The extreme carboxyl-terminal amino acid sequence of the $\gamma$ chain of fibrinogen is involved in the binding of this adhesive protein to the platelet integrin glycoprotein (GP) IIb-IIIa, and synthetic peptides corresponding to this region inhibit fibrinogen as well as fibronectin and von Willebrand factor binding to platelets. A chemical cross-linking approach was used to characterize the interaction of a 16-amino acid fibrinogen $\gamma$ chain peptide with platelets and to localize the site of its binding to GPIIb-IIIa. This peptide became specifically cross-linked to GPIIb, and platelet stimulation selectively enhanced its cross-linking to this $\alpha$ subunit. The cross-linking reaction was specifically inhibited by fibrinogen and an Arg-Gly-Asp peptide but not by an unrelated protein or a substituted peptide. Utilizing a combination of immunomapping, enzymatic and chemical digestions, and amino acid sequencing, the cross-linking site of the $\gamma$ chain peptide in GPIIb was localized to a stretch of 21 amino acids. The identified region, GPIIb 294-314, contains the second putative calcium binding domain within GPIIb. The primary structure of this region is highly conserved among $\alpha$ subunits of other integrin adhesion receptors. These results identify a discrete region of GPIIb that resides in close proximity to a ligand binding site within GPIIb-IIIa. The homologous region may be involved in the functions of other integrin receptors.

The integrin family is comprised of structurally homologous heterodimeric cell surface receptors which mediate a variety of cell-cell and cell-matrix interactions via their recognition of specific ligands (1-4). GPIIb-IIIa is the most prominent integrin of platelets and is involved in both platelet-platelet and platelet-substrata interactions (5,6). As a member of the cytoadhesive subfamily ($\beta_1$), it has the same (or a very similar) $\beta$ subunit (GPIIb-IIIa) as the broadly distributed vitronectin receptor (7,8). The $\alpha$ subunit, GPIIb, of GPIIb-IIIa, however, appears to be platelet-specific although it is structurally similar to the $\alpha$ subunit of the vitronectin receptor as well as to the $\alpha$ subunits of other integrins (9). As some but not all of the ligands that bind to GPIIb-IIIa also interact with the vitronectin receptor (10-12) (e.g. fibronectin binds to GPIIb-IIIa but not to the vitronectin receptor) (12,13), the $\alpha$ subunits must play a critical role in determining the fine differences in the recognition specificities of these two integrins. The importance of the $\alpha$ subunits in establishing ligand specificity is also apparent in the VLA ($\beta_1$) subfamily of integrins in which several receptors with distinct ligand specificities are derived from the combination of different $\alpha$ subunits with a single common $\beta$ subunit (14-16).

GPIIb-IIIa can interact with several adhesive proteins including fibrinogen, fibronectin, and von Willebrand factor (3,6). In order for GPIIb-IIIa to bind soluble forms of these ligands, the platelet must be activated first by agonists such as ADP, PMA, or thrombin (17). Two linear peptide sequences have been implicated in mediating the interactions of the ligands with GPIIb-IIIa. One sequence is Arg-Gly-Asp (RGD) which is found in fibrinogen, fibronectin, and von Willebrand factor. RGD containing peptides inhibit the binding of all three adhesive proteins to platelets (18-21), and GPIIb-IIIa interacts directly with these peptides (12). When RGD peptides are cross-linked to activated platelets, they couple predominantly to GPIIIa (22,23). Recently, we have pinpointed an amino acid sequence in GPIIIa, residues 100-171, to which RGD peptides become cross-linked (24). This sequence is, therefore, likely to reside in close proximity to the ligand binding site of the receptor. This 63-amino acid stretch of GPIIIa is highly conserved among the integrin $\beta$ subunits and may participate in the recognition of RGD sequences within several ligands recognized by different integrin receptors. It is noteworthy that an overlapping region was independently identified as the RGD cross-linking site in the vitronectin receptor (25). The second sequence involved in the binding of ligands to GPIIb-IIIa is referred to as the fibrinogen $\gamma$ chain peptide. This peptide, HHLGGAKQAGDV (H12), or smaller derivatives thereof, corresponds to the sequence at the carboxyl terminus of the $\gamma$ chain of fibrinogen. Although this sequence is unique to fibrinogen, the $\gamma$ chain peptide inhibits fibronectin and von Willebrand factor as well as fibrinogen binding to platelets (26-28). This peptide also interacts directly with GPIIIa (29) and binds to platelets in an activation-dependent manner (30). Although the $\gamma$ chain and RGD peptides appear to interact with the same or mutually exclusive sites on GPIIIa (29,31), in contrast to the cross-linking of RGD peptides to GPIIIa, Santoro and Lawing (32) reported that a $\gamma$ chain peptide, labeled with a photoaffinity probe, became cross-linked predominantly to GPIIb. This differential cross-linking provides an opportunity

