Platelet Glycocalicin

I. ORIENTATION OF GLYCOPROTEINS OF THE HUMAN PLATELET SURFACE*

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The orientation of proteins and glycoproteins of the platelet surface has been studied using various surface probes and labeling reagents. A fourth major glycoprotein has now been detected in platelet plasma membranes by sodium dodecyl sulfate-gel electrophoresis in addition to the previously recognized glycoproteins I, II, and III. Glycoprotein IV \( M_w \) = approximately 87,000 appears to be present on the inner aspect of the membrane or buried within it since it is not accessible to surface probes such as lactoperoxidase-catalyzed iodination, radiolabeling with transglutaminase and \([^{14}C]\)glycine ethyl ester, or proteolytic enzymes. The ratio of these four major membrane-bound glycoproteins is approximately 10:4:2:3.

Contrary to previous reports, only one glycoprotein, glycoprotein III, is accessible to lactoperoxidase-catalyzed iodination in intact platelets. Differences in the rate of destruction of glycoprotein II in intact platelets by trypsin suggests that two components may be migrating in this region.

Examination of the soluble fraction obtained following platelet homogenization showed the presence of a single soluble glycoprotein of molecular weight 148,000 comprising about 10% of total platelet sialic acid. Treatment of intact platelets with neuraminidase resulted in the quantitative loss of sialic acid from the soluble glycoprotein, and it was strongly labeled in the intact platelet by \([^{14}C]\)glycine ethyl ester in the presence of transglutaminase. Treatment of intact platelets with chymotrypsin which does not cause the platelet release reaction, caused the rapid conversion of the soluble glycoprotein to a macroglycopeptide. These results indicate a surface origin for the soluble glycoprotein rather than a cytoplasmic or granular origin. The term glycocalicin is suggested for this glycoprotein in view of its origin in the platelet glycocalyx.

Intact blood platelets possess a carbohydrate-rich outer coat, or glycocalyx, on their surface, the presence of which has been demonstrated by a number of techniques, including the use of specific staining reagents in electron microscopy (1, 2), the modification of platelet properties by treatment with neuraminidase (3, 4) and the isolation of defined glycopeptides from intact platelets (5) and isolated platelet membranes (6, 7). This glycoprotein coat appears to be involved in the reaction of platelets with lectins (8, 9) and may also play a role in their reaction with thrombin (10) and with ADP and serotonin (11).

Despite their probable importance in platelet function the orientation of proteins and glycoproteins at the platelet surface is still imperfectly understood. Based on gel electrophoretic studies, platelet plasma membranes have been reported to contain three glycoproteins, designated glycoproteins I, II, and III (12, 13) with approximate molecular weights of 150,000, 120,000, and 100,000, respectively.

We have now been able to show that the platelet membrane contains a fourth glycoprotein and that the glycoproteins of the platelet surface may be divided into three groups based on their orientation to the platelet exterior: glycoprotein IV is probably located on the inner aspect of the platelet membrane or buried within it, while glycoproteins I, II, and III are located on the outer aspect of the membrane; another glycoprotein is loosely associated with the outer surface of the platelet and is released in soluble form following platelet homogenization. This glycoprotein, for which the name glycocalicin is proposed based on its origin in the platelet glycocalyx, may be the receptor for both thrombin and ristocetin in the aggregation of blood platelets (14).

EXPERIMENTAL PROCEDURE

Materials

Phenylmethylsulfonyl fluoride, neuraminidase (Clostridium perfringens; 0.8 unit/mg of protein) and apyrase (potato; 2 units/mg of protein) were purchased from Sigma Chemical Co., St. Louis. Chymotrypsin (50 units/mg) and tosylphenylalanylchloromethane-treated trypsin (195 units/mg) were from Worthington Biochemicals, Freehold, N. J. \([^{14}C]\)Glycine ethyl ester (8 mCi/mmole) and Na\(^{125}\)I (carrier-free) were from New England Nuclear, Boston, Mass.

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Platelet membranes and the platelet soluble fraction were isolated by the glycerol-lysis technique (15) or after homogenization by the use of ultrasonicator (Branson Soniter, Branson Sonic Power Co., Plannview, N. Y.; output control setting 6) followed by sedimentation in the same way onto a sucrose cushion.

