Interactions between platelet-surface glycoproteins

Identification of glycoproteins capable of binding to platelet surfaces

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1. Platelets have been isolated from plasma and their surface glycoconjugates radioactively-labelled using galactose oxidase and NaB^3H_4 . 2. Conditions have been defined for optimal labelling of glycoproteins and a membrane fraction enriched in plasma membrane has been prepared and characterized by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 3. Desialylated glycoproteins that act as receptors to peanut agglutinin and lentil lectin have been purified from a detergent extract of plasma membrane. 4. Two glycosylated polypeptides that are able to bind to the surfaces of platelets have been identified and some characteristics of the binding have been investigated.

The platelet surface plays a fundamental role in the function of the cells in haemostasis. In particular, the glycoproteins of the plasma membrane have been implicated in adhesion to the subendothelium, intercellular adhesion and aggregation phenomena (Baumgartner, 1973; George, 1978; Hagen *et al.*, 1980; Phillips, 1980).

As a first step in studying the molecular basis of adhesion and aggregation, we have investigated the potential interactions that occur between platelet-surface glycoproteins. The approach we have chosen involves surface-labelling the glycoproteins of the plasma membrane, preparing a solubilized extract of these membranes and following any interactions that may occur between the solubilized glycoproteins and the surfaces of platelets. This report presents data on the methodology of the labelling technique, the characterization of specific glycoproteins of the plasma membrane using immobilized-lectin affinity chromatography and the identification of two glycoproteins that are able to bind to receptors on the platelet surface.

Materials and methods

Preparation and surface-labelling of platelets

Platelet concentrates were kindly provided by the local Regional Blood Transfusion Service from stocks (blood group A) that had been stored for 18 h at 20°C. Platelets were recovered from the concentrates by centrifugation (1500 *g* for 8 min) and freed of plasma and other blood-cell contamination

Abbreviations used: TCA, trichloroacetic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulphate; PAS, periodic acid/Schiff.

by three additional wash cycles (50 ml/1 ml of packed cells per cycle) using platelet wash buffer (buffer A): 10 mM-Tris/HCl/0.8% NaCl/10 mM-EDTA, pH 7.2, containing 1% (v/v) aprotinin (Sigma; sp. activity 28.3 trypsin inhibitor units). Visual examination of the platelet suspension by phase-contrast microscopy indicated that contamination by leucocytes and red blood cells was negligible. The cells were finally suspended in buffer A at 1×10^6 cells/ml and incubated concurrently with galactose oxidase (Sigma type V) and sialidase (Sigma type IX) for 15 min at 37°C. The cells were then washed once as before, resuspended in 0.5 ml of buffer A containing 1 mCi of NaB^3H_4 (Amersham; sp. radioactivity 10 Ci/mmol) and incubated for 10 min at room temperature, after which they were further washed (three cycles).

Isolation of membrane and preparation of a detergent-solubilized extract

Surface-labelled cells were resuspended in 10 ml of buffer A and sonicated for 2×15 s bursts (Dawe sonicator fitted with a microprobe). The sonicated material was centrifuged at 10 000 *g* (r_{av} , 9.0 cm) for 20 min at 4°C, and the resultant supernatant further centrifuged at 150 000 *g* (r_{av} , 6.0 cm) for 1.5 h at 4°C. The pellet was resuspended in phosphate-buffered saline, pH 7.2 (buffer B) containing 1% (v/v) aprotinin and sonicated to give a total membrane suspension. Suspensions were made 1% (v/v) with Triton X-100 to a final detergent excess of 10-fold that of the protein present, and after further sonication were centrifuged at 150 000 *g* (r_{av} , 6.0 cm) for 1.5 h at 4°C. The solubilized material was diluted

with buffer B to a final concentration of 0.1% (v/v) detergent, aprotinin was added to maintain the mixture at 1% (v/v), CaCl_2 was added to 1 mM and MgCl_2 to 2 mM final concentrations.

