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Relationship between glycocalicin and glycoprotein Ib of human platelets

(Factor VIII-related von Willebrand factor receptor/platelet glycoproteins/peanut agglutinin affinity chromatography/radioactive peptide map)

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ABSTRACT **Asialoglycoprotein Ib and asialoglycocalicin have been isolated from the membranes and from the supernatant, respectively, after sonication of neuraminidase-treated platelets, by lectin affinity chromatography on peanut agglutinin. The isolated asialoglycoprotein Ib had an apparent molecular weight of 160,000 when not reduced and 150,000 when reduced, whereas the asialoglycocalicin had an apparent molecular weight of 150,000, both reduced and unreduced, on sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Both preparations contained only trace amounts of impurities. The asialoglycoprotein Ib and asialoglycocalicin in both the unreduced and reduced states were separated by gel electrophoresis, radioiodinated in gel slices, and digested with trypsin, and the digests were analyzed by two-dimensional high-voltage electrophoresis and thin-layer chromatography followed by autoradiography. The tryptic peptide maps showed great similarities between glycocalicin and glycoprotein Ib, with the latter (both unreduced and reduced) containing additional peptides, supporting the idea that glycocalicin is derived from glycoprotein Ib. The unreduced glycoprotein Ib contained additional peptides compared to the reduced due to the disulfide-bond-linked β component. There were also slight differences between unreduced and reduced glycocalicin, indicating that at least one intramolecular disulfide bond is present.**

Considerable evidence indicates that the platelet receptor involved in platelet aggregation induced by ristocetin and dependent on human Factor VIII-related von Willebrand factor (FVIIIR:WF) or by bovine von Willebrand factor is a membrane glycoprotein. Glycocalicin, an easily solubilized glycoprotein, has been proposed as the platelet receptor for both ristocetin-induced and thrombin-induced platelet aggregation (1) and indeed platelets that have lost glycocalicin no longer respond to bovine von Willebrand factor (2), or to ristocetin and FVIIIR:WF (3). However, platelet membranes also contain an integral glycoprotein, GPIb, known to consist of two glycoprotein chains linked by an intermolecular disulfide bridge (4), which disappears from the platelet surface under the same conditions that glycocalicin appears in the supernatant (5, 6). We have recently presented evidence that the platelet receptor for bovine von Willebrand factor is an integral membrane glycoprotein (7) that differs from glycocalicin in binding strongly to phenyl-Sepharose, indicating that it contains a hydrophobic moiety while glycocalicin does not (8). Glycocalicin has lectin-binding properties similar to those of GPIb and has a molecular weight similar to that of reduced GPIb on $NaDodSO_4$/polyacrylamide gel electrophoresis (9).

Recently, crossed immunoelectrophoresis studies have shown that platelets contain two components reacting with an anti-glycocalicin antiserum (3, 6, 10), glycocalicin itself and a slower-migrating component. The slower-migrating component binds to phenyl-Sepharose, whereas glycocalicin does not (10). Antisera both against glycocalicin and against a glycoprotein fraction containing a mixture of GPIa and GPIb inhibit ristocetin/FVIIIR:WF-induced platelet aggregation (3).

All this evidence points strongly to a close relationship between glycocalicin and GPIb. It was therefore unexpected that the tryptic peptide maps of glycocalicin and the GPI complex [both isolated by lectin affinity chromatography on wheat germ agglutinin (WGA)] were totally different, indicating that there can barely be any relationship between these glycoproteins (11). This induced us to repeat these experiments under carefully controlled conditions with defined starting materials.

Studies on the binding of ^{125}I-labeled lectins to platelet membrane glycoproteins separated by polyacrylamide gel electrophoresis (12) have indicated that peanut agglutinin (PNA) binds only GPIb (from neuraminidase-treated platelets), whereas WGA binds to more glycoproteins (from untreated platelets). Treatment with neuraminidase is necessary in order to expose the PNA-binding site.

