gly-Asp (RGD) sequence. The platelet glycoprotein IIb-IIIa complex (GP IIb-IIIa) is a member of the integrin receptor family that recognizes adhesive proteins containing the Arg-Gly-Asp (RGD) sequence. In the present study the binding characteristics of the synthetic hexapeptide Tyr-Asn-Arg-Gly-Asp-Ser (YNRGDS, a sequence present in the fibrinogen \( \gamma \)-chain at position 570–575) to purified GP IIb-IIIa were determined by equilibrium dialysis. The binding of \( ^{125}I \)-YNRGDS to GP IIb-IIIa was specific, saturable, and reversible. The apparent dissociation constant was \( 1.0 \pm 0.2 \) \( \mu \)mol, and the maximal binding capacity was 0.92 ± 0.02 mol \( ^{125}I \)-YNRGDS/mol of GP IIb-IIIa, indicating that GP IIb-IIIa contains a single binding site for RGD peptides. The binding of \( ^{125}I \)-YNRGDS to purified GP IIb-IIIa showed many of the characteristics of fibrinogen binding to activated platelets: the binding was inhibited by fibrinogen, by the monoclonal antibody AsA1, and by the dodecapeptide from the C terminus of the fibrinogen \( \gamma \)-chain. In addition, the binding of \( ^{125}I \)-YNRGDS to GP IIb-IIIa was divalent cation-dependent. Our data suggest that two divalent cation binding sites must be occupied for YNRGDS to bind: one site is specific for calcium and is saturated at 1 \( \mu \)mol free Ca^{2+}, whereas the other site is less specific and reaches saturation at millimolar concentrations of either Ca^{2+} or Mg^{2+}. The results of the present study support the hypothesis that the RGD domains within the adhesive proteins are responsible for their binding to GP IIb-IIIa.

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‡ The abbreviations used are: GP IIb-IIIa, glycoprotein IIb-IIIa complex; YNRGDS, Tyr-Asn-Arg-Gly-Asp-Ser; HHLGGAKQAGDV, His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val (dodecapeptide); HGDS, His-Gly-Asp-Ser; RADS, Arg-Ala-Asp-Ser; AES, ami-noethylylglycine; EGT, [ethylenebis(oxyethyleneimino)]tetraacetic acid; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography.
EXPERIMENTAL PROCEDURES

Peptides and Peptide Affinity Matrix—Peptides were synthesized by the classical technique (19) using various coupling procedures and a combination of acid-labile protecting groups. The peptides were purified by preparative high performance liquid chromatography (HPLC) using a LiChrosorb RP-18 column (Merck, Darmstadt, FRG). The purity of each peptide exceeded 95% as assessed by thin layer chromatography, analytical HPLC, mass spectroscopy, and amino acid analyses using a LiQuimat III analyzer (Labotron, Munich, FRG).

The peptide Tyr-Asn-Arg-Gly-Asp-Ser (YNRGDS) was labeled with $^{125}$I (2 mCi) by the chloramine-T method (20). The labeled material was injected into a C18 HPLC column (Vydac) pre-equilibrated in 0.1% aqueous trifluoroacetic acid and acetonitrile (95:5, v/v). The hexapeptide, the mono- and the di-iodinated hexapeptide were separated by applying a gradient of 5 to 30% acetonitrile. The monoiiodinated form of YNRGDS was finally lyophilized from a solution containing 2% d-lactose, 0.05% bovine serum albumin, and 3 mM butanesulfonic acid in 100 mM sodium phosphate buffer. The $^{125}$I-labeled YNRGDS was stored at 4°C and used within 2 weeks. The specific activity was 2200 C/mmole peptide (81 TBq/mmole).

The affinity matrix was prepared by incubating 120 mg of amine-ethylglycine-Arg-Gly-Asp-Ser (Aeg-RGDS) with 10 ml of CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden) according to the method described by Pytel et al. (9). The spacer aminooxyethylcine (Aeg) was introduced to facilitate coupling.