Affinity chromatography using immobilized lectins

Peanut agglutinin was prepared from peanuts (var. *Jumbo*) as previously described (Bowles *et al.*, 1979) using ϵ -aminohexanoyl- β -D-galactopyranosylamine-Sepharose. Lentil lectin was prepared using Sephadex G-150, from red lentil beans (Entlicher *et al.*, 1969). The lectins were immobilized on Sepharose 4B (3 mg/ml of Sepharose) by using the CNBr procedure and the capacity of the affinity systems was standardized by using iodinated desialylated fetuin. Columns (5 ml) of lectin-Sepharose were routinely used to prepare lectin receptors from detergent-solubilized extracts of desialylated radioactively-labelled platelet membranes. The columns were developed in buffer B containing 0.1% (v/v) Triton X-100 at 4°C, and were eluted with 0.2 M-galactose (peanut-agglutinin-Sepharose) or 0.2 M- α -methyl mannoside (lentil-lectin-Sepharose) in the same buffer. The eluate was monitored for radioactivity and peak fractions were precipitated with 6% (w/v) TCA, and analysed.

Binding assays

Washed platelets were treated with formaldehyde [2% (v/v) in buffer B] for 18 h at room temperature and then further washed with buffer B (six cycles). The fixed cells were incubated in duplicate under a variety of conditions with the detergent-solubilized membrane extract in a final assay volume of 1 ml. After incubation (on an Eppendorf shaker unit), the suspension in 1.5 ml tubes was centrifuged for 4 min at 10000 g (Eppendorf microfuge). The supernatant was removed and the platelet pellet was resuspended in 100 μ l of phosphate-buffered saline containing 0.1% Triton X-100, 1 mM- CaCl_2 and 2 mM- MgCl_2 (buffer C). This suspension was loaded into a 500 μ l tube containing a cushion composed of 250 μ l of 5% (w/v) BSA (Sigma) in buffer C and centrifuged for 1 min (9000 g) (Beckman Microfuge). The ends of the tubes were then cut off, the pellet was resuspended in buffer A and either transferred directly to a scintillation vial for determination of radioactivity or processed for elution of the radioactively labelled material.

When conditions of the standard assay were varied, the additives and detergent-solubilized extract were pre-incubated for 15 min on a shaker before addition of formaldehyde-treated platelets.

For the elution of radioactively labelled glycoproteins from the platelets, the cells were first washed by resuspension in 1 ml of buffer A and centrifuged to remove the bulk of associated BSA.

After resuspension of the pellet in 500 μ l of buffer A, SDS was added to a final concentration of 2% (w/v), the cells were incubated at room temperature for 10 min and re-centrifuged. The supernatant containing >80% of the total radioactivity was removed and analysed.

To determine the effect on binding of modifying the platelet surface, formaldehyde-treated cells were treated with a variety of enzymes. In all instances, 10^9 cells were incubated at 37°C for 30 min in a final volume of 0.5 ml of buffer B containing 1 mg of Pronase/ml (Sigma; type VI), 1 mg of chymotrypsin/ml (Sigma; type VII) or a mixture of 7.5 units of galactose oxidase and 0.054 Sigma units of sialidase. After the incubations, the cells were washed through six cycles with buffer B and resuspended in a final volume of buffer B to give identical cell counts per treatment.

Gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out under either reducing or non-reducing conditions on 5–15% gradient slab gels by using a modification of the system used by Laemmli (1970). Molecular-weight markers (Boehringer) include soya-bean trypsin inhibitor (mol.wt. 21000), BSA (68000), RNA polymerase α -chain (39000), β -chain (155000), β' -chain (165000). In addition, to obtain a series of mol.wts. in the 80000–180000 range, a sample of characterized pig kidney microvillus-membrane protein (a gift from Dr. A. J. Kenny) was used for further calibration. The molecular weights of the unknown radioactively-labelled platelet glycoproteins were calculated by using a plot of $\log(\text{molecular weight})$ versus $\log[\text{acrylamide}(\%)]$ for the series of molecular-weight markers stated above. For determination of radioactive incorporation, 2 mm slices of the slab-gel tracks were solubilized by incubation at 56°C in 0.5 ml of H_2O_2 .

