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Fibrinogen Hershey IV: A Novel Dysfibrinogen with a γ V411I Mutation in the Integrin α Ib β 3 Binding Site

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Summary

The carboxyl terminal segment of the fibrinogen γ chain from γ 408–411 plays a crucial role in platelet aggregation *via* interactions with the platelet receptor α Ib β 3. We describe here the first naturally-occurring fibrinogen point mutation affecting this region and demonstrate its effects on platelet interactions. DNA sequencing was used to sequence the proband DNA, and platelet aggregation and direct binding assays were used to quantitate the biological effects of fibrinogen Hershey IV. The Hershey IV proband was found to be heterozygous for two mutations, γ V411I and γ R275C. Little difference in aggregation was seen when fibrinogen Hershey IV was compared to normal fibrinogen. However, less aggregation inhibition was observed using a competing synthetic dodecapeptide containing the V411I mutation as compared to the wild-type dodecapeptide. Purified fibrinogen Hershey IV also bound to purified platelet α Ib β 3 with a lower affinity than wild-type fibrinogen. These findings show that the γ V411I mutation results in a decreased ability to bind platelets. In the heterozygous state, however, the available wild-type fibrinogen appears to be sufficient to support normal platelet aggregation.

Keywords

Fibrinogen/fibrin; GP IIb/IIIa; inherited coagulation disorders; integrins; platelet glycoproteins

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Introduction

Fibrinogen plays a critical role in clot formation. Synthesized in the liver, it circulates as a heterodimer comprised of two copies of each of 3 chains, A α , B β , and γ (1). The γ chain of fibrinogen has several important functions (2, 3). FXIIIa mediates γ chain cross-linking through glutamine residues at γ 398 and 399 and the lysine residue at γ 406 (4, 5). The γ chain also plays a key role in platelet aggregation via its interaction with platelet integrin α IIB β 3. This interaction occurs *via* the carboxyl terminal region of the fibrinogen γ chain, particularly the last 4 amino acids (γ 408–411, AGDV) (6–10). Fibrinogen binding to α IIB β 3 leads to platelet aggregation, a process that is stimulated by several different agonists (11). The γ' variant of fibrinogen, which arises from alternative mRNA processing (12, 13), contains a different 20 amino acid sequence in place of γ 408–411 and does not bind α IIB β 3 (9, 10, 14, 15).

Fibrinogen defects can be either quantitative or qualitative. A quantitative defect in fibrinogen, either its absence or a significant decrease in circulating protein, is typically associated with bleeding (16). Qualitative defects lead to dysfibrinogenemia, with alterations in fibrinogen structure (17). The majority of mutations affect fibrinopeptide cleavage or fibrin polymerization, with a minority involving FXIII cross-linking (18). To date, over 350 mutations have been reported (19), with the most frequent sites of mutation at the fibrinopeptide A cleavage site and the γ chain arginine 275.

To the best of our knowledge, no naturally occurring single amino acid mutations have been previously reported in the platelet-binding domain at the carboxy terminal end of the γ chain. In this manuscript, we characterize a dysfibrinogen from a subject who is heterozygous for two γ chain mutations, R275C and V411I, termed fibrinogen Hershey IV. Although the contribution of the latter mutation to clinical bleeding is unclear, our studies show that the 411I mutation decreases fibrinogen's ability to bind α IIB β 3.

Materials and methods

Materials

All chemicals were obtained from Fisher Scientific (Fairlawn, NJ) unless otherwise specified.

DNA sequencing

Genomic DNA was purified from whole blood clot specimens using the QIAamp DNA blood kit (Qiagen, Valencia, CA). DNA sequencing of the coding regions of each of the three fibrinogen genes as well as the intron-exon boundaries was performed as described previously (20). The entire DNA sequence of the coding regions was obtained for each of the three fibrinogen genes from both strands.

Fibrinogen purification

Citrate-anticoagulated plasma was collected from the index patient with fibrinogen Hershey IV and the dysfibrinogen was purified by glycine precipitation (21), as was control fibrinogen from an anonymous donor. The purified fibrinogens were analyzed by

polyacrylamide gel electrophoresis as described previously (20). The fibrinogen concentration in the plasma was determined using an ELISA described previously (22).

