A Cluster of Basic Amino Acid Residues in the γ370−381 Sequence of Fibrinogen Comprises a Binding Site for Platelet Integrin $\alpha_{IIb}\beta_3$ (Glycoprotein IIb/IIIa)[†]

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ABSTRACT: Adhesive interactions of platelet integrin $\alpha_{IIb}\beta_3$ with fibrinogen and fibrin are central events in hemostasis and thrombosis. However, the mechanisms by which $\alpha_{IIb}\beta_3$ binds these ligands remain incompletely understood. We have recently demonstrated that $\alpha_{IIb}\beta_3$ binds the γ365−383 sequence in the γC-domain of fibrin(ogen). This sequence contains neither the AGDV nor the RGD recognition motifs, known to bind $\alpha_{IIb}\beta_3$, suggesting the different specificity of the integrin. Here, using peptide arrays, mutant fibrinogens, and recombinant mutant γC-domains, we have examined the mechanism whereby $\alpha_{IIb}\beta_3$ binds γ365−383. The $\alpha_{IIb}\beta_3$-binding activity was localized within γ370−381, with two short sequences, γ^{370}ATWKTR^{375} and γ^{376}WYSMKK^{381}, being able to independently bind the integrin. Furthermore, recognition of $\alpha_{IIb}\beta_3$ by γ370−381 depended on four basic residues, Lys^{373}, Arg^{375}, Lys^{380}, and Lys^{381}. Simultaneous replacement of these amino acids and deletion of the γ^{408}AGDV^{411} sequence in the recombinant γC-domain resulted in the loss of $\alpha_{IIb}\beta_3$-mediated platelet adhesion. Confirming the critical roles of the identified residues, abnormal fibrinogen Kaiserslautern, in which γLys^{380} is replaced by Asn, demonstrated delayed clot retraction and impaired $\alpha_{IIb}\beta_3$ binding. Also, a mutant recombinant fibrinogen modeled after the naturally occurring variant Osaka V (γArg^{375} → Gly) showed delayed clot retraction and reduced binding to purified $\alpha_{IIb}\beta_3$. These results identify the γ370−381 sequence of fibrin(ogen) as the binding site for $\alpha_{IIb}\beta_3$ involved in platelet adhesion and clot retraction and define the new recognition specificity of this integrin.

Integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa) is a platelet receptor for fibrinogen, critically involved in the formation of the primary hemostatic plug upon vascular injury. This receptor also participates in the subsequent thrombus stabilization and its remodeling (clot retraction). Adhesive interactions of $\alpha_{IIb}\beta_3$ with fibrinogen and its clotting product, fibrin, also play a role in many pathological conditions, and, therefore, this integrin is an important target for antithrombotic therapy (*1*−*3*). However, the mechanisms by which $\alpha_{IIb}\beta_3$ binds soluble fibrinogen and, especially, insoluble fibrin remain to be understood.

Fibrinogen (340 kDa) is an abundant plasma protein consisting of two pairs of polypeptide chains, Aα, Bβ, and γ, organized into three structural regions, the central E and two peripheral D (*4*, *5*). Several sequences in fibrinogen have been designated as the $\alpha_{IIb}\beta_3$ recognition sites. They are the RGD-based sequences at 95−98 (RGDF) and 572−575 (RGDS) in the Aα chains, and ^{400}HHLGGAKQAGDV^{411} (H12) in the γ-chains (for details see reviews, refs *6* and *7*). Synthetic peptides that duplicate these sequences and mAbs directed against the corresponding epitopes in fibrinogen inhibit different aspects of $\alpha_{IIb}\beta_3$ adhesive function. The primary binding sites for $\alpha_{IIb}\beta_3$ in *soluble* plasma fibrinogen, involved in agonist-stimulated platelet aggregation, reside in the peripheral D regions. In electron micrographs, isolated $\alpha_{IIb}\beta_3$ contacted one of the globular nodules in the D regions (so-called γC-domains) (*8*), and, consistent with this observation, studies with recombinant human fibrinogens showed that the COOH-terminal sequence ^{408}AGDV^{411} in the γC-domain (γC)[1] is the binding site for $\alpha_{IIb}\beta_3$ (*9*, *10*).

Binding of fibrinogen to $\alpha_{IIb}\beta_3$ is a multiphasic process with initial reversible contacts followed by irreversible fibrin(ogen) binding that stabilizes large platelet aggregates and may involve additional ligand−receptor sites (*11*−*16*). Also, as the thrombus formation proceeds, the interactions of $\alpha_{IIb}\beta_3$ with the growing fibrin clot engage new contacts that lead to clot retraction. The nature of binding sites for $\alpha_{IIb}\beta_3$ in

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[1] Abbreviations: γC, globular COOH-terminal domain of the γ-chain of fibrinogen; BSA, bovine serum albumin; GST, glutathione *S*-transferase; TBS, Tris-buffered saline; PBS, phosphate buffered saline; isotonic HEPES buffer, 20 mM HEPES, pH 7.3, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3.3 mM NaH_2PO_4; spot synthesis, parallel synthesis of many peptides on a continuous cellulose membrane within defined synthesis areas (spots).

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the platelet-bound fibrinogen, as well as those in fibrin, is largely unknown. In contrast to platelet aggregation, the γAGDV sequence is not required for fibrin clot retraction. Furthermore, RGDs in the Aα chains do not contribute to $\alpha_{IIb}\beta_3$-mediated clot retraction. In this regard, Rooney et al demonstrated (*10*, *17*) that recombinant human fibrinogen in which all Aα chain RGDs were mutated and AGDVs in the γC-domains were truncated exhibited normal clot retraction. In addition, in vivo studies demonstrated that mice with fibrinogen missing the γ^{407}QAGDV^{411} sequence, although manifesting impaired platelet aggregation, still had normal clot retraction (*18*). These observations indicate that the site(s) involved in the initial binding of fibrinogen to $\alpha_{IIb}\beta_3$ during platelet aggregation are different from those that participate in the interaction of platelets with fibrin during the thrombus growth and clot retraction.

We have recently identified the new sequence, γ^{365}NGIIWATWKTRWYSMKKTT^{383} (P3), in the γC-domain of fibrinogen that may function as the binding site for $\alpha_{IIb}\beta_3$ during clot retraction (*19*). The peptide duplicating this sequence was a potent inhibitor of platelet-mediated clot retraction and platelet adhesion. Furthermore, mutational analyses have demonstrated that P3 is the binding site for $\alpha_{IIb}\beta_3$ and suggested that both γ^{408}AGDV^{411} and P3 are required for the full adhesive activity of γC for platelets (*19*). It appears, though, that the mechanism by which $\alpha_{IIb}\beta_3$ binds P3 differs from AGDV recognition. First, adhesion of platelets to fibrinogen fragments containing P3 does not require their prior stimulation, whereas the engagement of the AGDV site is activation-dependent (*19*). Therefore, P3 and other sequence(s) (*20*, *21*) might hypothetically be responsible for adhesion of nonactivated platelets to immobilized fibrinogen, a mimic of fibrin (*22*). Moreover, since binding of resting $\alpha_{IIb}\beta_3$ to fibrin can initiate clot retraction (*23*), this interaction might also involve P3. Second, part of P3, the segment γ373−383, is fibrin-specific in that its exposure on the surface of the molecule is regulated by the transformation of fibrinogen to fibrin (*24*). In contrast, the AGDV sequence is well exposed. Finally, P3 contains no sequences resembling the AGDV or RGDX motifs and, thus, appears to define a previously unknown recognition specificity of $\alpha_{IIb}\beta_3$.

