Characterization of a Novel Self-Associating Mr 40,000 Platelet Glycoprotein

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A novel platelet glycoprotein has been purified and characterized. This glycoprotein, designated Pltgp40, is an acidic sialylated 40,000-dalton protein that bears both O-linked and N-linked oligosaccharides. Treatment of Pltgp40 with neuraminidase resulted in a 5,000-dalton reduction in its Mr and a 1.5 Unit alkaline shift in the isoelectric point, indicating the presence of a large number of sialic acid residues. A similar size reduction and change in pl were observed after treatment of Pltgp40 with O-glycase showing that sialic acids are present on O-linked oligosaccharides. Digestion of Pltgp40 with N-glycanase reduced the Mr to approximately 20,000 daltons but did not affect the isoelectric point, suggesting that Pltgp40 contains six to seven nonsialylated N-linked carbohydrate chains. High Mr proteins were observed in affinity purified Pltgp40 and were identified as detergent-stable protein oligomers consisting of multiple 40,000-dalton monomers. Immunodepletion and direct binding studies indicated that Pltgp40 was not equivalent to Ig Fc receptor type II, another 40,000-dalton glycoprotein expressed on platelets. However, Pltgp40 copurified with Fc receptor type II when platelet extracts were loaded onto human IgG affinity columns, raising the possibility that Pltgp40 may associate with Fc receptors or Fc receptor-Ig complexes. Amino acid sequence analysis of the N-terminus of Pltgp40 was performed and confirmed that Pltgp40 is a novel platelet glycoprotein. Epitopes on Pltgp40 appear to be widely expressed because monoclonal antibodies against Pltgp40 also reacted with a variety of myeloid, lymphoid, and epithelial cells. Pltgp40 was detected on activated but not resting platelets, indicating that Pltgp40 is a platelet activation marker.

The prototype sialoglycoprotein on eukaryotic cells is glycoporin, a major erythrocyte membrane glycoprotein. Glycoporin is a transmembrane protein of 131 amino acids bearing one N-linked and 15 O-linked oligosaccharides.1,2 Sialoglycoproteins have also been purified from the membranes of rat and mouse mammary carcinoma cells3-4 and from normal human plasma. Very few sialoglycoproteins have been detected on human leukocytes. Sialoporphin (CD43) is a major sialoglycoprotein expressed on monocytes, lymphocytes, and platelets6-9 whose defective expression is associated with the Wiskott-Aldrich syndrome.10 The amino acid composition and glycosylation pattern of sialoporphin is very similar to that of glycoporin.5,7,8,11 Platelets express a number of mucin-type glycoproteins, including the widely expressed GP IV (CD36) and sialoglycoprotein GP Ib (CDW16).12 CD44 (also designated Fgp-117 and Hermes18) is yet another leukocyte glycoprotein with mucin-like properties. CD44 is an acidic, sulfated glycoprotein containing N-linked carbohydrate in addition to a high content of O-glycosylation.13,14

All of the above described highly sialylated glycoproteins appear to have adhesion-related functions. Anti-sialoporphin monoclonal antibodies (MoAbs) induce homotypic adhesion of lymphocytes and monocytes22,23 Platelet GP IV serves as a receptor for thrombospondin and collagen and has been shown to mediate the early phase of interactions between platelets and collagen fibrils.14,24 Recently GP IV (CD36) was identified as the adhesion molecule mediating the binding of malaria parasite-infected erythrocytes to endothelial cells and platelets.25 CD44 (Hermes/Fgp-1) plays an important role in lymphocyte homing and appears to mediate the binding of lymphocytes to high endothelial venule cells in peripheral lymph nodes.19 The homology of CD44 with the fibroblast extracellular matrix receptor type III and the recently demonstrated close homology to cartilage link proteins suggests that CD44 may be involved in cell binding to subendothelial basement membrane matrix proteins.10,21,26

We have begun a series of experiments designed to identify and characterize novel adhesion-related platelet glycoproteins. In this report, MoAb5s raised against human splenic adherent cells have been used to identify and characterize a novel platelet glycoprotein. This molecule, designated Pltgp40, is an acidic 40-Kd protein bearing both O- and N-linked oligosaccharide chains. The molecule appears to be highly sialylated, but 50% of its mass is represented by nonsialylated N-linked carbohydrates. Pltgp40 purified by affinity chromatography forms detergent-stable oligomers consisting of two or more monomers. Amino acid sequence analysis of the N-terminus indicates that Pltgp40 is not related to any previously characterized platelet glycoprotein, including the 40-Kd Fc RI1 molecule. Pltgp40 appears to be a previously undescribed platelet glycoprotein sharing some properties with well-characterized but distinct highly sialylated membrane glycoproteins. MoAbs against Pltgp40 reacted with diverse cell types, including epithelial cells, suggesting that Pltgp40 or related structures are widely expressed. Furthermore, anti-Pltgp40 reacted with activated but not resting platelets, indicating that Pltgp40 is an activation marker for platelets.

MATERIALS AND METHODS

MoAbs. Hybridomas secreting MoAbs against the novel antigen described in this report were produced from spleen cells of Balb/c mice immunized with human splenic adherent cells. Spleen...
cells from the immunized mice were fused to P3x63.Ag8 myeloma cells as described. Hybridomas were for screened for secretion of antibodies by immunohistochemistry on human lymph node cryostat sections as previously described. Selected hybridomas were cloned and subcloned by limiting dilution. The eight MoAbs selected for study were H5G2, H2G2, H2D, H4F3, H5B7, H54, HSH2 (all IgGl,κ) and H4F8 (IgG2a,κ). Other MoAbs used in these studies have been described previously: H52, IgG1,κ (anti-CD18); IV.3, IgG2b (anti-Fc RII). MoAb IV.3 was kindly provided by Dr M. Fanger (Dartmouth Medical School, Hanover, NH). Ascites fluids containing high levels of IgG were produced by intraperitoneal injection of 10⁶ hybridoma cells into Balb/c mice previously injected intraperitoneally with 0.5 mL of tetrathymepentylidencane (Aldrich, Milwaukee, WI).