Mass spectrometry

Five to 10 µg portions of purified control and mutant forms of fibrinogen were dried by vacuum centrifugation and dissolved in 10 µl of 8M urea/1.0 M Tris/0.2 M methylamine. One µl of 0.2 M dithioerythritol was then added and the samples were incubated at 50° C for 15 minutes, followed by the addition of 2 µl of 0.5 M iodoacetamide and incubation at room temperature for 15 minutes. An additional 2 µl of 0.2 M dithioerythritol was then added, and samples were diluted to a final 40 µl volume with 5% formic acid. One µg of each reduced and alkylated sample was injected onto a micro protein trap cartridge (Michrom Bioresources, Inc., Auburn, CA), and fibrinogen subunits were separated by reverse phase chromatography using a 1.0 × 250 mm C4 column (214 MS C4, Vydac, Hesperia, CA). The column used a 2–60% acetonitrile gradient over 70 minutes in a mobile phase containing 0.1% formic acid and a 19 µl/minute flow rate. Spectra were collected in profile mode over a 600–2000 m/z range while averaging 20 µscans. Data collected during the elution of the fibrinogen γ chain were averaged and deconvoluted using BioMass Calculation and Deconvolution Software (Bioworks 3.2, ThermoFinnigan, San Jose, CA). Mass measurements with an error of less than 0.01% were confirmed using horse myoglobin as a standard.

Fibrinogen-αIIbβ3 binding assays

Fibrinogen binding to immobilized αIIbβ3 was quantitated using a modification of a method described previously (23). 250 ng of purified αIIbβ3 (Enzyme Research Labs, South Bend, IN) was immobilized per well on a polystyrene plate and incubated overnight at 4° C. Wells were then blocked with 1 mg/ml bovine serum albumin in saline, pH 7.4, and either control or Hershey IV fibrinogen was added at concentrations ranging from 31.25 ng/mL to 64 µg/mL. After incubation at 37° C for 1 hour, a horseradish peroxidase-conjugated sheep anti-human fibrinogen antibody (Innovative Research, Southfield, MI) was added. TMB supersensitive 1 component HRP microwell substrate and stop solution (BioFX Laboratories, Owings Mills, MD) were used for detection (22). The absorbance was read at 450 nm and the data were expressed as a percentage of the maximum binding measured with saturating quantities of control fibrinogen.

Platelet aggregation assays

Dodecapeptides were synthesized to mimic the carboxyl terminal 12 amino acids of the γ chain (Invitrogen, Carlsbad, CA). The γ 400–V411 peptide contained the wild-type sequence (HHLGGAKQAGDV), and the γ 400–I411 peptide contained the carboxyl terminal isoleucine mutation (HHLGGAKQAGDI). Platelets from a healthy donor were washed and the total platelet count adjusted to 250,000/mm³ prior to use in aggregation experiments. Aggregation studies were performed on a PAP-4 platelet aggregation profiler (Bio/Data Corporation, Horsham, PA). Platelets were gently mixed during incubation at 37° C with control fibrinogen (0.5 mg/mL, Enzyme Research Laboratories) and varying concentrations of peptide (50–500 µM). Platelets were activated with ADP (final concentration 5 µM, Sigma-Aldrich, St. Louis, MO) to minimize release of intracellular

platelet α -granule fibrinogen, and the optical density was measured at 37° C for 1 minute prior and 5 minutes post addition of agonist.

Platelet aggregation was also performed on washed platelets reconstituted with purified control or Hershey IV fibrinogen. Washed platelets were prepared as described above to remove endogenous fibrinogen. The platelets (final concentration 250,000/mm³) were incubated with either fibrinogen Hershey IV or control fibrinogen at concentrations of 125–500 nM prior to activation with ADP. Optical density was again measured on a PAP-4 platelet aggregation profiler.

Results

Clinical history

The proband for fibrinogen Hershey IV is a 52-year-old woman who gave a history of frequent, sometimes prolonged epistaxis as a child. In addition, she had menorrhagia, most likely secondary to uterine fibroids. A work-up prior to hysterectomy showed a prolonged thrombin time and low functional plasma fibrinogen. She received ten units of cryoprecipitate preoperatively; her surgery was uneventful. Further work-up confirmed a prolonged thrombin time (34 seconds), normal fibrinogen antigen levels of 277 mg/dL, and low functional plasma fibrinogen (55 mg/dL), consistent with a diagnosis of dysfibrinogenemia. One of her brothers was noted to have low functional plasma fibrinogen (~ 50 mg/dL). He bled excessively during nephrectomy and received cryoprecipitate. Two other siblings and two children had no bleeding complications. The proband's father died from severe bleeding during pancreatotomy for a malignant tumor.

