Localisation of a PIA1 Epitope to the Amino Terminal 66 Residues of Platelet Glycoprotein IIIa

By Ron D. Bowditch, Patricia H. Tani, Carol E. Halloran, Andrew L. Frelinger III, Robert McMillan, and Mark H. Ginsberg

A platelet glycoprotein (GP) IIIa epitope library was constructed by insertion of randomly cleaved GPIIla cDNA fragments in the prokaryotic expression vector pgt22 and screened with purified anti-PIA1 antibodies for clones expressing a PIA1 epitope. Five independent clones were isolated and characterized by nucleotide sequencing. The smallest anti-PIA1 reactive clone obtained encoded the amino terminal 66 residues of mature GPIIIa. Substitution of leucine33 (PIA1) with a proline33 (PIA2) by in vitro mutagenesis resulted in the loss of anti-PIA1 reactivity; however, this clone still reacted with anti-GPIIla polyclonal antibodies. These data indicate that a PIA1 alloantigenic epitope is located within a small, unglycosylated fragment of GPIIla containing the polymorphism responsible for the PIA1 phenotype. Furthermore, these results prove that small recombinant mimics of a PIA1 epitope may be synthesized and used for detection of these alloantibodies.

© 1992 by The American Society of Hematology.

MATERIALS AND METHODS

Anti-PIA1 Alloantibodies

Anti-PIA1 alloantibody from the plasma of four patients with PTP (plasma kindly provided by Dr S. Schinke, San Jacinto, CA [patient 1]; Dr G. Schmidt, City of Hope Medical Center, Duarte, CA [patient 2]; and Dr V. Blanquette, Hospital for Sick Children, Toronto, Ontario, Canada [patients 3 and 4]) was affinity-purified using GPIIb-IIa coupled to CNBr-sepharose-4BCL (Pharmacia, Piscataway, NJ).

Washed normal platelets were solubilized in isotonic citrate buffer, pH 6.5, containing 1% Triton X-100 (Sigma Chemical Co, St Louis, MO) for 30 minutes at 4°C followed by centrifugation at 100,000g for 60 minutes at 4°C. The supernatant was incubated overnight at 4°C with murine monoclonal anti-GPIIb (2A9; provided by Dr V. Woods, University of California at San Diego) coupled to CNBr-sepharose-4BCL. After washing with phosphate-buffered saline, the bound GPIIb-IIa was eluted with 0.1 mol/L diethylamine in 1% octaglucopyranoside (OPG) in phosphate-buffered saline, pH 10, and immediately dialyzed against 0.1 mol/L phosphate buffer containing 1% OPG. The purified GPIIb-IIa was then coupled to CNBr-sepharose-4BCL. The GPIIb-IIa sepharose was incubated overnight at 4°C with plasma, washed, bound alloantibody eluted with 0.1 mol/L glycine, pH 2.5, immediately neutralized with 1 mol/L Tris, pH 9, and dialyzed in phosphate-buffered saline. The antibody eluate was titered by an antigen capture assay.1

Epitope Library Construction and Screening

A 2.6-kb fragment encoding full-length GPIIla was isolated by cleavage of the plasmid BS3as with the restriction enzymes Spe1 and Sph1, and subsequent purification of the 2.6-kb band from an agarose gel. Five micrograms of the isolated band was then digested in 0.5 mL of 20 mmol/L Tris-C1, pH 7.5, and 1.5 mmol/L MgCl2, with 0.5 mg DNase I for 30 minutes at 37°C. The reaction was immediately stopped by phenol:chloroform extraction of the reaction mixture. After ethanol precipitation of the GPIIla DNA, the fragments were polished with T4 DNA polymerase, ligated with the SalI linker GGTGACCC, cleaved with the restriction enzyme SalI, and ligated into dephosphorylated SalI digested pgt22 (Promega Corp, Madison, WI). The ligated DNA was then packaged and the amplified library titered on Escherichia coli Y1090 and found to contain 2 × 106 total phage (84% recombinant).