GP IIb-IIIa Purification—Outdated, washed human platelets were lysed with 1% Triton X-100, 0.15 M NaCl, 20 mM Tris-HCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 0.02% NaN$_3$, 10 μM leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.3, for 15 h. The glycoproteins were isolated at room temperature by concanavalin A affinity chromatography according to Fitzgerald et al. (21) using 0.1% Triton X-100, 0.15 M NaCl, 20 mM Tris-HCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 0.05% NaN$_3$, pH 7.0, as column buffer (buffer A). The concanavalin A-retained glycoproteins were eluted with buffer A containing 100 mM α-methyl-d-mannose and applied onto an Aeg-RGDS-Sepharose column. The bound GP IIb-IIIa complexes were eluted by including RGDS (3 mM) in buffer A. To remove the tetrapeptide the purified GP IIb-IIIa was extensively dialyzed at 4°C versus buffer A. Removal of RGDS was monitored by including a trace amount of $^{125}$I-labeled YNRGDS in the dialysis bag.

For electrophoresis, aliquots of the platelet lysate, of different column fractions, and of the purified GP IIb-IIIa were treated with sodium dodecyl sulfate (SDS), reduced with β-mercaptoethanol, and examined on 7.5% SDS-polyacrylamide gels (22). The gels were stained with Coomassie Brilliant Blue R-250, and the relative amounts of GP IIb and GP IIIa were determined by densitometry using a TLC Scanner II (Camag, Switzerland) connected to a Sanyo personal computer.

Peptide Binding Assay—Binding of $^{125}$I-labeled YNRGDS to purified GP IIb-IIIa was determined by equilibrium dialysis at pH 7.4 using a 20-cell dialyzer (Dianorm, FRG). Dialysis membranes (Spectrapor) with a molecular weight cutoff of 12,000–14,000 were presoaked in buffer A were used to separate each Teflon cell into two 200-μl compartments. The GP IIb-IIIa complex was introduced in one cell half, whereas $^{125}$I-labeled YNRGDS (1–5 x 10$^3$ cpm) was added to both compartments in equal concentrations. In some experiments, various concentrations of monoclonal antibodies or other proteins such as fibrinogen (IMCO, Stockholm, Sweden), fibronectin (Sigma), or bovine serum albumin (Sigma) were added to the same compartment as the GP IIb-IIIa complex. Peptides, however, were routinely included in both compartments. The cells were slowly rotated for 4 h in a water bath at 25°C. Following dialysis the two compartments of each cell were emptied and the $^{125}$I radioactivity was determined in a γ-counter (Kontron, Switzerland). Before and after dialysis the protein concentrations were determined by the method of Bradford (23).

In addition, the total amino acid content of each GP IIb-IIIa stock solution was determined.

For the binding experiments in presence of various Ca$^{2+}$ concentrations, the GP IIb-IIIa complexes were dialyzed into buffer A without Mg$^{2+}$. The amounts of EGTA and Ca$^{2+}$ required to achieve the desired free Ca$^{2+}$ concentrations at pH 7.4 and 25°C, in the presence of absence of 1 mM Mg$^{2+}$, were calculated with an iterative computer program as described previously (24). The extent of dissociation of the GP IIb-IIIa complex into subunits was analyzed immediately following dialysis by sedimentation through linear 5 to 25% sucrose gradients as described by Fitzgerald and Philips (25).

Monoclonal Antibodies—The monoclonal antibody A2Ag was kindly provided by Drs. J. Bennett and S. Shattil, University of Pennsylvania School of Medicine, Philadelphia. The monoclonal antibody pl-21 was produced in our laboratory by standard techniques (26). Both monoclonal antibodies are directed against the GP IIb-IIIa complex and belong to the immunoglobulin G class.

Platelet Aggregation—Platelets were isolated from fresh human plasma by gel filtration on Sepharose CL-2B (Pharmacia, Uppsala) in Tyrode’s buffer containing 0.2% bovine serum albumin, 1 mM CaCl$_2$, and 1 mM MgCl$_2$ (27). The gel-filtered platelets (1.5 x 10$^9$/ml) were incubated in the presence of 0.6 μM fibrinogen and various amounts of RGDS or YNRGDS. Aggregation was initiated by addition of 10 μM ADP and measured in a dual-channel aggregometer (ELVI, Milan, Italy) at 37°C. The concentration of peptide required for 50% inhibition of aggregation was designated as IC$_{50}$.

