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IDENTIFICATION OF ECTOPROTEINS OF HUMAN PLATELETS

COMBINATION OF RADIOACTIVE LABELLING AND TWO-DIMENSIONAL ELECTROPHORESIS

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Summary

Two-dimensional gel electrophoresis combining isoelectric focussing of reduced or non-reduced proteins in the first dimension with electrophoresis in sodium dodecyl sulfate polyacrylamide gels in the second dimension enabled us to identify 25 ectoproteins in the non-reduced state and 32 in the reduced state. Gel electrophoresis in sodium dodecyl sulfate of non-reduced proteins in the first dimension followed by reduction and gel electrophoresis in sodium dodecyl sulfate in the second dimension was helpful in the identification of the major ectoproteins and indicated that at least seven additional components might be present in the region between 170 and 85 kdaltons. All major ectoproteins could readily be identified. Glycoprotein V showed only a small increase in apparent molecular weight on reduction. It was one of the most basic proteins with a p*I* value of 6.9.

The majority of the ectoproteins were located at an isoelectric point of 5.7 with glycoproteins Ib, IIc, VI and VII as the most acidic components. A good agreement was observed between the three labelling techniques which indicates that almost all ectoproteins are glycoproteins containing sialic acid.

Introduction

Ectoproteins of cells mediate important reactions such as adherence to surfaces and interactions with other cells. They also serve as receptors for

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substances that may regulate cell function. In human blood platelets, three major surface glycoproteins were first recognized, but improved techniques allowed first identification of seven glycoproteins [1,2] and, recently, of at least eight others [3,4].

The two major glycoproteins, IIb and III, are missing in Glanzmann's thrombasthenia which is characterized by absent platelet-platelet interactions [5]. Another glycoproteins, Ib, is diminished in the Bernard-Soulier Syndrome, in which platelets lack the receptor for the factor VIII-Von Willebrand complex which is required for optimal adhesion to the vessel wall [6,7]. Two glycoproteins have been implicated in the action of thrombin. One protein, glycocalicin, a hydrophilic fragment released from glycoprotein Ib by a calcium-dependent protease, inhibits thrombin binding competitively [8]. A second protein, glycoprotein V, is proteolytically cleaved from the platelet [3,4].

Recently, Clemetson et al. [9] have used the two-dimensional high-resolution gel electrophoresis technique of O'Farrell [10] for the study of platelet proteins. Employing periodic acid-Schiff base-staining, they could demonstrate all major glycoproteins. In this paper, we present our data on the combination of three different radioactive labelling techniques with two-dimensional gel electrophoresis for the analysis of platelet ectoproteins.

Materials and Methods

Chemicals. Carrier-free ^{125}I (approx. 17 Ci/mg) was obtained from the Radiochemical Center, Amersham, U.K. NaB[^{3}H]H_4 (25 Ci/mmol) was purchased from Centre d'Etude Nucléaire de Saclay, France. [^{125}I]Iodosulfanilic acid (more than 1000 Ci/mmol) was from New England Nuclear, Boston, MA. Acrylamide, bisacrylamide, $(NH_4)_2S_2O_8$ and *N,N,N',N'*-tetramethylethylene diamine were from Biorad Laboratories (Richmond, CA, U.S.A.). Sodium dodecyl sulfate (SDS), 2,5-diphenyloxazole (PPO), 2-mercaptoethanol and $NaIO_4$ were from Merck (Darmstadt, F.R.G.). Nonidet P-40 was from Fluka AG (Buchs, Switzerland). Glucose oxidase of fungal origin, grade I, was from Boehringer Mannheim GmbH (F.R.G.). Lactoperoxidase from milk (67 U/mg protein) was from Sigma (St. Louis, MO, U.S.A.). *N*-Ethylmaleimide was from Aldrich-Europe (Beerse, Belgium). Marker proteins for SDS gel electrophoresis were low molecular weight markers from Pharmacia Fine Chemicals (Uppsala, Sweden). Spectrin from human red blood cells (240 and 220 kdaltons) was added as additional marker. This was obtained from Dr. C. Mombers, Biochemistry Department, State University, Utrecht.