General analytical techniques

Radioactive incorporation into proteins was determined by precipitation with TCA, which was added to a final concentration of 6% (w/v), the precipitate was collected on glass-fibre discs, washed extensively first with ice-cold 5% (w/v) TCA and then with absolute ethanol, before drying and transferring to scintillant. Radioactive incorporation into lipids was determined by extraction of aqueous samples with chloroform/methanol (3:2, v/v) (final ratio of chloroform/methanol to sample was 1:0.1). After addition of 4 mM- MgCl_2 , the organic phase was washed extensively with Folch's upper phase before drying and addition of scintillant. Sialic acid release from formaldehyde-treated platelets or fetuin (Sigma; type IV) was assayed by the Aminoff procedure (Hatton & Regoezi, 1973). Proteolytic activity in the enzyme preparation was tested by

using both a colorimetric procedure (Azocoll; Calbiochem) and iodinated β -chain of insulin in a standard assay for endopeptidases (George & Kenny, 1973). Protein was determined by the Coomassie Blue dye procedure (Sedmarck & Grossberg, 1976) with BSA as standard.

Scanning electron microscopy

Platelet samples that had been treated with 2% (v/v) formaldehyde, either as a suspension in plasma or in buffer A for 18 h, were washed five times with buffer B, dehydrated using a 10–100% acetone series and critical-point dried (Sandri PV T3). Samples were viewed in a Cambridge scanning electron microscope after shadowing with gold.

Results

Variation in enzyme preparations

The use of sialidase and galactose oxidase are fundamental to the surface-labelling procedure of platelet glycoproteins utilizing NaB^3H_4 . Various commercial preparations of enzymes were investigated for their effectiveness. Although galactose oxidase marketed by Kabi had been used previously (Anderson & Gahmberg, 1978) the enzyme was no longer available in the U.K. Galactose oxidase, marketed by Sigma, was stored at -20°C and heated to 56°C for 30 min before use. Heat-treated enzyme exhibited no demonstrable proteolytic activity when tested using the Azocoll procedure, and trace activity when tested in the endopeptidase assay (0.132 nmol/min per ml of proteolytic activity per 7.5 Sigma units of enzyme).

Different commercial sialidase preparations (Koch–Light, Sigma, Boehringer) varied markedly, with respect to the release of sialic acid from fetuin or formaldehyde-treated platelets, the presence of contaminant proteinases and glycosidases, and radioactive incorporation after subsequent galactose oxidase/ NaB^3H_4 treatment of the platelets (results not shown). Since the *Chlostridium perfringens* sialidase from Sigma exhibited no demonstrable proteolytic activity with the Azocoll procedure and negligible activity with the endopeptidase assay (0.054 Sigma units exhibited 0.003 nmol/min per ml of proteolytic activity) and yet also caused the highest incorporation of ^3H into platelets, that commercial preparation was used for all further experiments. Different batches of the enzyme preparation were also found to vary in activity and eventually it was found necessary to standardize each new batch by assaying sialic acid release from formaldehyde-treated platelets.

Visual detection of surface-labelled platelets

The gross morphological effect on the platelet surface of the enzymic digestions and numerous

washing cycles was monitored by the use of scanning electron microscopy. Plate 1 shows the results. Unwashed platelets in plasma were used as the control (Plate 1a). The platelets were typically discoid. Washing the cells free of plasma by centrifugation and resuspension increased the proportion of cells with filopodia (Plate 1b), although when low-power fields were scanned the majority of the platelets were seen to have retained a smooth discoid appearance. The labelling procedure did not cause any further visible changes to the surface of the cells, as shown in Plate 1(c).

Preparation of radioactively-labelled membranes

Since a major cell-surface glycoprotein of platelets is released from the membrane into soluble form by sonication, the characteristics of both the pellet and the supernatant after centrifugation at 150 000 g were always investigated in parallel. Fig. 1 shows the polypeptides of the two subcellular fractions as visualized by Coomassie Blue staining after surface labelling of the cells and separation of components by SDS/polyacrylamide-gel electrophoresis on 5–15% slab gels under reducing conditions. Comparing gel (1) with (2) and (3), and gel (5) with (6) and (7), it can be seen that there is no variation in patterns of the isolated polypeptides after enzymic treatment of the intact cells. As an internal control for activation of endogenous proteinases of platelets, cells that had been treated with both enzymes and NaB^3H_4 were finally suspended in 10 mM-Tris/HCl/0.8% (w/v) NaCl, pH 7.2, and CaCl_2 was added to a final concentration of 2 mM after sonication of the cells. The effect of Ca^{2+} is clearly demonstrated by comparing membrane-bound proteins in gel (3) with those in gel (4) and soluble proteins in gel (7) with those in gel (8).