Platelets. Heparinized venous blood was centrifuged at 400g for 10 minutes. The platelet-rich plasma (PRP) was removed and centrifuged at 1,500g for 20 minutes. The pelleted platelets were then washed twice with modified Tyrode's buffer (12 mmol/L NaHCO₃, 138 mmol/L NaCl, 2.7 mmol/L KCl, 0.36 mmol/L Na₂HPO₄, 1 mmol/L MgCl₂, 5.5 mmol/L dextrose, pH 7.3) containing 6.6 mmol/L EDTA. The platelets were resuspended in Tyrode's-EDTA buffer. Platelets were also isolated from nontransfusable units by differential centrifugation followed by two washes with 10 vol of Tyrode's-EDTA. For use in activation studies and some immunoprecipitation studies, platelets were isolated by chromatography on Sepharose 2B columns. Briefly, venous blood from normal volunteers was mixed with 0.13 mol/L citrate (9:1) and spun at 860g for 20 minutes. The PRP was collected and loaded onto a 12x-vol Sepharose 2B column equilibrated with Tyrode's-EDTA buffer. The column was eluted with Tyrode's-EDTA buffer and the peak platelet-containing fractions were pooled. In some studies prostaglandin E₁ was included in all buffers to prevent premature activation of the platelets. Platelets were activated by adding thrombin (Boehringer-Mannheim, Indianapolis, IN) to a final concentration of 0.1 to 1.0 U/mL followed by incubation for 5 to 45 minutes at 23°C.

Preparation of MoAbs. Small-scale purification of MoAbs from ascites fluid was performed by ion-exchange high performance liquid chromatography (HPLC) as previously described, except that a 45% ammonium sulfate fractionation was performed before HPLC purification. Large-scale purifications were performed by 45% ammonium sulfate fractionation of ascites fluid followed by ion exchange chromatography on DEAE Affi-Gel Blue (BioRad, Richmond, CA). Antibody purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified MoAbs were derivatized with biotin or fluorescein isothiocyanate as previously described. Human IgG (Cappel, Malvern, PA) at a concentration of 10 mg/mL in phosphate-buffered saline (PBS) was aggregated by incubating at 60°C for 20 minutes. Gross aggregates were removed by centrifugation at 50,000g for 30 minutes.

Preparation of affinity columns. Purified MoAb or aggregated human IgG (aggIgG) in 50 mmol/L Na borate, pH 8.0 was mixed with carbonyl-dimimidazole-activated agarose (ReactiGel 6X; Pierce, Rockford, IL) at a ratio of 5 mg IgG/mL of packed beads and incubated with constant mixing for 36 hours at room temperature. Unreacted sites were then neutralized by incubation in 2.0 mol/L ethanalamine, pH 8.0, for 2 hours. Coupling efficiency was 95% to 99%.

Preparation of F(ab')₂ fragments. F(ab')₂ fragments of MoAb were prepared essentially as previously described. Briefly, ascites fluids were dialyzed against 0.1 mol/L Na citrate, pH 3.5, before adding pepsin (Sigma, St Louis, MO) to a final concentration of 100 μg/mL. The mixtures were then incubated for 6 to 18 hours at 37°C before dialysis against 20 mmol/L TrisHCl, pH 8.5. The F(ab')₂ fragments were then purified by ion-exchange HPLC as described for IgG. Preparations containing no whole IgG were determined by SDS-PAGE analysis.

Affinity purification. Platelets were isolated from 35 to 50 nontransfusable units as described above. Contaminating red blood cells were lysed by resuspending platelets in 50 mmol/L TrisHCl, 150 mmol/L NaCl, pH 7.3 containing 140 mmol/L NH₄Cl for 10 minutes at 37°C. The platelets were then washed twice by resuspending in Tyrode's-EDTA buffer followed by centrifugation at 1,500g for 15 minutes. TEN (50 mmol/L TrisHCl, 2 mmol/L EDTA, 150 mmol/L NaCl, pH 7.5) containing 2% Triton X-100 and protease inhibitor cocktail (0.5 mmol/L phenylmethylsulfonylfluoride [PMSF], 1 μg/mL aproatin, 1 μg/mL leupeptin, 0.5 μg/mL antipain, 0.5 μg/mL chymotatin, and 1 μg/mL soybean trypsin inhibitor) was then added to the pellet for a final detergent to protein ratio of 5:1 (wt/wt). The mixture was stirred 15 hours at 0°C before centrifugation at 100,000g for 45 minutes at 4°C. The supernatant was collected and diluted to 1% Triton X-100 with TEN before loading onto aggIgG and MoAb (H52C6) affinity columns connected in series in the order stated. After washing with 20 column vol of TEN, 1% Triton X-100, the columns were separated and the H5C6 column was washed sequentially with: (1) 125 mmol/L Na₂HPO₄, 1.0 mol/L NaCl, 0.5% Triton X-100, pH 8.0 (10 column vol); (2) 1.0 mol/L TrisHCl, 0.5% Triton X-100, pH 10.0 (10 column vol); and (3) 20 mmol/L TrisHCl, 0.5% octyl glucoside, pH 7.5 (10 column vol). Bound proteins were eluted with 50 mmol/L glycine, 1.0 mol/L NaCl, 0.1% octyl glucoside, pH 2.5. Fractions were immediately neutralized by adding 0.05 vol of 10% Tris, pH 12. The aggIgG column was washed with 20 column vol of PBS, 0.1% Triton X-100 followed by an equal volume of PBS, 0.5% octyl glucoside. Bound proteins were then eluted and neutralized as described for the H5C6 column. Eluted proteins from both columns were analyzed by SDS-PAGE and fractions containing proteins were pooled and concentrated by vacuum-dialysis against PBS, 0.5% octyl glucoside. Purified proteins were stored at −70°C.