In order to establish what the relationship is between glycocalicin and GPIb we have isolated the asialoforms of these glycoproteins by PNA affinity chromatography and, after radiolabeling, we have compared their tryptic peptide maps.

MATERIALS AND METHODS

Isolation of Human Blood Platelets. Platelets were isolated from citrate-treated blood collected for the Central Laboratory of the Blood Transfusion Service of the Swiss Red Cross, within 20 hr after collection (13). The buffy coats were siphoned into a buffered glucose solution to give platelet-rich plasma containing about 20 mM glucose, 12 mM sodium phosphate buffer (pH 6.8), and about 4×10^9 platelets per ml (14). The platelet-rich plasma was washed three times in 0.01 M Tris·HCl/0.15 M NaCl/10 mM EDTA, pH 7.4; the platelets were resuspended to 8–9 $\times 10^9$ per ml in neuraminidase buffer [0.1 M sodium acetate (pH 5.6)/0.34 M NaCl/9 mM $CaCl_2$]; neuraminidase [*Vibrio cholerae*, 1 unit per 2×10^{12} platelets (Behringswerke) (1 international unit of neuraminidase will split off 1 μmol of *N*-acetylneuraminic acid from human α_1-acid glycoprotein in 1 min at 37°C and pH 5.5)] was added; and the platelet suspension was incubated at 37°C for about 45 min. The neuraminidase was certified by the manufacturers as containing less than 0.1 milliunit/ml of protease activity (1 international unit of protease activity will split 1 μmol of benzoyl-DL-arginine *p*-nitroanilide·HCl per min at 37°C). The efficiency of removal of sialic acid was checked by measuring the agglutination response of aliquots of platelets to PNA. For isolation of glycocalicin, the

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Abbreviations: GP, glycoprotein; FVIIIR:WF, Factor VIII-related von Willebrand factor; WGA, wheat germ agglutinin; PNA, peanut agglutinin.

platelets were centrifuged and resuspended in phosphate-buffered saline, pH 7.4; for isolation of GPIb after centrifugation the platelets were resuspended in 0.01 M Tris·HCl/0.15 M NaCl/1 mM EDTA, pH 7.4. In both cases a ratio of 1 part platelets to 3 parts buffer, vol/vol, was used. The platelet suspensions were then cooled in an ice bath to 4°C and sonicated with a B-30 sonifier (Branson Sonic Power Company, Danbury, CT) for 2 min (output control, 7; 50% duty cycle; pulsed mode). Membranes and sonication supernatant were obtained by differential centrifugation (9000 × *g* for 30 min, then 100,000 × *g* for 1 hr). Supernatants were used fresh and membranes either fresh or after freezing at −70°C and thawing once.

PNA Affinity Chromatography. PNA was isolated from raw peanuts (kindly donated by Astra, Steffisburg, Switzerland) by affinity chromatography of a crude saline extract (15) on lactose coupled to Sepharose (16) and was coupled to polyacrylic hydrazide-Sepharose by the method of Wilchek and Miron (17) at 1.7 mg/ml.

Asialoglycocalicin was prepared by loading the 100,000 × *g* supernatant (≈60 ml) from the sonication of platelets onto a PNA-polyacrylic hydrazide-Sepharose column (50 × 3.5 cm) and eluting with phosphate-buffered saline. When the A_{280} of the effluent returned to the baseline the column was eluted with 3.6% lactose in phosphate-buffered saline. The asialoglycocalicin eluted as a broad peak. The fractions were pooled, dialyzed against water, and lyophilized. The yield from 70 units (33 liters) of blood averaged about 30 mg of asialoglycocalicin.

Asialo GPIb was prepared by solubilizing membranes prepared from neuraminidase-treated platelets (4 ml, 50 mg protein) in 1% sodium deoxycholate, centrifuging at 100,000 × *g* for 1 hr, and loading the supernatant directly on a column of PNA-polyacrylic hydrazide-Sepharose (10 × 2.5 cm) that had been equilibrated with 0.5% sodium deoxycholate. The column was eluted with 0.5% sodium deoxycholate until the A_{280} of the effluent returned to baseline and then the eluant was changed to 3.6% lactose in 0.5% sodium deoxycholate. The fractions containing the A_{280} peak that eluted were pooled, dialyzed against 0.05 M ammonium acetate at pH 8, and then lyophilized. Yield averaged about 2 mg.