To gain an understanding of the molecular basis for the recognition of P3 by $\alpha_{IIb}\beta_3$, we have analyzed binding of $\alpha_{IIb}\beta_3$ to the P3-based substitutional peptide libraries, to recombinant mutant γC-domains, and to mutant natural and recombinant fibrinogens. We have found that the reactivity of P3 is confined to γ370−381, that it contains two independent recognition signals, and that its binding to $\alpha_{IIb}\beta_3$ depends on several discontinuous basic residues. Since the mechanism by which P3 binds to $\alpha_{IIb}\beta_3$ is different from that of RGD, these results imply that the binding site for P3 in $\alpha_{IIb}\beta_3$ is unlike that utilized by RGD or AGDV.

EXPERIMENTAL PROCEDURES

Proteins, Peptides, and Monoclonal Antibodies. Human thrombin and fibrinogen, depleted of fibronectin and plasminogen, were obtained from Enzyme Research Laboratories (South Bend, IN). Fibrinogen Kaiserslautern (with a substitution of γ^{380}Lys to Asn) was purified from plasma obtained from a homozygous individual as described previously (*25*).

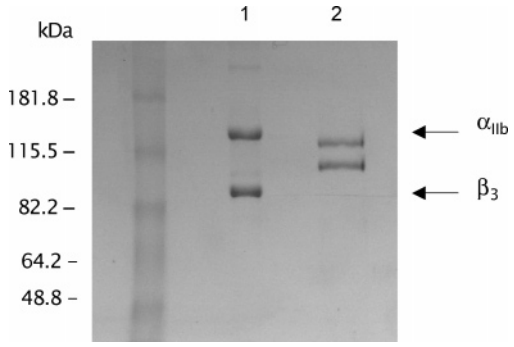

FIGURE 1: SDS−PAGE of the $\alpha_{IIb}\beta_3$ preparation. The integrin was purified from outdated platelet lysate as described under Experimental Procedures. The purity of $\alpha_{IIb}\beta_3$ was assessed by Coomassie blue staining of 7.5% polyacrylamide gels run under nonreducing (lane 1) and reducing conditions (lane 2).

The platelet integrin $\alpha_{IIb}\beta_3$ was isolated according to previously published procedures (*26*, *27*). Briefly, washed, outdated platelets (The Blood Center, New Orleans, LA) were lysed overnight at 4 °C in TBS, containing 2% Triton X-100, 1 mM $CaCl_2$, 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 10 μM leupeptin. The lysate was centrifuged and diluted in the same buffer to obtain a final Triton X-100 concentration of 0.2%, and passed over Con-A-Sepharose (Amersham Biosciences). Proteins were eluted with a 20 mM Tris buffer, pH 7.4, containing 250 mM NaCl, 0.2% Triton X-100, 2 mM $CaCl_2$, and 100 mM α-methyl-D-mannoside (Sigma). The eluted fraction was additionally purified from fibrinogen and thrombospondin on a Mono Q column (Amersham Biosciences) equilibrated with a 20 mM Tris buffer, pH 7.4, containing 2 mM $CaCl_2$ and 0.2% Triton X-100 reduced (Calbiochem). The integrin was eluted with a 0.05−1.0 M NaCl gradient. The purity and integrity of the integrin were assessed by SDS−PAGE (Figure 1). Approximately 85−90% of $\alpha_{IIb}\beta_3$ obtained by this procedure is in the inactive state as verified by fractionation by affinity chromatography on KYGRGDSPK-Sepharose (*27*). Purified integrin was labeled with ^{125}Iodine using IODO-GEN (Pierce). Iodinated protein was dialyzed against PBS and stored at −20 °C. Mab 4A5 directed against the C-terminal end of γC, γ406−411 (*28*), was a gift from Dr. G. Matsueda (Bristol-Meyers Squibb).

The peptide duplicating the fibrinogen sequence γ365−383, NGIIWATWKTRWYSMKKTT, and the mutant peptide, ATWATDWYSMDATT, with substitutions of K373A, R375D, K380D, and K381A, were synthesized using Fmoc chemistry and purified by HPLC on a preparative C18 Vydac column using a 5−90% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Authenticity and purity of the peptides were confirmed by mass spectroscopy.

Expression of Recombinant γC-Domains and Mutagenesis. The recombinant γC-domains were expressed as fusion proteins with glutathione *S*-transferase as described previously (*29*). Briefly, the coding region for the wild-type γC-domain (residues Ile^{145}-Val^{411}) was amplified using a template plasmid p674 (*30*) consisting of full-length cDNA encoding the human fibrinogen γ-chain. The accuracy of the DNA sequence was verified by sequencing. The recombinant proteins were purified from soluble fractions of *Escherichia coli* lysates by affinity chromatography using glutathione-agarose. The intactness of the C-terminus of the wild-type

γC-domain was confirmed by Western blot analysis using mAb 4A5. A deletion mutant without the γ397−411 C-terminal part and a series of γC-domains with selected point mutations within the P3 site were produced using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA). Concentrations of γC-domains were determined using Micro BCA Protein Assay Reagent kit (Pierce, Rockford, IL).

Expression of Recombinant Fibrinogen. Chinese hamster ovary cells expressing the human fibrinogen Aα and Bβ polypeptide chains (AαBβ-CHO cells) were cotransfected with wild-type and mutated pMLP-γ vectors containing the entire human γ-chain cDNA as described (*31*).[2] To construct a mutant expression vector containing the γ-chain Arg375Gly mutation corresponding to the variant fibrinogen Osaka V (*32*), the pMLP-γ vector was altered by oligonucleotide-directed mutagenesis using QuikChange Site-Directed Mutagenesis kit (Sratagene, La Jolla, CA). Wild-type and mutant plasmids were cotransfected into the AαBβ-CHO cells with the histidinol selection plasmid (pMSVhis). Colonies were selected with histidinol and G418, and screened for fibrinogen synthesis by ELISA as described (*31*). The individual clones with the highest expression levels of normal and mutant fibrinogens were used to seed roller bottles. Every 5 days serum-free media from roller bottles were collected, pooled, and stored at −70 °C until the fibrinogen was purified. Fibrinogen production continued for 3 months. Fibrinogens were purified by a two-step method as reported previously (*33*). Briefly, the proteins were concentrated by ammonium sulfate precipitation and then purified by immunoaffinity chromatography using anti-fibrinogen mAb IF-1 conjugated to Sepharose 4B. Purified proteins were dialyzed against 20 mM HEPES buffer, pH 7.4, 150 mM NaCl and stored at −70 °C.

Synthesis of Cellulose-Bound Peptide Libraries. The P3-derived peptide libraries assembled on cellulose membranes were prepared by parallel spot synthesis according to a protocol developed by Frank et al (*34*, *35*). The 9-fluorenylmethoxycarbonyl (Fmoc)-protected and pentafluorophenyl (Pfp) activated amino acids were purchased from Bachem (King of Prussia, PA). Pfp-activated Trp was obtained from Novabiochem (San Diego, CA). The following side-chain protecting groups were used: trityl for Cys, His, Asn, and Gln; *tert*-butyl for Asp, Glu, Ser, and Thr; *tert*-butoxycarbonyl for Lys, Trp; and pentamethylchroman sulfonyl for Arg. All other reagents were of the highest quality and were used without further purification. Peptides were COOH-terminally attached to cellulose via a $(\beta\text{-Ala})_2$ spacer and were acetylated N-terminally.