Radioiodinations. Purified proteins were labeled with ¹²⁵I using the chloramine-T method as previously described. Iodinations were performed in the presence of 0.5% octyl glucoside. The specific activity of radioiodlated purified proteins was 10 to 30 x 10⁸ cpm/μg. Platelets isolated as described above were vectorially labeled with ¹²⁵I using lactoperoxidase as previously described except that glucose oxidase was used instead of H₂O₂. Briefly, 20 to 50 μL of packed platelets were taken in 200 μL of Tyrode's buffer to which was added 10 U lactoperoxidase. The mixture was then incubated 20 minutes at 0°C before washing the platelets three times with 15 mL of Tyrode's buffer.

Immunoprecipitation. Radioiodinated platelets were lysed by incubation in 1 mL of TEN containing 1% NP-40 and protease inhibitor cocktail for 45 minutes on ice. Detergent insoluble material was removed by centrifugation at 100,000g for 45 minutes at 4°C. The extract was depleted of nonspecific binding proteins by adding 100 μL of 10% fixed Staphylococcus aureus (SaC) followed by removal of the SaC by centrifugation after 45 minutes at 4°C. Purified MoAbs (2.5 μg) were added to 10 to 50 x 10⁶ cpm of labeled platelet extract or labeled purified proteins and the mixtures were then incubated for 4 to 12 hours on ice. Ten to 50 micrograms of purified rabbit antimouse IgG (RAM; Jackson ImmunoResearch, West Grove, PA) was added and the incubation continued for 1 to 4 hours before adding 50 μL of SaC. After 20 minutes the SaC was pelleted and washed sequentially with (1) TEN, 2M KCl, 1% NP-40; and (2) 50 mmol/L Tris, pH 8.0, 0.5% NP-40. The pellets were taken up in SDS-PAGE sample buffer with or without 0.75 μL 2-mercaptoethanol, incubated at 100°C for 3 minutes, and the SaC removed by centrifugation. The eluted...
proteins were then analyzed by SDS-PAGE followed by autoradiography using Kodak XAR film (Eastman Kodak, Rochester, NY).

One- and two-dimensional PAGE. One-dimensional SDS-PAGE was performed as described by Laemmli. Two-dimensional gel analysis consisting of isoelectric focusing (IEF) followed by SDS-PAGE was performed by the method of O’Farrell under reducing conditions (0.75 mol/L 2-mercaptoethanol) in both dimensions. Samples were first run on pre-electrophoresed tube gels containing 10% ampholines (Pharmacia, Piscataway, NJ) and then separated by size in the second dimension on 10% polyacrylamide slab gels.

Peptide mapping studies. Peptide mapping was performed on SDS polyacrylamide slab gels using a modification of a previously described method. Affinity-purified radiolabeled glycoproteins (5 to 106 cpm) were separated by SDS-PAGE, and after autoradiography the protein bands were excised from dried gels. The polypeptides were eluted from ground gel slices into 0.1% SDS and mixed with 2.5 to 5 μg of Staphylococcus protease V8 (Boehringer-Mannheim) and electrophoresed on 12% SDS polyacrylamide slab gels under standard conditions.

Glycosidase digestions. Affinity purified radiolabeled proteins in PBS, 0.5% octyl glucoside were first treated with 20 mmol/L dithiothreitol (DTT) for 2 hours at 37°C. For neuraminidase treatment, 50 μL of DTT-treated protein (2 × 106 cpm) were mixed with 0.1 U of neuraminidase (Vibrio cholerae; Sigma) and incubated for 2 hours at 37°C. For O-glycanase treatment 50 μL of radiolabeled protein in PBS, 0.5% octyl glucoside, was mixed with 5 μL of O-glycanase (970 mU/mL; Genzyme, Boston, MA) and incubated for 12 hours at 37°C. For N-glycanase treatment 75 μL of DTT-treated radiolabeled protein was mixed with 1 μL of β-mercaptoethanol, 54 μL of 0.55 mol/L NaHPO4, pH 8.6, 15 μL of 100 mmol/L phenanthroline, 25 μL of 7.5% NP-40, and 6 μL of N-glycanase (250 U/mL, Genzyme) and incubated for 15 hours at 37°C. Samples to be treated with O-glycanase after N-glycanase treatment were first treated with neuraminidase after digestion with N-glycanase. Endoglycosidase H (endo H) digestion was performed by mixing 50 μL of heat-denatured radiolabeled protein in 0.5% SDS, 0.1 mol/L β-mercaptoethanol with 50 μL of 100 mmol/L Na citrate, 1% SDS, 1 mmol/L PMSF, and 10 mU of endo H (Boehringer-Mannheim) followed by incubation at 37°C for 15 hours.

Western blotting. Immunoblotting was performed as described previously. Briefly, proteins were separated on SDS-PAGE gels and transferred onto nitrocellulose filters. After blocking with bovine serum albumin, the filter strips were incubated with biotin-conjugated MoAb [F(ab')2, 10 μg/mL] for 15 hours at 4°C and washed with 50 mmol/L TrisHCl, 150 mmol/L NaCl, 0.05% NP-40, pH 7.5. The strips were then incubated with 125I-purified goat anti-biotin IgG (5 × 106 cpm) for 2 hours at 4°C. The strips were washed and bound antibodies detected by autoradiography.