Radiolabeling and Tryptic Peptide Mapping. Asialo GPIb and asialoglycocalicin, in both the reduced and the unreduced states, were separated on 7.5% gels by discontinuous polyacrylamide gel electrophoresis (Laemmli system). The gels were stained with Coomassie blue and individual bands were cut out, labeled with ^{125}I, and digested with trypsin, essentially as described by Elder *et al.* (18). The digested lyophilized samples were dissolved in 20 μl of electrophoresis solution (acetic acid/formic acid/water, 3:2:16, vol/vol) and 4 μl (approximately 10^6 cpm) was spotted at the origin on a 20 × 20 cm cellulose-coated thin-layer chromatography plate [0.1 mm layer (Merck, Darmstadt, Federal Republic of Germany]. The first-dimension electrophoresis was carried out using a flat-bed high-voltage electrophoresis apparatus [CAMAG (Muttenz, Switzerland)] in electrophoresis solution at 1 kV for about 60 min. Electrophoresis was followed by using acid fuchsin spotted in the opposite corner of the plate (migration is in the opposite direction) and was carried out until the first component of the acid fuchsin had migrated a fixed distance. The plates were dried and second-dimension chromatography was carried out in 1-butanol/pyridine/acetic acid/water, 6.5:5:1:4, vol/vol. After drying, indirect autoradiography was carried out with Kodak X-Omat R film and Cronex Lightning Plus intensifier screens (Du Pont).

RESULTS

Asialoglycocalicin isolated by PNA affinity chromatography gave one band on $NaDodSO_4$/polyacrylamide in both the unreduced and reduced state, with both Coomassie blue and periodic acid/Schiff's reagent staining, at a higher apparent molecular weight (150,000) than glycocalicin that had not been treated with neuraminidase and that was isolated by WGA affinity chromatography (140,000). When glycocalicin isolated by WGA affinity chromatography was treated with neuraminidase it moved to a position corresponding to a higher apparent molecular weight, identical to that of the PNA-isolated asialoglycocalicin (Fig. 1).

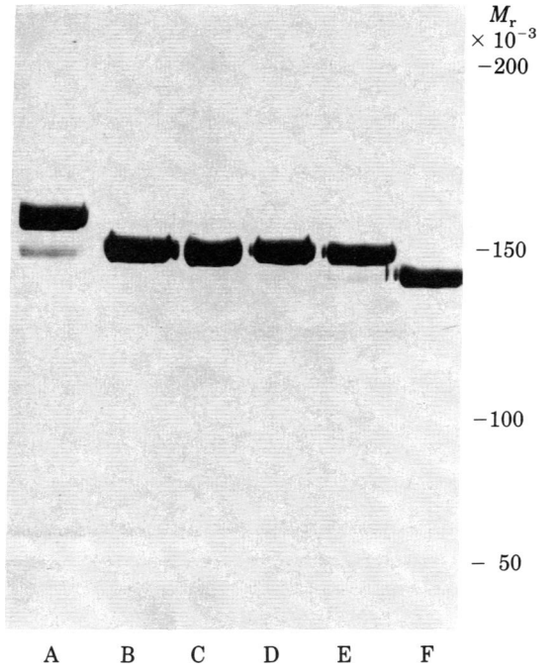

FIG. 1. Discontinuous $NaDodSO_4$/polyacrylamide gel electropherogram stained with periodic acid/Schiff's reagent and Coomassie blue. Lane A, asialo-GPIb isolated on a PNA affinity column, unreduced. Lane B, as in lane A, reduced. Lane C, asialoglycocalicin isolated on a PNA affinity column, unreduced. Lane D, as in lane C, reduced. Lane E, glycocalicin isolated on a WGA affinity column, after neuraminidase treatment, reduced. Lane F, glycocalicin isolated on a WGA affinity column, reduced.