Screening of the P3-Derived Peptide Libraries for $\alpha_{IIb}\beta_3$ Binding. The membranes with covalently coupled peptides were incubated for 1 min in methanol and then washed with TBS buffer. After being blocked with 1% BSA for 2 h at 22 °C, the membranes were incubated with 10 μg/mL ^{125}I-labeled $\alpha_{IIb}\beta_3$ (10^5cpm/mL) in HEPES buffer containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ for 3 h at 22 °C using a shaker platform and then washed with TBS containing 0.05% Tween 20. The membranes were dried, and $\alpha_{IIb}\beta_3$ binding was visualized by autoradiography and analyzed by densitometry.

[2] The Synthesis of Recombinant Fibrinogen, multimedia presentation, available upon request from Dr. S. Lord.

$\alpha_{IIb}\beta_3$-Mediated Fibrin Clot Retraction. Clot retraction assays were performed as described previously (*19*). Briefly, whole blood was collected from healthy volunteers and anticoagulated by adding ACD (acid/citrate/dextrose) in the presence of 2.8 μM PGE_1. Platelets were isolated by differential centrifugation followed by gel filtration on Sepharose 2B. The reaction mixtures, with a total volume of 1.0 mL consisted of 3×10^8 platelets, 0.25 mg/mL fibrinogen, 1 mM $CaCl_2$ and 1 U thrombin in isotonic HEPES buffer (20 mM HEPES, pH 7.3, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3.3 mM NaH_2PO_4). Clot retraction assays with mutant fibrinogen Osaka V were performed using mixtures consisting of 0.1 mg/mL fibrinogen and 1×10^8/mL platelets (a volume of 100 μL). The progress of clot retraction was monitored by taking photographs of clots at selected time intervals using a digital camera. Clot retraction was expressed as a percentage of the cross-sectional area occupied by fibrin clot in the absence of platelets (determined as described) (*19*).

Adhesion Assays. Platelet adhesion was performed essentially as described previously (*19*). Briefly, the wells of 96-well tissue culture plates (Costar, Cambridge, MA) were coated with different concentrations (0−10 μg/mL) of recombinant γC-domains or fibrinogens for 3 h at 37 °C and post-coated with 1% BSA inactivated at 75 °C. Platelets were labeled with 10 μM Calcein AM (Molecular Probes, Eugene, OR) for 30 min at 37 °C, washed in isotonic HEPES buffer, and resuspended at 1×10^8/mL in the same buffer supplemented with 0.1% BSA, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. Aliquots (100 μL) of cells were added to the wells and incubated at 37 °C for 50 min. The nonadherent cells were removed by two washes with PBS and fluorescence was measured in a Cytofluor II fluorescence plate reader (Applied Biosystems, Framingham, MA). The number of adherent cells was determined using the fluorescence of aliquots with a known number of labeled cells.

Solid-Phase Binding Assays. To test the binding of solubilized $\alpha_{IIb}\beta_3$ to the immobilized fibrinogens, microtiter wells of Immulon 4BX Removawell strips (Dynatech Laboratories, Chantilly, VA) were coated with different concentrations of various preparations of plasma and recombinant fibrinogens, and blocked with 0.5% poly(vinylpyrrolidone) for 1 h at 22 °C. A total of 100 μL of ^{125}I-$\alpha_{IIb}\beta_3$ with a specific radioactivity of ∼0.002 μCi/μg in TBS containing 1 mM $MnCl_2$ and 0.1% BSA was added to each well. The plates were incubated for 3 h at 37 °C. Unbound integrin was washed out with TBS + 0.05% Tween 20, and radioactivity was counted in a γ counter. Nonspecific binding was defined as the binding to the wells coated with 0.5% poly(vinylpyrrolidone) and was subtracted from the total binding.

RESULTS

Localization of Amino Acid Residues in the P3 Peptide Involved in $\alpha_{IIb}\beta_3$ Binding. To narrow down the active part of the γ365−383 peptide, we prepared a scanning peptide library consisting of 6-mer overlapping peptides with a one-residue offset covering γ365−383 (Figure 2A). The membrane with covalently coupled peptides was screened for binding of ^{125}I-$\alpha_{IIb}\beta_3$. The results of autoradiography revealed that peptides 4 through 14 spanning the γ368−383 sequence

