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# PLATELET GLYCOCALICIN

## ITS MEMBRANE ASSOCIATION AND SOLUBILIZATION IN AQUEOUS MEDIA

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## Summary

Glycocalicin has been extracted from human platelets by 3 M KCl and purified using affinity chromatography on columns of Sepharose-coupled wheat germ agglutinin as the most efficient step. Rabbit antiserum to the purified protein agglutinated human platelets and inhibited the agglutination induced by bovine Factor VIII-related protein. Crossed immunoelectrophoresis of Triton X-100 extracts of platelets in Triton X-100-containing agarose revealed the presence of two glycocalicin-related components of different electrophoretic mobilities giving a continuous double-peak immunoprecipitate with this antiserum. The fast-moving component, which represented the minor peak of the immunoprecipitate, corresponded to purified soluble glycocalicin. Crossed hydrophobic interaction immunoelectrophoresis did not demonstrate binding of the purified glycocalicin or the fast-moving component to phenyl-Sepharose CL-4B as hydrophobic matrix. The slow-moving component, which represented the major peak of the immunoprecipitate, showed a strong binding to the hydrophobic matrix. Immunoelectrophoretic quantitation of glycocalicin present in the aqueous media demonstrated that the presence of EDTA, *N*-ethylmaleimide and iodoacetamide during lysis of platelets significantly reduced the solubilization of glycocalicin. At the same concentrations these inhibitors strongly inhibited the calcium-activated protease of platelet sonicates. Sialic acid determination after acid hydrolysis of aliquots from the soluble fractions showed that their content of sialic acid was considerably higher when lysis was performed in the absence, rather than in the presence, of EDTA and that glycocalicin contributes significantly to the total platelet sialic acid.

## Introduction

A one-chain glycoprotein derived from the carbohydrate-rich surface-coat of platelets (glycocalix) was first purified by Lombart et al. [1] and given the name glycocalicin [2]. This protein was described by others [3—5] in terms like GPS or GP Is which imply that it is found in the soluble fraction of platelet homogenates, and that it is a component of the so-called 'GP I fraction' of glycoproteins observed on sodium dodecyl sulphate polyacrylamide gel electrophoresis of reduced samples of whole platelets. The idea that glycocalicin is derived from the surface membrane coat is mainly based on the finding that it can be labelled in intact platelets by [$^{14}$C]glycine ethyl ester and transglutaminase [2], or by diazotized [$^{125}$I]diiodosulphanilic acid [4], and that it has an unusually high content of carbohydrate [6]. The observation that agglutination of human platelets by bovine factor VIII related protein depends on the presence of glycocalicin also supports this view [7].

Purified, soluble glycocalicin has been characterized biochemically by Okumura et al. [6] as to molecular weight, amino acid and carbohydrate composition and susceptibility to proteolytic enzymes. For the present work, glycocalicin was extracted at high ionic strength and purified by an alternative procedure using affinity chromatography on wheat germ agglutinin-Sepharose as the most efficient step. The aim of the study has been to gain further information regarding its membrane association and solubilization.

## Materials and Methods

*Human blood.* Human blood (9 parts) anticoagulated with 54 mM EDTA in 120 mM NaCl (1 part) was obtained from registered blood donors immediately before it was used for the experiments. Platelet-rich plasma was obtained from this by centrifugation at 320 × *g* for 15 min at 4°C. Platelets were isolated from the platelet-rich plasma and washed in a solution consisting of 148 mM NaCl, 5 mM glucose, 0.6 mM EDTA and 20 mM Tris-HCl (pH 7.4, 280 mosM) as described previously [20].

*Glycocalicin.* Glycocalicin was extracted from human platelets and purified as follows. Washed platelelets were sedimented by centrifugation, resuspended in 3 M KCl ($5 \cdot 10^9$ cells/ml), incubated for 15 min at 37°C and centrifuged at 2000 × *g* for 10 min. The glycocalicin-containing supernatant (KCl extract) was removed, recentrifuged at 8000 × *g* for 4 min, and dialyzed at 4°C for approx. 18 h against large volumes of 0.2 mM EDTA solution which also contained 15.3 mM $NaN_3$, pH 7.0. These agents were used to prevent proteolysis and bacterial growth. During dialysis a glycocalicin-containing precipitate formed. This was isolated by centrifugation at 2000 × *g* for 10 min. The sediment was then stirred at 37°C for 10 min in a solution containing 154 mM NaCl, 0.2 mM EDTA and 15.3 mM $NaN_3$ using approx. 1 ml per $5 \cdot 10^9$ platelets originally counted, and centrifuged at 2000 × *g* for 10 min. Sodium dodecyl sulphate gel electrophoresis followed by staining of the gels with the periodic acid Schiff's reagent revealed glycocalicin as the absolutely predominant glycoprotein in solution at this step. A portion of the supernatant (usually 15—20 ml) was applied to a column of wheat germ lectin-Sepharose 6-MB