In most cases, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, was carried out under standard conditions (16) using 6% polyacrylamide and 2.5% cross-linking agent or, preferably, 10% polyacrylamide with 0.67% cross-linking agent for 2.5 h at 25 mA/gel for the first 15 min increased to 50 mA/gel for the remaining period, gel size, 0.5 x 7.5 cm. In experiments designed to obtain a higher resolution of the glycoprotein components, the absence of sodium dodecyl sulfate, was carried out under standard conditions for maximum resolution, SDS-gel electrophoresis of the platelet plasma membrane fraction gave four major, clearly resolved bands which stained with the PAS reagent (Fig. 1A). Three of these bands corresponded to glycoproteins I, II, and III and in the soluble fraction (glycocalicin) at positions corresponding to apparent molecular weights of 150,000, 120,000, and 100,000 as determined from previous work (12, 13). A fourth glycoprotein, glycocalicin IV, was resolved under these conditions and had a molecular weight of 87,000 by extrapolation from its congeners. The ratio of these individual glycoproteins, as determined from their stability with the PAS reagent, was 51:21:11:16 averaged from 13 samples.

The soluble fraction showed a single glycoprotein of estimated molecular weight 148,000 when examined under identical conditions (Fig. 1B). Similarly, a single band of PAS-positive material was observed on gel electrophoresis in the absence of SDS. The ratio of the soluble glycoprotein to membrane-bound glycoprotein for intact platelets was approximately 1:3 when corrected for membrane yield or when calculated as the ratio between the soluble fraction and total insoluble fraction of the platelet homogenate.

The debris fraction remaining after the separation of the soluble and membrane fractions from the platelet homogenate, and comprising platelet granules and mitochondria as well as unlysed platelets, showed no glycoprotein bands in addition to "The abbreviations used are: SDS, sodium dodecyl sulfate; PAS, periodic acid-Schiff."
Platelet Glycocalicin: I. Surface Orientation

Fig. 1. SDS-polyacrylamide gel electrophoresis of platelet fractions. Conditions as described under “Materials and Methods.” PAS stain. A, membrane fraction (glycoproteins I, II, III, and IV); B, soluble fraction (glycocalicin).

those in the membrane and soluble fractions. This result indicates that there are no significant amounts of glycoprotein associated with subcellular compartments other than the platelet membrane and glycocalyx.

Accessibility of Glycocalicin to Neuraminidase: Intact platelets were treated with neuraminidase and the lysate was separated into soluble and membrane fractions. When subjected to electrophoresis in the absence of SDS, the rate of migration of glycocalicin in the soluble fraction was greatly reduced compared with that of the control (Fig. 2A). That the decrease in electrophoretic mobility was due to charge alone and did not arise from contaminating activities such as proteases in the neuraminidase was shown by the fact that both the desialylated glycocalicin and the control had identical mobilities in the presence of SDS (Fig. 2B). The decreased reaction of glycocalicin in each of the gels with the PAS-stain following treatment with neuraminidase is a reflection of the sensitivity of this procedure to the presence of sialic acid in glycoproteins, since similar amounts of native and desialylated glycocalicin were applied to the gels in each case.

Accessibility of Glycocalicin to Chymotrypsin—In order to determine whether glycocalicin was present on the outer surface of the platelet or in the platelet cytoplasm, experiments were carried out with chymotrypsin, which does not induce the platelet release reaction. Electrophoresis of the membrane fraction obtained after chymotrypsin treatment of intact platelets showed the glycoprotein I had been completely removed and that only glycoprotein IV remained in the membrane (Fig. 3A). Similarly, no glycoprotein corresponding to glycocalicin could be detected in the soluble fraction remaining after lysis of the washed chymotrypsin-treated platelets (Fig. 3B).

Lactoperoxidase-catalyzed Iodination—When subjected to lactoperoxidase-catalyzed iodination and SDS-gel electrophoresis under standard conditions, the pattern of radiolabeling was generally similar to that found by others (12, 13) with the maximum incorporation being in the area of glycoprotein

Fig. 2. Effect of neuraminidase on glycocalicin in intact platelets. Intact platelets were treated with neuraminidase and the soluble fraction of the homogenate was subjected to polyacrylamide gel electrophoresis in the absence of SDS (panel A) and in its presence (panel B). Glycocalicin from control platelets (——), from neuraminidase-treated platelets (- - -).

Fig. 3. Electrophoresis of platelet fractions obtained following treatment with chymotrypsin. A, membrane fraction; B, soluble fraction. Control (——), chymotrypsin-treated, (---).
III and lesser incorporation in the area of glycoprotein I and II (Fig. 4A). Under these conditions, virtually none of the radiolabel is incorporated into glycocalicin (Fig. 4B).