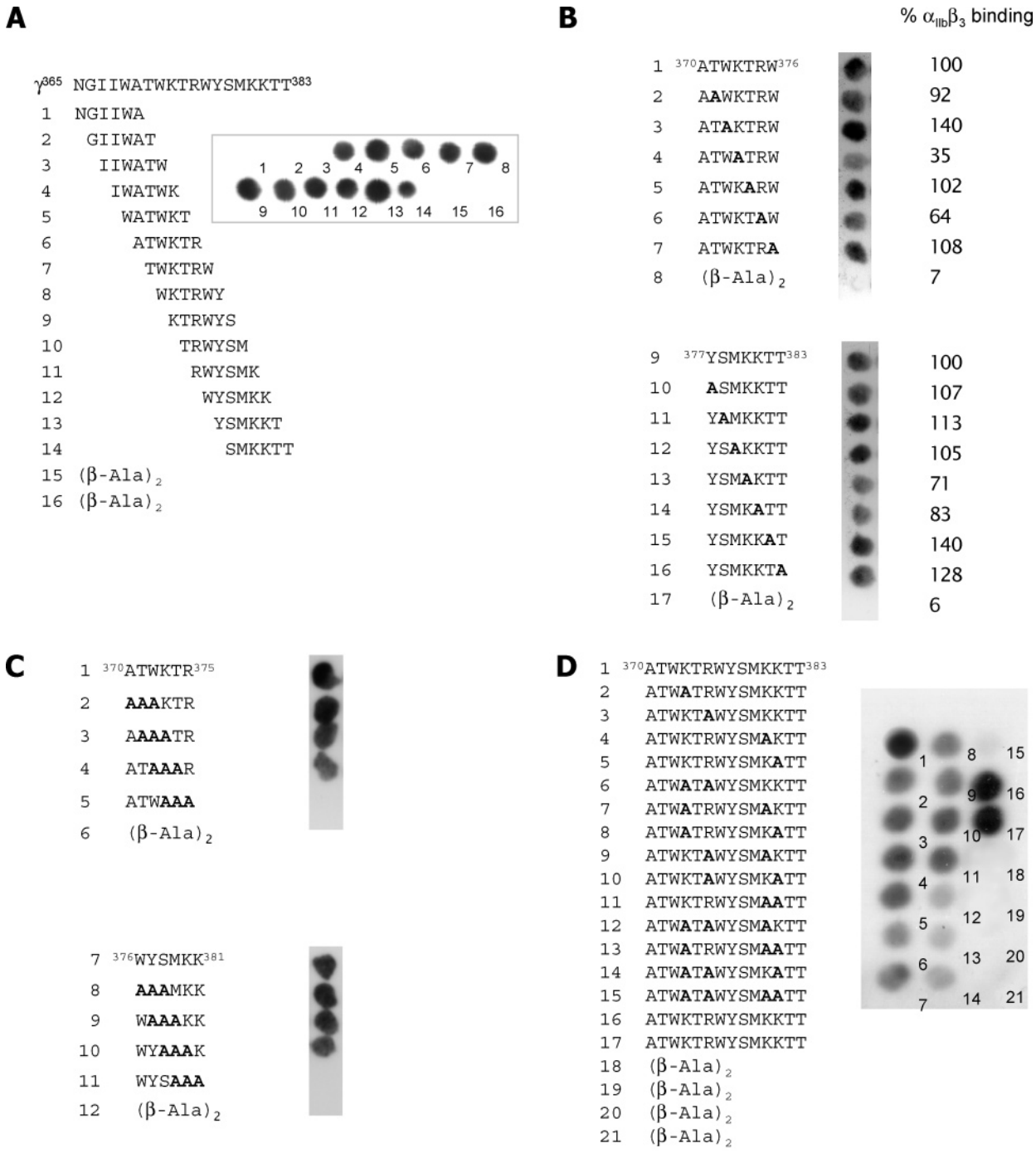

FIGURE 2: Binding of $\alpha_{IIb}\beta_3$ to the cellulose-coupled peptide libraries derived from the P3 peptide. (A) The membrane with bound 6-mer peptides derived from P3 (^{365}NGIIWATWKTRWYSMKKTT^{383}) was synthesized by parallel spot synthesis as described under Experimental Procedures. The membrane was incubated with 10 μg/mL ^{125}I-labeled purified $\alpha_{IIb}\beta_3$ for 3 h at 22 °C. Bound $\alpha_{IIb}\beta_3$ was visualized by autoradiography. No binding to spots 1−3 was detected. Spots 15 and 16 contain only the (β-Ala)$_2$ spacers. (B) Peptide libraries derived from the two active parts of γ370−383, ^{370}ATWKTRW^{376} (spot 1) and ^{377}YSMKKTT^{383} (spot 9), in which all residues were consecutively substituted to Ala. The sequences of wild-type and mutant peptides are shown on the left. The numbers on the right show the relative binding of receptor to peptides, and they represent a percentage of the intensity of the spot containing wild-type peptide as analyzed by densitometry. (C) Peptide libraries of ^{370}ATWKTR^{375} and ^{376}WYSMKK^{381} in which three consecutive residues were mutated to Ala. (D) Binding of $\alpha_{IIb}\beta_3$ to the combinatorial alanine scanning γ370−383 peptide library in which Lys^{373}, Arg^{375}, Lys^{380}, and Lys^{381} were substituted to Ala, and single or different combinations of two, three, and four mutated residues were introduced in peptides. Spots 1, 16, and 17 contain wild-type γ370−383, and spots 18−21 are $\alpha_{IIb}\beta_3$ binding to control spots that contain the (β-Ala)$_2$ spacer.

bound the receptor, while peptides derived from the N-terminal part (spots 1−3) were not active. Consistent with this result, γ^{366}GIIW^{369} is not exposed on the surface of γC (*36*) and thus presumably cannot participate in $\alpha_{IIb}\beta_3$ binding. Therefore, the activity is apparently confined within the γ370−383 sequence. Furthermore, because nonoverlapping peptides from the N-terminal part and C-terminal part of P3 (for example, peptides in spots 5 and 13) can bind $\alpha_{IIb}\beta_3$, γ370−383 may contain at least two independent binding regions. To identify residues critical for receptor binding, we prepared two scanning libraries covering the N- and C-terminal parts of γ370−383, ^{370}ATWKTRW^{376} and

^{377}YSMKKTT^{383}, in which each residue was mutated to Ala (Figure 2B). Densitometry analyses of scans demonstrated that individual substitution of ^{373}Lys and ^{375}Arg in γ370−376 (spots 4 and 6) and of two lysines, 380 and 381, in γ377−383 (spots 13 and 14) decreased receptor binding by ∼40−70% of that to wild-type peptides. The results suggested that these amino acids participate in $\alpha_{IIb}\beta_3$ binding and that simultaneous mutations of two residues might be required to abrogate function. Since mutations of two C-terminal threonines did not alter the interaction with the receptor, two 6-mer peptides, γ370−375, ATWKTR (P3-1), and γ376−381, WYSMKK (P3-2), were analyzed further by screening substitutional peptide libraries in which three consecutive residues in each peptide were changed to Ala. As shown in Figure 2C, changes of Lys^{373} and R^{375} in P3-1 (spot 5) and substitutions of two neighboring lysines (^{380}KK^{381}) in P3-2 (spot 11) resulted in the loss of $\alpha_{IIb}\beta_3$ binding. Because peptides 3 and 4 from the P3-1 library, which contain either Lys^{373} or Arg^{375} substituted to Ala, respectively, are still capable of $\alpha_{IIb}\beta_3$ binding, simultaneous substitution of two basic residues in ^{370}ATWKTR^{375} seems to be necessary to abolish binding. Likewise, the ability of peptide WYAAAK (spot 10 in the P3-2 library) to bind $\alpha_{IIb}\beta_3$ suggests that two lysines are required for binding. To substantiate the conclusion that positively charged residues in the entire γ370−383 contribute to its activity, we have synthesized the additional P3-based substitutional library in which different combinations of Lys^{373}, Arg^{375}, Lys^{380}, and Lys^{381} were changed to Ala. Analyses demonstrated that single, double, and triple mutations, although they reduced $\alpha_{IIb}\beta_3$ binding, did not destroy it completely (spots 2−5, 6−11, 12−14, respectively), and only when all selected residues were mutated to Ala (spot 15) was $\alpha_{IIb}\beta_3$ binding lost (Figure 2D). To determine how substitutions of side-chains of the identified critical basic and other residues within γ370−381 affect its ability to bind receptor, we have generated the libraries in which each position within P3-1 and P3-2 was changed in turn to all 20 L-amino acids (Figure 3). By contrast with Ala mutagenesis, substitution of only one positively charged residue within either P3-1 or P3-2 to Asp or Glu resulted in the complete loss of $\alpha_{IIb}\beta_3$ binding. Similar to Ala mutagenesis, some decrease was noted in $\alpha_{IIb}\beta_3$ binding to P3-1 in which Arg^{375} was substituted to Pro and Ala (Figure 3A) and to P3-2 in which Lys^{380} and Lys^{381} were substituted to other residues (Figure 3B, last two lines). Also, substitutions of Thr^{371} in P3-1 to Asp or Glu (Figure 3A, spots 3 and 4 in line 2) decreased receptor binding. At the same time, changes of key basic residues and all other residues to different polar, hydrophobic, and aromatic residues did not alter the interaction with receptor. Although the semiquantitative nature of these analyses precludes definitive conclusions about the contribution of Thr^{371} and other residues, these results indicate that basic residues within the P3 site are critical for receptor binding and that substitutions of side-chains of other residues can be tolerated.

To test the functional role of identified key residues, we have synthesized the mutant peptide, ATW<u>A</u>T<u>D</u>WYSM-<u>DA</u>TT, in which basic residues were substituted to Ala or Asp, and tested its ability to inhibit clot retraction. (A mutant peptide with four critical residues mutated to Ala was also prepared; however, it could not be tested because of poor solubility). As shown in Figure 4, at 300 μg/mL (200 μM) the mutant peptide was inactive, whereas the same concentration of wild-type γ370−383 inhibited clot retraction completely. Also, the mutant peptide immobilized onto the microtiter plates did not support platelet adhesion (both stimulated and nonstimulated), while wild-type γ370−383 promoted strong cell adhesion (not shown). Thus, these results indicate that functional activity of γ370−383 depends on four basic residues, Lys^{373}, Arg^{375}, Lys^{380}, and Lys^{381}.

