The glycoprotein (GP) IIb–IIIa complex was isolated from human platelet membranes by immunoaffinity chromatography using a monoclonal antibody specific for GP IIb–IIIa. GP IIb and IIIa were further separated in the presence of sodium dodecyl sulfate (SDS) by gel filtration high-performance liquid chromatography (HPLC). Two cycles of this procedure yielded almost complete separation of homogeneous preparations of GP IIb and IIIa. Each protein was then digested with lysyl endopeptidase (Achromobacter protease I), which cleaves at the carboxyl side of lysine residues, and the resulting oligopeptides from GP IIb and IIIa were fractionated with HPLC using a C18 reverse-phase column. Comparison of the elution profiles showed no obvious homology between the two proteins. Amino acid sequences of selected oligopeptides from each glycoprotein were determined using a gas-phase protein sequencer. Sixty amino acid residues (26 residues for IIb and 34 residues for IIIa) were identified.

**Materials and Methods**

Production and purification of a monoclonal antibody. The production and specificity of the murine monoclonal antibody HPL-1 (IgG1) used in this study have been described in detail elsewhere. In brief, this hybridoma was derived from (BALB/c x C57BL/6) female mice immunized with human platelets. By immunoprecipitation experiments with 3H-labeled platelet lysates, HPL-1 precipitated the antigen molecules of 127 and 104 kd under reduced conditions and antigen molecules of 138 and 90 kd under unreduced conditions. This antibody inhibited platelet aggregation induced by ADP or collagen and did not react with platelets of thrombasthenia patients. Thus, the antigen recognized by HPL-1 antibody was considered the GP IIb–IIIa complex. HPL-1 was purified from ascites fluid by precipitation with saturated ammonium sulfate followed by diethylaminoethanol (DEAE) cellulose column chromatography. The purified antibody was diazylated against coupling buffer (borate buffer pH 8.3 containing 0.5 mol/L of NaCl).

Imunoaffinity purification of the GP IIb–IIIa complex. Ten milligrams of purified HPL-1 antibody was coupled to 2 mL of cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscatway, NJ) according to the manufacturer's directions. Platelets (5 x 10^9) were washed three times in phosphate-buffered saline (PBS) pH 7.4, containing 2.5 mmol/L of EDTA; whole platelets were then solubilized in 50 mL of extraction buffer (10 mmol/L of Tris-HCl, pH 7.4, 0.15 mol/L of NaCl, 1 mmol/L of phenylmethyisulfonyl fluoride (PMSF), 2.5 mmol/L of EDTA, and 0.5% Lubrol PX) for 45 minutes at 0 to 4 °C with periodic vortexing. An additional 50 mL of the extraction buffer was added, and the sample was incubated for another 30 minutes to permit complete solubilization. The lysate was centrifuged at 100,000 g for 30 minutes at 4 °C to remove particulate matter, and the supernatants containing platelet membrane protein were pooled. The platelet lysate was first applied to a column made of bovine serum albumin (BSA)-conjugated Sepharose 4B and equilibrated in 10 mmol/L of Tris-HCl, pH 7.4, 0.15 mol/L of NaCl, and 0.5% Lubrol. The throughout fraction, from which materials adsorbed nonspecifically to Sepharose and/or BSA had been removed, was applied to a HPL-1-Sepharose 4B affinity column (2 mL) equilibrated with the same buffer as described above. The column was washed extensively with a high-salt buffer (10 mmol/L of Tris-HCl, pH 7.4, 1 mol/L of NaCl and 0.5% Lubrol), and the retained protein was eluted with 50 mmol/L of diethylamine, pH 11.5, containing 0.1% Lubrol. The eluate was immediately neutralized by the addition of 200 μL of 2 mol/L of Tris-HCl, pH 7.2, and stored until further use at −80 °C. Protein concentration was determined using the Bio-Rad (Richmond, CA) Protein Assay kit.
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Approximately 20 to 30 μg of the purified protein was electrophoresed in an 8% polyacrylamide slab gel according to the method of Laemmli. Reduced samples contained 5% β-mercaptoethanol. Gels were stained with Coomassie brilliant blue. The following proteins were electrophoresed as mol wt standards: Escherichia coli RNA polymerase containing subunits of β’ (160,000), β (150,000), σ (90,000), α (40,000); BSA (67,000).