From the Committee on Vascular Biology and the Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA.

Submitted October 31, 1991; accepted December 27, 1991.

Supported by National Institutes of Health Grants HL28825 and AR27214 (M.H.G.), and HL37945 (R.M.). R.D.B. was supported by a research fellowship from the American Heart Association, California Affiliate. This is publication number 7021-CVB from the Scripps Research Institute.

Address reprint requests to Ron D. Bowditch, PhD, The Scripps Research Institute, Committee on Vascular Biology, 10666 N Torrey Pines Rd, La Jolla, CA 92037.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1992 by The American Society of Hematology.
Screening of λgt22 Recombinant Phage

Five thousand plaques per plate of the recombinant λgt22-GPIIIa epitope library were plated on E.coli Y1090. The lacZ gene was induced with isopropyl-β-D-thiogalactoside (IPTG) saturated filters as described. The filters were then screened with either polyclonal rabbit anti-GPIIIa antibodies (1:250 dilution) preabsorbed with a lysate of λgt22 infected E.coli Y1090, preabsorbed normal rabbit serum (1:250 dilution), or purified anti-PIA1 antibodies (1:100 dilution). Bound antibodies were detected by reaction with a peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) or anti-human IgG secondary antibody and developed with the chromogenic substrate o-phenylenediamine. The purified anti-GPIIIa and anti-PIA1 positive phage were screened by plating 100 to 200 plaques of a 1:1 mixture of λgt22 and a recombinant λgt22 construct on E.coli Y1090. The lacZ gene was induced, the filters were cut into pieces, and screened with preabsorbed anti-GPIIIa antibodies, normal rabbit serum, anti-PIA1 antibodies, and normal human serum.

Sequencing of GPIIIa Inserts

Recombinant λgt22 DNA was isolated as described, cut with the restriction enzymes Not I and EcoRI, and subcloned by standard techniques into the plasmid Bluescript II SK +/- (Stratagene, La Jolla, CA). Both strands were then sequenced using the T7 and T3 primers (Stratagene) by the dideoxy sequencing method to determine the boundaries of the DNA fragments. The plasmid subclone of the smallest anti-PIA1 positive GPIIIa fragment (292 bp) was completely sequenced and subsequently used for mutagenesis.

In Vitro Mutagenesis

Oligonucleotide-directed mutagenesis was performed using a combination of the primer extension method and the strand selection method, which provides strong biological selection against the nonmutagenized strand. The single T → C substitution at base 196 of GPIIIa was introduced into the 292-bp frag-
LOCALIZATION AND EXPRESSION OF A PI^4 EPITOPE

Fig 2. Anti-PI^4 antibodies do not react with a recombinant GPIIIa fragment encoding the PI^2 phenotype. Recombinant λgt22 phage plaques expressing the 66-amino terminal residues of GPIIIa with leucine^33 (A and C) and proline^33 (B and D) were screened with purified anti-PI^4 alloantibodies (A and B) and polyclonal anti-GPIIIa antibodies (C and D). Nonrecombinant λgt22 phage, mixed at a 1:1 ratio, are present in the background as negative controls.

ment using the oligonucleotide GGTGAGCCCGAG-GCAGGGCCTCA. Inserts with the expected mutation were screened for the appearance of an Nci I site and then completely sequenced. The insert with the T → C substitution was then subcloned back into λgt22.