Isolation of blood platelets. Human blood platelets were obtained as buffy coat from the local blood blank within 1½ h from blood donation. These buffy coats were prepared by centrifugation (4°C, 2700 × *g*, 10 min) of 450 ml blood anticoagulated with 50 ml of 0.113 M disodium citrate containing 0.128 M glucose. Red and white blood cells were removed by centrifugation (10 min, 250 × *g*, room temperature) and the supernatant platelet-rich plasma was applied to a Sepharose 2B column equilibrated with Tris-buffered saline/EDTA (10 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, pH 7.40). The platelets eluting in the void volume were washed once in phosphate-buffered saline (1 vol. 0.1 M sodium phosphate buffer, pH 7.40, 9 vol. 0.154 M NaCl)

(10 min, 1000 × *g*, room temperature) and resuspended in the buffer required for labelling. To prevent any contamination by red cell membranes, red stained parts of the pellet were discarded.

Radioactive labelling

Lactoperoxidase labelling [11]. $5 \cdot 10^9$ platelets were resuspended in 0.5 ml of phosphate-buffered saline with 20 mM glucose, 10 μl of lactoperoxidase in the same buffer (1 mg/ml), 10 μl of glucose oxidase also in the same buffer (25 μg/ml) and 5 μl of ^{125}I (approx. 0.5 mCi) were added. The mixture was incubated for 60 min at room temperature. The platelets were then washed four times, first with phosphate-buffered saline, thereafter once with Tris-buffered saline + 0.5% bovine serum albumin and then twice with Tris-buffered saline and finally resuspended in 100 μl Tris-buffered saline and divided into two portions. SDS and β-mercaptoethanol were added to one sample to final concentrations of 1 and 5%, respectively. To the second portion SDS and *N*-ethylmaleimide were added to final concentrations of 1% and 30 mM, respectively. Both samples were heated for 5 min at 100°C and then stored at −80°C until further use.

[^{125}I]Iodosulfanilic acid labelling. This was performed according to the manufacturer's instructions with some modifications. 0.5 mCi of [^{125}I]iodosulfanilic acid together with 10 μl of 50 mM sulfanilic acid were evaporated until near-dryness under a stream of N_2 in a glass tube. The tube was put in melting ice and 10 μl of a fresh solution of 0.05 M $NaNO_2$ and 10 μl of 0.1 M HCl were added and this mixture was incubated for 5 min in the dark; 100 μl of 0.1 M sodium phosphate buffer, pH 7.35, at room temperature were added and the mixture was transferred to room temperature. 0.5 ml of phosphate-buffered saline containing $5 \cdot 10^9$ platelets was added to this mixture and incubated for 15 min at room temperature in the dark. The platelets were washed and stored as described for the lactoperoxidase iodination.

Periodate/[^3H]borohydride labelling [12]. $5 \cdot 10^9$ platelets were resuspended in 1 ml phosphate-buffered saline and incubated for 5 min in ice. 10 μl of 100 mM $NaIO_4$ were added and the mixture was kept in ice for another 5 min. 10 ml of phosphate-buffered saline containing 1 mM EDTA at room temperature were added and this suspension was centrifuged (10 min, 1000 × *g*, room temperature). The platelets were washed again in phosphate-buffered saline and resuspended in 500 μl of the same buffer. 25 μl NaB[^3H]H_4 (approx. 0.5 mCi) were added and this suspension was incubated for 30 min at room temperature. The platelets were washed three times; once with phosphate-buffered saline and twice with Tris-buffered saline and stored as described for the lactoperoxidase iodination.

Two-dimensional electrophoresis

Two-dimensional electrophoresis using isoelectric focussing. This was performed in the first dimension according to the technique of O'Farrell [10] as modified by Clemetson et al. [9]. The sample volume was 100 μl, β-mercaptoethanol was used instead of dithiothreitol and solid urea was added to a final concentration of 9 M. Before application, the sample was spun for 10 min at 1000 × *g* (room temperature) to remove undissolved material. The cylindrical

gels used for isoelectric focussing had a diameter of 3 mm and a length of 10 cm. In general, 600 μg of protein were applied to the first dimension. Isoelectric focussing was carried out for 18 h at 300 V followed by 1 h at 400 V. The second dimension was performed in 7.5% polyacrylamide slab gels (2.6% cross-linking) with 1 cm 3% stacking gel (2.6% cross-linking) at 25 mA/gel (constant current). The gel was 12 cm high, 14 cm wide and 0.75 mm thick. The gels were stained with Coomassie brilliant blue, destained, and prepared for autoradiography and fluorography according to the method of Bonner and Laskey [13].