Table 1 compares the yields of membrane protein and ^3H -labelled TCA-precipitable components during a characteristic preparation of membrane fractions. Incubation of cells with NaB^3H_4 alone routinely gave very low incorporation into TCA-precipitable components. In further experiments, the proportion of ^3H in glycolipids was compared with that in glycoproteins; the results are shown in Table 2. Incubation with NaB^3H_4 alone led to preferential labelling of glycolipids of the membranes, which was only slightly affected by prior incubation with galactose oxidase. Removal of sialic acid by sialidase led to a shift of incorporation of ^3H into glycoproteins both in the soluble and membrane-bound fractions.

The distribution of radioactivity into glycoproteins of the fractions was determined after separation of components by SDS/polyacrylamide-gel electrophoresis and measurement of radioactivity in gel slices. Initially, plasma-membrane fractions isolated from platelets that had been only incubated with NaB^3H_4 or with NaB^3H_4 plus galactose oxidase

Fig. 1. *A comparison of polypeptides in the soluble and plasma-membrane-enriched fractions of platelets, after surface-labelling intact cells*

Polypeptides from each fraction were separated by SDS/polyacrylamide-gel electrophoresis under reducing conditions on 5–15% slab gels as described in the Materials and methods section. Gels (1)–(4) are plasma-membrane-enriched fractions; gels (5)–(8) are soluble fractions. M = myosin, A = actin. Treatment of the intact cells used to prepare the fractions is described in detail in the Materials and methods section, and involves: gels (1) and (5), pre-incubation with NaB^3H_4 ; gels (2) and (6), pre-incubation with galactose oxidase + NaB^3H_4 ; gels (3) and (7), pre-incubation with sialidase + galactose oxidase + NaB^3H_4 ; gels (4) and (8), as gels (3) and (7), but CaCl_2 added to 2 mM final concentration before centrifugation of the homogenate to prepare the fractions; 40 μg of protein was loaded per gel. Polypeptides were visually detected by staining with Coomassie Brilliant Blue.

Table 1. *Comparison of radioactive incorporation from NaB^3H_4 and recovery of protein after surface-labelling of platelets and preparation of subcellular fractions*

Fractions were prepared from 10^{10} platelets/treatment as described in the Materials and methods section.

Treatment of cells . . .	NaB^3H_4		Galactose oxidase + NaB^3H_4		Sialidase + galactose oxidase + NaB^3H_4	
	$10^{-4} \times \text{TCA-precipitated radioactivity (c.p.m.)}$	Protein (mg)	$10^{-4} \times \text{TCA-precipitated radioactivity (c.p.m.)}$	Protein (mg)	$10^{-4} \times \text{TCA-precipitated radioactivity (c.p.m.)}$	Protein (mg)
Homogenate	19.84	8.33	26.26	8.55	330.95	7.85
20 000 g pellet	8.62	2.0	13.0	2.0	116.16	1.85
150 000 g supernatant	6.19	3.23	6.39	2.98	61.85	3.32
150 000 g pellet	4.18	1.35	5.50	1.30	75.4	0.98
Recovery (%)	95.6	79	94.8	73.5	76.5	78.3
Recovery as 150 000 g pellet (%)	21.1	16.2	21.0	15.2	20.3	12.4

were examined; the very low incorporation of ^3H into TCA-precipitable components was found to spread throughout the range of polypeptides present (results not shown). Fig. 2(a) shows the distribution

of radioactively-labelled glycoproteins from the membrane-bound fraction and the soluble fraction of platelets incubated with sialidase, galactose oxidase and NaB^3H_4 . The radioactivity recovered in the

EXPLANATION OF PLATE 1

Scanning electron micrographs of platelets

Platelets were treated with formaldehyde after different stages of the radiolabelling procedure and processed for microscopy as described in the Materials and methods section. (a) Platelets were fixed in plasma. (b) Plasma was removed from the cells by four successive wash cycles. (c) Washed cells were treated with sialidase, galactose oxidase and NaB^3H_4 . Magnification bar, $1\text{ }\mu\text{m}$.