(gel dimensions, 12 mm diameter and 135 mm height) followed by a solution of 154 mM NaCl, 0.2 mM EDTA and 15.3 mM $NaN_3$, pH 7.0. Fractions of 2.3 ml were collected and monitored for absorbance at 280 nm. After the unadsorbed proteins had been washed off the column, and a stable baseline of $A_{280nm}$ had been established (usually after 25 fractions), the adsorbed material was eluted with 113 mM *N*-acetylglucosamine in a solution of 154 mM NaCl, 0.2 mM EDTA and 15.3 mM $NaN_3$. This resulted in an immediate peak in $A_{280nm}$ followed by a slow return to baseline values. The fraction corresponding to the peak in absorbance (usually 12 fractions) and the same number of the immediately following fractions were pooled into two main fractions. Each were dialyzed extensively against distilled water, lyophilized and dissolved in 300 $\mu$l of 154 mM NaCl. Both contained purified glycocalicin but in varying quantities. The column procedure and dialysis were carried out at 4°C.

*Triton X-100 extracts of platelets.* Extracts were prepared by suspending washed platelets in a buffer containing 1% Triton X-100, 38 mM Tris and 100 mM glycine, pH 8.7. The suspension was sonicated using a Branson sonifier at setting 4 for three periods of 5 s each with cooling of the container in ice, followed by centrifugation at 100 000 × *g* for 1 h in a SW 39 rotor of a Beckman ultracentrifuge.

*Antisera.* Antisera against glycocalicin were obtained by four subcutaneous injections in rabbits of approx. 75 $\mu$g of the purified material mixed with an equal volume of Freund's incomplete adjuvans in two week intervals followed by a monthly intramuscular booster dose of approx. 25 $\mu$g of the purified glycocalicin without adjuvans. The antisera were either used directly or after absorption with the soluble fraction obtained by freeze-thawing of platelets in the presence of 3.6 mM EDTA which selectively inhibits the solubilization of glycocalicin, or after isolation of the immunoglobulin fraction. Absorbed antiserum was further heated at 56°C for 30 min and adsorbed with $Al(OH)_3$ gel (6 mg/ml, 5 min) prior to use to remove fibrinogen and clotting factors. Antiserum to cold-insoluble globulin (plasma fibronectin) was prepared as described by Fyrand and Solum [8].

*Immunoquantitation of glycocalicin.* This was performed according to Laurell [9] as described by Weeke [10]. Four 7-$\mu$l samples of purified glycocalicin ranging from 20 to 70 $\mu$g/ml were used to construct a reference line.

*Crossed immunoelectrophoresis of hydrophilic proteins.* This was performed as described by Weeke [10]. Crossed immunoelectrophoresis of membrane proteins dissolved in Triton X-100 was performed using Triton X-100-containing agarose gels [11], and crossed hydrophobic interaction immunoelectrophoresis with phenyl-Sepharose CL-4B as hydrophobic matrix as described by Bjerrum [12]. Normally, only 20% of phenyl-Sepharose was used in the agarose gel.

*SDS-polyacrylamide gel electrophoresis.* This procedure was carried out as a modification of the method of Weber and Osborn [13] on gels containing urea and EDTA. The electrophoresis system, sample preparation and staining of gels with Coomassie brilliant blue G and periodic acid Schiff's reagent are described in detail elsewhere [3].

*Agglutination of platelets and platelet fragments.* This was performed in an aggregometer or in microtitre wells as previously described [7] with highly-

purified bovine Factor VIII-related protein [7]. Freeze-thawing of platelets was performed by incubation of the platelet suspension in an acetone solid $CO_2$ mixture for 5 min followed by incubation at 37°C for 5 min. Normally, the freeze-thawing cycle was carried out three times.

*Proteolysis.* Proteolysis due to proteolytic enzymes in platelet sonicates was studied as described by Phillips and Jakabova [14] using azocasein as substrate.

*Sialic acid.* Sialic acid was determined according to the method of Warren [15] using *N*-acetylneuraminic acid as standard.

*Total protein.* Total protein was determined according to Miller's [16] modification of the method of Lowry et al. [17] except with Triton X-100-containing solutions where the Bio-Rad protein assay was used essentially as described by Bradford [18].

*Commercial materials.* Those used were neuraminidase (EC 3.2.1.18) from *Vibrio comma* purchased from Behringwerke, Marburg, F.R.G., *N*-acetylneuraminic acid, *N*-ethylmaleimide, iodoacetamide, *N*-acetyl-D-glucosamine, azocasein and Triton X-100 from Sigma, St. Louis, MO, U.S.A., wheat germ lectin-Sepharose 6MB and phenyl-Sepharose CL-4B from Pharmacia, Uppsala, Sweden, and agarose from Litex, Denmark.

## Results

Glycocalicin was purified from 3 M KCl extracts of washed platelets. The glycoprotein patterns obtained after SDS-polyacrylamide gel electrophoresis of unreduced samples from control platelets and platelets which were extracted with 3 M KCl are shown in Fig. 1. This figure demonstrates that one of the predominant glycoprotein bands seen with the control platelets was almost totally absent from the gels containing the samples of KCl-treated platelets (Fig. 1). Highly-purified glycocalicin showed one band located at the same position in SDS-polyacrylamide gel electrophoresis whether the gels were stained with Coomassie brilliant blue or the periodic acid-Schiff reagent (Fig. 2). No difference in migration was seen between reduced (Fig. 2) and unreduced samples (not shown). The glycocalicin band corresponded to that located immediately below that which is absent from the KCl-extracted platelets (see Fig. 1). This indicates that glycocalicin is a smaller molecule than the membrane glycoprotein which represents the disappearing band.

