GPIIIa-Related PLA1 Antigens With Different Molecular Weights: Studies in Platelets, Endothelial Cells, and Megakaryocytes

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The mol wt of the glycoprotein(s) carrying the PLA1 antigen was examined on platelets, megakaryocytes and endothelial cells by immunoblotting with a human polyclonal anti-PLA1 antibody (BE), as well as on four different monoclonal antibodies (MoAbs; DEK-1, DEK-2C, DEK-10, and DEK-16) raised against GPIIIa. The 100,000-mol wt platelet glycoprotein known to carry the PLA1 antigen, BE reacted with PLA1 positive but not with PLA1 negative platelets. DEK-1 reacted strongly with PLA1 positive platelets but weakly with PLA1 negative platelets. The remaining three MoAbs reacted equally with PLA1 positive as well as negative platelets. BE, DEK-1, DEK-10, and DEK-16 reacted with a 120,000- as well as 100,000-mol wt band on immunoblot of PLA1 positive platelets. The 120,000-mol wt band copurified with affinity purified 100,000-mol wt GPIIIa. Megakaryocytes had a prominent 120,000- as well as 105,000-mol wt band that reacted with BE on immunoblot (the 100,000-mol wt band was not detectable). Umbilical cord endothelial cells from presumed PLA-positive infants had a prominent 100,000-mol wt band that reacted with BE, DEK-16, and DEK-1 (the 120,000-mol wt band was not visualized). The 120,000- and 100,000-mol wt PLA1-positive bands could be digested with proteolytic enzymes to 55,000- to 65,000-mol wt-resistant fragments that retain PLA1 epitopes. Further digestion with endoglycosidase-H lowered the apparent mol wt by approximately 2,000 to 6,000 daltons without affecting PLA1 reactivity. We conclude that the PLA1 antigen is present on a 120,000- as well as 100,000-mol wt glycoprotein of platelets and megakaryocytes, a 105,000-mol wt band of endothelial cells, and a 100,000-mol wt glycoprotein of megakaryocytes, and a 100,000-mol wt glycoprotein of endothelial cells. We postulate that the 120,000-mol wt glycoprotein, which shares three or more epitopes with the 100,000-mol wt GPIIIa, may be a post-translational precursor of this species.

POST-TRANSFUSION purpura (PTP) and neonatal isoimmune thrombocytopenic purpura (NIPT) are two clinical platelet disorders that arise from an incompatibility between the platelets of a donor and a recipient, or a mother and her fetus, respectively. The incompatibility arises from the presence or absence of a specific antigen, usually PLA1 (also designated Zw). PLA1 is present on a platelet membrane glycoprotein designated GPIIIa, which has an apparent mol wt of 100,000. For purposes of studying the PLA1 antigen on GPIIIa, we have produced four monoclonal antibodies (MoAbs) against GPIIIa (one against the PLA1 epitope) and have obtained a high titer polyclonal antibody against PLA1 from a patient with PTP.

The purpose of this communication is to report the presence of the platelet PLA1 antigen on a 120,000-mol wt as well as 100,000-mol wt GPIIIa band on platelets, a 100,000-mol wt band on endothelial cells, and a 120,000-mol wt and 105,000-mol wt band on megakaryocytes when visualized on immunoblot. The 100,000-mol wt and 120,000-mol wt band on platelets share antigenic determinants with GPIIIa as well as PLA1. Enzymatic digestion of both the 120,000- and 100,000-mol wt species produces glycopeptide fragments of apparent mol wt of 55,000 to 65,000 that retain both the PLA1 and GPIIIa epitopes.

MATERIALS AND METHODS

MoAb product. MoAbs were raised against GPIIIa by modification of the method of Kohler and Milstein. GPIIIa was obtained following mechanical elution from a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) slab gel of purified platelet membranes. The antigen (50 μg) was emulsified with complete Freund’s adjuvant (Sigma Chemical Co, St. Louis) and injected intraperitoneally (IP) into Balb/c mice (Taconic, Germantown, NY), followed by three weekly injections of 10 μg of antigen in incomplete Freund’s adjuvant (Sigma). Spleens were harvested five days after the last injection, minced, and the cells fused with P3U1 mouse myeloma cells (kindly supplied by Dr V. Nussensweig, New York University Medical School). Supernatants were assayed for antibody reactivity against platelets by an enzyme-linked immunosorbent assay (ELISA) procedure (see below).

ELISA assay of hybridoma supernatants on platelets. Human platelets were washed three times in a modified human Ringer’s solution containing 2 μmol/L EDTA and then applied to U-bottom microtiter plates (Dynatech, Chantilly, VA) at a concentration of 107 platelets in 40 μL of Ringer’s solution. The plates were blocked for one hour at 37°C with 1% bovine serum albumin (BSA) in 0.01 mol/L phosphate-buffered saline (PBS) pH 7.4. Forty microliters of cell culture supernatant was then placed in each well and assayed by an ELISA employing an antiserum Ig-alkaline phosphatase conjugate (Sigma).