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to identify a second region which might be involved in or proximal to the ligand binding site of the receptor. In our efforts to continue to define the topography of GPIIb-IIIa and that of the integrins in general, we have sought to further characterize the cross-linking of the γ chain peptide to GPIIb-IIIa and to locate the site of its cross-linking within GPIb.

**Experimental Procedures**

**Peptides—**A 16-amino acid fibrinogen γ chain peptide (K16), the sequence of which was KYGGHHLGGAKQAGDV, was prepared by solid phase synthesis on an Applied Biosystems model 430 peptide synthesizer (Foster City, CA) using standard Fmoc/LBoc chemistry and t-butyloxycarbonyl amino acids purchased from Applied Biosystems. The peptide was analyzed for homogeneity by high performance liquid chromatography using a C18 μBondapak column with a linear gradient of 0-100% acetonitrile in 0.1% trifluoroacetic acid and was found to be >85% homogeneous. The amino acid composition of the K16 peptide was determined on 24-h hydrolysates in 6 N HCl, and the results were consistent with theoretical yields. The peptide was dissolved in PBS (0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.3) and radiiodinated with a modified lactoperoxidase-glucose oxidase method as previously described (23, 29). Glucose (40 μg in 50 μl of 0.2 M sodium phosphate, pH 7.4), carrier-free Na125I (15 μCi), and Enzymobead reagent (Bio-Rad) were added to 10-12 μg of the peptides. The iodinated peptide was separated from free Na125I by gel filtration on a Bio-Gel P-2 column (Bio-Rad). The concentration of the labeled peptide was determined by absorbance at 280 nm using an extinction coefficient derived from its amino acid composition. The specific activity was 5-5 μCi/μM. Other peptides utilized in this study were also synthesized and characterized by the procedures described above.

**Platelet Binding and Cross-linking—**Platelets were isolated from fresh human blood by differential centrifugation followed by gel filtration on Sepharose 2B in divalent ion-free Tyrode’s buffer, pH 7.5, containing 0.1% bovine serum albumin. Platelet binding of K16 peptide followed the protocols previously described from our laboratory for measuring platelet interactions with adhesive proteins and synthetic peptides. The native peptides (13, 18, 23). Briefly, platelets were suspended in 4 × 10^9/ml in divalent ion-free Tyrode’s-albumin buffer. Ca^2+ was added to a final concentration of 1 μM. The platelet stimuli used were 10 μM ADP, 0.5 unit/ml α-thrombin, or 100 μM PMA. In assays in which fibrinogen and thrombin were present, the enzyme was inactivated with 30 μM D-phenylalanyl-L-prolyl-arginine ketone (Calbiochem) 5 min after the addition of thrombin to the platelets and 5 min prior to the addition of the fibrinogen. The radiolabeled K16 peptide was added at a concentration of 30 μM, and binding proceeded for 45 min at 22°C. The primary cross-linking agent used in this study was bis(sulfosuccinimidyl)suberate (BS^3- (32) (Pierce Chemical Co.). The cross-linked complexes were terminated after 10 min at 22°C by addition of 10 mM Tris, pH 7.0. Processing of samples for SDS-PAGE analyses were performed as previously described (23, 24). Briefly, the cell-bound ligand was recovered by centrifugation through 20% sucrose, and the cells were extracted in PBS containing 1% Nonidet P-40 and 10 mM N-ethylmaleimide (Sigma). Extracted proteins were treated with 10% trichloroacetic acid, and the pellet obtained after centrifugation was washed three times with cold 85% ethanol.

Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate in vertical slab gels in the buffer system of Laemmli (33). Samples in Laemmli sample buffer (33) were treated with 5% 2-mercaptoethanol for disulfide bond reduction. Analytical gels were dried, and autoradiograms were developed with Kodak X-Omat AR films. Molecular weights were estimated on the basis of electrophoretic mobility relative to prestained standards obtained from Diversified Biotech. The relative mobility (Rr) was determined by measuring the migration of the 125I-K16-GPIB band relative to the migration of the ovalbumin marker protein in the same gel. In some experiments, autoradiograms were scanned using a Zeineh soft laser scanning densitometer coupled with a Bio-Image Systems Model 475A for NH*-terminal sequencing. To optimize peptide sequencing on the PDVF membranes, the above procedures were carried out using freshly made buffers, reagents, chemicals, and H2O of the highest purity, and organic washes in the sequencer were decreased to approximately 20% of the time used in the standard cycles. Initial use of amino acids reagents from 17-21 μmol, and repetitive yields were in the 86-97% range. The sensitivity of the instrument was approximately 0.1 pmol.

**Results and Discussion**

The γ chain peptide utilized in our cross-linking studies had the structure KYGGHHLGGAKQAGDV and was designated K16. The carboxyl-terminal 12 amino acids of K16 correspond to residues 400-411 (H12) of the fibrinogen γ chain; and, to this sequence, we added two glycine residues as spacers, a tyrosine residue for radiodination, and a lysine residue to facilitate cross-linking. K16 and H12 were of similar potency in inhibiting 125I-fibrinogen binding to ADP-stimulated platelets; as determined from dose titration curves, the IC50 of K16 and H12 were 45 and 70 μM, respectively. In a previous study (30), we had determined that a γ chain peptide were dissolved in 250 μl of a previously described immunoprecipitation buffer containing EDTA (24, 35). The lysates were supplemented with 1% bovine serum albumin containing 10 μl of each of the monoclonal antibodies. Samples were incubated overnight at 4°C, and Protein A reagent was then added. The recovered immunoprecipitates were washed three times by centrifugation, solubilized at 100°C for 3 min in Laemmli sample buffer, and then subjected to SDS-PAGE. Immunoblot membranes containing intact or fragmented GPIIb were resolved on SDS-PAGE. After electrophoresis, the proteins were transferred onto nitrocellulose filters (Bio-Rad) as previously described (36). The filters were probed with PMI-1 (34) or with a rabbit antiserum raised to peptide sequences from the heavy and light chains of GPIIb (37). The bound antibodies were detected with horseradish peroxidase (Bio-Rad) and 4-chloro-1-naphthol as substrate. Filters were autoradiographed to detect radioactive proteins.

**Preparative Recovery of K16 Cross-linked to GPIb—**The position of the GPIb-K16 complex on 7.5% gels was established from the autoradiogram, and the region of the gel was excised and soaked for 40 h at 22°C in 0.2 M Tris containing 2% SDS, pH 7.8. 100 μM DTT was added in the soaking buffer to prevent oxidation and NH2-terminal blockage (24). The extracted GPIb-K16 complex was concentrated by precipitation in 80% cold acetone. This procedure also removed residual SDS. The precipitate obtained after centrifugation at 10°C was dried in a 37°C oven, and the resulting precipitate was reconstituted in minimal amounts of PBS containing 0.1% SDS and 100 μM DTT.