Antisera to the purified glycocalicin agglutinated washed human platelets and inhibited agglutination induced by bovine Factor VIII-related protein (Fig. 3). The pre-immunization serum of the same rabbit and a high-titred antiserum to cold-insoluble globulin (plasma fibronectin) served as negative controls in these experiments.

The purified glycocalicin showed one immunoprecipitate peak with the antiserum to glycocalicin in all of the three systems of crossed immunoelectrophoresis described in Materials and Methods (Figs. 4 and 5). No binding to the hydrophobic matrix, phenyl-Sepharose CL-4B, could be observed characterizing the purified form of the molecule as a hydrophilic protein (Fig. 5 and 6). Removal of sialic acid by incubation with neuraminidase did not change this behaviour (Fig. 6) but reduced the electrophoretic mobility in the first dimension gel (Fig. 4).

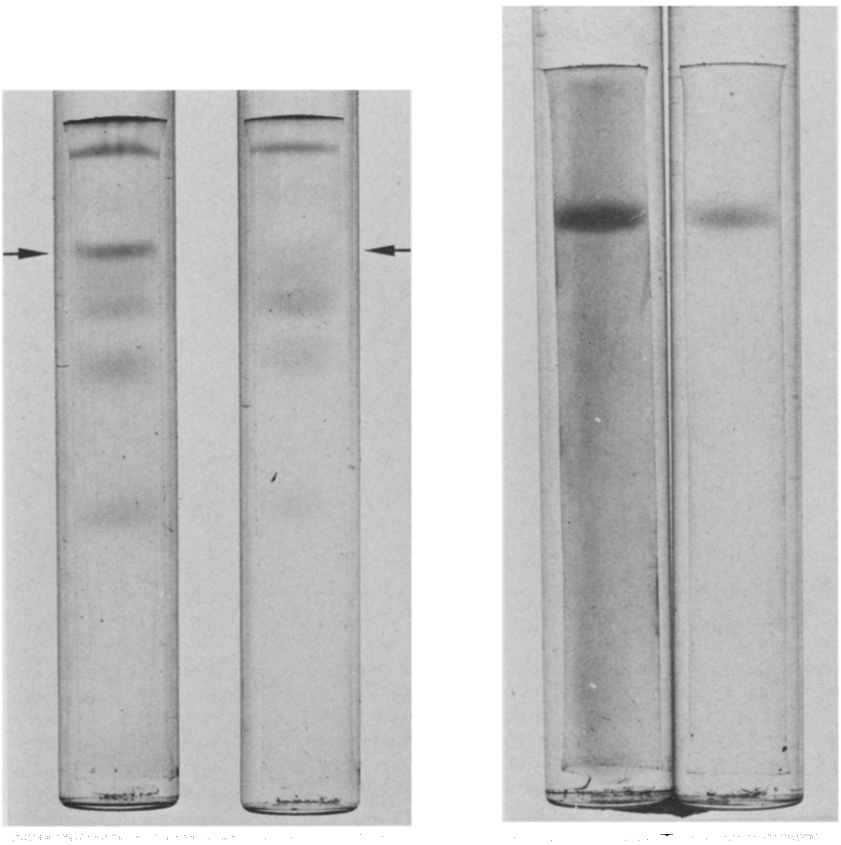

Fig. 1. SDS-polyacrylamide gel electrophoresis of (left) untreated control platelets and (right) particulate material remaining after extraction of platelets in 3 M KCl. Washed platelets were suspended in medium A (see legend to Table I) or in 3 M KCl to a platelet count of $4.8 \cdot 10^9$ platelets/ml. The platelet suspension in 3 M KCl was incubated at 37° C for 15 min and sedimented by centrifugation at 2000 × *g* for 10 min. The sediment (particulate material after KCl extraction) was resuspended in medium A to the original volume. Aliquots from each suspension corresponding to $2.4 \cdot 10^8$ platelets were diluted one third in SDS solution [3] to dissolve all particulate material, incubated at 37° C for 2 h and applied on gels for electrophoresis at 1.5 mA per gel for 15 h whereafter the gels were stained with the periodic acid-Schiff reagent. Standard electrophoresis procedure was employed [3]. Note that the lower band on each gel represents an opaque zone and not a true glycoprotein band. Unreduced sample.

Fig. 2. Polyacrylamide gels after electrophoresis in SDS of reduced samples of purified glycocalicin (approx. 7 μg on each gel) stained with (left) Coomassie brilliant blue or (right) periodic acid-Schiff reagent.