Two-dimensional electrophoresis (non-reduced/reduced). This was performed according to the method of Phillips and Agin [2] using a 5% gel in the first dimension and a 7.5% gel in the second dimension. As a rule, 150 μg of protein were applied to the first dimension using the platelets treated with SDS and *N*-ethylmaleimide. Cylindrical gels with a length of 10 cm and a diameter of 3 mm were used for the first dimension. Electrophoresis was carried out at 50 V during stacking and at 110 V (constant voltage) until the tracking dye reached the bottom of the gel. The gel for the second dimension was 15 cm high, 14 cm wide and 0.75 mm thick and was run at 25 mA/gel (constant current). A stacking gel of 3% was used for both dimensions.

Results

Coomassie blue staining

The two-dimensional pattern of reduced proteins of whole platelets is shown in Fig. 1. The overall pattern conforms well to that published by Clemetson et al. [8]. The metachromasia of the proteins, which they termed A, was not apparent under our staining conditions as this is only seen after prolonged staining (Clemetson, K.J., personal communication). The resolution of reduced gels was in general much better than that of non-reduced gels which showed more streaking on the basic side, also less protein seemed to have penetrated into the gels.

Autoradiography and fluorography of reduced samples

The patterns obtained with all three labelling techniques are essentially similar which warrants a combined discussion. Fig. 2 shows the pattern of lactoperoxidase labelling and Fig. 3 shows the compound diagram. For the sake of comprehensibility we have divided the whole gel into four regions. (I) Top—180 kdaltons, (II) 180—80 kdaltons, (III) 80—43 kdaltons, (IV) 43 kdaltons—front. To facilitate discussions, we have numbered the various proteins.

Region I. Nine relatively minor radioactively labelled components were found in this region. Two had a more basic location; all others were concentrated around an apparent p*I* of 5.7. Eight proteins demonstrated considerable charge heterogeneity as seen by the linear spots. Protein 5 showed up as a round spot. Protein 6 was variable in labelling intensity and sometimes absent. Protein 7 was not seen with borohydride and iodosulfanilic acid labelling. Protein 3 was strongly labelled with borohydride. Protein 1, immediately beneath the top of the gel, was variable in labelling intensity.

Region II. This region contains the main 'classic' glycoproteins (Ia, Ib, Ic, IIa, IIb, III and IV in the nomenclature of Phillips and Agin [2]). We found 11 components in this region. The major proteins were: 10 (175 kdaltons) which is probably identical to Ia, 12 (150 kdaltons) which represents Ib, 14 (130 kdaltons) which is IIb, 16 (105 kdaltons) which is III and 20 (85 kdaltons) which is IV. The increased resolution of the techniques used allows the identification of three proteins (13, 14 and 15) instead of only one in the region of IIb. IIb (14) and III (16) are the major 'Coomassie blue spots'. Ib (12) is also seen as a 'Coomassie blue spot' but is much more vague. One additional minor component, protein 17, was observed at the acidic side of glycoprotein III. This protein was not labelled with iodosulfanilic acid but a similar spot was seen in borohydride labelling. For all other proteins present in this region, iodosulfanilic acid labelling was similar to lactoperoxidase labelling, but protein 12 (Ib) was less strongly labelled. The borohydride pattern also showed the same proteins, though proteins 13, 15 and 20 (IV) were more stained than with the lactoperoxidase method.