Under conditions yielding a higher resolution of the components of the membrane (Fig. 5), it is seen that radioactivity and PAS-staining are congruent only for glycoprotein III and that there is no peak of radioactivity clearly associated with any one of the other three glycoproteins.

**Radiolabeling with Transglutaminase**—Washed intact platelets were treated with [³¹⁴C]glycine ethyl ester and transglutaminase under conditions described under "Materials and Methods," and the membrane and soluble fractions were subjected to SDS gel electrophoresis. The soluble fraction (Fig. 6A) showed a major peak of radiolabeling corresponding to the soluble glycoprotein, and another major peak that showed a small degree of PAS-staining and which had an estimated molecular weight of 80,000. The membrane fraction (Fig. 6B) showed a major broad peak of labeling with a maximum in the region of glycoprotein II and with essentially no labeling in the regions of glycoprotein III and IV. Three minor peaks of radiolabeling in the membrane with this technique, which were not associated with glycoprotein components, had estimated molecular weights of 85,000, 50,000, and 30,000, respectively.

**Time Course of Proteolysis of Intact Platelets**—The glycoproteins present in the intact platelet showed varying degrees of susceptibility to proteolytic digestion with trypsin and chymotrypsin. Trypsin resulted in the quantitative destruction of both glycocalicin and membrane-bound glycoprotein I within 1 min (Fig. 7A). About one-half of glycoprotein II was destroyed within the same time but digestion then proceeded very slowly and only about another 20% was removed by the end of the experiment (60 min). Glycoprotein III was completely destroyed within 60 min, while glycoprotein IV was not affected under these conditions. The amount of platelet macroglycopeptide reached a maximum in the enzyme supernatant fraction at 1 min, corresponding to the complete destruction of glycoprotein I and glycocalicin and no electrophoretic change was seen thereafter, nor did other glycopeptide components appear in this fraction as determined by gel electrophoresis.

Proteolytic degradation with chymotrypsin proceeded in the same sequence but at a slower rate, particularly in the case of glycocalicin and glycoprotein I which required 60 min for complete degradation (Fig. 7, right).

When isolated platelet membranes were treated with trypsin or chymotrypsin, all of the surface glycoproteins, including glycoprotein IV, were readily cleaved showing that the resistance of the latter to proteolytic digestion in intact platelets is not due solely to the lack of linkages susceptible to the two proteases used in this experiment.

**Immunological Studies**—Macroglycopeptides were obtained by tryptic digestion of isolated platelet plasma membranes (7) and of the soluble glycoprotein (19). These macroglycopeptides...
Three of these appear to correspond to glycoproteins I, II, and III which have been previously described. Glycoprotein IV, which has been resolved from glycoprotein III under improved electrophoretic conditions, appears to be inaccessible to a variety of surface probes and reagents. Similarly, the band corresponding to glycoprotein II appears to contain two components differing in their accessibility to proteolytic enzymes. Moreover, unlike previous reports that indicated that all the surface glycoproteins of the platelet were iodinated in the presence of lactoperoxidase we find that only glycoprotein III is accessible to this reagent.

In addition to these four membrane-bound glycoproteins, we find that homogenization of intact platelets leads to the release of a soluble glycoprotein containing about 10% of the total platelet sialic acid.

The subcellular location of this glycoprotein is of particular importance. The present experiments show that it is accessible to neuraminidase and to chymotrypsin in intact platelets and, in addition, it is rapidly degraded by trypsin and chymotrypsin. Taken together, these data strongly support a surface location for this glycoprotein in intact platelets. Finally, this surface glycoprotein is labeled with [14C]glycine ethyl ester in the presence of transglutaminase. The high molecular weight of this enzyme (85,000; Ref. 20) and the fact that it labels only the exterior of the PM2 virion, for which this method was originally developed (17), makes it unlikely that internal labeling of the platelet occurs with this technique and further supports the presence of glycocalcin in a site exterior to the platelet membrane itself.

Glycocalcin and the membrane-bound glycoprotein I appear to be similar in terms of electrophoretic mobility in the presence of SDS. In addition, chicken antiglycocalcin antiserum is able to cause the aggregation of both isolated platelet membranes which lack glycocalcin and washed platelets. Furthermore, the macroglycopeptides derived from the membrane-bound and soluble glycoproteins give reactions of immunological identity. Although these data might suggest that glycocalcin and glycoprotein I are identical, resolution of this question will have to await the isolation of the membrane-bound and soluble components in pure form: the purification of soluble glycocalcin is reported in the following paper (19).