Table 2. Comparison of radioactive incorporation from NaB^3H_4 into glycolipids and glycoproteins in subcellular fractions of platelets

Fractions were prepared from 0.6×10^{11} platelets/treatment as described in the Materials and methods section. Values in parentheses indicate the TCA-precipitated radioactivity/lipid radioactivity ratio.

Treatment of cells . . .	NaB^3H_4		Galactose oxidase + NaB^3H_4		Sialidase + galactose oxidase + NaB^3H_4	
	$10^{-4} \times \text{Radioactivity (c.p.m.)}$		$10^{-4} \times \text{Radioactivity (c.p.m.)}$		$10^{-4} \times \text{Radioactivity (c.p.m.)}$	
	TCA precipitate	Lipid	TCA precipitate	Lipid	TCA precipitate	Lipid
150 000 g supernatant	2.79 (6.2)	0.45	4.42 (8.2)	0.52	38.91 (81.1)	0.48
150 000 g pellet	1.23 (0.56)	2.19	3.68 (0.99)	3.72	50.87 (21.7)	2.34

Fig. 2. A comparison of radioactively-labelled glycoproteins in the soluble and plasma-membrane-enriched fractions of platelets, after surface-labelling intact cells. Intact cells were treated with sialidase, galactose oxidase and NaB^3H_4 as described in the Materials and methods section. Polypeptides from each fraction were separated by SDS/polyacrylamide-gel electrophoresis under reducing conditions on 5–15% slab gels and the gels were sliced and radioactivity in the segments determined as described in the Materials and methods section. (a) Soluble fraction; (b) plasma-membrane-enriched fraction. A range of molecular-weight markers is included.

although the total number of polypeptides in that fraction is high (Fig. 1, gel 7), only one of those components is radioactively labelled and is therefore both glycosylated and localized at the surface of the platelets. In Fig. 2(b) the pattern of ^3H incorporation into membrane-bound polypeptides is shown. By using a 5–15% gradient single-dimension gel under reducing conditions, the major zone of radioactivity is associated with four polypeptides in the apparent mol.wt.-range 100 000–160 000. Below a mol.wt. of 100 000, the pattern is more resolvable and clearly distinct peaks can be seen in the apparent-mol.wt.-range 20 000–100 000.

Detergent solubilization of membranes and isolation of seed-lectin receptors

Although the pattern of radioactive incorporation into membrane components separated by SDS/polyacrylamide-gel electrophoresis was reproducible, the marked similarity in apparent molecular weights of the polypeptides in the mol.wt.-range 100 000–160 000 made it necessary to purify certain components from the membrane to use as internal standards for the localization of specific glycoproteins on gels. When detergent-solubilized membrane components were separated from the particulate residue by centrifugation at 150 000 g, 90% of the radioactively-labelled glycoproteins were extracted (results not shown). Fig. 3(a) indicates that the distribution of the different glycoproteins in the detergent extract was essentially the same as in the total membrane fraction (cf. Figs. 2b and 3a), except for the slightly decreased quantity of a glycoprotein of apparent mol.wt. 209 000. Fig. 3(b) shows the purified receptor in platelet membranes for lentil lectin. The major radioactively labelled component is a polypeptide of apparent mol.wt. 135 000, which would migrate as the fast-moving shoulder to the major peak of radioactivity in the total membrane fraction (cf. Figs. 3b and 3a). Previous studies on the lentil receptor in platelet plasma membranes have shown that the major glycoprotein recognized by the lectin is 2B (Clemetson *et al.*, 1977). Fig. 3(c) shows the purified receptor in platelet membranes to peanut agglutinin, and indicates that desialylated glyco-

soluble fraction is associated with a single polypeptide of apparent mol.wt. approx. 150 000. Thus,

Fig. 3. A comparison of radioactively-labelled polypeptides in a detergent-extract of a plasma-membrane-enriched fraction with the purified platelet-surface receptors to lentil lectin and peanut agglutinin