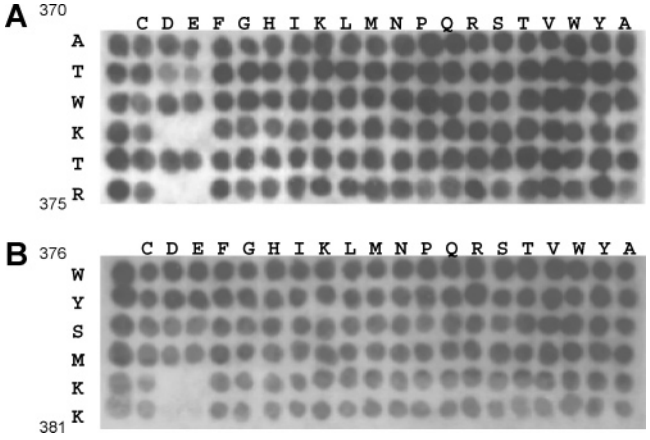

FIGURE 3: Full substitutional analyses of two parts of γ370−381 (γ370−375 and γ376−381). The residues (on the left of each panel) in wild-type peptides γ^{370}ATWKTR^{375} (A) and γ^{376}WYSMKK^{381} (B) were substituted to all other L-amino acids (rows). For example, spots in the first horizontal row in panel A contain mutant peptides in which Ala in the ATWKTR sequence was substituted to all 20 L-amino acids. Each spot in the first vertical row contains wild-type peptides γ^{370}ATWKTR^{375} and γ^{376}WYSMKK^{381} in panels A and B, respectively. The constructed peptide libraries were tested for $\alpha_{IIb}\beta_3$ binding as described above.

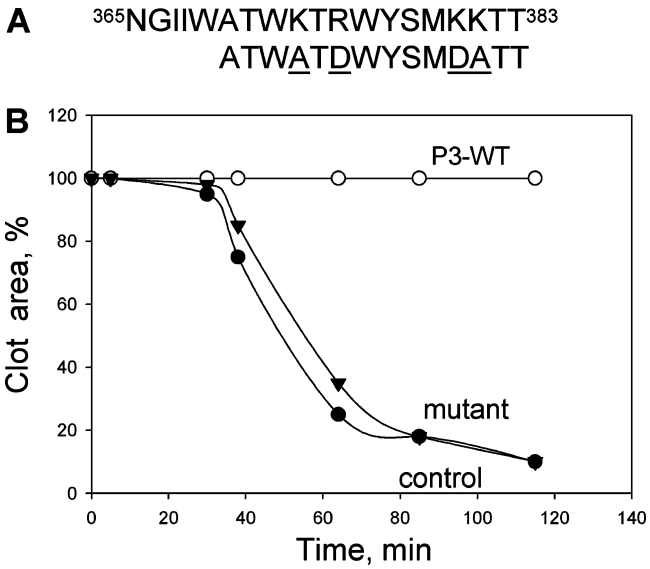

FIGURE 4: Effect of the P3 and mutant peptides on platelet-mediated fibrin clot retraction. (A) P3 and P3-mutant sequences with four mutated residues underlined. (B) Gel-filtered platelets were mixed with 0.25 mg/mL fibrinogen in isotonic HEPES buffer containing 1 mM $CaCl_2$ and 300 μg/mL of the wild-type P3 peptide (open circles) or P3-quadruple mutant (triangles). Fibrin clots were formed by adding 1 U/mL thrombin at 22 °C. Clot retraction was observed by taking photographs at different times (0−110 min). Retraction was expressed as a percent of control (clot area occupied by a clot formed in the absence of platelets). Filled circles, clot retraction mediated by platelets in the absence of peptides. A representative experiment is shown.

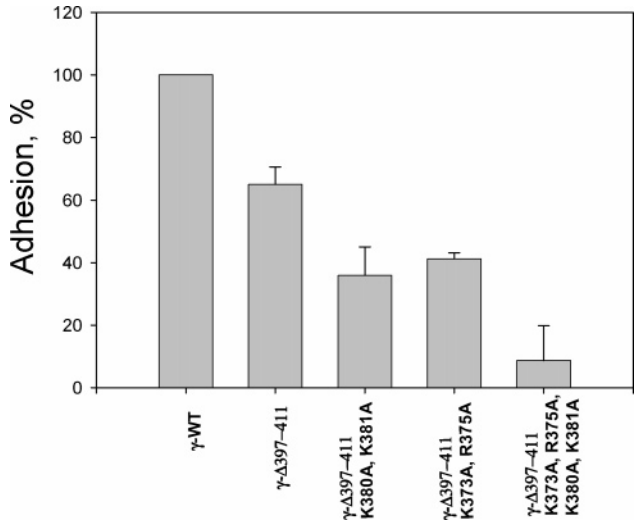

FIGURE 5: Adhesion of platelets to recombinant wild-type and mutant γC-domains. Gel-filtered nonstimulated platelets were labeled with calcein AM and added to microtiter wells coated with wild-type and mutant γC-domains. Cell adhesion was measured as described under Experimental Procedures. Data are expressed as a percent of control (adhesion to wild-type γC) and are the mean ± SE of four individual experiments performed with triplicate determinations in each experiment.

Recombinant γC-Domain with Mutations of Critical Amino Acid Residues in the P3 Site and with ^{408}AGDV^{411} Deleted Does Not Support Platelet Adhesion. The capacity of the identified residues to contribute to $\alpha_{IIb}\beta_3$ binding was verified by using recombinant γC-domains in which critical residues were mutated to Ala. Inspection of the three-dimensional structure of γC (*36*) demonstrated that side-chains of selected residues are exposed on the surface of the γC-domain and do not interact with neighboring residues. Thus, their substitution to Ala is unlikely to perturb the conformation, and the proteins fold. To exclude the contribution of the second $\alpha_{IIb}\beta_3$ binding site at γ^{408}AGDV^{411}, mutations were introduced in γC in which the C-terminal part, γ397−411, was deleted. As expected, platelets adhered strongly to wild-type γC. Adhesion partially decreased after removal of the AGDV site (62 ± 7%) (Figure 5). Additionally mutating two residues, Lys^{373} and Arg^{375}, or Lys^{380} and Lys^{381}, resulted in a further decline of the γC's ability to support platelet adhesion. Finally, γC-ΔAGDV carrying a quadruple mutation was completely inactive. Thus, these data show that the two sequences, AGDV and P3, constitute the γC-domain functional epitope.

γC Mutation at Lys380Asn in Abnormal Fibrinogen Kaiserslautern Impairs its Ability to Bind $\alpha_{IIb}\beta_3$. It is known that patients with dysfibrinogenemia have fibrinogen with point mutations in different chains and exhibit various defects in hemostatic function. Since Lys^{380} was identified in the present study as one of the critical residues in the P3 site involved in $\alpha_{IIb}\beta_3$ binding, we have analyzed platelet-mediated clot retraction in the presence of fibrinogen Kaiserslautern. In this mutant, Lys^{380} is substituted to Asn resulting in additional carbohydrate being appended at this residue (*25*). As shown in Figure 6A, clot retraction with fibrinogen Kaiserslautern was delayed compared to that by normal fibrinogen. Thus, whereas clot retraction by normal fibrinogen began after 35 min, fibrin clots made from mutant fibrinogen did not start to retract until ~110 min. Analyses of kinetic parameters indicated that the V_{max} of clot retraction in the presence of fibrinogen Kaiserslautern decreased by ~4-fold compared to that mediated by normal fibrinogen (0.72 ± 0.1 versus 2.9 ± 0.1%/min, respectively). It has been reported (*25*) that fibrinogen Kaiserslautern displays prolonged thrombin-induced fibrin polymerization. Although fibrin polymerization is not a rate-limiting step in the overall process of clot retraction, we have tested whether the observed difference in clot retraction is due to delayed clot formation by fibrinogen Kaiserslautern. Since the aberrant fibrin polymerization of fibrinogen Kaiserslautern can be normalized by increased calcium concentration (*25*), we performed clot retraction in the presence of 5 mM $CaCl_2$. Addition of Ca^{2+} did not correct clot retraction mediated by fibrinogen Kaiserslautern (not shown), suggesting that the defective binding of mutant fibrinogen to $\alpha_{IIb}\beta_3$ on platelets might be responsible for the delay.