Asialo-GPIb isolated by PNA affinity chromatography gave a strong band with an apparent molecular weight of 160,000 and a fainter band of 150,000 by $NaDodSO_4$/polyacrylamide gel electrophoresis in the unreduced state. Both stained with periodic acid/Schiff's reagent and Coomassie blue in the same relationship. In the reduced state the asialo-GPIb gave a single band of molecular weight 150,000 (Fig. 1).

The tryptic peptide maps of asialo-GPIb and asialoglycocalicin in both the reduced and unreduced states were very similar; however, some differences could be seen (Fig. 2). Unreduced asialo-GPIb gave several additional peptide spots in the tryptic map (Fig. 2*a*) compared to reduced asialo-GPIb (Fig. 2*b*), one of which was quite major. Comparison with the tryptic maps from asialoglycocalicin (Fig. 2 *c* and *d*) showed that the reduced asialo-GPIb and the asialoglycocalicin maps were almost, but not quite, identical. There were several minor differences, both qualitative and quantitative (indicated in Fig. 2*e*). Comparison of the peptide map from unreduced asialoglycocalicin with that from reduced asialoglycocalicin again showed patterns that were not absolutely identical but presented minor differences. A composite diagram of the different tryptic peptide maps is shown in Fig. 2*e* with the various differences indicated. Tryptic peptide maps of glycocalicin isolated by WGA affinity chromatography and then desialylated were identical to those for

