Epithelial Membrane Glycoprotein PAS-IV Is Related to Platelet Glycoprotein IIIb Binding to Thrombospondin But Not to Malaria-Infected Erythrocytes

By Bruno Catimel, John L. McGregor, Thomas Hasler, Dale E. Greenwalt, Russell J. Howard, and Lawrence L.K. Leung

Glycoprotein (GP) IIIb (also termed GPIV or CD36) is an integral platelet membrane protein, and has been identified as a binding site for thrombospondin, collagen, and malaria-infected erythrocytes. PAS-IV is an integral membrane protein found in lactating mammary epithelial cells and capillary endothelial cells. The N-terminal sequence of PAS-IV is nearly identical to that of GPIIIb and monospecific anti-PAS-IV antibody reacts with GPIIIb, indicating that PAS-IV is structurally related to GPIIIb. In this study, human platelet GPIIIb and bovine epithelial PAS-IV were compared in terms of structural, immunologic, and functional characteristics.

The two-dimensional tryptic peptide map of both intact and deglycosylated PAS-IV was highly similar but not identical to that of GPIIIb. PAS-IV and GPIIIb reacted to an equal extent with monoclonal antibodies OKM5 and OKM8 by enzyme-linked immunosorbent assay. GPIIIb bound to surface immobi-lized thrombospondin (TSP) in a concentration-dependent and saturable manner, with 60% reduction in binding in the presence of EDTA. PAS-IV bound to TSP with similar characteristics except that maximum binding was consistently >50% of that of GPIIIb and binding was not inhibited by EDTA. GPIIIb supported adhesion of Plasmodium falciparum-infected erythrocytes (PRBC) in a dose-dependent manner while no significant adhesion of PRBC to PAS-IV was observed. Our data demonstrate that while epithelial PAS-IV and platelet GPIIIb are structurally and immunologically related, there are significant differences in their functional properties. Whether this result is due to different posttranslational glycosylation modifications or that PAS-IV and GPIIIb represent a family of related cell adhesive protein receptors remains to be determined.

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MATERIALS AND METHODS

Carrier-free [125I]iodide iodine was purchased from New England Nuclear (Boston, MA). Iodobeads were obtained from Pierce Chemical Co. CM-Sepharose, heparin-Sepharose, Mono-S, and a fast flow liquid chromatography (FPLC) system were purchased from Pharmacia (Piscataway, NJ). Triton X-114, Tween 20, phenylmethylsulfonylfluoride (PMSF), aprotinin, bovine serum albumin (BSA), and p-nitrophenyl phosphate (pNPP) were from Sigma Chemical Co (St Louis, MO). Alkaline-phosphatase-conjugated goat-antimouse (GAM-AP) was from Bio-Rad (Richmond, CA). MoAbs OKM5 and OKM8 were generous gifts from DNAX Research Institute (Palo Alto, CA). All reagents were of analytical grade.

Purification of bovine PAS-IV. PAS-IV was purified from bovine milk according to the method of Greenwalt and Mather with that the two proteins are closely related but also found significant functional differences between them.
modifications. Briefly, fresh raw cream was diluted 1:2 with 0.1 mol/L sodium citrate, 0.1 mol/L sodium chloride, pH 7.4. The diluted cream was homogenized with a blender until it turned to butter. The homogenate was filtered with cheesecloth and ultracentrifuged (Beckman L8M) at 26,500 rpm. The milk-fat-globule membrane pellets were resuspended and recentrifuged using the homogenization buffer. The membrane pellets were extracted in 1% Triton X-114 in 20 mmol/L Tris, 0.15 mol/L NaCl, pH 7.4 (TBS) containing 0.5 mmol/L PMSF, 1% aprotinin, and 1 mmol/L EDTA. The detergent phase of the extract was loaded onto a CM-sepharose column connected to an FPLC and eluted with a Tris-Tween buffer containing 0.5 mmol/L PMSF.

Membrane pellets were resuspended and recentrifuged using the same buffer, pH 6.0. The peak eluted at 0.15 mol/L NaCl was pooled and observed under both reduced and nonreduced conditions (Fig 1). A major protein band of Mr = 75,000 was observed under both reduced and nonreduced conditions (Fig 1, lane 3). A minor band of Mr = 145,000 was also observed that has been ascribed to a dimeric form of PAS-IV.