Intact cells were surface-labelled, plasma-membrane-enriched fractions were prepared and SDS/polyacrylamide-gel electrophoresis was carried out as described in the Materials and methods section. Receptors to the lectins were purified from a total detergent extract as described in the Materials and methods section. (a) Total detergent-solubilized plasma-membrane-enriched extract; (b) purified receptor to lentil lectin; (c) purified receptor to peanut agglutinin.

protein 1B is the major glycoprotein recognized by the lectin. Since, under reducing conditions, desialylated glycoprotein 1B has an apparent mol.wt. of 155 000, it would migrate as the slightly slower-moving shoulder/major peak of radioactivity in the total membrane fraction (cf. Figs. 3c and 3a). In addition to glycoprotein 1B, there is a fast-moving glycolipid component that migrates ahead of the buffer front on the SDS/polyacrylamide-gel-electrophoretic system.

Binding assays between solubilized glycoproteins and platelet surfaces

When detergent-solubilized extracts of surface-labelled platelet membranes were incubated with formaldehyde-treated platelets, radioactive material bound to the platelets. Fig. 4(a) indicates that the binding was time-dependent, with a plateau in maximal binding occurring after 30 min. To investigate whether the radioactively-labelled material that was bound by formaldehyde-treated platelets could be identified with discrete components of the plasma membrane, the bound material was eluted with 2% (w/v) sodium dodecyl sulphate and, after reduction, was subjected to SDS/polyacrylamide-gel electrophoresis. Fig. 4(b) shows that only two main glycosylated polypeptides were bound to the platelet surface, those of apparent mol.wts. 90 000 and 21 000. Unreduced eluates were also found to give the same two peaks of radioactivity (results not shown). When the amount of radioactively-labelled extract incubated with the platelets was varied, the results shown in Fig. 4(c) were obtained. Up to the limits of the amount of radioactively-labelled extract added, no saturation in total radioactivity bound was observed. However, it can be clearly seen that the two polypeptides characterized in Fig. 4(b) are minor radioactively-labelled components of the plasma membrane (cf. Figs. 4b and 3a) and therefore the absence of saturation in Fig. 4(c) is probably due to the low concentration of these polypeptides in the detergent extract.

As a preliminary means of investigating the nature of the binding, the standard assay was varied. In Table 3, the result of each variable is expressed as the percentage value of ^3H bound in a standard assay. Pre-treatment of the cells with proteolytic enzymes greatly increased the amount of ^3H bound, whereas the effect of sialidase/galactose oxidase was negligible. It is possible that the binding was the result of charge interactions between the desialylated membrane extract and the native sialylated surface of the formaldehyde-treated cells. However, since removal of sialic acid by sialidase treatment of the cells did not alter the amount of ^3H bound, such an explanation is unlikely. The addition of fibrinogen to the standard assay caused a decrease in ^3H bound, whereas the effect of heparin and gelatin

Fig. 4. Features of the binding interaction between solubilized membrane extract and the surface of formaldehyde-treated platelets

(a) Incubations were for the periods of time indicated. The assay and determination of radioactivity bound to the cells are described in the Materials and methods section. (b) Analysis of radioactively-labelled binding components eluted from formaldehyde-treated platelets by SDS as described in the Materials and methods section. SDS/polyacrylamide-gel electrophoresis on 5–15% gradient gels under reducing conditions and determination of radioactivity in gel segments is as

were negligible. The presence of 10mM-EGTA decreased the ^3H bound. Although high concentrations of the non-ionic detergent Triton X-100 and the ionic detergent potassium deoxycholate impaired binding, SDS was the most effective detergent inhibitor. Other substances that were also tested and found to have negligible effect included blood-group A, B and H glycopeptides at final concentrations of 1 mg/ml, and glucose, galactosamine hydrochloride, glucosamine hydrochloride and arginine at final concentrations of 100mM. Lowering the incubation temperature from 30°C to 0°C reduced ^3H -bound to 23% of that in the standard assay.

Discussion

Galactose oxidase and NaB^3H_4 have been used previously to surface-label platelets, but the procedure was not quantitatively characterized for isolated membrane fractions, and the comparative distribution of radioactivity into glycoproteins and glycolipids was not analysed (Phillips & Poh Agin, 1977a; Andersson & Gahmberg, 1978; Kinoshita *et al.*, 1979).