Crossed immunoelectrophoresis of Triton X-100 extracts of whole platelets in Triton X-100-containing agarose showed one continuous immunoprecipitation line appearing as two peaks (Fig. 4). The rear peak was always the major one. Addition of purified glycocalicin to the platelet extracts showed that the front peak corresponded to soluble glycocalicin (Fig. 4). In contrast to the two peaks seen with the regular crossed immunoelectrophoresis, the crossed hydrophobic interaction immunoelectrophoresis showed only one peak which was small and corresponded to purified glycocalicin (Fig. 5). To establish the correlation between the patterns of the regular crossed immunoelectrophoresis and

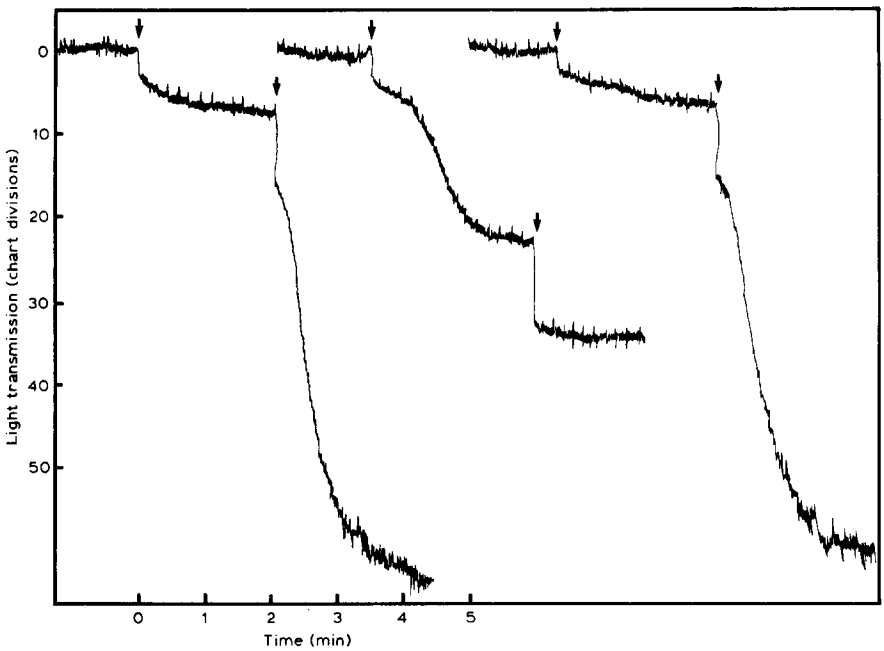


Fig. 3. Effect of antiserum to glycocalicin on human platelets and their agglutination by bovine Factor VIII-related protein. Aggregometer tracings. In an aggregometer cuvette, 500 μl of washed pre-warmed platelets (37°C) in medium A were stirred magnetically for 75 s. Then, 50 μl of rabbit serum were added (first arrow) followed 150 s later by 75 μl of highly-purified bovine Factor VIII related protein (final concn., 2 μg/ml) (second arrow). The rabbit sera were: (left trace) pre-immunization serum as a negative serum control; (middle trace) antiserum to glycocalicin; (right trace) antiserum to cold-insoluble globulin as a negative antiserum control (see Materials and Methods).

the crossed hydrophobic interaction immunoelectrophoresis, the electrophoretic separation was allowed to take place as a regular first dimension electrophoresis followed by a second dimension immunoelectrophoresis in which the proteins had to pass through an intermediate gel which contained phenyl-Sepharose (Fig. 7). Also, this procedure gave only one small immunoprecipitate peak which corresponded to purified glycocalicin (Fig. 7). This strongly indicates that the protein which is responsible for the major rear peak in the regular crossed immunoelectrophoresis of the extracts of whole platelets in Triton X-100 is strongly bound to phenyl-Sepharose. Thus, this protein represents a glycocalicin-related component which is clearly more hydrophobic than glycocalicin itself.

Glycocalicin is solubilized during lysis of platelets by freeze-thawing (Table I). The glycocalicin thus solubilized behaved identically to the glycocalicin purified from 3 M KCl extracts of whole platelets, both in the polyacrylamide gel electrophoresis and in the immunoelectrophoresis systems described above. The metal chelating agent, EDTA, and the sulphydryl-blocking agents, *N*-ethylmaleimide and iodoacetamide, strongly inhibited the solubilization of glycocalicin whereas their effects on the solubilization of total protein were very low (Table I). Lysis of platelets by freeze-thawing in the absence of any inhibitors led to the disappearance of the same glycoprotein band on SDS-polyacrylamide gel electrophoresis as described above for the extraction with