Immunoblot assay. Platelet membranes were prepared as described previously. Whole platelet lysates were prepared by solubilization of washed platelets in 10% SDS in 60 mmol/L Tris buffer, pH 6.8, or in 1% Triton X-100 in 0.15 mol/L NaCl, pH 7.4. PLA1 negative platelets were obtained from females who had given birth to children with neonatal isoimmune thrombocytopenic purpura. The PLA1 negative phenotype was determined by two different laboratories using specific anti-PLA1 antibodies. Platelet membranes or whole platelet lysates were electrophoresed on 10% SDS-PAGE gels and then transferred to nitrocellulose paper. The nitrocellulose paper was blocked with BLOTTO before incubation with either polyclonal anti-PLA1

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antibody at a dilution of 1:5,000 or MoAbs DEK-1, DEK-16, DEK-10, DEK-2c at dilutions of 1:50 to 1:1,000 in BLOTTO for two hours on a shaker at 37°C. The unadsorbed antibody was washed away and the bound antibody detected with an alkaline-phosphatase conjugate of goat-antimouse Ig (1:150, Sigma) or F(ab')2 fragments of goat antihuman IgG (gamma chain specific; 1:1,000, Sigma), which were developed with the enzymatic substrate provided by Bio-Rad Laboratories, Richmond, CA.

Human umbilical endothelial cells were a gift of Dr Richard Levin, New York University Medical Center. They were passed one to two times, grown to near confluence, washed three times with Tyrode's-HEPES buffer, solubilized in 0.5% Triton, and run on immunoblot. The preparation was devoid of platelets.

Human megakaryocytes were a gift of Dr Richard F. Levine, Veterans Administration Hospital, Washington, DC. Megakaryocytes were separated from normal bone marrow aspirates by velocity sedimentation followed by cell elutriation to a purity of approximately 95% megakaryocytes by cell number. 1 x 107 megakaryocytes were solubilized in 10% SDS and run on immunoblot.

Silver staining. This was performed with reagents (Gelcode) supplied by Pierce Chemical Co, Rockford, IL, according to their instructions.

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MoAb affinity columns. DEK-16 was purified by diethyl aminoethyl (DEAE) chromatography and coupled to an Affi-gel-10 column (Bio-Rad) at an antibody concentration of 5 to 4 mg/mL. Coupling was performed as suggested by the manufacturer after washing with iso-propanol, ice-cold distilled water, and 0.2 M NaHCO3 buffer, pH 8.0. Antibody, dialyzed against the same bicarbonate buffer, was added to the Affi-gel and incubated overnight at 4°C on a rocker. Remaining active binding sites were inactivated by incubation with 0.1 mol/L Tris for four hours at room temperature. The Affi-gel was then washed once in human Ringer's 2 mol/L EDTA solution and 4 to 5 mL of the platelet lysate (in 0.1 mol/L NaCl, 5 mmol/L EDTA, 0.1% Triton X-100) applied to a column or mixed with the Affi-gel, rocking overnight at 4°C. The column or slurry was then washed extensively with 0.01 mol/L Tris, 0.5 mol/L NaCl, 0.2% triton, 5 mmol/L EDTA, followed by 0.01 mol/L Tris, 0.15 mol/L NaCl, 0.1% Triton. The adsorbed platelet membrane antigen(s) was then eluted with 0.2 N acetic acid in 0.15 mol/L NaCl, 0.1% triton, pH 2.5, and the eluate neutralized with 1.5 mol/L Tris.

Protease digestions. Proteases were incubated with platelet preparations or affinity purified GPIIia in PBS, pH 7.6, at 37°C for one to 24 hours. The enzymes employed were chymotrypsin TLCK (Sigma, type VII), trypsin DPC (Sigma, type XI) at an enzyme platelet protein concentration ratio of 1:10 by weight; Subtilisin Carlsberg (Sigma, type VIII) at a ratio of 1:20; Staphylococcus aureus, strain V8 (Sigma, type VII) at a ratio of 1:10; and plasmin (Sigma) at a ratio of 1:100. All reactions were stopped with 0.1 mol/L PMSF, final concentration.