RESULTS

A platelet GPIIIa epitope library was constructed by insertion of randomly cleaved GPIIIa cDNA fragments in the prokaryotic expression vector λgt22. This library expresses random fragments, averaging 100 to 200 residues in length, of GPIIIa fused to β-galactosidase. The λgt22-GPIIIa epitope library was screened with purified anti-PI^4 antibodies for clones expressing a PI^4 epitope. Five independent clones that reacted with purified anti-PI^4 antibodies from patient number 1 were isolated, and sequence analysis of the DNA from each of the recombinant phage localized a PI^4 epitope to the amino-terminal end of GPIIIa (Fig 1A). Fragments from throughout the GPIIIa sequence were isolated by screening the λgt22-GPIIIa epitope library with polyclonal anti-GPIIIa antibodies. However, these other regions of GPIIIa failed to react with the anti-PI^4 antibodies from four patients (data not shown). The smallest fragment containing the PI^4 antigen expressed the amino-terminal 66 residues of mature GPIIIa and the 26-residue signal sequence (Fig 1B). All of the clones obtained overlapped this region and reacted with purified anti-PI^4 antibodies from four individuals, indicating that a PI^4 epitope was located within the amino terminal 66 residues of GPIIIa.

Residue 33 of mature GPIIIa is responsible for the two PI^4 phenotypes. The PI^2 (proline^33) phenotype was constructed in the λgt22 clone expressing the amino terminal 66 residues of GPIIIa by a T → C substitution. The replacement of leucine^33 with a proline resulted in loss of anti-PI^4 antibody reactivity; however, reactivity with the polyclonal anti-GPIIIa antibodies remained intact (Fig 2). Therefore, residue 33 was important in formation of this PI^4 epitope in a 7-Kd amino terminal fragment of GPIIIa.

DISCUSSION

The results of this work have two major implications. Firstly, an alloantigenic epitope for anti-PI^4 antibodies is located in the amino-terminal 66 residues of mature GPIIIa. Previous studies have presented evidence that the leucine^33/proline^33 amino acid polymorphism is responsible for the two PI^4 phenotypes; however, identification of the polymorphism does not define the epitope(s) for the alloantibodies of clinical interest. There have been conflicting reports as to the location of the PI^4 epitope by using proteolytic methods. Although the evidence suggested that a PI^4
epitope is located in proximity to the polymorphism, recent studies using synthetic peptides that spanned the polymorphism (Fig 1B) failed to bind anti-PIA\(^{11}\) antibodies and antipeptide antibodies failed to distinguish the two phenotypes.\(^6\) The data presented here indicate that an epitope for anti-PIA\(^{11}\) antibodies resides in the amino terminal 7 Kd of GPIIIa and within 33 residues of the actual polymorphism responsible for the PIA\(^{11}\) phenotype.

The second major finding in this study is that a PIA\(^{11}\) epitope can be expressed in a prokaryotic system. Utilization of a prokaryotic expression system for characterizing a PIA\(^{11}\) epitope preserves the disulfide bonds necessary for expression of the reduction sensitive epitope.\(^7\) Seven cysteines exist in the small fragment identified in this study (Fig 1B), indicating that local sulfhydryl bonding between these residues\(^8\) is involved in formation of this epitope and that the cysteine-cysteine\(^{45}\) disulfide bond is not required. These results agree with the model of the PIA\(^{11}\) alloantigen recently proposed by Newman.\(^19\) Furthermore, as suggested by biochemical studies,\(^11\) the synthesis of this PIA\(^{11}\) epitope in \(E\) coli proves that it does not depend on glycosylation. The ability to produce small recombinant mimics of a PIA\(^{11}\) epitope in large amounts will allow for the development of simple detection assays for these alloantibodies.

ACKNOWLEDGMENT

We thank Dr Joe Loftus for supplying the GPIIIa cDNA clone and for his most excellent and helpful discussions.

REFERENCES

17. Beer J, Coller BS: Evidence that platelet glycoprotein IIIa has a large disulfide-bonded loop that is susceptible to proteolytic cleavage. J Biol Chem 264:17564, 1989
Localization of a PIA1 epitope to the amino terminal 66 residues of platelet glycoprotein IIa

RD Bowditch, PH Tani, CE Halloran, AL 3d Frelinger, R McMillan and MH Ginsberg