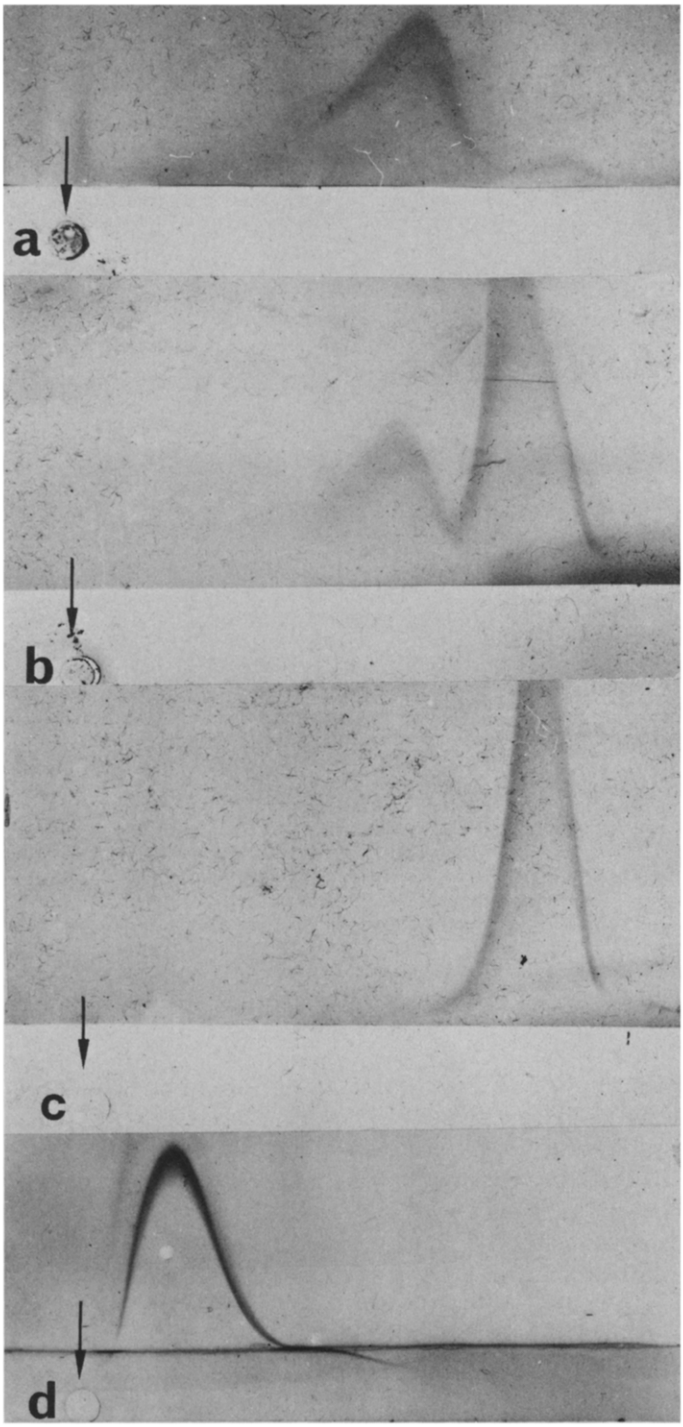


Fig. 4. Crossed immunoelectrophoresis in 1% (w/v) agarose gels which contain 1% (v/v) Triton X-100 of: (a) Triton X-100 extract of whole platelets (78 μg); (b) a mixture of the same Triton X-100 extract (65 μg) and purified glycocalicin (0.4 μg); (c) purified glycocalicin (0.4 μg) and (d) purified glycocalicin incubated at 37° C for 20 min with neuraminidase (0.75 units/ml) in acetate-buffered saline (pH 5.5) containing 9 mM $CaCl_2$ (negative control with incubation in the buffer alone not shown). The second dimension gels contained 15% (v/v) of antiserum to purified glycocalicin. The buffer (pH 8.7) used both in gels and electrophoresis chambers was a 1 : 1 mixture of the Tris-glycine buffer of Fig. 5 and the barbital buffer of Fig. 6. Field strength, 2 V/cm in second dimension. The arrows mark the application wells in each of the four electrophoreses.