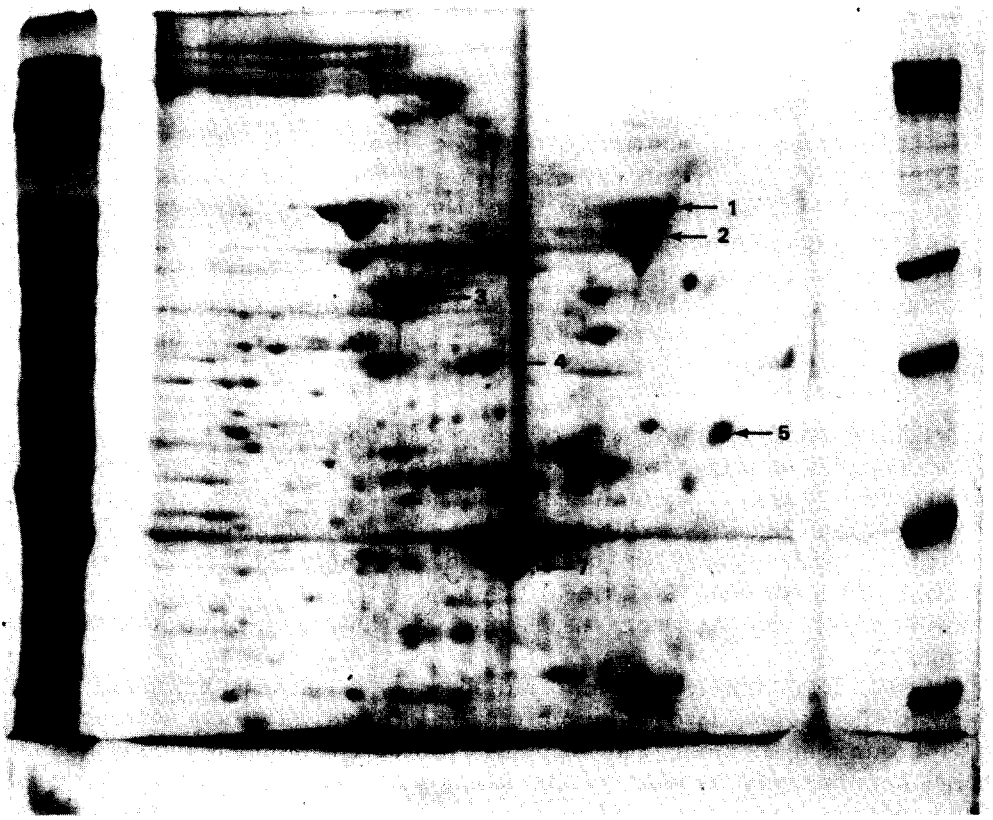

Fig. 1. High-resolution two-dimensional gel of reduced platelet proteins. On the left, a simultaneously run one-dimensional gel is shown. On the right, markers are shown of 240, 220, 94, 67, 43 and 30 kdaltons. Components indicated are: 1, glycoprotein IIb; 2, glycoprotein III; 3, 'protein group A'; 4, albumin; 5, tubulin; 6, fibrinogen chain; 7, actin.

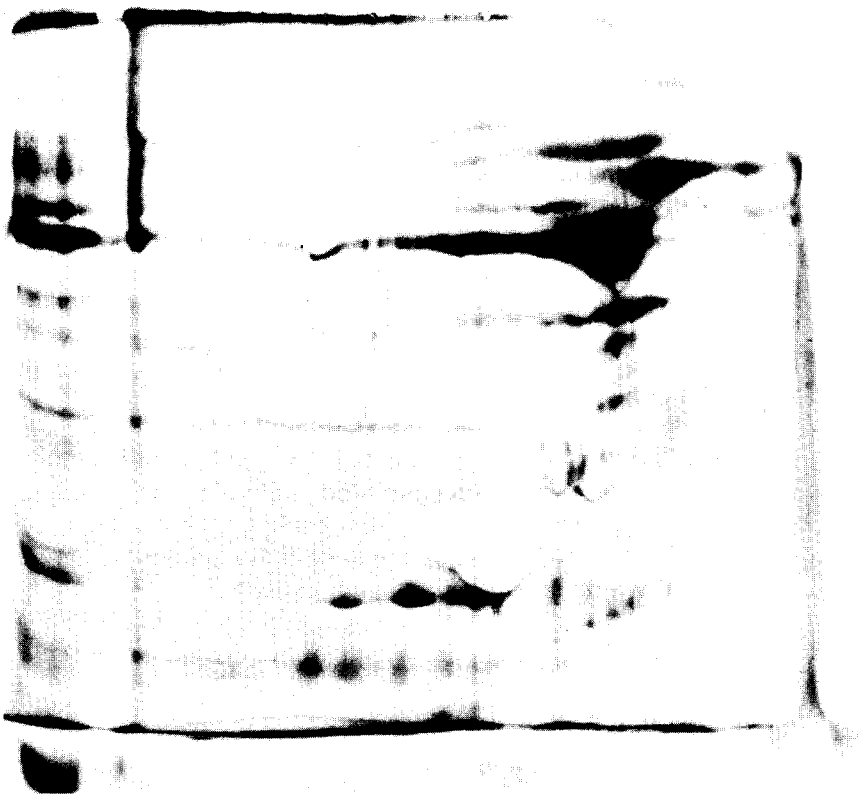

Fig. 2. Autoradiogram of lactoperoxidase-labelled reduced platelets. A one-dimensional gel, simultaneously run, is shown on the left.