**Purification of platelet GPIIb.** GPIIib was purified from Triton X-114 platelet membrane extract under nondenaturing conditions by tandem anion exchange and size exclusion chromatography using FPLC as recently described.7 Purified GPIIib had an apparent molecular weight (MW) of 88 Kd by SDS-PAGE under both reduced and nonreduced conditions (Fig 1, lane 1).

**Purification of TSP.** TSP was purified from thrombin-stimulated (1 U/mL) platelet releasates in the presence of 2 mmol/L CaCl2. Thrombin was inhibited with PPACK. The supernates were applied onto a heparin-sepharose column connected to an FPLC and, after washing with 0.25 mol/L NaCl, eluted with 0.55 mol/L NaCl as previously described.

**Endoglycosidase digestion of GPIIib and PAS-IV.** The purified proteins were dissolved in 10 mmol/L Tris-HCl, pH 6.0, and 0.1% Lubrol. To reduce the tertiary structure of the proteins, SDS (10 mg/mL) and 1% mercaptoethanol were added and the samples boiled for 15 minutes at 100°C. The SDS in the denaturing samples was diluted to 1 mg/mL and 1% Triton-X 100 added to stabilize the enzyme. The samples were incubated with 0.5 units of endo F overnight at 37°C for GPIIib and 4 hours at 37°C for PAS-IV.

**Enzyme-linked immunosorbent assays (ELISA).** Microtiter plates were coated with PAS-IV, GPIIib, and control proteins (albumin, transferrin, and fibronectin) at 4 μg/mL in bicarbonate coating buffer, pH 9.5 by incubating overnight at 4°C. The plates were washed with Tris-Tween buffer (20 mmol/L Tris-HCl, 0.05% Tween-20, pH 7.4) and nonspecific binding sites blocked with Tris-Tween buffer containing 0.5% BSA for 30 minutes at room temperature. After washing, OKMS or OKM8 (2 μg/mL) in Tris-Tween/BSA buffer was added and incubated for 2 hours at 37°C. After washing, GAM-AP IgG (1:7,500) in Tris-Tween/BSA was added and incubated for 1 hour at 37°C. After washing, the enzyme reaction was developed by adding the substrate (pNPP) and absorbance at 405 nm read using a Titertek photometer. To determine the actual amount of PAS-IV and GPIIib coated on the microwells,101-labeled PAS-IV and GPIIib bound on the plate were detected and quantified using OKM8 and GAM-AP as described above.

**Two-dimensional tryptic peptide map analysis of PAS-IV and GPIIib.** The purified proteins, both the intact and the deglycosylated forms, were excised from one-dimensional SDS-PAGE and labeled with 125I using the chloramine-T technique and treated overnight with trypsin (50 μg/mL) in 0.05 mmol/L NH4CO3 buffer, pH 7.8, at 37°C. The supernates were lyophilized and analyzed by two-dimensional high voltage electrophoresis and thin layer chromatography.

**Binding of P falciparum-infected erythrocytes to PAS-IV and GPIIib.** The assay was performed as previously described for parasitized red blood cell (PRBC) binding to TSP.36 PAS-IV and GPIIib were diluted with 10 mmol/L phosphate, 150 mmol/L NaCl, pH 7.4 (phosphate-buffered saline [PBS]) to the appropriate concentrations and petri dishes (Falcon 1007) were coated with 10 μL spots of dilute protein overnight at 4°C. Uncoated sites were blocked at room temperature with 1% BSA in 50 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L CaCl2, pH 7.8 for 30 minutes. The dishes were incubated with a 1% suspension of PRBC (Anos monkey-derived parasites, FVO strain, 10% trophozoites) in RPMI 1640, 25 mmol/L HEPES, 10% fetal calf serum (FCS) at pH 6.8 for 1 hour at 37°C without shaking. The dishes were washed three times with RPMI-HEPES and the adherent cells fixed with 1% glutaraldehyde in PBS for 2 hours and finally stained for 30 minutes with 1% Giemsa. Bound PRBC were then counted using light microscopy.

**RESULTS**

**Tryptic peptide maps of purified intact and deglycosylated bovine mammary epithelial PAS-IV and platelet membrane GPIIib.** The N-terminal sequences of both human and bovine mammary epithelial PAS-IV are nearly identical to that of human platelet GPIIib, suggesting that the two proteins are structurally related.30 To further characterize this relationship, the two-dimensional tryptic peptide maps of purified bovine PAS-IV and platelet GPIIib were compared. The peptide map of PAS-IV was highly similar to...
that of GPIIb (Fig 2). However, there was one heavily labeled peptide fragment present in PAS-IV that was not present in GPIIb (Fig 2, arrow), as well as a few minor spots that were not shared. The data suggest that the bovine epithelial PAS-IV is structurally closely related to but not identical with human platelet GPIIb.