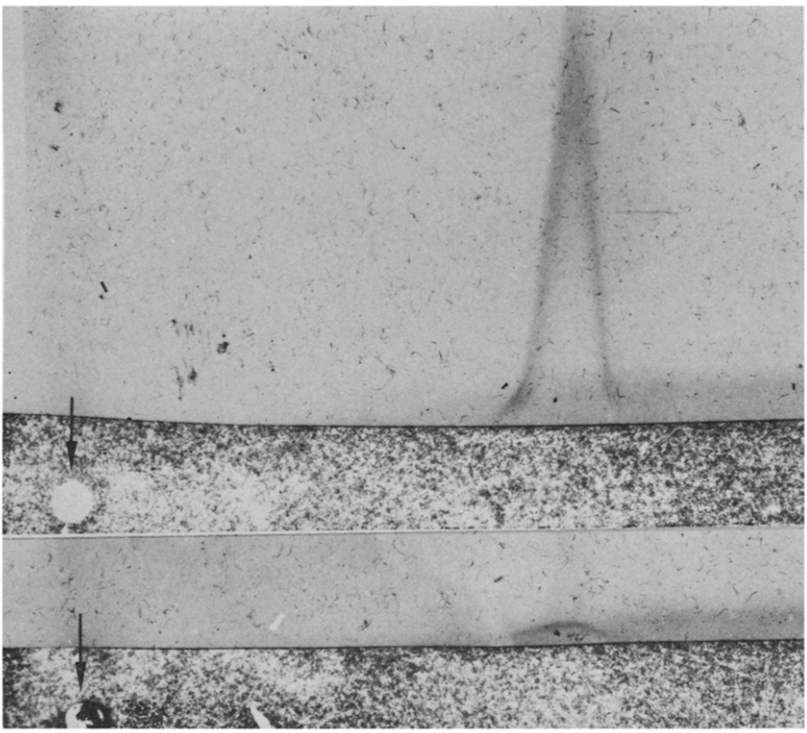

Fig. 5. Crossed hydrophobic interaction immunoelectrophoresis of: (upper panel) purified glycocalicin (0.3 μg) and (lower panel) Triton X-100 extract of whole platelets (78 μg). Gel support in the first dimension was 1% (w/v) agarose containing 20% (v/v) phenyl-Sepharose as hydrophobic matrix. Gel support in the second dimension was 1% (w/v) agarose which contained 1% (v/v) Triton X-100 and 15% (v/v) of antiserum to purified glycocalicin. The buffer used both in the gels and the electrophoresis chambers contained 100 mM glycine and 38 mM Tris, pH 8.7. Field strength 10 V/cm in the first dimension and 2.5 V/cm in the second dimension. The arrows mark the application wells in each of the two electrophoreses.

3 M KCl (see Fig. 1). The presence of the inhibitors prevented the disappearance of this band (not shown). The particulate platelet material remaining after freeze-thawing could easily be resuspended in buffer, but could not be agglutinated by bovine Factor VIII-related protein if freeze-thawing had taken place in the absence of inhibitors. This property was preserved if freeze-thawing were performed in the presence of EDTA, *N*-ethylmaleimide or iodoacetamide (Table I). These same inhibitors in the same concentrations as used in Table I also inhibited the calcium-activated protease of platelet sonicates as studied with azocasein as substrate (not shown).

Glycocalicin has a high content of sialic acid and this may contribute significantly to the total platelet sialic acid. The total sialic acid which could be liberated by acid hydrolysis (0.05 M $H_2SO_4$, 80°C, 1 h) was therefore determined separately for whole platelets and for the soluble and particulate fractions obtained after freeze-thawing of platelets in the absence or presence of EDTA (Table II). The mean value for whole platelets in this series was 7.8 μg sialic acid per mg protein (Table II). In the samples obtained by lysis in the absence of EDTA the acid-labile sialic acid of the whole platelets was fully