RESULTS

Purification of GP IIb-IIIa—Concanavalin A and Aeg-RGDS affinity chromatography were used to purify GP IIb-IIIa from outdated human platelets. Fig. 1 shows the SDS-polyacrylamide gel separations of various fractions. Densitometric scans of the material eluted from the Aeg-RGDS affinity column showed that GP IIb and GP IIIa represented >96% of the Coomassie-stained protein (Fig. 1, lane 5). Most of the GP IIb-IIIa present in the Triton X-100 lysate bound to the concanavalin A-Sepharose column. Of the GP IIb and GP IIIa present in the concanavalin A-retained material (Fig. 1, lane 3) between 5 and 10% were bound by the Aeg-RGDS-Sepharose column as determined by densitometry. When the flow-through of the Aeg-RGDS column (Fig. 1, lane 4) was
reapplied onto another Aeg-RGDS column of the same size, 20 times less GP IIb-IIIa were bound by this second column than by the first column. This indicates that 90–95% of the GP IIb-IIIa eluted from the concanavalin A affinity column was incapable of binding to the Aeg-RGDS column.

For the binding studies, purified GP IIb-IIIa was dialyzed to remove the tetrapeptide RGDS used for elution from the Aeg-RGDS column. When the dialyzed protein was reapplied onto an Aeg-RGDS column more than 95% of GP IIb-IIIa rebound. Analysis of the dialyzed protein by sedimentation through linear 5–25% sucrose gradients showed that more than 95% of GP IIb and GP IIIa sedimented as the heterodimer complex (data not shown).

Inhibition of Platelet Aggregation by RGDS and YNRGDS—The peptide YNRGDS, present in the fibrinogen a-chain at position 570–575 (28), was used as a prototype of the RGD-containing peptides. To compare the inhibitory activity of YNRGDS with that of RGDS, ADP-induced aggregation of gel-filtered platelets was performed. In presence of 0.6 μM fibrinogen, 32 μM RGDS and 39 μM YNRGDS were required for 50% inhibition (IC50), indicating that platelets had similar affinities for both RGD-containing peptides.

125I-YNRGDS Binding Assay—The 125I-labeled YNRGDS was routinely added to both compartments of the dialysis cells, and dialysis was performed for 4 h at 25 °C as described under "Experimental Procedures." Control experiments showed that equilibrium was reached within 3 h, when the labeled peptide was introduced in only one cell half.

Nonspecific binding of 125I-YNRGDS was measured in the presence of an excess of unlabeled peptide. In presence of 0.82 μM 125I-YNRGDS a 50-fold excess of unlabeled peptide inhibited total binding by 90%, whereas in presence of 0.12 μM 125I-labeled peptide a 200-fold excess was required to approximate the level of nonspecific binding (Fig. 2). Therefore, according to the 125I-YNRGDS concentration used, a 50–200-fold excess of unlabeled peptide was used to determine nonspecific binding. Specific 125I-YNRGDS binding to purified GP IIb-IIIa was calculated by subtracting nonspecific binding from total binding.

To determine whether iodination altered the binding characteristics of unlabeled YNRGDS, GP IIb-IIIa was incubated with various ratios of 125I-YNRGDS to unlabeled YNRGDS, while maintaining the total peptide concentration constant (Fig. 3). The control value (100% bound) was obtained with undiluted 125I-YNRGDS. The linear relationship (correlation coefficient = 0.997) demonstrated that the moniodinated and the unlabeled peptide bound to GP IIb-IIIa with similar association constants (29).

The reversibility of 125I-YNRGDS binding to GP IIb-IIIa was determined by adding excess unlabeled YNRGDS to preformed protein-peptide complexes. Purified GP IIb-IIIa (40 μg/ml) was first incubated with 0.82 μM 125I-YNRGDS for 4 h. Unlabeled YNRGDS or buffer A was then added to both compartments, and dialysis was continued for another 16 h. Analysis of the data showed that 98 ± 3% of the specific 125I-YNRGDS binding was reversed by 50 μM unlabeled YNRGDS. Thus, the binding of 125I-YNRGDS to purified GP IIb-IIIa was fully reversible.