Because GPIIb and PAS-IV are glycosylated to different extents, the observed differences in peptide maps may be due to different accessibility of the tyrosine residues for labeling. Both proteins were therefore deglycosylated with endo-F to remove the N-linked sugars, which on SDS-PAGE analysis showed a similar apparent molecular mass of 60 Kd (Fig 1, lanes 2 and 4). However, peptide maps of the deglycosylated proteins showed that the prominent spot noted previously was still present in the deglycosylated PAS-IV but absent in deglycosylated GPIIb (Fig 2), suggesting that it was not due to a glycosylation effect.

Binding of MoAbs OKM5 and OKM8 to PAS-IV and GPIIib. Using an ELISA system, the binding of OKM5 and OKM8 to surface immobilized PAS-IV and GPIIib was tested. OKM5 demonstrated specific binding to GPIIib as compared with control albumin, in agreement with published results. The extent of GPIIib binding was similar to that of PAS-IV (Fig 3). Interestingly, both proteins exhibited much higher reactivity with OKM8. Using 125-I-labeled GPIIib and PAS-IV, it was shown that the amounts of proteins coated on the microtiter plates were equivalent, indicating that PAS-IV and GPIIib showed similar immunologic reactivity with each MoAb.

Binding of TSP to PAS-IV and GPIIib. Using OKM8 as a probe in an ELISA binding system, the binding of PAS-IV and GPIIib to surface immobilized TSP was studied. GPIIIib bound to TSP in a concentration-dependent and saturable manner, in agreement with published results (Fig 4). Control proteins with albumin, transferrin, and fibrinectin were negative, demonstrating the specificity of binding (data not shown). PAS-IV showed similar binding characteristics except that at saturation, the extent of PAS-IV binding was consistently ~50% of that of GPIIib. Because both proteins showed similar reactivity to OKM8 (Fig 3), the data suggested that the amount of PAS-IV bound on TSP was approximately 50% of that of GPIIib.

One interpretation of these data is that there are two GPIIib binding sites on TSP, one Ca2+-dependent and the other Ca2+-independent. PAS-IV, being homologous but not identical with GPIIib, binds to the Ca2+-independent sites but not to the Ca2+-dependent sites. With the limitation of the ELISA binding technique, it is not possible to obtain a precise stoichiometry for the PAS-IV and GPIIib interaction with TSP.
To determine whether PAS-IV would support binding of P. falciparum-infected red blood cells, infected red blood cells adhere to human cells that express surface CD36 but not to those lacking this surface and purified GPlllb were detected and measured by the sequential addition of OKM8, GAM-AP, and enzyme substrate.

Binding of malaria-infected erythrocytes to PAS-IV and GPlllb. GPlllb has been known as a receptor site for the binding of P. falciparum-infected red cells.\textsuperscript{5,22}\textsuperscript{1} Infected red cells adhere to human cells that express surface CD36 but not to those lacking this surface molecule\textsuperscript{5,22} and pure GPlllb coated on plastic supports specific adherence of P. falciparum PRBC.\textsuperscript{5,22} To determine whether PAS-IV would support binding of malaria-infected red blood cells as observed for GPlllb, adherence of P. falciparum-infected red blood cells to purified PAS-IV and GPlllb immobilized on plastic was performed (Table 1). GPlllb supported PRBC binding in a dose-dependent manner (7,500 \pm 700 PRBC/mm\textsuperscript{2} at 0.4 \mu g/mL GPlllb coating concentration) while no significant binding of PRBC to PAS-IV was observed even when PAS-IV was coated at 10-fold higher concentration than GPlllb (90 \pm 100 PRBC/mm\textsuperscript{2} at 4.0 \mu g/mL PAS-IV coating concentration).