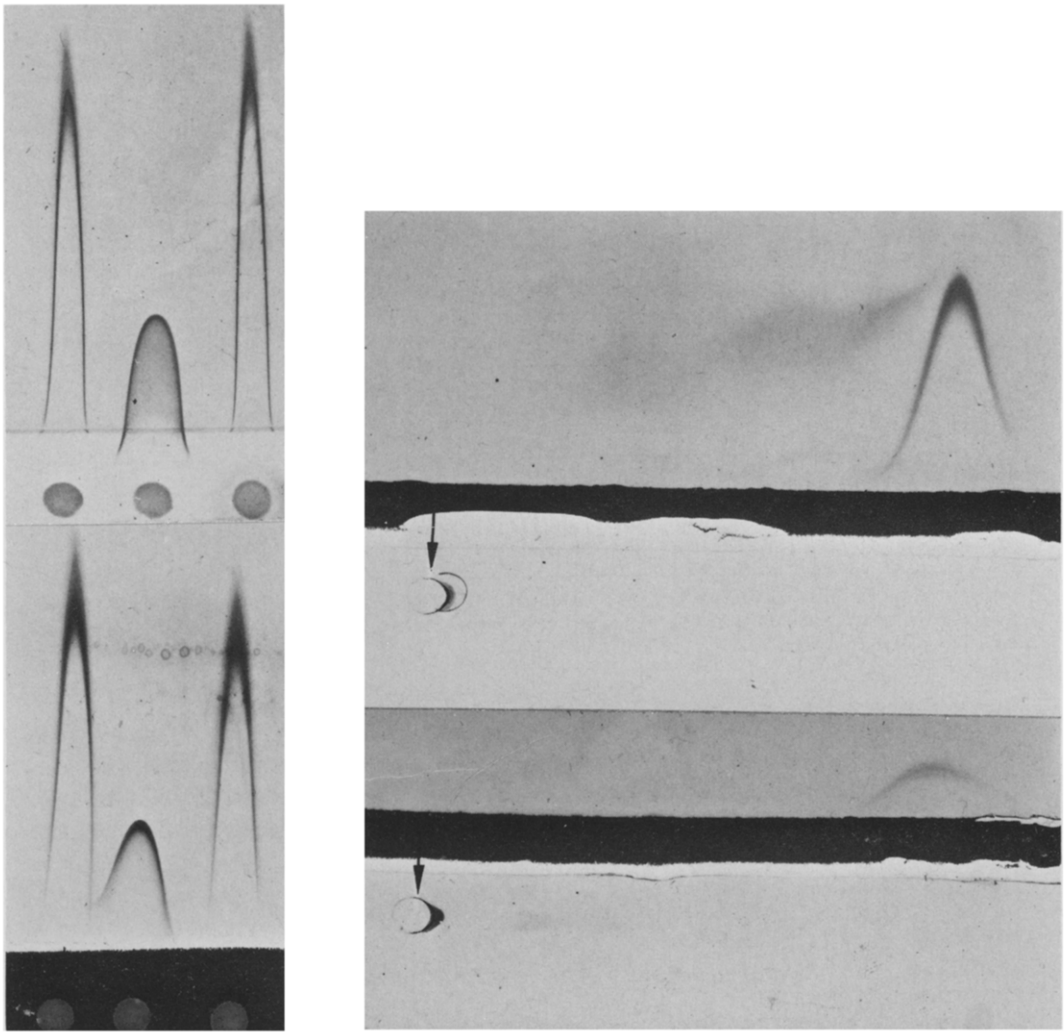

Fig. 6. Immunoelectrophoresis (rocket technique) of purified glycocalicin (0.3 μg) after incubation at 37° C for 10 min with: (left) Tris-buffered saline (pH 7.4); (middle) neuraminidase (1 unit/ml) in acetate-buffered saline (pH 5.5) containing 9 mM $CaCl_2$ and (right) acetate-buffered saline (pH 5.5) containing 9 mM $CaCl_2$. The samples were applied in gels consisting of (upper panel) 1% (w/v) agarose and (lower panel) 1% (w/v) agarose plus 20% (v/v) phenyl-Sepharose, and run into gels consisting of 1% (w/v) agarose and 20% (v/v) antiserum to purified glycocalicin. A 24 mM barbital buffer (pH 8.6) was used both in the gels and electrophoresis chambers. Field strength, 2 V/cm.

Fig. 7. Combination of crossed immunoelectrophoresis and hydrophobic interaction electrophoresis of: (lower panel) Triton X-100 extract of whole platelets (99 μg) and (upper panel) a mixture of the same Triton extract (80 μg) and purified glycocalicin (0.4 μg). Electrophoresis as in Fig. 4 except that an intermediate gel consisting of Agarose and phenyl-Sepharose CL-4B as given in Fig. 5 was used in the second dimension. The arrows mark the application wells.

recovered in the soluble and the particulate fractions (64 and 44% of whole platelets, respectively), whereas only 75% of the total was found in the two fractions when prepared in the presence of EDTA (22% and 53%, respectively). Total protein in the soluble fraction prepared in the presence of 3.6 mM EDTA

TABLE I

EFFECT OF EDTA, *N*-ETHYLMALEIMIDE AND IODOACETAMIDE ON THE SOLUBILIZATION OF GLYCOCALICIN DURING FREEZING AND THAWING OF PLATELETS

Washed platelets were suspended in Tris-buffered saline (148 mM NaCl, 20 mM Tris-HCl, pH 7.4, 280 mosM) containing the inhibitors stated. After removal of aliquots for protein determinations, the platelets were lysed by three cycles of freezing (solid $CO_2$/acetone, 5 min) and thawing (37°C, 5 min) and centrifuged at 8000 × *g* for 10 min. Aliquots from the supernatants were removed for determination of total protein and immunoquantitation of glycocalicin (Laurell technique). The sediments were resuspended in half the original volume of medium A (148 mM NaCl, 5 mM glucose, 3.6 mM EDTA and 20 mM Tris-HCl, pH 7.4, 280 mosM) and aliquots were transferred to the wells of a microtitre plate and tested for agglutination by bovine Factor VIII-related protein. For details see Materials and Methods.

| Inhibitor | Inhibitor concentration (mM) | Total protein in soluble fraction after freezing and thawing % of control * | Glycocalicin in soluble fraction after freezing and thawing % of control * | Agglutination response of particulate material remaining after freezing and thawing |
|---|---|---|---|---|
| None | — | 100 | 100 | negative |
| EDTA | 3.6 | 89 ± 10 [5] | 13 ± 12 [5] | positive |
| *N*-Ethylmaleimide | 4.0 | 80 ± 17 [4] | 35 ± 20 [5] | positive |
| Iodoacetamide | 4.0 | 85 ± 12 [5] | 43 ± 24 [5] | positive |

* Absolute values for total protein (mg/$10^9$ platelets) and glycocalicin ($\mu$g/$10^9$ platelets) in the controls were 2.1 ± 1.7 [5] and 67 ± 22 [5], respectively, corresponding to 25.8 ± 8.3 [5] $\mu$g of glycocalicin per mg whole platelet protein.

was only slightly lower than in that prepared in the absence of EDTA (6% lower as a mean of six experiments). Free sialic acid after hydrolysis was considerably lower in soluble fractions prepared in the presence of EDTA than in its absence (Table II). The difference corresponded to 42% (64% − 22%) of the total acid-labile platelet sialic acid (Table II).