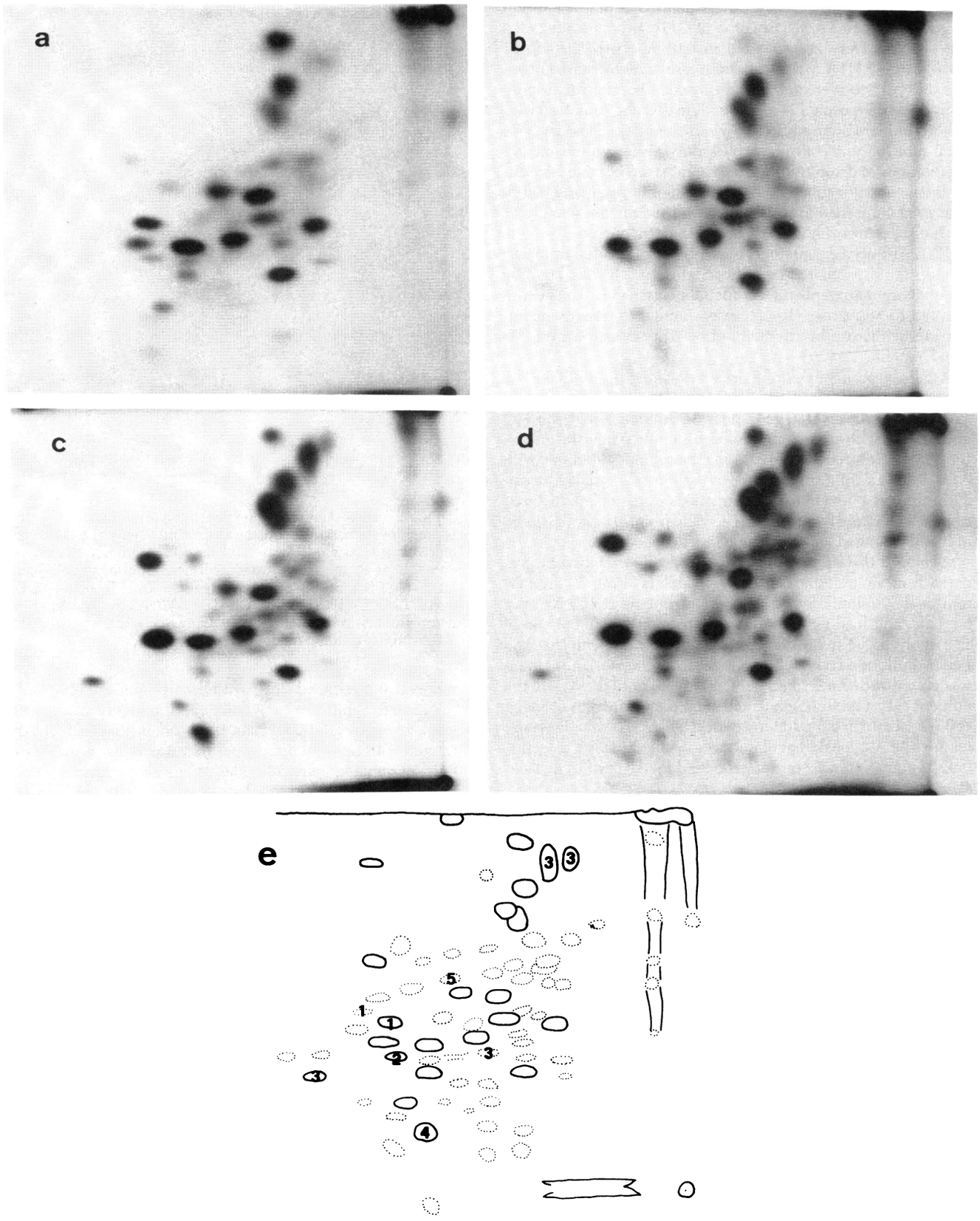

FIG. 2. Autoradiograms of ^{125}I-labeled tryptic peptide maps. The origin (application point of tryptic digest) is in the bottom right corner. High-voltage electrophoresis was in the horizontal direction with the anode on the right. Thin-layer chromatography was then carried out in the vertical direction. (*a*) Asialo-GPIb, unreduced. (*b*) Asialo-GPIb, reduced. (*c*) Asialoglycocalicin, unreduced. (*d*) Asialoglycocalicin, reduced. (*e*) Composite drawing of tryptic peptide maps showing differences. Numbers indicate: 1, Peptides present only in the map of unreduced asialo-GPIb. 2, Peptides present only in unreduced and reduced asialo-GPIb. 3, Peptides present or very much stronger only in asialoglycocalicin maps. 4, Peptides very much stronger in map from unreduced asialoglycocalicin. 5, Peptides present only in map from reduced asialoglycocalicin.

asialoglycocalicin isolated by PNA affinity chromatography (data not shown).

DISCUSSION

PNA affinity chromatography allows the isolation of desialylated GPIb and glycocalicin without significant contamination in a one-step procedure. The products thus obtained are therefore superior starting materials for structural studies of the two glycoproteins compared to other preparations, which often contain contaminants with similar molecular weight as judged by discontinuous polyacrylamide gel electrophoresis.

The increase in the apparent molecular weights of glycocalicin and of GPIb after treatment with neuraminidase, which has been noted previously (19, 20), is presumably due to loss of negative charges of sialic acid without increased binding of $NaDodSO_4$ to these glycoproteins.

Tryptic peptide mapping of asialoglycocalicin and asialo-GPIb clearly shows that these molecules are closely related. It has previously been shown that GPIb consists of two chains, Ibα and Ibβ, linked by a disulfide bridge (4). The additional peptides found in the peptide map of unreduced asialo-GPIb compared to that of reduced asialo-GPIb are probably derived from the small subunit Ibβ, which is lost on reduction. Glycocalicin has a molecular weight, both unreduced and reduced, very similar to that of reduced GPIb. However, the conditions under which it is released from the membrane appear to favor cleavage, by calcium-induced proteases, rather than reduction as the operating mechanism, and release is inhibited by thiol-protease inhibitors such as *N*-ethylmaleimide (10) and by calcium chelators such as EDTA (2). The fact that the peptide map of reduced asialo-GPIb is not completely identical to that of asialoglycocalicin supports this hypothesis.