Protein sequencing. Concentrated affinity purified protein was further purified by size exclusion HPLC on a TSK-3000PWHR column (Altex, Fullerton, CA) using 0.1 mol/L NaHPO4, pH 6.9, 0.2% octyl glucoside as running buffer. The major peak eluting in the void volume was collected and concentrated in centrifugal microconcentrators (Amicon). The protein (1 nmol) was then sequenced using an automated gas-phase microsequenator (Applied Biosystems, Foster City, CA), and the phenylthiohydantoin (PTH) amino acids were identified by reverse-phase HPLC.

MoAb binding studies. Flow microfluorimetry analysis and indirect liquid-phase cell binding assays using MoAbs and fluorescently-conjugated or radioiodinated second antibodies were performed exactly as previously described.

RESULTS

MoAbs recognize an Mr 40,000 platelet protein. In a previous study we produced a large panel of MoAbs against human splenic adherent cells. The MoAbs were screened initially for binding to frozen sections of human lymph...
and fluoresceinated or radioiodinated second antibodies exactly as bodies were tested for binding to a diverse panel of with most of the myeloid, lymphoid, and epithelial cells platelets. Eight MoAbs have been identified that show reactivity in these immunohistochemistry assays for binding to human and H54. Immunoprecipitation and SDS-PAGE analysis of no assays: H2G1, H4F8, H4H3, H5B7, H5C6, H5D2, H5H2, strong reactivity against platelets in solid phase radioimmu- node. We have tested all of the MoAbs showing reactivity in these immunohistochemistry assays for binding to human platelets. Eight MoAbs have been identified that show strong reactivity against platelets in solid phase radioimmunoassays: H2G1, H4F8, H4H3, H5B7, H5C6, H5D2, H5H2, and H54. Immunoprecipitation and SDS-PAGE analysis of the antigen recognized by the antibodies showed a broad heterodisperse protein band centered at approximately 40 Kd (designated Pltgp40) (Fig 1). Results are shown for four of the MoAbs, but similar results were obtained with all eight. The immunoprecipitates from fresh and outdated platelets were identical and, in addition to the 40-Kd protein, also contained a weaker 33-Kd protein band. The platelets after thrombin activation (Fig 2). Expression of Pltgp40 on various cell types is widely distributed. To further examine the expression of Pltgp40 on platelets, platelets were isolated by gel filtration and analyzed for expression of Pltgp40 by flow microfluorimetry in the presence or absence of thrombin. Pltgp40 was not detected on the surface of resting cells, but was expressed strongly on the platelets after thrombin activation (Fig 2). Expression of Pltgp40 was analyzed under reducing conditions were also present after analysis under nonreducing conditions (data not shown), showing that the polypeptides were not linked by disulfide bridges. However, the presence of intrachain-bound proteins (not shown). These results indicated that Pltgp40 was present within resting platelets and that Pltgp40 was mobilized to the surface rapidly on activation. These results also suggested that the platelets used in Fig 1, purified by differential centrifugation from heparinized blood, had been activated during purification. This was confirmed in immunoprecipitation studies using platelets isolated by gel filtration. Pltgp40 was immunoprecipitated from surface-labeled thrombin-treated platelets, but little if any antigen was detected in immunoprecipitates from resting platelets (data not shown).

**Purification of Pltgp40.** Based on the results of the immunoprecipitation studies, outdated platelets were used as starting material for purification of Pltgp40. A total of 680 µg of purified Pltgp40 was obtained from 1,800 mg of starting material. The purified protein consisted of polypeptides of 40 Kd and 33 Kd as seen in the immunoprecipitates from labeled platelets, but additional bands with molecular weights of approximately 80, 100, and 200 Kd were also seen (gp40, Fig 1). All of the bands observed when purified Pltgp40 was analyzed under reducing conditions were also present after analysis under nonreducing conditions (data not shown), showing that the polypeptides were not linked by disulfide bridges.

### Table 1. Expression of Pltgp40 on Various Cell Types

<table>
<thead>
<tr>
<th>A. Cells that react with anti-Pltgp40 MoAbs</th>
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<tbody>
<tr>
<td>Myeloid: Platelets, granulocytes, monocytes, macrophages, U-937 (monocytic leukemia) and HL-60 (promyelocytic leukemia)</td>
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<tr>
<td>Lymphoid: MLA-144 (gibbon T lymphoma); T-cell leukemia (A3.01, HSB-2)</td>
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<tr>
<td>Erythroid: K-562 (erythroleukemia)</td>
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<tr>
<td>Epithelial: MCF-7 (mammary carcinoma); HeLa (cervical carcinoma); Cos-7 (monkey kidney epithelium); 98 (sarcoma)</td>
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<table>
<thead>
<tr>
<th>B. Cells that do not react with anti-Pltgp40 MoAbs</th>
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</thead>
<tbody>
<tr>
<td>Lymphoid: Peripheral blood T cells; peripheral blood B cells; EBV-transformed</td>
</tr>
<tr>
<td>B cells: T-cell leukemia (Jurkat, Molt-4)</td>
</tr>
<tr>
<td>Erythroid: Human erythrocytes</td>
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*Indirect binding assays were performed using anti-Pltgp40 MoAbs and fluoresceinated or radioiodinated second antibodies exactly as previously described.*

**Flow microfluorimetry analysis of Pltgp40 expression on resting and activated platelets.** Platelets were isolated by gel filtration and incubated for 15 minutes at 23°C in the presence (A) or absence (B) of 1.0 U/mL of thrombin. The platelets were then incubated with FITC-conjugated F(ab') fragments (25 µg/mL) of anti-Pltgp40 MoAb (HSC6) or an irrelevant control antibody (AIM.20) for 30 minutes, and analyzed on a Coulter Profile II cytometer (Coulter, Hialeah, FL).
disulfide bridges was indicated by an increase in the electrophoretic mobility of all the bands after treatment with DTT (see below).