Four possibilities seem to exist: one is that the soluble and membrane-bound glycoproteins are identical and that the soluble form is released from the membrane by the effects of platelet homogenization and membrane isolation. However, further amounts of the membrane-bound glycoprotein were not solubilized following prolonged sonication of isolated membranes. The second possibility is that the macroglycopeptide portions of the membrane bound and soluble forms are identical but that the (non-glycopeptide “tail” portions differ so that this portion of the soluble glycoprotein is more loosely bound to the membrane and therefore more readily displaced during homogenization and isolation. The third possibility, which is closely related to the second, is that the structural differences in the soluble and membrane-bound forms are sufficiently great that the tail portion is not inserted within the membrane itself but that the soluble glycoprotein is loosely adsorbed to the platelet surface. The fourth possibility is that the soluble and membrane-bound forms are structurally identical but that individual molecules are bound to regions of somehow differing affinity in the membrane perhaps reflecting regions capable of varying degrees of lipophilic interaction.

The two latter possibilities are, perhaps, supported by the fact that the ratio of radiolabeling with [14C]glycine ethyl ester...
to PAS stainability is much higher in the soluble glycoprotein than in the equivalent membrane-bound glycoprotein, suggesting that these portions of the molecule are more accessible in the former case. On the other hand, both glycopacin and glycoprotein I appear to be completely inaccessible to lactoperoxidase-catalyzed iodination in the intact platelet but the former can be readily iodinated in both the peptide and macroglycopeptide portions when isolated in pure form.

This question of accessibility of surface components is particularly important since widespread use of the lactoperoxidase-catalyzed iodination procedure has tended to indicate that only glycoproteins are accessible at the surface of platelets (13) and other cells (21). The application to platelets of the transglutaminase reaction with $[14C]$glycine ethyl ester, originally developed for analysis of a viral coat protein (17), shows that a number of other components, both glycoprotein and non-glycoprotein in nature, are available at the cell surface. This observation emphasizes that the orientation of individual membrane components cannot be determined by a single labeling procedure and that differences in chemical composition and protein conformation must be taken into account as well as accessibility to the reagent itself.

While the actual radioactive counts incorporated with the transglutaminase procedure is relatively low in the present work, this is due to the much lower specific activity of $[14C]$glycine ethyl ester used (8 mCi/mmol) compared with $[125I]$ used for the iodination procedure (12 Ci/mmol). Taking this into account, the transglutaminase procedure is about 50 times more efficient in modifying accessible components of the platelet surface.

The fluid mosaic model of membrane structure (22) characterizes membrane surface proteins as intrinsic or extrinsic depending on the degree to which they are bound to the membrane bilayer. Although these proteins are thought to differ in their structural and functional characteristics in the fluid mosaic model, the present findings suggest that closely similar, if not identical, glycoproteins may have the properties of both intrinsic and extrinsic molecules.

A tentative model for the orientation of the various components of the platelet surface may be suggested on the basis of the present work. The most loosely-bound components, which are released from the platelet surface in soluble form following platelet homogenization, include glycopacin and another component both of which are labeled by the transglutaminase reagent in the intact platelet. However, glycopacin is not accessible in its entirety to membrane-labeling reagents, since differences have been found in the susceptibility to radiolabeling of the glycoprotein before and after solubilization.

Glycoproteins I, II, and III appear to be integral components of the platelet membrane and to be present on its outer surface, based on their accessibility in varying degrees to proteolytic enzymes. Glycoprotein III appears to be the only glycoprotein labeled with lactoperoxidase reagent. On the other hand, glycoprotein II is most strongly labeled by the transglutaminase method in the intact platelet. Glycoprotein I is only slightly labeled although its analog, glycopacin, is strongly labeled under the same conditions. Four other membrane-bound proteins also appear to be accessible to this reagent.

Glycoprotein IV is not radiolabeled by either the transglutaminase or lactoperoxidase methods. It is not accessible to trypsin or chymotrypsin in the intact platelet, but is susceptible to proteolytic digestion in isolated plasma membranes, suggesting that it is present on the inner aspect of the membrane, or is buried within the membrane of the intact platelet but is exposed during isolation procedures. It may be noted that the membrane changes which are associated with the platelet release reaction induced by trypsin are not sufficient to expose glycoprotein IV to proteolytic attack in intact platelets.

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