Table 1. Anti-GPIIia and PLA1 Antibodies Employed

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Specifity</th>
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<tbody>
<tr>
<td>BE</td>
<td>Polyclonal IgG</td>
<td>PLA1* + 120 Kd</td>
</tr>
<tr>
<td>DEK-1</td>
<td>Monoclonal IgM-k</td>
<td>PLA1* + 120 Kd</td>
</tr>
<tr>
<td>DEK-2c</td>
<td>Monoclonal IgG2a-k</td>
<td>GPIIia</td>
</tr>
<tr>
<td>DEK-10</td>
<td>Monoclonal IgG1-k</td>
<td>GPIIia + 120 Kd</td>
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<tr>
<td>DEK-16</td>
<td>Monoclonal IgG1-k</td>
<td>GPIIia + 120 Kd</td>
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*Both antibodies differentiate between PLA1-positive and PLA1-negative platelets. BE has no reactivity with PLA1-negative platelets. DEK-1 reacts weakly with PLA1-negative platelets and strongly with PLA1-positive platelets.
PLA1 positive platelets. The 120,000-mol wt band was shown not to be reduced GPIIb, employing a MoAb 3B2 that is specific for GPIIb and the GPIIb-GPIIIa complex. MoAb DEK-2c (lane B2) reacted very weakly or not at all with the 120,000-mol wt band. In addition, some of the antibodies occasionally reacted with a 70,000-mol wt band on the immunoblot. Reactivity of all the antibodies was seen at the top of the gel, which probably reflects the platelet Fc receptor, since these bands were also visible when the alkaline-phosphatase-conjugated second antibody was used alone. Following reduction of the platelet membrane with 5 mmol/L dithiothreitol, none of the five antibodies reacted on immunoblot.

Figure 3 demonstrates that the 120,000-mol wt band copurifies with the 100,000-mol wt GPIIIa band on a monoclonal DEK-16 affinity column. Note that the 120,000-mol wt band was not seen following Coomassie blue staining (lane B), whereas it was seen following silver staining (lane D) of the gel, indicating its relatively low concentration compared to the 100,000-mol wt GPIIIa band.

Figure 4 demonstrates the effect of chymotrypsin digestion of a platelet membrane preparation (panel A) as well as an affinity purified GPIIIa/120,000-mol wt band preparation (panel B) following digestion for one-half hour to 24 hours at 37°C. Note the disappearance of the 120,000-mol wt band (lanes A1 and B1), partial to complete disappearance.
The chymotrypsin digest fragments (lane C). Note the decrease in mol wt of the 100,000 species (lane B), as well as the 58,000- to 71,000-mol wt digest fragments (lane D). All the lanes were developed with polyclonal anti-PLA1 antibody BE. Thus carbohydrate is retained on the chymotrypsin digest containing the PLA1 epitope (lane C), and that which is removed is not required for reactivity of the epitope with anti-PLA1 antibody (lane D).

Figure 6 demonstrates the presence of PLA1 antigenic determinants on platelets (lane A2), megakaryocytes (lane A1), and endothelial cells (panel B). On platelets the PLA1 antigen is usually seen on immunoblot as a 100,000-mol wt and 120,000-mol wt band (the 120,000-mol wt band is not well visualized due to a transfer artifact). On megakaryocytes it is seen as a 105,000-mol wt and 120,000-mol wt band.
(as well as possible higher mol wt bands, which may represent Fc receptors). On endothelial cells it is seen as a 100,000-mol wt band with BE (lane B1), DEK-16 (lane B2), and DEK-D1 (lane B3).

**DISCUSSION**

DEK-1 differentiates PLA1 positive from negative platelets by its relatively strong reactivity with PLA1 positive compared to PLA1 negative platelets. Its weak reactivity with PLA1 negative platelets is of interest. It is conceivable that the PLA1 epitope requires components on PLA1 negative as well as positive platelets for full reactivity.

A MoAb reagent capable of differentiating PLA1 positive from negative platelets would be clinically valuable in predicting the risk of PLA1 isoimmune neonatal purpura in a pregnant woman, a condition associated with severe fetal thrombocytopenia, cerebral palsy, and occasional intratranterine death. Such a reagent would also be valuable in preventing post-transfusion purpura by avoiding the transfusion of PLA1 positive platelets into PLA1 negative recipients.

The 120,000-mol wt band seen on immunoblot is a consistent finding that does not appear to be related to proteolysis or methods of platelet preparation. The findings were reproducible following meticulous collection of blood and separation of platelets by gel filtration in the presence of an array of protease inhibitors. The 120,000-mol wt band was seen with three of four MoAbs raised against the 100,000-mol wt GPIIIa as well as a polyclonal anti-PLA1 antibody. It is unlikely that it is due to partially reduced 100,000-mol wt GPIIIa (which is known to raise its apparent position on SDS-PAGE due to intramolecular disulfide bonds) because none of our anti-PLA1 antibodies react with the epitope in the fully reduced state. However, we cannot rule out the possibility that the apparent 120,000-mol wt band may be a GPIIIa precursor that does not have its full complement of intradisulfide bonds and is therefore partially reduced. This could, in turn, be responsible for its aberrant mobility on SDS-PAGE. An alternative possibility is that the 120,000-mol wt band represents a different glycoprotein adhesive protein receptor with homology for GPIIIa. It is not due to dimerization of a 55,000-mol wt chymotrypsin digest product because the preparation is prepared in the presence of protease inhibitors and the 110,000-mol wt dimer reported by Kornecki et al is different from the 120,000-mol wt band that we are describing (data not shown).