Specificity of the Interaction between 125I-YNRGDS and GP IIb-IIIa—The specificity of 125I-YNRGDS binding to purified GP IIb-IIIa was examined in three ways. First, we investigated the effect of peptides, structurally related to YNRGDS, on the binding of 125I-YNRGDS to GP IIb-IIIa. As shown in Table I, the tetrapeptide RGDS had a similar inhibitory activity as the hexapeptide YNRGDS, IC50 = 3.8 and 4.2 μM, respectively. However, substitutions of one amino acid of the tetrapeptide, histidine for arginine or alanine for glycine, decreased the inhibitory activity by a factor of 8 for HGDS and more than 10 for RADS. The relative inhibitory potency of these synthetic peptides was similar in the platelet aggregation assay (Table I). Second, the inhibitory activities of two RGD-containing ligands of GP IIb-IIIa, fibrinogen and fibronectin, were compared (Fig. 4). Fibrinogen, at a concentration of 1 μM, inhibited 72% of specific 125I-YNRGDS binding, whereas 1 μM fibronectin only inhibited 21% of this binding. Bovine serum albumin at a concentration of 1 μM was without any effect (data not shown). Third, we investigated the effect

![Fig. 2. Inhibition of total 125I-YNRGDS binding with excess unlabeled peptide.](image)

![Fig. 3. Binding of 125I-YNRGDS at various ratios of 125I-labeled to unlabeled YNRGDS.](image)

**Table I**

| Peptide | Concentration required for 50% inhibition of 125I-YNRGDS binding | Platelet aggregation |
|---------|---------------------------------------------------------------|---------------------|
| YNRGDS  | 4.2 μM                                                       | 39                  |
| RGDS    | 3.8 μM                                                       | 32                  |
| HGDS    | 31 μM                                                        | 420                 |
| RADS    | >50 μM                                                       | >1000               |
Ca$^{2+}$-dependent Binding of an RGD Peptide to GP IIb-IIIa

Fig. 4. Inhibition of specific $^{125}$I-YNRGDS binding to GP IIb-IIIa by fibrinogen and fibronectin. Purified GP IIb-IIIa (44 μg/ml) was incubated with 0.63 M $^{125}$I-YNRGDS and various concentrations of fibrinogen or fibronectin. Nonspecific binding was determined by the inclusion of 50 μM unlabeled YNRGDS.

Fig. 5. Effect of two monoclonal antibodies directed against GP IIb-IIIa on the specific binding of $^{125}$I-YNRGDS to GP IIb-IIIa. Purified GP IIb-IIIa was preincubated for 30 min at room temperature with various concentrations of the monoclonal antibodies $A_2A_9$ or pl-21. These mixtures were then transferred to the dialysis cells and incubated with $^{125}$I-YNRGDS for 4 h. The final concentrations were 59 μg/ml GP IIb-IIIa and 0.67 M $^{125}$I-YNRGDS. To determine nonspecific binding, 48 μM unlabeled YNRGDS was included in the incubation mixture. The final concentrations of the two monoclonal antibodies are indicated.

of two monoclonal antibodies directed against GP IIb-IIIa ($A_2A_9$ and pl-21) on the binding of $^{125}$I-YNRGDS. It has been shown by Bennett et al. (30) that $A_2A_9$ inhibits fibrinogen binding to ADP-stimulated platelets. Fig. 5 shows that the antibody $A_2A_9$ inhibited the specific binding of $^{125}$I-YNRGDS to purified GP IIb-IIIa in a dose-dependent manner; 161 nM $A_2A_9$ inhibited 88% of this binding. These data reinforce the possibility that the binding sites for fibrinogen and for the synthetic fibrinogen Aα-chain sequence YNRGDS are identical or at least interdependent. The antibody pl-21 inhibited platelet aggregation with a similar IC50 as $A_2A_9$ (data not shown). However, this antibody had no effect on $^{125}$I-YNRGDS binding, indicating that pl-21 does not recognize the same epitope on GP IIb-IIIa as $A_2A_9$, although they both inhibit platelet aggregation.

Equilibrium Binding Constants—The equilibrium binding constants for specific $^{125}$I-YNRGDS binding to purified GP IIb-IIIa were determined by incubating GP IIb-IIIa with various concentrations of $^{125}$I-YNRGDS. The dissociation constant ($K_d$) was 1.0 ± 0.2 μM and the maximal binding capacity ($B_{max}$) was 0.92 ± 0.02 mol of $^{125}$I-YNRGDS/mol of GP IIb-IIIa complex as determined by Scatchard analysis (31) of three independent experiments. The results of a typical experiment are shown in Fig. 6.