To examine this possibility, we tested binding of isolated integrin to fibrinogen. In solid-phase binding assays, the ability of immobilized fibrinogen Kaiserslautern to bind ^{125}I-labeled $\alpha_{IIb}\beta_3$ was reduced (Figure 6B). Although the defect in integrin binding might not be simply due to the presence of Asn but carbohydrate in this position, the results demonstrate that this mutation caused a small but significant decrease in the ability of fibrinogen to bind $\alpha_{IIb}\beta_3$, thus supporting the role of Lys^{380}. However, since the process of clot retraction was not arrested completely, other residues likely participated in the interaction between $\alpha_{IIb}\beta_3$ and fibrin.

Studies with Recombinant Fibrinogens. A fibrinogen variant with mutation of another critical residue, Arg^{375}, is known as fibrinogen Osaka V (Arg → Gly) (*32*). We have generated recombinant fibrinogen in which Arg^{375} was substituted to Gly and compared its adhesive properties with those of wild-type recombinant fibrinogen. Normal recombinant fibrinogen and mutant fibrinogen Osaka V were purified to homogeneity (Figure 7A), and their interactions with $\alpha_{IIb}\beta_3$ were tested in solid-phase binding assays. As shown in Figure 7B, both immobilized proteins bound integrin. However, mutant fibrinogen bound less $\alpha_{IIb}\beta_3$ compared to wild-type recombinant fibrinogen. Moreover, clot retraction mediated by fibrinogen Osaka V was significantly delayed compared to wild-type fibrinogen (Figure 7C). Because of the limited quantity of recombinant Osaka V fibrinogen, kinetic parameters of the reaction could not be determined. Also, because the mutation caused a substantial effect on clot retraction, but only a small effect on $\alpha_{IIb}\beta_3$ binding, it might be a reflection of the lower (100 μg/mL) than normal concentration of fibrinogen used in the assay. Neither single point mutation variant showed a complete loss of clot retraction as would be expected given that the simultaneous substitution of four residues in γC was required to ablate platelet adhesion. However, generation of mutant recombinant fibrinogen with four mutations (and two mutations) was not feasible as such molecules were not secreted from cells (unpublished data, Podolnikova, N. P.). Nevertheless, the results with both mutant fibrinogens substantiate the roles of two basic residues in P3, Arg^{375} and Lys^{380}, in $\alpha_{IIb}\beta_3$-mediated adhesive reactions.

DISCUSSION

In a previous study, we described the new γ365−383 sequence in the γC-domain of fibrinogen (*19*) which binds

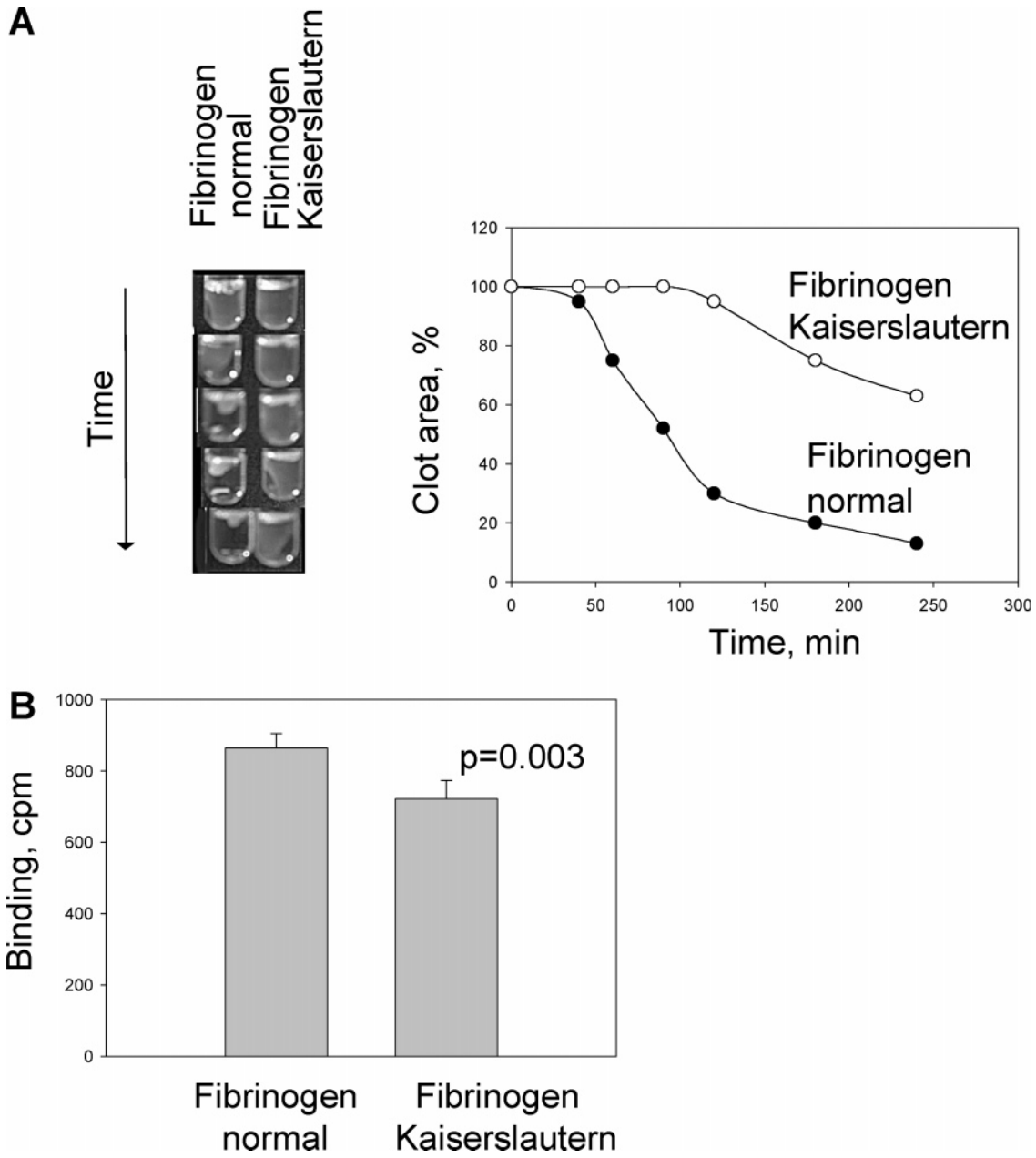

FIGURE 6: Interactions of $\alpha_{IIb}\beta_3$ with mutant fibrinogen Kaiserslautern (Lys380Asn). (A) Platelet-mediated fibrin clot retraction supported by fibrinogen Kaiserslautern. Platelets were mixed with 0.25 mg/mL normal fibrinogen or mutant fibrinogen Kaiserslautern in isotonic HEPES buffer containing 1 mM $CaCl_2$. Fibrin clot formation and retraction were initiated by adding 1 U/mL of thrombin, and clot retraction was monitored by taking photographs (shown on the left) at selected times. Clot retraction is expressed as a percent of clot area in the control tube without platelets (0 time). A representative experiment is shown. (B) Binding of purified $\alpha_{IIb}\beta_3$ to immobilized fibrinogens. ^{125}I-labeled $\alpha_{IIb}\beta_3$ (50 μg/mL) was added to microtiter wells coated with recombinant normal fibrinogen or fibrinogen Kaiserslautern. A representative experiment with binding of receptor to 10 μg/mL immobilized fibrinogens is shown. Values displayed are the means ± SE of 16 determinations.