Separation of glycoproteins IIb and IIIa by gel filtration HPLC. The affinity-purified GP IIb-IIIa complex was concentrated by lyophilization and then redisolved in water containing 1 mmol/L of EDTA and 0.1% SDS. The mixture was warmed for 60 minutes at 37 °C to achieve complete solubilization of protein complexes. To dissociate and purify the individual glycoproteins, the sample was applied to HPLC (Spectra-Physics, San Jose, CA) using a 60-cm TSK 4000SW gel filtration column equilibrated in 50 mmol/L of Tris-HCl, pH 7.2, 1 mmol/L of EDTA, and 0.1% SDS as described by Newman and colleagues. Chromatography was performed at a flow rate of 0.5 mL/min, and the protein was monitored by measuring the absorbance at 280 nm. Peak fractions containing GP IIb or GP IIIa were collected separately. Each fraction was rechromatographed under conditions of the first chromatography to achieve homogeneous preparations of each GP. Fractions were pooled and lyophilized.

Removal of SDS from GP IIb and GP IIIa preparations after gel filtration HPLC. Although nonionic detergents such as NP-40 or Lubrol do not inhibit proteolytic digestion, SDS affects the proteinase activity of lysyl endopeptidase. Therefore, removal of SDS from protein samples was necessary prior to enzymatic digestion of GP IIb and GP IIIa. To achieve complete extraction of SDS, we used the method described by Henderson and co-workers. In brief, the extraction solvent was a freshly prepared mixture of anhydrous acetone:triethylamine:acetic acid:water (85:5:5:5; vol/vol/vol/vol). Lyophilized GP IIb and GP IIIa samples containing SDS were dissolved directly in 1 mL of the extraction solvent containing 1 μg of lysyl endopeptidase. The solution was kept at −20 °C for at least 1 hour. The proteins were precipitated by centrifugation (5,500 g for 45 minutes at −5 °C). The enzyme and the substrate were co-precipitated in this way to give a subsequent more reproducible digestion. To insure complete removal of SDS, the protein precipitate was washed twice with fresh extraction solvent. Finally, the precipitate was washed twice with acetone to remove traces of the extraction solvent. Residual acetone was removed by lyophilization.

Digestion of GP IIb and GP IIIa polypeptides with lysyl endopeptidase. The SDS-free lyophilized samples were each redissolved in 500 μL of 50 mmol/L of Tris-HCl, pH 9.2, 0.1% Lubrol. An additional 1 μg of lysyl endopeptidase was added to each sample. The mixtures were incubated at 37 °C for 24 hours and then lyophilized. Lysyl endopeptidase (Achromobacter protease I, Wako, Japan) is a proteolytic enzyme from A. lyticus M 497-1. This enzyme hydrolyzes peptide bonds at the carboxyl side of lysine residues in a manner similar to that of trypsin. This enzyme attacks the Lys-Pro bond, which is resistant to trypsin, and does not attack the arginyl bond, which is easily hydrolyzed by trypsin. Moreover, this enzyme also cleaves aminomethyl cysteine-X bonds.

Purification and sequencing of oligopeptides digested with lysyl endopeptidase. Oligopeptides were fractionated by HPLC on a 4 × 250-mm reverse-phase (RP) column (Symchrop RPP C18 column, pore size 300 Å, Synchym, Linden, IN). The peptides were eluted with a linear gradient of 0% to 45% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 0.7 mL/min for 120 minutes. Absorbance at 214 nm was monitored, and peaks were collected manually. Fractions obtained in this manner were not rechromatographed because they were already pure for subsequent analysis even after one cycle of chromatography. Oligopeptides eluted as sharp peaks from RP-HPLC were selected as samples for amino acid sequencing because broad peaks occasionally contained two oligopeptides. Selected samples were lyophilized, and their amino acid sequences were determined on an Applied Biosystems (Foster, CA) Model 470A protein sequencer according to published procedures. Phenylthiohydantoin (PTH) amino acids were analyzed by RP-HPLC.