The physiologic significance of this 120,000-mol wt band seen on immunoblot of PLA1-positive platelets and that shares three or more epitopes with GPIIIa as well as an epitope with PLA1 remains to be determined. However, it is of interest that Coller et al have subsequently also noted this band on immunoblot in a study of control platelets employing our antibodies BE and DEK-16, as well as platelets from Glanzmann's thrombasthenic in Iraqi Jews employing their polyclonal rabbit anti-GPIIIa antibody. One of the patients studied had an absent GPIIIa band in the presence of a higher mol wt band that was 20,000 dalton greater than their GPIIIa band. These data are consistent with the postulate that the 120,000-mol wt glycoprotein may be a precursor of GPIIIa. It is also of interest that Stricker et al7 have recently reported the presence of an autoantibody against a 120,000-mol wt band in a patient with post-transfusion purpura. The 120,000-mol wt antigen was present on PLA1 positive as well as negative platelets. The properties of their 120,000-mol wt antigen are both similar and dissimilar to the one that we described. It is similar with respect to its presence on Glanzmann's thrombasthenic platelets and its apparent presence on PLA1 negative platelets. However, it is dissimilar with respect to its reactivity with polyclonal anti-PLA1 antibody: we cannot detect the antigen in the reduced state, whereas they can; they cannot demonstrate the sharing of epitopes for the 120,000- and 100,000-mol wt GPIIIa glycoproteins, whereas we can. Nevertheless, it is still possible that we are both observing the same 120,000-mol wt glycoprotein, since the polyclonal antibody that they have employed is different from ours.

The 105,000-mol wt band seen on immunoblot of megakaryocyte preparations is of interest and could represent the presence of a signal peptide on GPIIIa prior to its processing. However, this remains to be determined. The 120,000-mol wt band is similar to that seen on platelets and is consistent with the postulate that it may be a precursor of GPIIIa.

The presence of the PLA1 epitope on endothelial cells was established using polyclonal (BE), monoclonal (DEK-1), and monoclonal (DEK-16) antibodies. The PLA1 epitope is present on a protein with the same mol wt as GPIIIa of platelets. The presence of the PLA1 epitope is consistent with the description of GPIIIa on endothelial cells. A recent abstract has similarly reported PLA1 on endothelial cells. The absence of the 120,000-mol wt band on three different endothelial cell preparations is consistent with recent data suggesting that the endothelial GPIIIa gene may be different from the platelet GPIIIa gene. For example, Giltay et al have noted normal synthesis and expression of GPIIIa on endothelial cells of Glanzmann's thrombasthenic platelets. Thus the 120,000-mol wt band appears to be tissue specific.

The PLA1-reactive chymotrypsin, subtilisin, or trypsin digest products of 55,000 to 65,000 mol wt were noted following one hour of digestion and were resistant to further digestion by these enzymes for an additional 23 hours. However, we are unable to confirm the presence of a PLA1 epitope on endothelial cells of Glanzmann's thrombasthenic platelets that involves intramolecular sulfhydryl groups. Endoglycosidase H had no effect on PLA1 antigenicity, confirming the observation of Newman et al, who also noted no effect following treatment with endoglycosidases 0 and D. Our data extend these observations by noting a loss of mol wt of 2,000 to 6,000 daltons following endoglycosidase-H digestion. However, we are unable to confirm the presence of a 17,000-mol wt PLA1 epitope reported by Newman et al following trypsin digestion. In our hands trypsin had no further effect on the 55,000- to 65,000-mol wt PLA1 epitope after one to 24 hours of digestion at room temperature. The reason for this discrepancy is unclear. However, one proce-
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dure employed direct immunoblotting, whereas the procedure of Newman et al\textsuperscript{5} employed indirect immunoprecipitation of antigen–antibody complexes with \textit{Staphylococcus aureus} cells.

The precise relationship between the 120,000- and 100,000-mol wt glycoproteins as well as the location of the PLA1 antigen on these molecules will have to be determined by amino acid sequence analysis of peptide fragments. Such studies are currently in progress.

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GPIIla-related PLA1 antigens with different molecular weights: studies in platelets, endothelial cells, and megakaryocytes

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