A close structural relationship between platelet GPIIIb and epithelial PAS-IV is suggested by the recent observation that these glycoproteins share nearly identical N-terminal peptide sequences.\textsuperscript{25} This finding is supported by the findings of the present study. PAS-IV and GPlllb showed highly similar two-dimensional tryptic peptide maps (Fig 2) and both proteins demonstrated similar immunoreactivity with the MoAbs OKM5 and OKM8 (Fig 3). Both proteins also bound specifically to TSP (Fig 4). However, it is interesting that structural and functional differences between PAS-IV and GPlllb are observed. A major tryptic peptide was not shared between the two proteins on the peptide maps and this difference was present even after deglycosylation (Fig 2). PAS-IV binding to TSP was approximately 50\% less than that of GPlllb and the PAS-IV binding was not dependent on Ca\textsuperscript{2+} (Figs 4 and 5). Finally, while purified GPlllb clearly supported PRBC binding, it is striking that PAS-IV did not support PRBC at all (Table 1).

There are a number of possibilities that may account for the observed differences. It is possible that the purified PAS-IV used in this study represents a partially degraded or deglycosylated product of epithelial cell GPlllb that occurs during the purification procedure. This possibility is unlikely because protease inhibitors were used during purification and, more importantly, MoAbs raised against PAS-IV reacted specifically with a similar 76-Kd protein in detergent lysates of intact milk-fat-globule membrane by immunoblot analysis.\textsuperscript{27} Secondly, it is possible that the observed differences are species related because bovine epithelial PAS-IV was used in this study in the absence of sufficient quantity of human material. Human PAS-IV has a Mr of 80,000, which is very close to bovine PAS-IV, and N-terminal sequence analyses of human and bovine PAS-IV showed identical sequences.\textsuperscript{25} However, this possibility cannot be excluded until human epithelial PAS-IV is examined directly.

A likely explanation for the observed differences between these proteins is that different posttranslational glycosylation modifications exist in the two cell types. There is a marked difference in their sialic acid contents, with 25.5 mol/mol for GPIIb and 3.65 mol/mol for PAS-IV.\textsuperscript{27,29} We have also noticed that PAS-IV and GPIIib have different chromatographic behavior. PAS-IV bind strongly to a cation exchange column (CM-sepharose, Mono-S) at pH 6.0 while GPIIib was purified with anion exchange chroma-
tography (Q-Fast flow, Mono-Q) at pH 7.4. These results indicate that different charged groups are carried by these glycoproteins. Recent data demonstrate the presence of multiple forms of immunoreactive GPIIIb-like proteins in different tissues. An 85-Kd protein is specifically detected by monoclonal anti-PAS-IV antibodies in the microsomal fractions of heart and lung tissues. Similarly, a developmentally regulated 78-Kd GPIIIb-like protein has been found on erythroblast cell membrane. With a monospecific polyclonal antibody raised against human platelet GPIIIb, we have found that human peripheral blood monocytes synthesize a 94-Kd GPIIIb-like protein, roughly 6,000 daltons larger than platelet GPIIIb (unpublished observation, J.L. McGregor and L.L.K. Leung, June 1988). It is quite possible that all these different molecular forms of GPIIIb-like proteins can be explained on the basis of cell type-specific glycosylation differences. The different glycosylation may also account for the functional differences in terms of binding to TSP and malaria-infected red cells. Altered glycosylation leading to decreased adhesion of the cellular fibronectin receptor has recently been reported.

Because of the persistence of one major tryptic peptide difference, as observed by the peptide maps (Fig 2), it remains possible that there are some subtle differences in the peptide sequences of PAS-IV and GPIIIb. GPIIIb and PAS-IV may belong to a family of related cell adhesive protein receptors, with similar but not identical binding characteristics, thus analogous to the integrin superfamilies. It would be informative to study the functional properties of human PAS-IV and GPIIIb after protein deglycosylation and directly compare the primary peptide sequences of PAS-IV and GPIIIb as determined from their respective cDNAs.

In this context, it is noteworthy that the placental CD36 cDNA expressed on COS cells binds malaria-infected red cells but not TSP, while binding of TSP to platelet and monocyte GPIIIb has been demonstrated using a variety of in vitro experimental approaches. One possible explanation, among others, for this discrepancy is that the CD36 cDNA translation product in transfected COS cells does not undergo the proper posttranslational modifications necessary for TSP binding.

It is interesting that TSP is found in substantial amounts in human colostrum and milk. Based on its percentage of total protein, TSP in milk and breast secretion is 100- to 1,000-fold higher than that of plasma. Cytosols from malignant breast tissues contain an even higher amount of TSP. TSP plays an autocrine role in the control of vascular smooth muscle cells. The potential interaction of TSP with the mammary epithelial cell PAS-IV in terms of modulation of cell growth as well as mediating the interaction of epithelial cells with the cell matrix preserves the movement of malignant breast tissues.

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