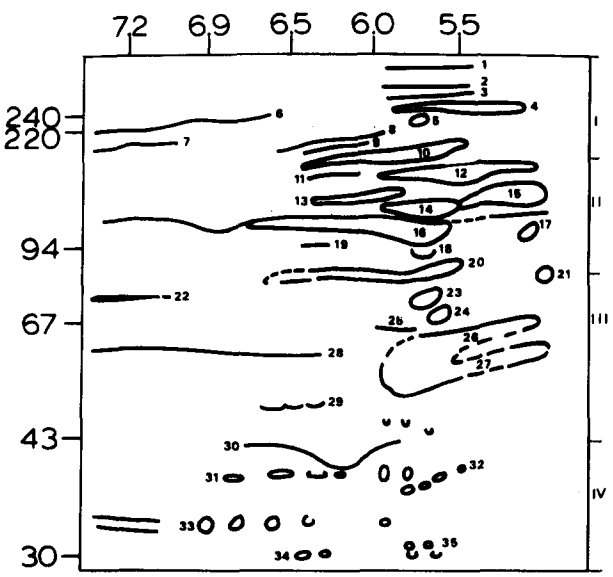

Fig. 3. Composite diagram of radioactively labelled ectoproteins detected in autoradiographs of reduced platelets subjected to high-resolution two-dimensional gel electrophoresis. For components indicated see text. On the right, the four regions mentioned in the text are indicated. (I) Top—180 kdaltons, (II) 180—80 kdaltons, (III) 80—43 kdaltons, (IV) 43 kdaltons—front.

Region III. This region (80—43 kdaltons) contains nine proteins, two of which are basic (22 and 28). Proteins 28 and 29 varied in labelling intensity and were only weakly visible after iodosulfanilic acid labelling. Protein 28 was not labelled with borohydride and protein 29 was only just visible. The two proteins probably represent the α- and γ-chains of fibrinogen which were released during the labelling procedure and remained stuck to the membrane during the subsequent washing. Proteins 22 and 23 are localized at the level of glycoprotein V, the thrombin substrate protein recently described by Phillips and Agin [3] and Mosher et al. [4]. Separate experiments (not shown) were performed to identify glycoprotein V. Thrombin treatment after borohydride labelling removed protein 22. A radioactively labelled protein with an apparent molecular weight of 59 000 was found in the supernatant. This protein was even more basic than protein 22.

Proteins 23, 24 and 26/27 are the major proteins in this region. Proteins 26/27 have a characteristic vertical cross striation in the lactoperoxidase labelling caused by two non-radioactive proteins. Iodosulfanilic acid labelling leads to stronger labelling of protein 22 and a minor extra spot on the basic side of protein 23 is not indicated. Borohydride labelling shows a similar pattern but proteins 23 and 24 are usually only weakly labelled.

Region IV. Six proteins are present in this region. Almost all of them are characterized by the presence of rows of discrete spots representing isoproteins. Protein 30 is present as a sharp line at the front of actin because it has been pushed forward by the large protein mass of actin. Proteins 33 and 34 are absent with borohydride labelling.

Autoradiography and fluorography of non-reduced samples

Fig. 4 shows the radioactive labelling pattern of non-reduced samples. The composite diagram is shown in Fig. 5.

Region I. (Top—180 kdaltons). This contained seven proteins of which proteins 4 and 5 were strongly labelled with borohydride.

Region II (180—80 kdaltons). This contained nine proteins: proteins 8, 9, 11, 12, 13 and 16 were the major ones. Proteins 12 and 16 correspond to Coomassie blue spots indicating that they represent glycoproteins IIb and III. Glycoprotein IV was included in protein 16.

Region III (80—43 kdaltons). This contained six proteins. Protein 17 is glycoprotein V as shown by thrombin treatment. Proteins 18 and 20 were not always seen but this was not dependent on the labelling technique.

Region IV (43 kdaltons—front). This contained three proteins characterized by their appearance as rows of discrete spots. Protein 25 was quite variable in its location.