TABLE II

ACID-LABILE SIALIC ACID IN WHOLE PLATELETS AND PLATELET FRACTIONS OBTAINED BY FREEZING AND THAWING

Washed platelets were resuspended in 0.15 M NaCl to $2 \cdot 10^9$ platelets/ml. Aliquots were withdrawn for determination of total acid-labile sialic acid (0.05 M $H_2SO_4$, 80°C, 1 h). Other aliquots were withdrawn, centrifuged at 2000 × *g* for 10 min and the supernatants replaced by the same volumes of Tris-buffered saline (pH 7.4) with or without 3.6 mM EDTA (see legend to Table I). The resuspended platelets were lysed by freezing and thawing as in Table I, and the soluble and particulate fractions generated by centrifugation at 2000 × *g* for 10 min. The soluble fractions were removed and replaced by the same volumes of 0.15 M NaCl as in the original aliquots. The soluble fractions and particulate suspensions, which all corresponded to $2 \cdot 10^9$ platelets/ml, were diluted with an equal volume of 0.1 M $H_2SO_4$ and hydrolysed at 80°C for 1 h. Free sialic acid in the hydrolysates was measured according to the method of Warren [15] using *N*-acetylneuraminic acid as standard. The absolute values for acid-labile whole platelet sialic acid were 7.8 ± 2.4 [7] given as $\mu$g/mg protein or 14.3 ± 4.5 [6] given as $\mu$g/$10^9$ platelets (corresponding values found in a previous work [20] were 7.4 ± 0.6 [11] and 19.2 ± 5.6 [11].

| Fraction | EDTA present or absent during freezing and thawing | Sialic acid after acid hydrolysis % of whole platelet sialic acid |
|---|---|---|
| Whole platelets | — | 100 |
| Soluble fraction | absent | 64 ± 18 [4] |
| Soluble fraction | present | 22 ± 15 [5] |
| Particulate fraction | absent | 44 ± 6 [7] |
| Particulate fraction | present | 53 ± 14 [7] |

## Discussion

Glycocalicin is defined as a one-chain glycoprotein present in the soluble fraction after platelet homogenization, which on SDS-polyacrylamide gel electrophoresis moves as a polypeptide of approx. 145 000 daltons [3,6]. In the present work, the protein was isolated after extraction from whole platelets by 3 M KCl. Glycocalicin purified by Okumura et al. [6] was found to contain 60% by weight of carbohydrate with galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine and sialic acid as the principal sugars in relative molar proportions of 2 : 1 : 1 : 2, as well as small amounts of glucose, mannose and fucose [6]. The results of the crossed hydrophobic interaction immunoelectrophoresis of the present work demonstrate that glycocalicin shows the characteristics of a hydrophilic protein.

As reviewed in the introduction, glycocalicin is considered to be located on the surface of the intact platelet. The observation that antiserum to purified glycocalicin agglutinates intact platelets represents additional support to this idea. It is difficult to comprehend, however, that this hydrophilic protein would fulfill the requirements of an integral membrane protein. It may be of considerable interest, therefore, that the glycoprotein patterns obtained after SDS-polyacrylamide gel electrophoresis of unreduced samples from whole platelets, KCl extracts and soluble fractions can be interpreted to mean that glycocalicin is derived from a larger protein than the soluble protein itself. This has also been observed by other groups [4]. The present observation that Triton X-100 extracts of whole platelets contain the bulk of the glycocalicin-related material in the form of a more hydrophobic protein than the previously known hydrophilic protein, is of further interest as the hydrophobic component is more likely to represent the membrane-associated form of the molecule.

Solubilization of glycocalicin occurs during cell lysis but the degree of solubilization obviously depends on the experimental conditions. As found in this study, solubilization can be inhibited by EDTA, *N*-ethylmaleimide and iodoacetamide in concentrations which will also inhibit the calcium-activated protease of platelet sonicates previously described by Phillips and Jakabova [14]. Recently, we have also observed that the protease inhibitor, leupeptin, has a similar effect in all of the systems related to glycocalicin solubilization described in this paper (unpublished observations). A simple hypothesis emerging from these observations is that the glycocalicin found in soluble fractions after cell lysis represents a hydrophilic proteolysis product derived from a more complex and hydrophobic integral membrane glycoprotein.

More observations indicate that the presence of glycocalicin on the platelet surface is a prerequisite to the agglutination of human platelets by bovine Factor VIII-related protein as well as by the combination of the corresponding human protein and ristocetin. This concept is further supported by the present observations that solubilization of glycocalicin is accompanied by a loss of agglutination ability and that certain enzyme inhibitors can prevent both the loss of this ability and the solubilization of glycocalicin. Such observations have led to the hypothesis that membrane-bound glycocalicin may be involved in the receptor mechanism for this protein (for review, see Refs. 7 and 19). One con-

sequence of the present work is that the glycoprotein of interest in this respect probably is the hydrophobic glycocalicin-related membrane protein described here and not the soluble glycocalicin as such.

As demonstrated in the present study, as much as 64% of the total acid-labile sialic acid of whole platelets could be found in the soluble fraction after lysis of the platelets by freeze-thawing in the absence of inhibitors. As demonstrated by the glycoprotein patterns observed after SDS-polyacrylamide gel electrophoresis of such soluble fractions, these contain glycocalicin as the major glycoprotein in addition to a glycoprotein solubilized from lysed granules [3]. The sialic acid in glycocalicin therefore contributes significantly to the total sialic acid content of the platelets. This may explain the reduced electrophoretic mobility of the platelets of Bernard-Soulier patients [20,21] which lack glycocalicin [7].

## Acknowledgement

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