**Glycosidase digestion of Pltgp40.** The glycosylation of Pltgp40 was examined by digestion of the purified protein with specific glycosidases. Treatment of Pltgp40 with O-glycanase, which removes serine- or threonine-linked oligosaccharides, resulted in a decrease in apparent Mr of the 40- and 80-Kd polypeptides by 5 and 10 Kd, respectively (Fig 3). Pltgp40 was also treated with N-glycanase, which catalyzes the hydrolysis of all common asparagine-linked oligosaccharides. N-glycanase treatment of the glycoprotein yielded major polypeptide bands of 20 and 35 Kd, with a minor band at approximately 15 Kd (Fig 3). The latter band was not consistently observed and may represent the product of contaminating proteases in the glycosidase preparation. Similar bands were obtained when N-glycanase was used in combination with O-glycanase and neuraminidase (Fig 3), suggesting that the 20- and 35-Kd proteins may represent core protein structures. This result indicated that the 40-Kd glycoprotein contains six to seven N-linked oligosaccharide chains. Treatment of Pltgp40 with neuraminidase alone increased the electrophoretic mobility of each component polypeptide band by approximately 10%, suggesting a significant degree of siylation (Fig 3). Pltgp40 was also treated with DTT, which resulted in an increase in the electrophoretic mobility of all polypeptide bands by 5% to 10%, suggesting that the molecules contain at least one intramolecular disulfide bridge (Fig 3). Pltgp40 was treated with endo H before and after SDS heat denaturation but was resistant to digestion under both conditions (data not shown), indicating that the N-linked sugars are of the complex or hybrid type. To rule out changes in the properties of Pltgp40 oligosaccharides during purification Pltgp40 was immunoprecipitated from thrombin-activated platelets and treated with N-glycanase and neuraminidase. The immunoprecipitates showed the same bands (Fig 4) as similarly treated immunoprecipitated Pltgp40, indicating that the purification process did not alter the oligosaccharide structures.

**Two-dimensional IEF-SDS-PAGE analysis of Pltgp40.** Affinity purified Pltgp40 was radioiodinated and subjected to two-dimensional IEF-SDS-PAGE analysis after treatment with various glycosidases. All of the polypeptide bands in the Pltgp40 preparation focused within a pH range centered at approximately 4.8 (Fig 5; Control). After treatment of the purified Pltgp40 with neuraminidase, all of the glycoproteins shifted by 1 to 1.5 pH U to 6.0 to 6.5 (Fig 5), indicating that all of the glycoproteins contain similar molar amounts of sialic acid. After N-glycanase treatment of the Pltgp40 the pH range of the resulting 20- and 35-Kd polypeptide bands as well as the higher bands was virtually identical to that of the untreated material (Fig 5), indicating that the N-linked oligosaccharides did not contain sialic acid residues. To confirm the restriction of sialic acid residues to O-linked oligosaccharides, Pltgp40 was analyzed by IEF-SDS-PAGE after treatment with O-glycanase. As shown in Fig 6, after O-glycanase treatment all of the polypeptides in affinity purified Pltgp40 focused within a pH range of 5.9 to 6.3. This result closely approximated that obtained with neuraminidase, confirming that the negative charge of the polypeptides was primarily due to sialic residues on O-linked oligosaccharides. This result also indicated that the O-linked sugars may be atypical because prior removal of sialic acid residues is usually required for efficient O-glycanase digestion. 

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**Fig 3.** Glycosidase digestion of affinity purified Pltgp40. Purified Pltgp40 was radioiodinated and treated with various glycosidases as described in Materials and Methods in the order indicated. Control and treated samples were then analyzed by SDS-PAGE on a 10% polyacrylamide gel under reducing conditions. For DTT treatment radioiodinated Pltgp40 was incubated for 2 hours at 37°C in 0.1% SDS containing 20 mmol/L DTT. Bands were visualized by autoradiography (Neuram, neuraminidase). Lines indicate the shift in molecular weights of the individual bands after glycosidase treatment or reduction.
Pltgp40 forms oligomers. The purified Pltgp40 contained bands with molecular weights that were approximately multiples of 40 Kd (40, 80, 120, and 160 Kd), and all of these bands focused in two-dimensional IEF-SDS-PAGE within identical pH ranges. These observations suggested that the Pltgp40 may self-associate to form oligomers. To test for oligomer formation purified Pltgp40 was radiiodinated and the component glycoproteins separated by SDS-PAGE. The individual bands were visualized by autoradiography, excised from dried gels, and subjected to SDS-PAGE analysis on a second slab gel. The isolated 40-Kd glycoprotein lane showed a strong band at 80 Kd in addition to the expected 40-Kd band (Fig 7). Similarly, the isolated 80-Kd protein lane showed a strong band at 80 Kd but also contained a band of equal intensity at 40 Kd. The 120-Kd glycoprotein lane showed the expected 120-Kd band, but polypeptides at 40 and 80 Kd were also seen (Fig 7). This result clearly demonstrated that the 40-Kd glycoprotein formed SDS-stable oligomers represented by the higher Mr proteins in the purified Pltgp40. The 20-Kd band obtained after N-glycanase digestion of Pltgp40 also showed a band at 38 Kd when isolated and re-analyzed by SDS-PAGE, indicating that the N-linked oligosaccharides of Pltgp40 are not required for self-association (Fig 7). The 33-Kd protein band present in immunoprecipitates and in some preparations of purified Pltgp40 was also isolated and re-analyzed by SDS-PAGE. As shown in Fig 7, the 33-Kd protein lane showed a band at approximately 60 to 63 Kd, clearly demonstrating dimer formation by this protein as well. Pltgp40 was also analyzed by Western blot analysis with MoAbs. The anti-Pltgp40 MoAb reacted with polypeptides bands migrating at 33, 40, and 80 Kd. Protein bands at 63, 160, and greater than 200 Kd were also stained (data not shown). This result showed that the epitope recognized by the MoAb was present on the 40-Kd glycoprotein and higher Mr forms of Pltgp40, and was consistent with self-association of the 40-Kd protein and the suggestion that the 33-Kd band is a proteolytic fragment of the 40-Kd molecule.