**Enzymatic and Chemical Digestion—**GPIIb-K16 complex was fragmented in the presence of enzymes α-chymotrypsin and Staphylococcus aureus V8 protease ( Worthington). Limited digestion of GPIb-K16 in the presence of α-chymotrypsin was carried out for 2 h at 22°C using the 1:0.4 (w/w) substrate to enzyme ratio in PBS containing 0.1% SDS and 100 μM DTT. Digestion with S. aureus V8 protease was performed at 1:1 (w/w) substrate to enzyme ratio for 16 h at 22°C in PBS containing SDS and DTT. Following digestion, samples were boiled for 5 min to inactivate the enzymes. CNBr digestion of GPIIb was carried out on gel slices containing radioactive GPIb-K16 complex. Gel slices were made porous by hydration in H2O and then lyophilized. Lyophilized gel slices were exposed to flames of CNBr in 70% formic acid for 12 h at 22°C in a tightly closed jar (38). Following CNBr digestion, gel slices were throuroughly washed in H2O and repeatedly lyophilized. Gel slices containing CNBr-digested GPIb-K16 complex were then extracted in 0.2 M Tris containing 2% SDS, pH 7.8.

**Protein Sequencing—**Enzymatically and chemically digested GPIb-K16 complexes were mixed with Laemmli sample buffer containing 2-mercaptoethanol. Samples were boiled and electrophoresed on 10-20% gradient gels. Proteins were electrophoretically transferred to Immobilon PVDF membranes (Millipore) in 10 mM CAPS (Calbiochem) in 10% methanol, pH 11.0 (39) at 450 mA for 1 h. Transfers were briefly washed in water and then stained with Coomassie Blue. The membranes were autoradiographed to detect radioactive fragments of GPIb-K16. Peptide bands of interest were excised and directly applied into a gas phase sequenator (Applied Biosystem model 475A) for NH2-terminal sequencing. To optimize peptide sequencing on the PDVF membranes, the above procedures were carried out using freshly made buffers, reagents, chemicals, and H2O of the highest purity, and organic washes in the sequencer were decreased to approximately 20% of the time used in the standard cycles. Initial use of amino acids reagents from 17-21 μmol, and repetitive yields were in the 86-97% range. The sensitivity of the instrument was approximately 0.1 pmol.
bound to an activation-dependent site on platelets with a $K_d$ of $\sim$30 $\mu M$, and this concentration was used in the cross-linking analyses. $^{125}$I-K16 was bound to washed human platelets for 45 min at 22 $^\circ$C, and then the chemical cross-linking reagent BS3 was added. After 10 min, the BS3 was neutralized, the cells were washed and extruded, and the extracts were analyzed by SDS-PAGE. A representative autoradiograph of $^{125}$I-K16 cross-linked to thrombin-stimulated platelets is shown in Fig. 1A. The radioactivity migrated as a single major band with an electrophoretic mobility identical to GPIIb. Minimal radioactivity was detected at the GPIIIa position. A 50-fold excess of nonlabeled K16 abolished the cross-linking of the radiolabeled peptide to the cells, providing an initial indication of specificity. That the major radioactive band was authentic GPIIb was demonstrated by its immunoprecipitation with a monoclonal antibody to GPIIb (Fig. 1B) (40). This antibody, PM1-1, immunoprecipitated the major radioactive band within the platelet extract while numerous control antibodies, including a monoclonal of the same subclass to another platelet protein, failed to immunoprecipitate the radioactive band. During the course of numerous cross-linking experiments, variable amounts of radioactive material were found to accumulate on top of the gels. The immunoprecipitation experiment shown in Fig. 1B indicates that at least a portion of the high molecular weight radioactivity contained GPIIb antigen.