In the present study the effect of the labelling procedure on the platelets was examined both at the cellular level by scanning electron microscopy and at the molecular level by comparison of polypeptides from labelled and control cells. The gross morphology of labelled washed cells was found to differ negligibly from the control cells. Previous studies using an identical washing and labelling technique showed that the labelled platelets exhibited negligible release of [^{14}C]serotonin and normal response to ADP, collagen and thrombin (Phillips & Poh Agin, 1977b). A further indication that the labelling procedure caused little change to the native state of the surface membrane was provided by the polypeptide patterns after SDS/polyacrylamide-gel electrophoresis. Several methods have been used for the preparation of plasma-membrane-enriched fractions from platelets. For rapidity, in this study a modified version of the general procedure characterized fully by George *et al.* (1978) has been used. After Coomassie Blue staining of polypeptides separated by a single-dimension 5–15% gradient gel, no differences could be observed in the isolated fractions of the NaB^3H_4 -treated, or enzymes-plus- NaB^3H_4 -treated platelets. However, the susceptibility of platelet polypeptides to degradation by

described in the Materials and methods section. (c) The effect of varying the amount of radioactively-labelled extract incubated with a standard quantity of platelets (2.6×10^8 cells). Conditions of the assay are described in the Materials and methods section.

Table 3. *Conditions affecting the binding of detergent-solubilized ^3H -labelled platelet glycoproteins to platelet surfaces* Incubations, assays and determination of radioactivity were as described in the Materials and methods section. The concentrations given for the additions in (b) are final concentrations.

Variation of standard assay	^3H bound, expressed as a proportion of standard assay (%)
(a) Pretreatment of formaldehyde-treated platelets with:	
Pronase	197
Chymotrypsin	141
Sialidase/galactose oxidase	101
(b) Additions to standard assay	
Gelatin (1 mg/ml)	92
Heparin (1 mg/ml)	88
Fibrinogen (1 mg/ml)	49
EGTA (10 mM)	38
Triton X-100 (1%, v/v)	50
SDS (1%, w/v)	8
Deoxycholate (1%, w/v)	43

soluble proteinases has been noted before (Phillips & Jakabova, 1977) and when, in this system, endogenous proteinases were activated by addition of Ca^{2+} , considerable alterations to the polypeptides occurred. Not only the polypeptide range of mol.wt. >200 000 were affected, as previously reported (Phillips & Jakabova, 1977), but also lower-molecular-weight polypeptides disappeared with a concurrent appearance of new polypeptides in the soluble fraction. SDS/polyacrylamide-gel electrophoresis on a single-dimension 5–15% gradient gel resolved the tritiated polypeptides of the particulate fraction into distinct peaks of radioactivity that varied from nine to 12 in number between different preparations. In contrast, only one peak of radioactivity was ever observed after SDS/polyacrylamide-gel electrophoresis of the soluble fraction of surface-labelled platelets. The polypeptide in the soluble fraction is most probably glycocalicin, a surface-membrane glycoprotein previously shown to be released on sonication of platelets (Okumura *et al.*, 1976; Nachman *et al.*, 1979). Release of multiple copies of a specific surface glycoprotein on cell disruption is not a particular feature of platelets, since the major surface antigen of the biologically dissimilar cells of *Trypanosoma brucei* is also released into soluble form on cell rupture (Cross, 1975). However, release of the surface antigen of Trypanosomes can also be specifically induced by raising the intracellular concentrations of Ca^{2+} with ionophore A23187 (D. J. Bowles & P. H. Voorheis, unpublished work), whereas no effect of the ionophore on release of glycocalicin has been observed (Mosher *et al.*, 1979).