Characterization of Hershey IV mutations

DNA sequencing showed heterozygosity for two point mutations, both involving the fibrinogen γ chain. The first mutation, γ R275C, has been reported previously as a cause of dysfibrinogenemia (18). This mutation is located at the interface of polymerized fibrin between the D-domains, where it interferes with fibrin polymerization. The second mutation, γ V411I, is a novel mutation involving the last amino acid at the carboxyl terminal end of the γ chain. No point mutations in this region, which is critical for fibrinogen interactions with platelet integrin α IIb β 3, have been reported previously in humans. Fibrinogen was also purified from the proband's plasma using glycine precipitation. The A α , B β , and γ chains were all normal in size (Fig. 1), as expected for point mutations.

The mutations appeared to be present on different chromosomes, based on mass spectrometry of the γ chains. The γ chain from control fibrinogen gave a single peak of 48,945 Da (48,375 Da after correction for the alkyl groups on the Cys residues), which is within 2 Da of the previously reported mass of 48,377 Da (24). In contrast, the γ chains from Hershey IV gave a doublet with uncorrected masses of 48,948 and 48,957 Da. These masses are most consistent with a single mutation on each γ chain. An Arg to Cys mutation would increase the mass of a single γ chain by 4 Da, allowing for the alkyl group on the new Cys residue; a Val to Ile mutation would increase the mass by 14 Da. Therefore, the predicted masses caused by single point mutations on each γ chain in Hershey IV would have a 10 Da

difference, 48,949 and 48,959 Da, which are very close to the observed masses of 48,948 and 48,957 Da. In contrast, had the mutations been on the same γ chain, the predicted masses would have been 48,945 Da (the same as control) and 48,963 Da, which differ more from the observed masses of 48,949 and 48,959 Da.

Platelet aggregation studies

The interaction of fibrinogen Hershey IV with platelets was investigated using platelet aggregometry. To eliminate experimental variability in comparing the patient's own platelets to normal platelets, washed normal platelets were reconstituted with either purified control fibrinogen or purified fibrinogen Hershey IV, and aggregation was initiated using ADP to minimize release of endogenous fibrinogen from platelet α -granules. Little difference in the initial rate of aggregation was seen, but there was a slight detectable difference in the total extent of aggregation (Fig. 2), which is likely due to the fact that the proband for fibrinogen Hershey IV was heterozygous for the V411I mutation. Since fibrinogen contains two γ chains, 50% of the fibrinogen Hershey IV molecules should be heterodimers containing one γ V411I and one wild-type γ chain, 25% should be homodimers containing two γ V411I chains, and 25% should be homodimers containing two wild-type γ chains, assuming equal expression of both alleles and random assortment of chains during fibrinogen assembly. The 25% homodimer wild-type γ chains were likely to be sufficient to support normal platelet aggregation.

To examine the effect of the V411I mutation in the absence of competing wild-type γ chains, platelet aggregation inhibition studies were performed using synthetic dodecapeptides that mimicked the carboxyl terminal end of the γ chain. Previous work by Kloczewiak and colleagues used synthetic peptides to inhibit fibrinogen-platelet binding, and demonstrated that the carboxyl terminal dodecapeptide γ 400–411 inhibited fibrinogen binding to platelets (6). The introduction of mutations into this region generally decreased the binding affinity of synthetic dodecapeptides for the platelet α IIB β 3 receptor (7). Several mutant dodecapeptides in this previous study were synthesized to incorporate changes at position 411, including cysteine, phenylalanine, tyrosine, and leucine. All of these had lower binding affinities, with a 3–16 fold decrease in inhibition (7). In order to characterize any functional defects caused by the γ V411I mutation, two peptides were synthesized corresponding to the carboxyl terminal 12 amino acids of the γ chain, one with the wild-type sequence and one incorporating the isoleucine mutation at γ 411.

Platelet aggregation was performed using washed control platelets and either wild-type or mutant dodecapeptide. Exogenous normal fibrinogen was added, and inhibition of ADP-induced aggregation was measured with both wild-type and mutant peptide (Fig. 3A, B). The decreased inhibition by the mutant peptide suggested that the isoleucine mutant was less effective at binding platelets, and therefore more mutant dodecapeptide would be required to effectively compete with control fibrinogen.