to integrin $\alpha_{IIb}\beta_3$. This sequence mediates platelet adhesion to immobilized fibrinogen and potentially could be involved in platelet-mediated fibrin clot retraction. In the present study, we determined the mechanism by which $\alpha_{IIb}\beta_3$ binds this γC segment employing cellulose-bound peptide scans, mutagenesis of recombinant γC-domains, and analyses of mutant fibrinogens. The major conclusions drawn from these studies are (1) the active part of γ365−383 is contained within γ370−381 (designated P3); (2) within P3, ^{370}ATWKTR^{375} and ^{376}WYSMKK^{381} can independently bind $\alpha_{IIb}\beta_3$; and (3) the $\alpha_{IIb}\beta_3$-binding activity of γ370−381 depends on four basic residues, Lys^{373}, Arg^{375}, Lys^{380}, and Lys^{381}. Simultaneous mutations of these residues together with deletion of the AGDV site produced a recombinant γC-domain not capable of supporting platelet adhesion, indicating that these residues in P3 are sufficient for $\alpha_{IIb}\beta_3$ docking. The identified critical amino acid residues also can contribute to the interaction of fibrin with $\alpha_{IIb}\beta_3$ in platelet-mediated clot retraction. Mutation of γLys^{380} in the naturally occurring abnormal fibrinogen Kaiserslautern (Lys → Asn) (*25*) led to delayed clot retraction and reduced its ability to bind purified integrin. Furthermore, substitution of γArg^{375} in recombinant fibrinogen which typifies abnormal fibrinogen Osaka V (Arg → Gly) (*32*) also impaired binding of receptor

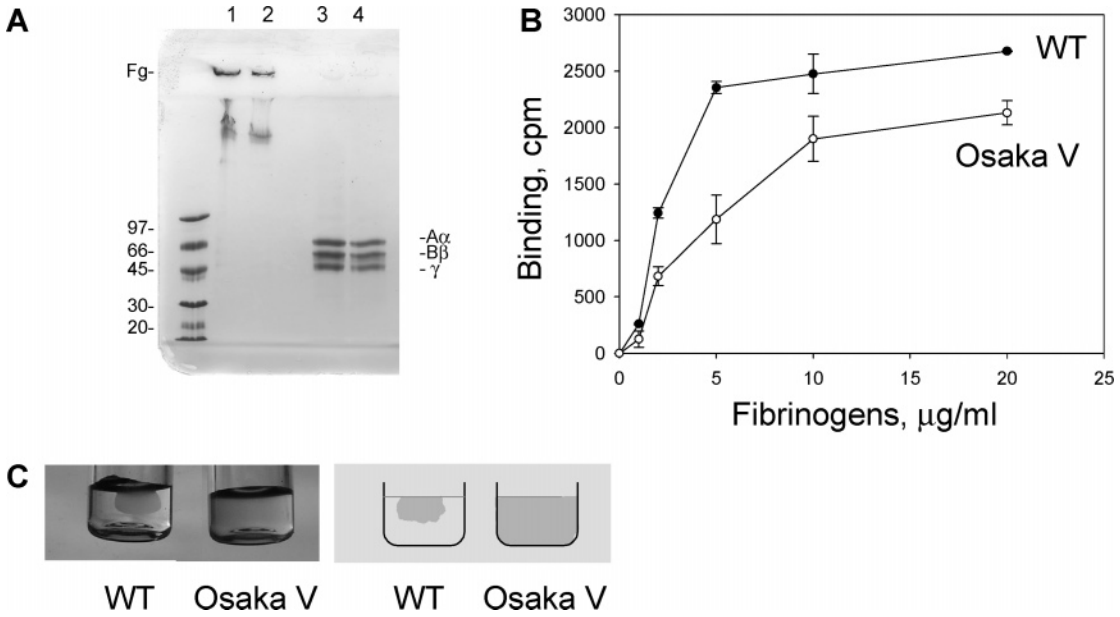

FIGURE 7: The interactions of $\alpha_{IIb}\beta_3$ with recombinant mutant fibrinogen Osaka V (Arg375Gly). (A) Characterization of recombinant fibrinogens by SDS−PAGE. Wild-type (lanes 1 and 3) and mutant (lanes 2 and 4) proteins were purified as described under Experimental Procedures and electrophoresed on 4−15% acrylamide gels under nonreducing (lanes 1 and 2) and reducing conditions (lanes 3 and 4). Molecular weight markers are shown on the left. Fg, indicates intact fibrinogen. Aα, Bβ, and γ indicate the constituent fibrinogen chains. (B) Binding of purified $\alpha_{IIb}\beta_3$ to immobilized fibrinogens. ^{125}I-labeled $\alpha_{IIb}\beta_3$ (50 μg/mL) was added to microtiter wells coated with various concentrations of recombinant wild-type (WT) or Osaka V fibrinogens. Values displayed are the means ± SE of three experiments with six determinations in each experiment. (C) Platelet-mediated fibrin clot retraction in the presence of wild-type (WT) and Osaka V fibrinogens. Clot retraction was performed as described in Figure 6 using mixtures (0.1 mL) consisting of 100 μg/mL fibrinogens and 1×10^8/mL platelets. Clots were photographed after 1.5 h (left panel). A drawing (right panel) depicts the location and shape of the retracted clot in the tube containing wild-type fibrinogen and nonretracted fibrin clot in the tube with fibrinogen Osaka V. One of two experiments performed is shown.

and delayed clot retraction. Thus, the present studies further establish P3 as a binding site for $\alpha_{IIb}\beta_3$ in fibrin(ogen) and define the new recognition specificity of this integrin.

It is generally accepted that the two fibrinogen peptides, γC ^{400}HHLGGAKQAGDV^{411} (H12) and the RGD-based peptides, define the recognition specificity of $\alpha_{IIb}\beta_3$ (reviewed in refs *2* and *7*). These peptides inhibit binding of soluble fibrinogen and other protein ligands to $\alpha_{IIb}\beta_3$ and block platelet adhesive reactions. In the XRGDX peptides, Arg and Asp are of critical importance for the peptides' abilities to bind $\alpha_{IIb}\beta_3$ and to function as the receptor antagonists (*37*−*39*). Furthermore, Gly, situated between Arg and Asp, and hydrophobic residues in the X position are required for the optimal interaction with $\alpha_{IIb}\beta_3$ (*38*, *40*, *41*). The mechanism by which H12 binds to $\alpha_{IIb}\beta_3$ has not been clarified at the structural level, although the fact that its activity is confined to the AGDV sequence (*9*, *10*, *18*) suggests that binding of H12 can involve Gly and Asp. The finding that the functional activity of P3 depends on basic residues with little contributions from other amino acids suggests that the mechanism by which $\alpha_{IIb}\beta_3$ binds this sequence is different from that of the two other fibrinogen recognition peptides. It is noteworthy that recognition of full-length P3 and short P3-derived peptides by $\alpha_{IIb}\beta_3$ appears to rely largely on the total peptide's charge rather than on specific combinations of amino acid residues. Consistent with this finding, partial neutralization of the charge by substitution of one basic residue to Glu or Asp, or removal of side-chains in both positively charged residues by substitutions to Ala, rendered the 6-mer peptides inactive (Figures 2 and 3). All other residues within P3 tolerated alterations to any other residue (Figure 3). However, particular positioning of critical residues is needed as their shuffling in scrambled P3 produced peptide with no ability to inhibit clot retraction and with decreased adhesion promoting capacity (*19*).