Effect of the Dodecapeptide from the Carboxyl Terminus of the Fibrinogen γ-Chain—It has recently been shown that the dodecapeptide from the C terminus of the fibrinogen γ-chain (HHLGGAKQAGDV) can elute solubilized GP IIb-IIIa that is bound to an RGDS affinity column (11). We therefore determined whether increasing concentrations of the dodecapeptide have an effect on the binding of $^{125}$I-YNRGDS to purified GP IIb-IIIa. At a concentration of 30 μM the dodecapeptide inhibited the specific binding by 70% (Fig. 7). The concentration required for 50% inhibition of specific $^{125}$I-YNRGDS binding (IC50) was 14.2 μM for the dodecapeptide as compared with 4.0 μM for YNRGDS.

Effect of Ca$^{2+}$ and Mg$^{2+}$ on $^{125}$I-YNRGDS Binding to GP IIb-IIIa—Fibrinogen binding to activated platelets requires the presence of approximately 1 mM of either Ca$^{2+}$ or Mg$^{2+}$ ions (35, 36). To determine the binding of $^{125}$I-YNRGDS to purified GP IIb-IIIa is also divalent cation-dependent, we studied the effect of various free Ca$^{2+}$ concentrations in the presence or absence of 1 mM Mg$^{2+}$ on specific $^{125}$I-YNRGDS binding (Fig. 8). In the presence of 1 mM Mg$^{2+}$, $^{125}$I-YNRGDS binding at 10 and 1 μM free Ca$^{2+}$ was slightly higher than the control value and decreased to 37% at 0.1 μM Ca$^{2+}$. However, in the absence of Mg$^{2+}$, $^{125}$I-YNRGDS binding was completely abolished at Ca$^{2+}$ concentrations of 10 μM or lower. Immediately following the equilibrium dialysis the protein-containing samples were analyzed by sedimentation.
plexes. In all the samples incubated at Ca\(^{2+}\) concentrations of appropriate quantities of fibrinogen, fibronectin, von Willebrand factor, was therefore not caused by dissociation of the GP IIb-IIIa complex. The reduced binding of \(^{125}\text{I}-\text{YNRGDS}\) at \(10 \text{ mM Ca}^{2+}\) concentrations indicated were obtained by the addition of appropriate quantities of \(\text{Ca}^{2+}\) and EGTA. The amount of residual binding was expressed as percentage of the control value determined in the standard buffer containing 1 mM \(\text{Ca}^{2+}\) and 1 mM \(\text{Mg}^{2+}\). This is apparent from the Scatchard analysis of the binding data which indicated that the maximum binding capacity was obtained. A similar \(K_d\) (0.38 \(\mu\text{M}\)) has been reported for the binding of an RGD-containing peptide, 13 amino acids in length, to 59,990 sites on thrombin-stimulated platelets (10). In another study, however, an RGD-containing peptide (14 amino acids) bound to about 350,000 sites with a \(K_d\) of \(11 \mu\text{M}\) (11).

Many of the properties of \(\text{YNRGDS}\) binding to purified GP IIb-IIIa were comparable with those of fibrinogen binding to stimulated platelets.

(i) The number of binding sites on GP IIb-IIIa for fibrinogen and the hexapeptide YNRGDS appears to be identical. This is apparent from the Scatchard analysis of the binding data which indicated that the maximum binding capacity was 0.92 mol of \(^{125}\text{I}-\text{YNRGDS}\)/mol of GP IIb-IIIa. When the purified and dialyzed GP IIb-IIIa was reassembled onto the AegrGD-Sepharose column, more than 90% of GP IIb-IIIa rebounds to this affinity matrix. This indicates that most of the GP IIb-IIIa used for the \(^{125}\text{I}-\text{YNRGDS}\) binding assays was capable of binding at least one RGD-containing peptide. Thus, it can be concluded that the GP IIb-IIIa complex contains a single binding site for RGD peptides. The binding of fibrinogen to GP IIb-IIIa also shows a 1:1 stoichiometry as calculated from the number of fibrinogen-binding sites (37) and of GP IIb-IIIa complexes on the surface of stimulated platelets (38).