Platelet activation markedly affected the cross-linking of $^{125}$I-K16 to GPIIb (Fig. 1C). ADP, PMA, and thrombin all increased the cross-linking of K16 to GPIIb relative to that observed with unstimulated platelets. The most pronounced enhancement of K16 cross-linking was observed with thrombin as the platelet stimulus. Relative to nonactivated platelets, thrombin stimulation increased the K16 cross-linking to GPIIb by 12-fold (four experiments). The augmentation of cross-linking was due to an increase in K16 binding to the stimulated cells as well as to an increase in the efficiency of the cross-linking reaction. The enhanced cross-linking of the peptide to GPIIb on stimulated platelets was also observed with two additional cross-linking reagents, 3,3′-dithiobis (sulfosuccinimidyl propionate) and dithiobis(succinimidyl propionate).

The specificity of the cross-linking of K16 to GPIIb was further explored by assessing the capacity of several nonlabeled peptides and proteins to inhibit the interaction. The effect of an RGD peptide on $^{125}$I-K16 cross-linking is shown in Fig. 2. GRGDSP markedly inhibited the cross-linking reaction. In contrast, GRGESP, which is considerably less potent that GRGDSP in inhibiting the binding of adhesive proteins to platelets (18), produced only a slight inhibition of the cross-linking reaction. To assess the relative inhibition of the cross-linking reaction by these peptides, the autoradiograms were subjected to densitometric scanning. GRGDSP produced 89% inhibition, whereas GRGESP produced only 12% inhibition. A GPIIb-IIIa ligand, fibrinogen, also inhibited K16 cross-linking (96% inhibition), whereas a similar concentration of an irrelevant protein, albumin, caused minimal inhibition (9% inhibition).

Densitometric scanning was also used to assess the divalent ion requirements for $^{125}$I-K16 cross-linking to GPIIb. The cross-linking of the peptide to GPIIb was similar in the presence of 1 $mM$ calcium or magnesium. When divalent ions were chelated with either 5 $mM$ EDTA or EGTA, the cross-linking reaction was markedly inhibited. At 22 $^\circ$C, EDTA and EGTA inhibited $^{125}$I-K16 cross-linking to GPIIb by 97 and 98.5%, respectively (relative to the signal obtained in the presence of calcium). The divalent ion dependence of the cross-linking reaction is consistent with the divalent ion requirement for the high affinity interaction of fibrinogen $\gamma$ chain peptides with platelets (30).

With the above data providing clear evidence for specific cross-linking of the K16 peptide to a relevant site, we sought to localize this site within the GPIIb subunit. The initial step was to determine whether $^{125}$I-K16 became associated with the heavy or light chain of GPIIb. $^{125}$I-K16 was cross-linked to thrombin-stimulated platelets. Radioactive bands of the GPIIb-K16 complexes were excised from gels run under nonreducing conditions, extracted, and rerun on gels under reducing conditions. Samples were then transferred from the gel onto a PVDF membrane and probed by immunoblotting with antibodies or subjected to autoradiography. Two previously described anti-peptide antibodies were used for immunoblotting: anti-V43 to the 17-amino acid peptide sequence at the carboxyl terminus of the heavy chain of GPIIb and anti-V41 to the 13-amino acid peptide sequence at the amino terminus of the light chain of GPIIb (37). The immunoblots developed with these two antibodies (Fig. 3) clearly indicate that, after cross-linking to K16, GPIIb could still be reduced into its heavy and light chain constituents. The autoradiograms of the sample indicated that all of detectable radioactivity migrated in the position of the heavy chain. When the regions of the gels containing the light and heavy chains were excised from the gel and counted, <2% of the radioactivity was in the position of the light chain and 98% in the position of GPIIb.