The resolution of nine to 12 membrane-bound glycoproteins in this study compares well with the identification of nine glycoproteins (Phillips & Poh Agin, 1977a), ten glycoproteins (Andersson & Gahmberg, 1978) and 11 glycoproteins (Kinoshita

et al., 1979) when galactose oxidase/ NaB^3H_4 has been used previously to label platelets. Seven glycoproteins were detected after using diazotized ^{125}I -labelled di-iodosulphanilic acid as a surface label (George *et al.*, 1978) and routinely only four to six glycoproteins can be detected by PAS-staining of gels (see, for example, Kinoshita *et al.*, 1979). Two glycosylated polypeptides (glycoproteins 1B and 2B) have been isolated by lectin affinity chromatography. Since desialylation was a necessary step in labelling the platelets, wheat-germ agglutinin, which recognizes sialic acid residues (Greenaway & Levine, 1973) could not be used as previously (Cooper *et al.*, 1979; Nachman *et al.*, 1979) for the purification of the glycoprotein 1 complex. Instead, immobilized peanut agglutinin, specific for β -D-galactopyranose residues (Lotan *et al.*, 1975), was found to bind to glycoprotein 1B, and immobilized lentil lectin, specific for α -D-mannopyranose residues, was used to purify glycoprotein 2B.

Although recent investigations have focused on the interaction between platelet plasma-membrane glycoproteins and the internal cytoskeletal systems (Phillips *et al.*, 1980), there is little information about the surface components that may mediate either directly or indirectly in the intercellular contact that occurs during aggregation. On aggregation, actin polymerization and membrane reorganization leads to the formation of filopodia and exposure of cryptic sites on the plasma membrane (Gitler *et al.*, 1980). Components of the storage granules, such as the glycoprotein of mol.wt. 149 000 (George *et al.*, 1980), also appear at the platelet surface and may be involved in the contact process either through the formation of direct intercellular bridging points or indirectly via their interaction with plasma proteins such as fibrinogen and cold insoluble globulin (Bowles, 1979; Marguerie *et al.*, 1979; Niewiarowski *et al.*, 1980, 1981; Kent Gartner *et al.*, 1980,

1981). As an initial entry point into this complexity, platelets have been pretreated with formaldehyde and used to provide immobilized recognition sites for solubilized glycoproteins to bind to. Since ligand binding to surface receptors generally involves lateral displacement of proteins in the plane of the fluid membrane, it is possible that the use of formaldehyde-treated platelets as a source of receptor sites is not optimal. However, radioactive material from the solubilized membrane extract did bind to the platelets and when analysed was found to consist of only two polypeptides of apparent mol.wt. 90000 and 21000. Both polypeptides are minor radioactively-labelled components of the total membrane extract, implying either that they are not highly glycosylated or that they are not present in large quantities on the platelet surface. The method used of eluting the radioactively-labelled binding proteins from the platelets with SDS led to a partial solubilization of proteins from the formaldehyde-treated cells; this made it impossible to directly calculate the specific activity of the binding proteins. Nor did it prove possible to measure protein in the gel associated with the radioactive peaks at mol.wts. 90000 and 21000 since the quantities present were undetectable by the methods used (results not shown). This suggests that the polypeptides are not major components of the native inactivated platelet surface and could further explain why no saturation in binding was observed within the limits of the amounts of total extract added to a constant number of platelets.

When the surface of the formaldehyde-treated platelets was altered by proteinase treatment a substantial increase in bound radioactivity was observed. Recently, both chymotrypsin and Pronase treatment have been found to lead to the exposure of cryptic fibrinogen binding sites as platelets (Niewiarowski *et al.*, 1981). However, in this study it is not as yet known whether the observed increase in radioactivity bound to the surface-digested platelets is due to enhanced binding by the polypeptides of mol.wts. 90000 and 21000 or to the induced binding of additional different polypeptides. Interestingly, a decrease in bound radioactivity was consistently observed when fibrinogen was pre-incubated with the solubilized-membrane extract before the incubation. In contrast, heparin, blood-group glycopeptides and a variety of simple sugars and amino acids had no effect. The inhibition in binding brought about by fibrinogen may indicate a role for the molecule in mediating the interaction of the 90000- and 21000-mol.wt. polypeptides with platelet surfaces. The involvement of Ca^{2+} is also implied by the effect of including EGTA in the incubation medium.

Recently, a polypeptide of mol.wt. 90000 has been implied to function in the process of platelet

activation and aggregation by thrombin (Phillips *et al.*, 1980). The relationship between that polypeptide and the binding protein described in the present study awaits further investigation.

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