α IIB β 3 binding studies

The decreased inhibitory activity of the V411I dodecapeptide towards platelet aggregation suggested that the mutation resulted in a decreased affinity for platelet integrin α IIB β 3. The

binding affinity of fibrinogen Hershey IV for the platelet $\alpha\text{IIb}\beta 3$ receptor was therefore measured using purified immobilized $\alpha\text{IIb}\beta 3$ and either control fibrinogen or fibrinogen Hershey IV. Fibrinogen Hershey IV binding to $\alpha\text{IIb}\beta 3$ was significantly decreased compared to control (Fig. 4). The K_d for control fibrinogen binding to $\alpha\text{IIb}\beta 3$ was $7.56 \pm 1.88 \mu\text{M}$ and the K_d for fibrinogen Hershey IV was $15.0 \pm 5.35 \mu\text{M}$. It should be noted that the K_d for fibrinogen Hershey IV reflects contributions from both the wild-type and mutant γ chains, since the Hershey IV proband was heterozygous, and the actual affinity for the V411I-containing γ chain is likely to be somewhat lower. However, these results confirmed that the V411I mutation resulted in decreased binding affinity for platelet $\alpha\text{IIb}\beta 3$.

Discussion

Fibrinogen Hershey IV is, to our knowledge, the first reported dysfibrinogen with a point mutation in the $\alpha\text{IIb}\beta 3$ binding domain. Our results suggest that the γ V411I mutation inhibits fibrinogen binding to $\alpha\text{IIb}\beta 3$. These results are consistent with previous evidence showing that the last four amino acids of the γ chain, AGDV, are critical residues for $\alpha\text{IIb}\beta 3$ binding (25). A genetically modified mouse with an altered fibrinogen γ chain lacking the last five carboxyl terminal amino acids (QAGDV) demonstrated defective $\alpha\text{IIb}\beta 3$ binding and platelet aggregation (26). However, no point defects in this region have been reported previously in humans. Platelet aggregation has been shown to be affected in at least one other γ chain dysfibrinogen, fibrinogen Paris I, although the mutation itself is not within the carboxyl terminus. Fibrinogen Paris I has a defect beginning at γ 351, with an insertion of 14 amino acids (27). Despite an intact $\alpha\text{IIb}\beta 3$ binding site, platelet aggregation in the presence of ADP was diminished by 30% in fibrinogen Paris I (27). The Paris I mutation, however, has not been associated with bleeding symptoms (28).

The γ R275C mutation, also present in fibrinogen Hershey IV, has been identified previously in a number of patients with dysfibrinogenemia and has been characterized extensively. The bleeding defect caused by the γ R275C mutation is due to a defect in fibrin polymerization, whereas no defects in platelet aggregation have been reported. While the proband suffered from post-operative hemorrhage, some other individuals with this mutation have presented with thrombosis, including post-partum DVT (29–31). Functional studies with other dysfibrinogens containing the R275C mutation have shown normal γ chain cross-linking but a disordered fiber network with numerous fiber terminations (32, 33). Polymerization of fibrin monomers in this mutation is delayed (34). These studies emphasize the critical importance of this region in D:D interactions. It is likely that in the absence of the γ R275C mutation, the γ V411I in Hershey IV would not have been detected by conventional clinical coagulation assays.

Our results suggest that the γ V411I mutation results in a decreased ability to bind $\alpha\text{IIb}\beta 3$ and consequently leads to decreased platelet aggregation. The clinical significance of this mutation is unclear, however, as in the heterozygous state, the amount of wild-type fibrinogen appears to be sufficient to support platelet aggregation.