Despite the similar apparent molecular weights of unreduced and reduced glycocalicin, the peptide maps show minor qualitative and quantitative differences, implying that this molecule contains at least one intramolecular disulfide bond. Reduction of this disulfide bond makes new sites accessible to trypsin and yields two peptides formerly present as a single disulfide-bond-linked entity. Evidence for this interpretation comes also from the work of Lawler *et al.* (21), who indicated that this internal bond was in the 45,000-dalton tryptic fragment of glycocalicin.

Recent results (3, 6, 10) have shown that platelets contain an amphiphilic membrane-bound protein that crossreacts immunologically with glycocalicin. In all cases minor amounts of glycocalicin were found to be present in the membrane preparations; this probably also accounts for the 150,000-dalton band found in this study in unreduced GPIb preparations. Cooper *et al.* (7) have shown that bovine von Willebrand factor receptor activity was associated with GPIa and GPIb rather than with glycocalicin.

The evidence presented here demonstrates that glycocalicin and GPIb are closely related and strongly supports the idea that glycocalicin is derived from the membrane-bound component GPIb.

It remains to be explained why our results differ from those obtained by Nachman *et al.* (11). It is known that WGA binds more than one glycoprotein in the GPI region (9), and these authors isolated the platelet membranes by using buffers that favor the release of glycocalicin. Hence it cannot be excluded that the GPI preparation that was used for comparison with glycocalicin was in fact largely devoid of GPIb.

Our results explain why in Bernard–Soulier syndrome, in which GPIb is absent (22) and platelets do not respond to ristocetin/FVIIIR:WF (23), glycocalicin is also absent (2). The immunoelectrophoresis evidence (10) indicates that GPIb disappears from the platelet membrane at the same time that the response to ristocetin/FVIIIR:WF is lost and glycocalicin appears in the ambient fluid. Together with the evidence from the tryptic peptide maps and what is already known about GPIb, the immunoelectrophoresis data enable the construction of the model of the GPIb molecule and ristocetin/FVIIIR:WF receptor and its relationship to glycocalicin shown in Fig. 3.

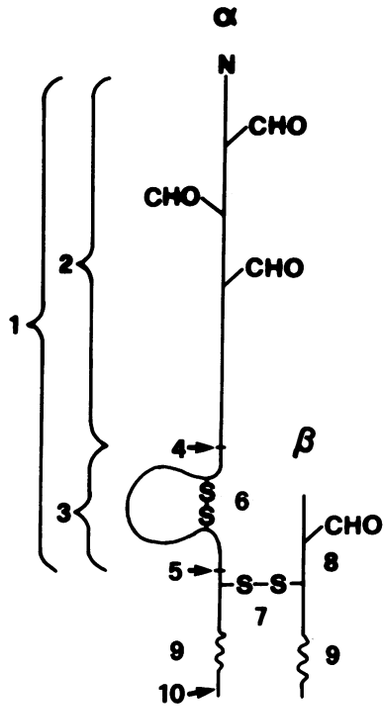

FIG. 3. The platelet von Willebrand factor receptor: GPIb. Features of the structure are indicated by numbers. 1, Glycocalicin (140,000 daltons). 2, Heavily glycosylated trypsin-resistant peptide (120,000 daltons). 3, Thrombin-binding (24) major tryptic fragment from glycocalicin (45,000 daltons). 4, Primary tryptic cleavage point. 5, Endogenous Ca^{2+}-activated protease cleavage point releasing glycocalicin. 6, Intramolecular disulfide bridge(s) within 3. 7, Intermolecular disulfide bridge(s) between α and β subunits. 8, β subunit (22,000 daltons), glycosylated. 9, Hydrophobic sequence penetrating membrane lipid bilayer present in one or both subunits. 10, Phosphorylation site on cytoplasmic side of membrane (25). N, NH_2 terminus of α subunit. CHO, carbohydrate side chains, number and position not yet known. A difference of 2000–3000 daltons between reduced GPIb and glycocalicin such as would be adequate to account for the structural features found between 5 and 10 would not be distinguishable by polyacrylamide gel electrophoresis.

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