![Fig. 1. Cross-linking of the $\gamma$ chain peptide (K16) to platelet integrin, GPIIb-IIIa. $^{125}$I-K16 peptide (50 $\mu M$) was bound to platelets ($6 \times 10^9$/ml) for 45 min at 22 $^\circ$C and cross-linked with BS3 (0.2 $mM$). Cross-linked samples were extracted and analyzed on SDS-PAGE under nonreducing conditions as previously described (23, 24), and autoradiographs of the gels are shown. In panel A, $^{125}$I-K16 cross-linking to thrombin stimulated platelets in the absence of inhibitor (left lane) or in the presence of a 50-fold molar excess of nonlabeled K16 peptide (right lane), a control for the specificity of the cross-linking reaction. Panel B, immunoprecipitation of a detergent extract of $^{125}$I-K16 cross-linked to thrombin-stimulated platelets with the GPIIb specific antibody, PM1-1 (40). Immunoprecipitation with a monoclonal antibody to a second platelet protein in parallel failed to precipitate any radioactive products. Panel C, $^{125}$I-K16 cross-linking to platelets in the presence of ADP (10 $\mu M$), PMA (0.1 $\mu M$), or thrombin (0.5 units/ml) (lanes 2–4) and in the absence of agonists (lane 1).

![Fig. 2. Specificity of K16 cross-linking to GPIIb-IIIa. $^{125}$I-K16 (50 $\mu M$) was cross-linked to thrombin-stimulated platelets in the absence of nonlabeled competitors (lanes 1 and 4) or in the presence of a 50-fold molar excess of nonlabeled GRGDSP (lane 2), GRGESP (lane 3), fibrinogen (lane 5), or albumin (lane 6).]
under nonreducing conditions. The samples were then rerun reduced in the presence of 2-mercaptoethanol on 10-20% gradient gels. Gel transfers were probed with an antibody to the GPIIb heavy chain with the anti-peptide antibody V43 (lane 1) or to the GPIIb light chain, the anti-peptide antibody V41 (lane 2). Lanes 3 and 4 are of the heavy chain. When the GPIIb-K16 extract was run on second gel under reducing conditions (RF = 0.32) and nonreducing conditions (RF = 0.28) of the radioactive band was similar to that observed in the second gel. When the second gel was of a lower acrylamide providing a control for the recovery of radioactivity in the GPIIb heavy chain under reducing conditions (see Fig. 3), the radioactive band was consistently observed (Fig. 5). The radioactive band was subjected to amino acid sequence analysis, and an unambiguous sequence was obtained for 14 residues of this band were determined and corresponded to the amino-terminal sequence of the GPIIb heavy chain. Similar analyses were performed on two additional preparations of the radioactive CNBr fragment, and, in each case, a sequence corresponding to the amino terminus of GPIIb was obtained. Control experiments were performed which indicated that GPIIb was not sensitive to formic acid cleavage under the conditions used in the CNBr reaction, restricting the CNBr cleavage sites to the methionyl residues of the protein. The first three methionyl residues are located at positions 285, 314, and 489 of GPIIb (9). CNBr cleavage at either of the first two sites would yield a fragment within the 30-40 kDa range (as there are two potential Asn-linked glycosylation sites within this region, precise molecular masses cannot be calculated), compatible with the 40-kDa fragment observed. CNBr cleavage at the third methionyl residue, on the other hand, would yield a fragment of ≥54 kDa. Therefore, the K16 cross-linking site appears to be restricted to a discrete region within the first 314 amino acid residues of GPIIb. Occasionally, a radioactive doublet was observed in the CNBr digest, with the higher band having an estimated molecular mass of 54 kDa. This second band is apparent in the CNBr digest in Fig. 4, lane 2. Instead, three lower molecular weight fragments were detected, and these did not align with any of the PMI-1 positive fragments. These results indicate that the 60-kDa region of GPIIb extending toward the amino terminus from the extreme carboxyl terminus of the heavy chain does not contain the K16 cross-linking site. Conversely, these data suggest that the cross-linking site must reside within the amino-terminal half of the GPIIb heavy chain.