Fig 4. Glycosidase digestion of immunoprecipitated Pltgp40. Platelets were isolated by gel filtration and activated by treatment with 1.0 U/mL of thrombin for 15 minutes. The platelets were then vectorially radioiodinated, and lysed with detergent. Pltgp40 was immunoprecipitated from the detergent lysate with MoAb H5C6 and eluted by boiling in 0.1% SDS for 5 minutes. The eluted Pltgp40 was then treated with N-glycanase or neuraminidase as described in Materials and Methods before analysis by SDS-PAGE under reducing conditions. 1, No treatment; 2, neuraminidase; 3, N-glycanase. The arrow indicates the 18- to 20-Kd band seen after N-glycanase treatment.

Fig 5. Two-dimensional IEF-SDS-PAGE analysis of Pltgp40. Control and glycosidase-treated radioiodinated Pltgp40 were focused in IEF polyacrylamide tube gels as previously described. The tube gels were then loaded onto 10% polyacrylamide slab gels for SDS-PAGE analysis under reducing conditions. Bands were visualized by autoradiography. The pH gradient of the IEF gels is shown at the top of the figure.
Fig 6. Two-dimensional IEF-SDS-PAGE analysis of O-glycanase treated Pltgp40. Affinity purified radioiodinated Pltgp40 was treated with O-glycanase as detailed in Materials and Methods. Control and treated Pltgp40 were focused in IEF tube gels, which were then loaded onto 10% polyacrylamide slab gels for SDS-PAGE analysis under reducing conditions. Bands were visualized by autoradiography. The pH gradient of the IEF gel is shown at the top of the figure.

Cleveland peptide mapping studies. The interrelationship of the Pltgp40 glycoproteins was further examined in peptide mapping studies. In these experiments SDS-PAGE-separated Pltgp40 bands were eluted from gels and loaded onto a second SDS-PAGE gel (12%) in the presence or absence of the *Staphylococcus* V8 protease as previously described. The 33- and 40-Kd glycoproteins were more sensitive to digestion than were the larger proteins with the gp120 showing the fewest peptide fragments (Fig 8). This may reflect limited protease access to certain regions of Pltgp40 when the molecule self-associates to form oligomers. The profile of peptides from the 33-Kd glycoprotein appeared to be a subset of the 40-Kd molecule. Interestingly, the 40-Kd peptides included a band at approximately 33 Kd (Fig 8). This result suggested again that the 33-Kd protein is a proteolytic fragment of the 40-Kd glycoprotein.

The peptides generated from the 120-Kd band appeared to be a subset of those generated from the 80-Kd glycoprotein (Fig 8). Moreover, peptides of identical molecular weights to those generated from the 80-Kd molecule were also present in the 40- and/or 33-Kd glycoprotein digests. These results confirmed the suggestion that the multiple protein forms present in the purified Pltgp40 material were all derived from the 40-Kd molecule.

Pltgp40 is not Fc receptor type II. The platelet IgG Fc receptor (FcγRII) is a major platelet surface glycoprotein with an Mr of 40 Kd. We examined whether Pltgp40 was related to FcγRII in sequential immunoprecipitation studies using an MoAb (IV.3) against FcγRII. The IV.3 antibody failed to immunoprecipitate radiolabeled affinity-purified Pltgp40 (Fig 9A). FcγRII was purified from plate-
Fig 8. Peptide mapping of Pgp40 glycoproteins using *Staphylococcus* V8 protease. Radioiodinated Pgp40 glycoproteins were separated by SDS-PAGE on a 7.5% polyacrylamide gel. The individual bands were eluted from gel slices into 0.1% SDS and loaded onto a 12% polyacrylamide SDS-PAGE slab gel in reducing sample buffer containing 0.25, or 5 μg of *Staphylococcus* V8 protease followed by electrophoresis under standard conditions. The peptides bands were visualized by autoradiography.