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References

1. Weisel JW. Fibrinogen and fibrin. *Adv Protein Chem.* 2005; 70:247–299. [PubMed: 15837518]
2. Mosesson MW. Fibrinogen γ chain functions. *J Thromb Haemost.* 2003; 1:231–238. [PubMed: 12871494]
3. Farrell DH. Pathophysiologic roles of the fibrinogen γ chain. *Curr Opin Hematol.* 2004; 11:151–155. [PubMed: 15257013]
4. Chen R, Doolittle RF. γ - γ cross-linking sites in human and bovine fibrin. *Biochemistry.* 1971; 10:4487–4491. [PubMed: 5168975]
5. Lorand L. Factor XIII: structure, activation, and interactions with fibrinogen and fibrin. *Ann N Y Acad Sci.* 2001; 936:291–311. [PubMed: 11460485]
6. Kloczewiak M, Timmons S, Lukas TJ, et al. Platelet receptor recognition site on human fibrinogen. Synthesis and structure-function relationship of peptides corresponding to the carboxy-terminal segment of the γ chain. *Biochemistry.* 1984; 23:1767–1774. [PubMed: 6326808]
7. Kloczewiak M, Timmons S, Bednarek MA, et al. Platelet receptor recognition domain on the γ chain of human fibrinogen and its synthetic peptide analogues. *Biochemistry.* 1989; 28:2915–2919. [PubMed: 2742819]
8. Cheresch DA, Berliner SA, Vicente V, et al. Recognition of distinct adhesive sites on fibrinogen by related integrins on platelets and endothelial cells. *Cell.* 1989; 58:945–953. [PubMed: 2673537]
9. Farrell DH, Thiagarajan P, Chung DW, et al. Role of fibrinogen α and γ chain sites in platelet aggregation. *Proc Natl Acad Sci U S A.* 1992; 89:10729–10732. [PubMed: 1438269]
10. Farrell DH, Thiagarajan P. Binding of recombinant fibrinogen mutants to platelets. *J Biol Chem.* 1994; 269:226–231. [PubMed: 8276798]
11. Bennett JS. Structure and function of the platelet integrin α IIb β 3. *J Clin Invest.* 2005; 115:3363–3369. [PubMed: 16322781]
12. Chung DW, Davie EW. γ and γ' chains of human fibrinogen are produced by alternative mRNA processing. *Biochemistry.* 1984; 23:4232–4236. [PubMed: 6091742]
13. Fornace AJ Jr, Cummings DE, Comeau CM, et al. Structure of the human γ -fibrinogen gene. Alternate mRNA splicing near the 3' end of the gene produces γ A and γ B forms of γ -fibrinogen. *J Biol Chem.* 1984; 259:12826–12830. [PubMed: 6092346]
14. Kirschbaum NE, Mosesson MW, Amrani DL. Characterization of the γ chain platelet binding site on fibrinogen fragment D. *Blood.* 1992; 79:2643–2648. [PubMed: 1586714]
15. Lawrence SO, Wright TW, Francis CW, et al. Purification and functional characterization of homodimeric γ B- γ B fibrinogen from rat plasma. *Blood.* 1993; 82:2406–2413. [PubMed: 8400290]
16. Neerman-Arbez M. Molecular basis of fibrinogen deficiency. *Pathophysiol Haemost Thromb.* 2006; 35:187–198. [PubMed: 16855369]
17. Roberts HR, Stinchcombe TE, Gabriel DA. The dysfibrinogenemias. *Br J Haematol.* 2001; 114:249–257. [PubMed: 11529842]
18. Ebert RF. Dysfibrinogenemia: an overview of the field. *Thromb Haemost.* 1991; 65:1317.
19. Hanss M, Biot F. A database for human fibrinogen variants. *Ann N Y Acad Sci.* 2001; 936:89–90. [PubMed: 11460527]
20. Flood VH, Al-Mondhiry HA, Farrell DH. The fibrinogen A α R16C mutation results in fibrinolytic resistance. *Br J Haematol.* 2006; 134:220–226. [PubMed: 16846481]