(ii) Binding of the hexapeptide YNRGDS to GP IIb-IIIa requires the presence of millimolar concentrations of either \(\text{Ca}^{2+}\) or \(\text{Mg}^{2+}\), which is consistent with the divalent cation requirement for fibrinogen binding to stimulated platelets (35, 36), to isolated platelet membranes (39), and to purified GP IIb-IIIa incorporated into phospholipid vesicles (40).
Therefore, it appears that the low affinity binding site(s) for these cations are probably located on GP IIb-IIIa and not on fibrinogen. The two cations either induce the binding sites for YNRRGDS and fibrinogen on GP IIb-IIIa or they are fibrinogen. The two cations either induce the binding sites class of higher affinity on GP IIb-IIIa that is specific for Ca$^{2+}$.

In addition to these low affinity binding sites we found a class of higher affinity on GP IIb-IIIa that is specific for Ca$^{2+}$. Incubation of purified GP IIb-IIIa in the presence of 1 mM Mg$^{2+}$ and varying concentrations of free Ca$^{2+}$ indicated that maximal binding of YNRRGDS required 1 μM Ca$^{2+}$, since at 0.1 μM Ca$^{2+}$ YNRRGDS binding decreased to 37%. Concomitantly with the loss of binding a significant extent of dissociation of the heterodimeric complex was observed. It has been reported that in the presence of Mg$^{2+}$ maximal fibrinogen binding to activated platelets (41) and isolated platelet membranes (39) also occurs at Ca$^{2+}$ concentrations ≥ 1 μM. However, in intact platelets the dissociation of the GP IIb-IIIa complex is not the case of the observed loss of fibrinogen binding at low Ca$^{2+}$ concentrations, because the binding experiments were performed at 22 °C (41), a temperature where Ca$^{2+}$ concentrations in the nanomolar range do not induce dissociation of the GP IIb-IIIa complex (25, 42, 43). Although the binding of YNRRGDS to GP IIb-IIIa could be regulated by these Ca$^{2+}$ ions identically to the binding of fibrinogen, we cannot exclude the possibility that the dissociation of the GP IIb-IIIa complex was causing the decrease in the YNRRGDS binding.

Brass and Shattil (44, 45) have reported that GP IIb-IIIa contains two high affinity (K$\text{d}$ = 9 nm) and approximately six low affinity (K$\text{d}$ = 400 nm) binding sites for Ca$^{2+}$. The present study suggests that GP IIb-IIIa might also contain at least one very low affinity binding site (K$\text{d}$ ≥ 200 μM) for either Ca$^{2+}$ or Mg$^{2+}$.

(iii) The binding sites on GP IIb-IIIa for fibrinogen and for the hexapeptide YNRRGDS appear to overlap. This conclusion is supported by the finding that the monoclonal antibody A$_2$A$_3$, which has previously been shown to inhibit fibrinogen binding to activated platelets (30), also inhibited YNRRGDS binding to purified GP IIb-IIIa. Recently it has been reported that the synthetic tetrapeptide RGDS does not inhibit A$_2$A$_3$ binding to stimulated platelets (46). The reason for this apparent difference is presently not known, but the size of the RGD-containing peptides used could be critical for its inhibitory effect.

(iv) The binding of 125I-YNRRGDS to GP IIb-IIIa was inhibited by the dodecapeptide HHLGGAQKAGDV as is fibrinogen binding to intact platelets (12, 13) and to purified GP IIb-IIIa incorporated into vesicles (40). There is convincing evidence that RGD-containing peptides and the dodecapeptide corresponding to the C terminus of the fibrinogen $\gamma$-chain are binding to GP IIb-IIIa in a mutually exclusive manner (11, 16, 17, 46). Our data confirm this finding. Two explanations for the mutually exclusive binding of the two peptides have been discussed in the literature (16, 46); either the binding of one peptide induces a conformational change in GP IIb-IIIa so that the other peptide cannot bind anymore or both peptides bind to the same or to an overlapping site on GP IIb-IIIa. Further studies are required to clearly differentiate between these two possibilities.

In summary, we have demonstrated that the hexapeptide YNRRGDS, corresponding to a sequence present in the fibrinogen $\alpha$-chain at position 570–575, binds to purified GP IIb-IIIa with properties very comparable with fibrinogen binding to activated platelets. Our data strongly suggest that the RGD-containing domains within the adhesive proteins are indeed responsible for the binding to GP IIb-IIIa. This conclusion is supported by the recently reported results that antibodies specifically recognizing the RGD-containing domains within von Willebrand factor (47) and fibrinectin (48) inhibit the binding of these adhesive proteins to activated platelets.

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