Fig 9. Peptide mapping of Pgp40 glycoproteins using *Staphylococcus* V8 protease. Radioiodinated Pgp40 glycoproteins were separated by SDS-PAGE on a 7.5% polyacrylamide gel. The individual bands were eluted from gel slices into 0.1% SDS and loaded onto a 12% polyacrylamide SDS-PAGE slab gel in reducing sample buffer containing 0.25, or 5 μg of *Staphylococcus* V8 protease followed by electrophoresis under standard conditions. The peptides bands were visualized by autoradiography.
and possibly to other well-characterized glycoproteins. Pltgp40, purified by MoAb affinity chromatography, was further purified by size-exclusion HPLC in the presence of 0.2% octyl glucoside. A single major peak eluted in the void volume and was shown by SDS-PAGE to include the 40-, 80-, 120-, and 160-Kd proteins seen in the starting material. The material in this peak was concentrated and 1 nmol (based on an Mr of 20 Kd) was subjected to automated amino acid sequence analysis. A single signal was obtained and identification of the PTH amino acids was possible through residue 22 (Table 2). Identical results were obtained using Pltgp40 from two independent affinity purifications. A search of available protein sequence data showed no homology between Pltgp40 and any well-characterized platelet or leukocyte glycoproteins. Although it is possible that some of the proteins in the purified Pltgp40 samples could have blocked N-termini, these results are entirely consistent with those obtained in the above studies showing that all of the glycoproteins in the affinity-purified Pltgp40 material are derived from a single polypeptide chain.

DISCUSSION

Pltgp40 is a novel 40-Kd sialylated platelet glycoprotein bearing both N- and O-linked oligosaccharides. Pltgp40 appears to be a previously unidentified component of platelets representing 0.037% of total detergent-extractable protein. Partial amino acid sequence analysis of the N-terminus of Pltgp40 suggests that Pltgp40 is not related to any of the previously characterized platelet glycoproteins. Recognition of a diverse panel of cells by anti-Pltgp40 antibodies showed that Pltgp40 or related structures are widely expressed.

An unusual feature of affinity-purified Pltgp40 was the presence of high Mr polypeptide bands after SDS-PAGE analysis that were not present in immunoprecipitates from radiolabeled platelets. These proteins had apparent molecular weights that were multiples of 40 Kd, suggesting oligomer formation by Pltgp40. Individual bands cut from gels showed the presence of higher or lower Mr forms of Pltgp40 when rerun on SDS-PAGE gels. Limited proteolysis of the individual Pltgp40 bands and analysis of the resulting peptides showed overlapping peptide profiles. On two-dimensional gel analysis control and glycosidase-treated high Mr forms of Pltgp40 focused within a pH range identical to that of the 40-Kd molecule analyzed under the same conditions. Western blot analysis of purified Pltgp40 using MoAbs showed that the high Mr bands also carried the epitopes recognized on the 40-Kd molecule. Partial N-terminus sequence analysis showed only a single PTH-amino acid signal in each cycle. All of these results indicate that the high Mr polypeptides observed in the purified Pltgp40 are SDS-stable oligomers of the 40-Kd molecule. The absence of oligomers in immunoprecipitates from radiolabeled platelets may indicate that Pltgp40 oligomer formation occurs only at relatively high concentrations. This idea is supported by the observation of much stronger gp80 and gp120 bands after SDS-PAGE analysis of concentrated affinity-purified Pltgp40 compared with the unconcentrated eluate fractions from the affinity columns (J.E.K. Hildreth, unpublished observations, June 1988). The appearance of the 40-Kd band after SDS-PAGE analysis of the higher Mr forms of Pltgp40 indicates that oligomerization is reversible and that the molecule may exist in equilibrium between the monomeric and oligomeric forms. We have not determined if the observed self-association of purified Pltgp40 is of physiologic importance in platelets. Although the function of Pltgp40 is not clear, the tendency of the molecule to form oligomers suggests that it may play a role in cytoskeleton-lipid bilayer interactions as has been proposed for other self-associating membrane proteins such as an abundant 28-Kd erythrocyte membrane glycoprotein.46 Oligomer formation by Pltgp40 may reflect structural similarities to sialoglycoproteins because self-association of glycophorin, the major erythrocyte sialoglycoprotein, has been reported.46

Pltgp40 is highly glycosylated and contains both O- and N-linked oligosaccharides as determined by digestion with glycosidases. Treatment of Pltgp40 with N-glycanase, which removes all asparagine-linked carbohydrate chains,44 reduced the Mr of the molecule from 40 Kd to approximately 20 Kd, suggesting that N-linked carbohydrate chains constitute approximately 50% of the total mass of the molecule. Based on the 3-Kd average Mr of N-linked oligosaccharides,49 Pltgp40 appears to contain six to seven such structures. Pltgp40 was resistant to digestion by endo H, indicating that the N-linked oligosaccharides are of the complex or hybrid type.45 The acidic pI of Pltgp40 was unchanged by treatment with N-glycanase, suggesting that

Table 2. Amino Acid Sequence of Pltgp40 N-Terminus

<table>
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<tr>
<th>Residue</th>
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<th>Experiment 2</th>
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<tr>
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<tr>
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<td>Leu</td>
</tr>
<tr>
<td>20</td>
<td>( )</td>
<td>Ala</td>
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</table>