RESULTS

Isolation of the platelet membrane GP IIb-IIIa complex. The GP IIb-IIIa complex was purified from solubilized human platelets by chromatography on HPLC-1-Sepharose 4B. Typical yields of the GP IIb-IIIa complex were ~500 μg/10^8 platelets. SDS-PAGE analysis of the immunoaffinity purified materials is shown in Fig 1A. The apparent mol wt of isolated GP IIb under unreduced and reduced conditions was 137,000 and 116,000, respectively; that of isolated GP IIIa under unreduced and reduced conditions was 90,000 and 100,000, respectively. These variations of apparent sizes of GP IIb and IIIa are in agreement with their previously reported behaviors. Densitometry scans of Coomassie brilliant blue-stained gels showed that GP IIb and IIIa at this stage were ~80% pure.

Separation of GP IIb and GP IIIa proteins. Although GP IIb and IIIa form a calcium-dependent heterodimer complex even in the presence of nonionic detergents, they can be dissociated with SDS and EDTA. Therefore, gel filtration HPLC was performed using a solution containing 0.1% SDS and 1 mmol/L of EDTA. As seen in Fig 2A, two GPs were well separated under the conditions used. Each protein peak was collected separately and rechromatographed to achieve complete purification (Fig 2B and C). SDS-PAGE indicated that the two proteins were separated from each other under these conditions and purified to apparent homogeneity (Fig 1B).

Proteolytic digestion of the GP IIb and GP IIIa polypeptides. After removal of SDS by solvent extraction, GP IIb and GP IIIa were digested with lysyl endopeptidase. The resulting oligopeptides were fractionated by RP-HPLC, and the peak fractions identified by ultraviolet (UV) absorbance were collected for subsequent amino acid sequence analysis. Repeated analysis of the peptide mapping gave the same result. As shown in Fig 3, the oligopeptide maps of these two glycoproteins appeared to be clearly different, confirming and extending previous observations of structural differences between GP IIb and GP IIIa.

Amino acid sequence analysis. The N-terminal amino acid sequences of several oligopeptides were determined using a gas-phase protein sequencer. The results are shown in Table 1. We obtained amino acid sequences identical to those of oligopeptides IIIa-3 that had been prepared independently. Therefore, we consider our analytical system reliable. Sixty amino acid residues (26 residues for IIb and 34 residues for IIIa) were identified.

DISCUSSION

During the last several years, much evidence derived from a number of laboratories has demonstrated that the human platelet GP IIb-IIIa complex serves as the receptor for
extracellular fibrinogen, a plasma protein required for platelet aggregation. GP IIb consists of two disulfide-linked subunits, GP IIbα (mol wt 116,000) and GP IIbβ (mol wt 23,000). In contrast, GP IIIa consists of a single polypeptide chain with multiple intrachain disulfide bonds (mol wt 105,000 as determined from SDS gels).34

Several different procedures for purifying the mixture of the two GPs have been reported. Copurification of these GPs has been reported by McEver and co-workers," using a monoclonal antibody affinity column; by Leung and co-workers," using Lens culinaris lectin affinity chromatography; and by Jennings and Phillips," using sucrose density gradient centrifugation and Sephacyl S-300 gel filtration chromatography. The determination of the amino acid sequence of a polypeptide requires a large quantity of highly purified protein samples. Immunoaffinity chromatography using specific monoclonal antibodies is often well suited for the initial stages of purification. As shown in Fig 1A, however, the immunoaffinity purified GP IIb–IIIa complex was contaminated with a small amount of actin, which may interact with GP IIb–IIIa.35 Furthermore, antibodies that are directed against only one component of the GP IIb–IIIa complex can copurify both glycoproteins.36 Therefore, fur-
Amino Acid Sequences of Oligopeptides From Human Platelet Membrane Glycoproteins IIb and IIIa