To further locate the K16 cross-linking site, the GPIIb-K16 complex, isolated from gels, was subjected to cleavage with CNBr. Upon re-electrophoresis and gel transfer, a major 40-kDa radioactive fragment was consistently observed (Fig. 4). The radioactive band was subjected to amino acid sequence analysis, and an unambiguous sequence was obtained for 14 cycles. This sequence (Fig. 5) corresponds precisely to the amino-terminal sequence of the GPIIb heavy chain. Similar analyses were performed on two additional preparations of the radioactive CNBr fragment, and, in each case, a sequence corresponding to the amino terminus of GPIIb was obtained. Control experiments were performed which indicated that GPIIb was not sensitive to formic acid cleavage under the conditions used in the CNBr reaction, restricting the CNBr cleavage sites to the methionyl residues of the protein. The first three methionyl residues are located at positions 285, 314, and 489 of GPIIb (9). CNBr cleavage at either of the first two sites would yield a fragment within the 30-40 kDa range (as there are two potential Asn-linked glycosylation sites within this region, precise molecular masses cannot be calculated), compatible with the 40-kDa fragment observed. CNBr cleavage at the third methionyl residue, on the other hand, would yield a fragment of ≥54 kDa. Therefore, the K16 cross-linking site appears to be restricted to a discrete region within the first 314 amino acid residues of GPIIb. Occasionally, a radioactive doublet was observed in the CNBr digest, with the higher band having an estimated molecular mass of 54 kDa. This second band is apparent in the CNBr digest in Fig. 5. The amino-terminal sequence of this upper band was also determined and corresponded to the amino-terminal sequence of GPIIb at each of the 10 positions determined. This fragment, therefore, must arise from CNBr cleavage at the methionyl residue at position 489. Together, the radioactivity within these two fragments accounted for 88% of the radioactivity applied to the gel shown in Fig. 5.

A limit digest of the GPIIb heavy chain-K16 complex with chymotrypsin yielded a single 7-kDa radioactive fragment (Fig. 5). Of the radioactivity within the GPIIb-K16 complex, ≥90% was recovered in this 7-kDa band. The amino-terminal 6 residues of this band were determined and corresponded to the GPIIb sequence commencing from residue 204. Sequence analyses of three separate preparations of the 7-kDa fragment yielded at least the 3-residue sequence AVT, an amino acid
sequence unique to residues 294–296 of GPIIb. A 7-kDa fragment beginning at residue 294 should contain approximately 60 amino acids and terminate in the vicinity of residue 350.

To corroborate this localization, *S. aureus* V8 protease was used to digest the GPIIb heavy chain-K16 complex. The peptide pattern of this digest was extremely complex; therefore, it was first subjected to high pressure liquid chromatography on a C4 column. The radioactive fractions were pooled, and the amino acid sequences obtained are indicated. The positions within the GPIIb sequence are from Ref. 9.

The methionyl residues at positions 285 or 314 are the two alternative sites for the carboxyl terminal of the 40-kDa fragment. Step 4 confined the cross-linking site to a 7-kDa chymotryptic fragment extending from residue 294. This result establishes that the radioactive CNBr-derived fragment must terminate at 314 rather than 285 and places the K16 cross-linking site within a stretch of 21 amino acids. Step 5 established that the cross-linking site resided within the 9-kDa *S. aureus* V8 protease fragment beginning at residue 253. This fragment is predicted to terminate in the vicinity of residue 350. As the 294–314 region is contained within the S. aureus V8 protease fragment, step 5 provides corroborating evidence for the localization deduced from step 4.

The sequence of the 21-amino acid stretch of GPIIb containing the γ chain cross-linking site is indicated in Table I. Inspection of the sequence indicates an absence of lysine residues within this region. While lysines are the preferred residues for BS3 cross-linking, reactions involving histidine and tyrosine residues have also been reported (41). Thus, the sequence contains at least two candidate sites. In the middle of this sequence is the second of the four putative Ca²⁺ binding sites within GPIIb (9). Residues 297–308 contain the appropriately spaced aspartic acid residues necessary to form an EF-hand structure. Divalent ions are required for the interaction of GPIIb-IIIa ligands (42), including the γ chain peptide (30), with the receptor. Lindag binding to GPIIb-IIIa induces conformational changes in the receptor (43, 44) which can also be elicited by treating the receptor with chelating agents in the absence of ligands (45). These observations raise the possibility that ligand binding may modulate the calcium-binding functions of GPIIb-IIIa. The close proximity of the cross-linking site to a potential site of divalent ion binding is compatible with this hypothesis.