*Affinity purified Pltgp40 was further purified by size exclusion HPLC and subjected to automated sequence analysis as detailed in Materials and Methods. Parentheses indicate that no PTH amino acid was identified.
the N-linked carbohydrate chains are not sialylated. In this respect Pltgp40 behaved like the lymphocyte homing receptor CD44 (Pgp-1), which bears both O-linked and N-linked sugars and whose acidic PI is unaffected by removal of N-linked sugars.18 The presence of sialic acids on Pltgp40 was demonstrated by digestion with neuraminidase, which reduced the Mr by about 5 Kd and shifted the pl from a range of 4.5/5.0 to 6.1/6.5. A similar reduction in Mr and shift in pl were observed when Pltgp40 was digested with O-glycanase, which removes O-linked carbohydrate chains,19 demonstrating that sialic acid residues are present on O-linked sugars. These results indicate that although Pltgp40 is heavily glycosylated and very acidic, it is probably not a true sialoglycoprotein because O-linked sugars constitute more than 50% of the mass of such proteins.6 Additionally, whereas removal of O-linked sugars from true sialoglycoproteins results in an increase in apparent Mr on SDS-PAGE analysis,6,9 the opposite effect was observed for Pltgp40. Pltgp40, like GPIV (CD36) and CD44, binds to wheat germ agglutinin suggesting the potential presence of clustered sialic acids.7 A 56-amino acid stretch of CD44 has been identified as an O-glycosylation (mucin) domain characterized by a high frequency of serine and threonine residues.20 Thus, Pltgp40, CD36, and CD44 may represent pseudosialoglycoproteins, N-glycosylated proteins that bear O-linked oligosaccharides in high density in restricted domains. Such mucin domains could potentially serve as “stalks” for other domains because polypeptides with a high content of O-glycosylation adopt an extended conformation with little secondary structure.21 Both of the previously characterized highly sialylated major platelet glycoproteins (GP Ib and CD36) have been shown to have adhesion functions.13,24 The potential role of Pltgp40, a third sialylated platelet glycoprotein, in adhesion-related functions remains to be determined.

Prior treatment of Pltgp40 with neuraminidase was not necessary to cleave the O-linked carbohydrates with O-glycanase. This suggests that the O-linked sugars on Pltgp40 may have an unusual structure because other studies indicate that the presence of sialic acids on O-linked sugars interfere with the action of O-glycanase.45 Alternatively, we could not rule out the presence of contaminating sialidase activity in the O-glycanase used in the present studies. The possible presence of unusual carbohydrate structures on Pltgp40 is also suggested by the observation that all of the MoAbs produced against Pltgp40 in this laboratory (13 antibodies from four independent fusions) appear to recognize an epitope on the carbohydrate chains (J.E.K. Hildreth, manuscript in preparation).

Pltgp40 is similar in size to the platelet Ig receptor, FcγRII, a single-chain 40-Kd glycoprotein that binds preferentially to immune complexes.26,29 The 40-Kd Pltgp40 molecule does not appear to be homologous to FcγRII based on several observations. First, the core protein of Pltgp40 appears to be approximately 20 Kd in size whereas the core protein of FcγRII has an Mr of approximately 35 Kd.29 Second, in contrast to Pltgp40, FcγRII is not highly glycosylated having only 2 N-linked oligosaccharides that constitute approximately 7% to 10% of the total mass of the molecule.50 Finally, direct evidence that Pltgp40 is not FcγRII was obtained in immunodepletion assays in which it was shown that depletion of FcγRII from platelet agglutinin binding proteins with an MoAb did not remove Pltgp40. The results of N-terminus amino acid sequence analysis also clearly indicate that Pltgp40 and FcγRII are distinct proteins.

Pltgp40 copurified with FcγRII when platelet extracts were passed through agglutinin affinity columns. While it is clear that Pltgp40 is structurally unrelated to FcγRII, this result suggests that Pltgp40 may interact directly with human IgG or FcγRII. Other investigators have not reported observing a molecule like Pltgp40 when FcγRII has been purified from platelet extracts using IgG affinity columns.8,31 A number of factors may account for the failure to detect Pltgp40 in other studies. In previous experiments extracts of small numbers of radiolabeled platelets were used and therefore the concentration of platelet glycoproteins in these extracts was probably much lower than in the present study. In addition, the incubation times of the extracts with the affinity columns in the previous studies were relatively short in comparison with those of the present study. Finally, we have used aggregated-human IgG to prepare affinity columns whereas in the previous studies monovalent IgG was used.

We have ruled out direct binding of Pltgp40 to human IgG or immune complexes using radiolabeled Pltgp40 in solid phase radioimmunoassays. Studies are currently underway to determine if Pltgp40 represents an FcR-associated protein and directly interacts with FcγRII. Based on recent studies of the high affinity IgE receptor it has been postulated that FcR-associated proteins may play important roles in the functions of FcRs.52 The MoAbs against Pltgp40 also react with monocytes, macrophages, and neutrophils. The molecule recognized on these cells is a 60-Kd glycoprotein that appears to have the same 20-Kd core polypeptide as Pltgp40 (D.O. Azorsa and J.E.K. Hildreth, unpublished result, July 1989). Thus, Pltgp40 may represent a new myeloid differentiation antigen. Moreover, because all mature myeloid cells express Fc receptors, we have begun studies to determine if Pltgp40 plays a common role in FcR-related functions on platelets and myeloid cells. The broad expression of Pltgp40 (or related structures) on diverse cell types suggests that this molecule may be involved in a common cell function such as transmembrane signalling or cell adhesion, as has been described for other widely expressed highly glycosylated proteins such as CD44 and CD36.13,19

Pltgp40 was expressed on the surface of platelets purified by gel filtration only after activation with thrombin. The expression occurred very rapidly and appeared to be due to mobilization of intracellular stores of the antigen based on staining of permeabilized resting platelets. These results are very similar to those reported for GMP-140, a 140-Kd α granule glycoprotein that is rapidly expressed on the surface of platelets after activation.53,54 We have not yet determined whether Pltgp40 is localized to α granules or...
lysosomes in platelets. However, the expression of Pltgp40 on a variety of cell types suggest that lysosomes may be the more likely intracellular vesicle containing Pltgp40. Interestingly, a 53-kD glycoprotein localizing to platelet lysosomes has been reported. This molecule, like Pltgp40, is expressed on activated but not on resting platelets. It will be of interest to determine the exact relationship between Pltgp40 and this platelet lysosomal protein.

ACKNOWLEDGMENT

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REFERENCES


Characterization of a novel self-associating Mr 40,000 platelet glycoprotein [see comments]

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