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino Acid Yield (pmol)</th>
<th>Amino Acid</th>
<th>Amino Acid</th>
<th>Amino Acid</th>
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<tbody>
<tr>
<td>IIb-1</td>
<td>X — Ala 173 Ser† 130 His 54 Asp 66 161</td>
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<tr>
<td>IIb-2</td>
<td>Asn 33 Ser† 74 Phe 200 Val 158 Asp 60 203</td>
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<tr>
<td>IIIa-1</td>
<td>X — Val 114 Thr† 15 Leu 196 Leu 152 233</td>
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<tr>
<td>IIIa-2</td>
<td>Pro 55 Gin 94 Ile 116 Thr† 13 Trp 42 29</td>
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<tr>
<td>IIIa-3</td>
<td>X — Leu 105 Lys 52 Leu 174 Ser† 14 50</td>
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<tr>
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<td>Asp 8 Ser† 23 — — Thr† 5 Gly 33 103</td>
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<tr>
<td>III-12</td>
<td>— — — — — Lys 11 — — —</td>
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</table>

(1) and (2) indicate experimental number.
X, not identified.
As in the case of IIIa-3, repeated analysis gave the same conclusion.
†Analysis for serine and threonine could not be accurately measured due to the presence of multiple peaks obtained during analysis of the phenylthiohydantoin (PTH) amino acids. Some of those peaks are known to reflect derivatives from serine and threonine, which were specifically detected as peaks at the different positions from other PTH amino acids.

The separation and purification are necessary for the structural analysis of these components.

We evaluated several different protocols for the separation of GP IIb from GP IIIa, including electrophoretic elution from polyacrylamide gel slices containing GP IIb or GP IIIa, proteolytic digestion of proteins within gel pieces, and finally, gel filtration HPLC. Although electrophoresis or proteolytic digestion of gel slices resulted in relatively low protein yields, two cycles of gel filtration HPLC achieved both good separation of GP IIb from GP IIIa and high protein recovery. The purity of the proteins isolated by this technique provided us with the opportunity to obtain large amounts of these two membrane glycoproteins for further study.

Because small amounts of SDS in the purified glycoprotein samples interfered significantly with subsequent proteolytic digestion, we removed SDS by a method of ion-pair extraction that resulted in reproducible digestion. Because removal of SDS from a sample may cause protein aggregation, Lubrol was added to the sample to prevent aggregation.

Nonionic detergents such as NP-40, Lubrol, etc. did not affect the enzymatic digestion.

Computerized analysis of the autoradiograms of the two-dimensional tryptic $^{31}$ peptide maps has indicated structural differences between GP IIb and GP IIIa. McEver and colleagues also demonstrated similar findings when comparing HPLC tryptic peptide maps of a mixture of $^{125}$I-IIb and $^{131}$I-IIIa. These findings are in agreement with the peptide maps of the individual glycoproteins obtained following digestion with lysyl endopeptidase. Because we did not use radioiodinated glycoproteins in these experiments, our results represent information about all oligopeptides, including those that contain no tyrosine residues. Purified oligopeptides were then successfully used as samples for amino acid sequencing. Together, our results are consistent with the hypothesis that GP IIb and GP IIIa are coded for by different structural genes that are coordinately expressed.

Amino acid sequences of the five peptides obtained in this study were compared with 7,687 protein sequences in the Protein Research Foundation/Sequence Data Base (PRF/SEQDB) to check for homology with other proteins. The computer search against all known protein sequences failed to reveal any significant similarity to the peptides derived from either GP IIb or GP IIIa.

The isolation of cDNA clones encoding GP IIb and GP IIIa should provide important additional insights about the structure of the human platelet fibrinogen receptor and the mechanism of regulation of its expression. A recent report documents the isolation of a lambda phage containing a 20-kilobase (kb) genomic library, which, on transfection, leads to the expression of the alpha chain of the OKM-1, LFA-1, and platelet GP IIb–IIIa molecules. Recently, a number of investigators were also successful in the isolation of mRNA species that encode human platelet GP IIb and IIIa. Studies are in progress in our laboratory to isolate mRNA from a megakaryoblastic leukemia cell line that expresses these glycoproteins and to establish a cDNA library. Several amino acid sequences obtained in this study have regions suitable for the synthesis of oligonucleotide probes. Probes such as these should be useful for the screening of this cDNA library.

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REFERENCES


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Purification and partial amino acid sequence of human platelet membrane glycoproteins IIb and IIIa

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