21. Kazal LA, Amsel S, Miller OP, et al. The preparation and some properties of fibrinogen precipitated from human plasma by glycine. *Proc Soc Exp Biol Med.* 1963; 113:989–994. [PubMed: 14073163]
22. Lovely RS, Falls LA, Al-Mondhry HA, et al. Association of $\gamma A/\gamma'$ fibrinogen levels and coronary artery disease. *Thromb Haemost.* 2002; 88:26–31. [PubMed: 12152671]
23. Wierzbicka I, Kowalska MA, Lasz EC, et al. Interaction of $\beta 3$ integrin-derived peptides 214–218 and 217–231 with $\alpha IIB\beta 3$ complex and with fibrinogen A α -chain. *Thromb Res.* 1997; 85:115–126. [PubMed: 9058485]
24. Meh DA, Siebenlist KR, Brennan SO, et al. The amino acid sequence in fibrin responsible for high affinity thrombin binding. *Thromb Haemost.* 2001; 85:470–474. [PubMed: 11307817]
25. Liu Q, Matsueda G, Brown E, et al. The AGDV residues on the γ chain carboxyl terminus of platelet-bound fibrinogen are needed for platelet aggregation. *Biochim Biophys Acta.* 1997; 1343:316–326. [PubMed: 9434121]
26. Holmback K, Danton MJ, Suh TT, et al. Impaired platelet aggregation and sustained bleeding in mice lacking the fibrinogen motif bound by integrin $\alpha IIB\beta 3$. *EMBO J.* 1996; 15:5760–5771. [PubMed: 8918453]
27. Denninger MH, Jandrot-Perrus M, Elion J, et al. ADP-induced platelet aggregation depends on the conformation or availability of the terminal γ chain sequence of fibrinogen. Study of the reactivity of fibrinogen Paris 1. *Blood.* 1987; 70:558–563. [PubMed: 3607287]
28. Cote HC, Lord ST, Pratt KP. γ -chain dysfibrinogenemias: molecular structure-function relationships of naturally occurring mutations in the γ chain of human fibrinogen. *Blood.* 1998; 92:2195–2212. [PubMed: 9746756]
29. Haverkate F, Samama M. Familial dysfibrinogenemia and thrombophilia. Report on a study of the SSC Subcommittee on Fibrinogen. *Thromb Haemost.* 1995; 73:151–161. [PubMed: 7740487]
30. Linenberger ML, Kindelan J, Bennett RL, et al. Fibrinogen Bellingham: a γ -chain R275C substitution and a β -promoter polymorphism in a thrombotic member of an asymptomatic family. *Am J Hematol.* 2000; 64:242–250. [PubMed: 10911375]
31. Siebenlist KR, Mosesson MW, Meh DA, et al. Coexisting dysfibrinogenemia (γ R275C) and factor V Leiden deficiency associated with thromboembolic disease (fibrinogen Cedar Rapids). *Blood Coagul Fibrinolysis.* 2000; 11:293–304. [PubMed: 10870810]
32. Mosesson MW, Siebenlist KR, DiOrio JP, et al. The role of fibrinogen D domain intermolecular association sites in the polymerization of fibrin and fibrinogen Tokyo II (γ 275 Arg→Cys). *J Clin Invest.* 1995; 96:1053–1058. [PubMed: 7635941]
33. Ishikawa S, Hirota-Kawadobora M, Tozuka M, et al. Recombinant fibrinogen, γ 275Arg→Cys, exhibits formation of disulfide bond with cysteine and severely impaired D:D interactions. *J Thromb Haemost.* 2004; 2:468–475. [PubMed: 15009465]
34. Schmelzer CH, Ebert RF, Bell WR. Fibrinogen Baltimore IV: congenital dysfibrinogenemia with a γ 275 (Arg→Cys) substitution. *Thromb Res.* 1989; 56:307–316. [PubMed: 2617471]

Figure 1. Purified fibrinogen Hershey IV

Purified fibrinogens were electrophoresed under reducing conditions on 10% polyacrylamide gels. Lane 1 shows a molecular weight standard (labeled in kDa), lane 2 contains 1 μ g of control fibrinogen, and lane 3 contains 1 μ g of purified fibrinogen Hershey IV.

Figure 2. Platelet aggregation with purified fibrinogens

ADP (5 μM) was added to $250,000/\text{mm}^3$ washed platelets in the presence of 250 nM fibrinogen. Little difference in platelet aggregation was seen with fibrinogen Hershey IV (top curve) when compared to control fibrinogen (bottom curve).

Figure 3. Platelet aggregation inhibition by γ chain dodecapeptides

ADP ($5 \mu\text{M}$) was added to $250,000/\text{mm}^3$ washed platelets in the presence of 0.5 mg/ml fibrinogen (data shown from representative experiments). A. Normal platelet aggregation in response to ADP. B. Platelet aggregation was inhibited by $500 \mu\text{M}$ control γ 400–411 dodecapeptide (top curve) and, to a lesser extent, by $500 \mu\text{M}$ mutant γ I411 dodecapeptide (bottom curve).

Figure 4. Fibrinogen binding to purified platelet α IIb β 3

Binding of fibrinogen Hershey IV (closed circles) and control fibrinogen (open circles) to immobilized platelet α IIb β 3 was measured over the indicated range of fibrinogen concentrations. Fibrinogen binding was detected using a horseradish peroxidase-conjugated sheep anti-human fibrinogen antibody, followed by a peroxidase substrate. The absorbance was read at 450 nm, and the data expressed as a percentage of the binding observed at saturating amounts of control fibrinogen. The mean values from three experiments are plotted $\pm$ SE.