All four key residues in P3 are situated in the extended loop in the crystal structure of γC (*36*), with their side-chains being accessible for receptor docking (Figure 8). Moreover, the formation of the γC−γC interface during the DD-dimer assembly appears to not affect their location (*5*). Nevertheless, P3 and the adjacent γ383−395 region, the binding site for leukocyte integrin Mac-1 (*42*, *43*), are poorly exposed in soluble fibrinogen and become available for integrin binding only after its conversion to fibrin and after deposition of fibrinogen onto surfaces or in the extracellular matrix (*44*). The structural basis for conformational changes which unmask this "integrin-prone" region in the γC-domain is unknown. However, shielding by the flexible γ397−411 tail and/or "pulling-out" of the β strand formed by γ380−390 have been proposed as possible mechanisms (*44*, *45*).

The distinct binding specificity of P3 suggests that it should bind to the site(s) on $\alpha_{IIb}\beta_3$ different from those for RGDX and H12. Recent studies demonstrated that $\alpha_{IIb}\beta_3$ with RGD bound is still capable of binding to soluble fibrinogen (*46*), presumably through its H12 sequence. Hence, $\alpha_{IIb}\beta_3$ contains at least two ligand binding pockets, one for RGD peptides and one for fibrinogen (*46*, *47*). Although the location of the binding site for H12 remains uncertain (*48*, *49*), it is not likely that P3 binds to the same site as H12. Since the interaction between $\alpha_{IIb}\beta_3$ and P3 occurs after transformation of soluble fibrinogen to fibrin or upon platelet adhesion to immobilized fibrinogen, it is possible that P3 binds to yet another previously unrecognized binding site(s) in $\alpha_{IIb}\beta_3$. The fact that binding of H12 and P3 is regulated

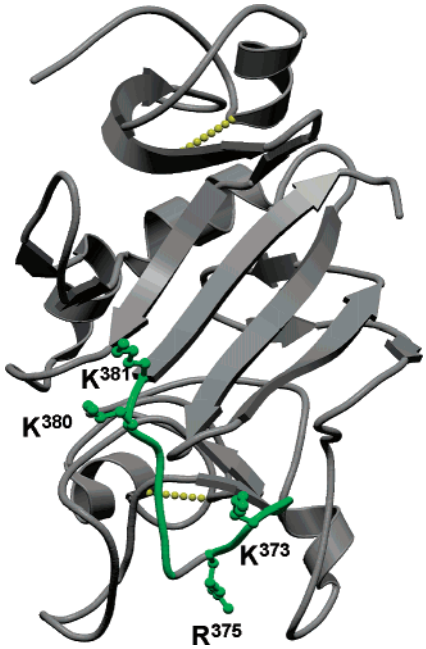

FIGURE 8: Positions of the identified critical residues in the P3 sequence in the three-dimensional structure of γC. The ribbon diagram of the fibrinogen γC-domain is based upon its crystal structure (PDB code 1fib) (*36*). Side-chain groups of Lys[373], Arg[375], Lys[380], and Lys[381] and the backbone of γ370−375 and γ376−381 are colored in green. Yellow dotted lines depict disulfide bonds. The figure was constructed using the computer programs MolScript, BobScript, and Raster (*63*−*65*).

independently by integrin activation (*19*) further argues for a model in which these sequences bind to separate sites on $\alpha_{IIb}\beta_3$. Whether the binding of fibrin-specific P3 to receptor shares the regions involved in RGD and H12 binding or it binds to separate site(s) is under investigation.

Among integrin family members, $\alpha_{IIb}\beta_3$ is regarded as the classic fibrinogen receptor. Nonetheless, this integrin is capable of binding many other ligands. These include both proteins that contain RGD/KGD adhesion motifs (fibronectin, C1-domain of vWF, vitronectin, thrombospondin, prothrombin, collagens, disintegrins, CD40L) (*50*−*52*) and those that lack an RGD sequence (plasminogen, ICAM-4, the A1-domain of vWF) (*53*−*55*). Thus, although $\alpha_{IIb}\beta_3$ exhibits the discernible specificity that is manifested in the activation-dependent binding of soluble fibrinogen through H12, it can also bind other sequence(s) within fibrin(ogen) and in other proteins through different specificities. The structural features that impart to $\alpha_{IIb}\beta_3$ the intrinsic property of binding a broad range of ligands is not known. The relatively limited requirements for the binding of P3 by $\alpha_{IIb}\beta_3$ suggest that other sequences, which contain analogous combinations of amino acid residues or have similar physicochemical properties, could function as the $\alpha_{IIb}\beta_3$ binding sites in fibrin. For example, the second homologous domain in the D region of fibrinogen, βC, contains a sequence highly homologous to P3 (β^{438}MNWKGSWYSMRKMS^{451}). Several sequences with adjacent basic residues are present in other fibrinogen domains and in other ligands. Therefore, it is tempting to speculate that such clusters can interact with $\alpha_{IIb}\beta_3$ and may determine its ability to bind a wide repertoire of ligands.

Broad recognition specificity is known to be inherent to other integrins and, in particular, to the most promiscuous member of the family, leukocyte integrin $\alpha_M\beta_2$ (Mac-1). In $\alpha_M\beta_2$, the inserted α_MI-domain is largely responsible for $\alpha_M\beta_2$'s ability to bind numerous proteins, including fibrinogen. Recently, we demonstrated that the α_MI-domain binds to multiple sites in the D region of fibrinogen in which it picks out the sequences enriched in basic and hydrophobic residues (*56*) (and unpublished data).[3] It is interesting that P3 binding by $\alpha_{IIb}\beta_3$ shares the same dependence on basic residues as that of ligand recognition by the α_MI-domain. Nevertheless, there is a certain distinction between the specificity exhibited by $\alpha_{IIb}\beta_3$ and $\alpha_M\beta_2$ since the α_MI-domain recognition peptide P2-C (γ^{383}TTMKIIPFNRLTIG^{395}) (*42*) is a poor inhibitor of clot retraction (*19*). Furthermore, α_MI-domain utilizes hydrophobic residues in its ligands, while $\alpha_{IIb}\beta_3$ binding to P3 appears to be insensitive to such residues. The contribution of basic residues in ligands to integrin recognition becomes persuasive. Not only do the I-domain containing integrins, such as $\alpha_M\beta_2$, $\alpha_X\beta_2$, and $\alpha_2\beta_1$, bind nonhomologous positively charged sequences (*57*, *58*), the non-I-domain integrins $\alpha_5\beta_1$ (*19*) and $\alpha_v\beta_3$ (unpublished data)[4] also can interact with P3. At present, the mechanisms responsible for differential specificity exhibited by $\alpha_{IIb}\beta_3$ in binding of the RGD, H12, and P3 sequences remain to be elucidated.

As a final point, the ability of $\alpha_{IIb}\beta_3$ to bind fibrin(ogen) through the P3 specificity bears upon the development of $\alpha_{IIb}\beta_3$ antagonists. The capacity of fibrinogen to serve as a cofactor in platelet aggregation has been targeted for antithrombotic therapy, and a number of effective $\alpha_{IIb}\beta_3$ antagonists were designed and approved for patients undergoing coronary artery intervention (reviewed in refs *2* and *3*). Since platelet binding to fibrin (and, by extrapolation, to fibrinogen deposited within atherosclerotic lesions) appears to involve regions on $\alpha_{IIb}\beta_3$ additional to those that mediate interactions with soluble fibrinogen, inhibition of platelet interactions with insoluble fibrin(ogen) may be a necessary and important property of $\alpha_{IIb}\beta_3$ antagonists (*3*). It is noteworthy, that numerous studies documented the ability of various positively charged compounds, including natural polyamines, to influence platelet aggregation (*59*−*61*) and to inhibit the fibrinogen binding to $\alpha_{IIb}\beta_3$ (*62*). Identification of the cationic site in the P3 sequence of fibrinogen suggests that these agents could potentially target the P3−$\alpha_{IIb}\beta_3$ interactions.

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