The sequence of the γ chain cross-linking site in GPIIb has been optimally aligned with those determined for other α subunits of human integrins in Table I. A high conservation of primary structure is evident. The sequence identity of this region of GPIIb ranges from 48% for the α subunit of Mac-1...
 Ligand Binding Site of an Integrin Receptor

TABLE I
Alignment of the fibrinogen γ chain peptide (K16) cross-linking site within GPIIb with other α subunits of the integrin family

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Sequence</th>
<th>Identity to K16 cross-linking region</th>
<th>Identity to overall GPIIb sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPIIb</td>
<td>AVTDVNGDGRHDL-LVGAPLYM</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>VnKα</td>
<td>AaTD1NGudyADV-1GAPfLM</td>
<td>(57)%</td>
<td>36%</td>
</tr>
<tr>
<td>VLA-2</td>
<td>csVDVkdDrtDtvL1VGAPfYM</td>
<td>(52)%</td>
<td>22%</td>
</tr>
<tr>
<td>VLA-4</td>
<td>csVDINaDGfSD-L1VGAPfQM</td>
<td>(48)%</td>
<td>24%</td>
</tr>
<tr>
<td>VLA-5</td>
<td>AaTDVNGDGlDlDL-LVGAPfLM</td>
<td>(81)%</td>
<td>38%</td>
</tr>
<tr>
<td>LFA-1</td>
<td>cgVDvDGet1LL1GAPfLy</td>
<td>(48)%</td>
<td>30%</td>
</tr>
<tr>
<td>Mac-1</td>
<td>csVDVsdGNgSt1DVL1GAPfYY</td>
<td>(48)%</td>
<td>25%</td>
</tr>
<tr>
<td>P150,95</td>
<td>csVDVtdGNgSt1DVL1GAPfYY</td>
<td>(52)%</td>
<td>25%</td>
</tr>
</tbody>
</table>

The sequences reported are taken from Refs. 9 and 45-54, in the order listed. Residues showing identity with specified amino acids in GPIIb are in upper case lettering, and those residues that differ from GPIIb are in lower case.

to 81% for the α subunit of VLA-5, the fibronectin receptor. The overall identity of the entire GPIIb to these other α subunits ranges from 22 to 38%. Such selective conservation of structure favors a role for this region in receptor function.

The goal of this study was to localize the K16 cross-linking site as a means of providing insight into the topography and function of GPIIb-IIIa and other integrin family members. The γ chain peptide sequence is directly involved in the binding of fibrinogen to platelets (30). Therefore, the GPIIb region comprised of residues 294-314 is either directly involved or resides in close proximity to a site in the receptor involved in adhesive protein binding. The conformation of this region of the receptor also appears to be altered by activation of the platelets based upon the increased binding and efficiency of the cross-linking of the peptide to stimulated cells. These observations, combined with our previous localization of the RGD cross-linking site to residues 100-171 of GPIIIa (24) and with the data of others (46, 47), implicate the amino-terminal aspects of the receptor in ligand binding functions. The 21-amino acid stretch of GPIIb now becomes a key target area for other approaches, such as site-directed mutagenesis or anti-peptide antibodies, to further define the structure-function relationships of GPIIb-IIIa and other integrins.

Acknowledgments—Susan Jepsen and Vicky Byers-Ward provided expert technical assistance on this project.

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Ligand Binding Site of an Integrin Receptor

The ligand binding site of the platelet integrin receptor GPIIb-IIIa is proximal to the second calcium binding domain of its alpha subunit.

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