Autoradiography and fluorography of reduced/non-reduced gels

The two-dimensional electrophoresis with SDS-polyacrylamide gel electrophoresis of non-reduced samples in the first dimension and SDS-polyacrylamide gel electrophoresis after reduction in the second dimension was performed in order to facilitate the identification of the major glycoproteins. Fig. 6a shows the pattern after staining with Coomassie blue and Fig. 6 shows the radioactive labelling pattern obtained by borohydride labelling. Comparison with the

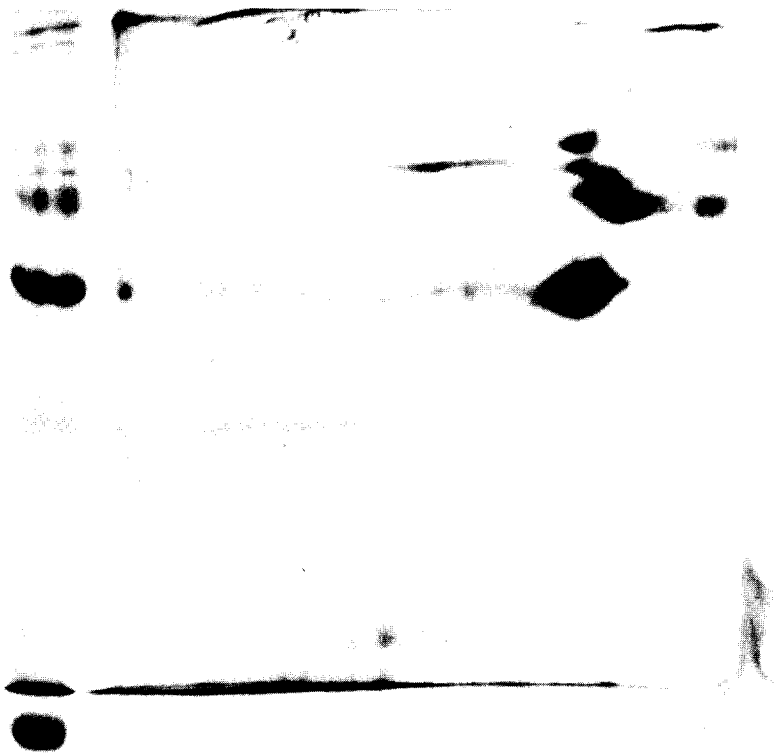

Fig. 4. Autoradiogram of lactoperoxidase-labelled non-reduced platelets. A one-dimensional gel, simultaneously run, is shown on the left.

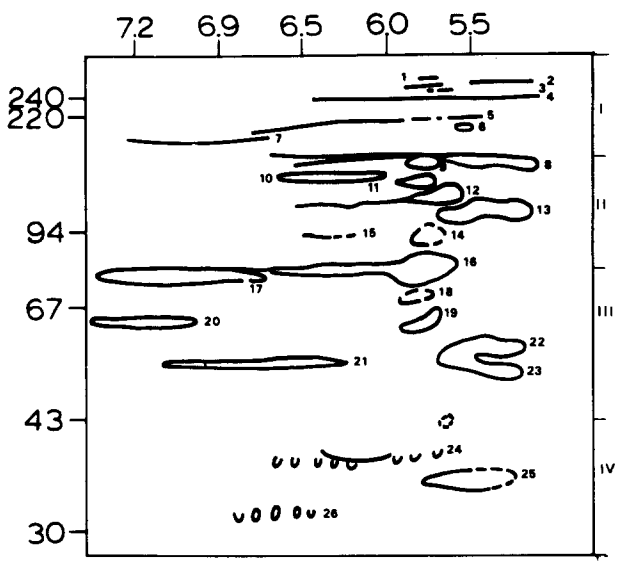

Fig. 5. Composite diagram of radioactively labelled ectoproteins from autoradiograms of non-reduced platelets subjected to high-resolution two-dimensional gel electrophoresis. For components indicated see text. On the right, the four regions mentioned in the text are indicated. (I) Top—180 kdaltons, (II) 180—80 kdaltons, (III) 80—43 kdaltons, (IV) 43 kdaltons—front.

patterns obtained by Phillips and Agin [2] showed that a good resolution of the major glycoproteins was obtained. Several additional spots were observed; however, this could be due to the higher label intensity that we attained.

The high molecular weight proteins (top—180 kdaltons) were not well labelled with lactoperoxidase and iodosulfanilic acid, but better labelling was obtained with borohydride. Six proteins were seen in this region. They were all located slightly above the diagonal, indicating the presence of intra-chain disulfide bonds. Eighteen proteins were observed between 170 and 90 kdaltons in the second dimension, indicating that the resolution of this method may be even better than that of the two-dimensional technique with isoelectric focussing in the first dimension. The proteins Ibβ, Icβ and IIbβ found by Phillips and Agin [2] were the only small molecules derived from proteins in this region. Icβ was not labelled in the lactoperoxidase procedure but was labelled with iodosulfanilic acid and weakly with borohydride. The glycopro-

Fig. 6a.

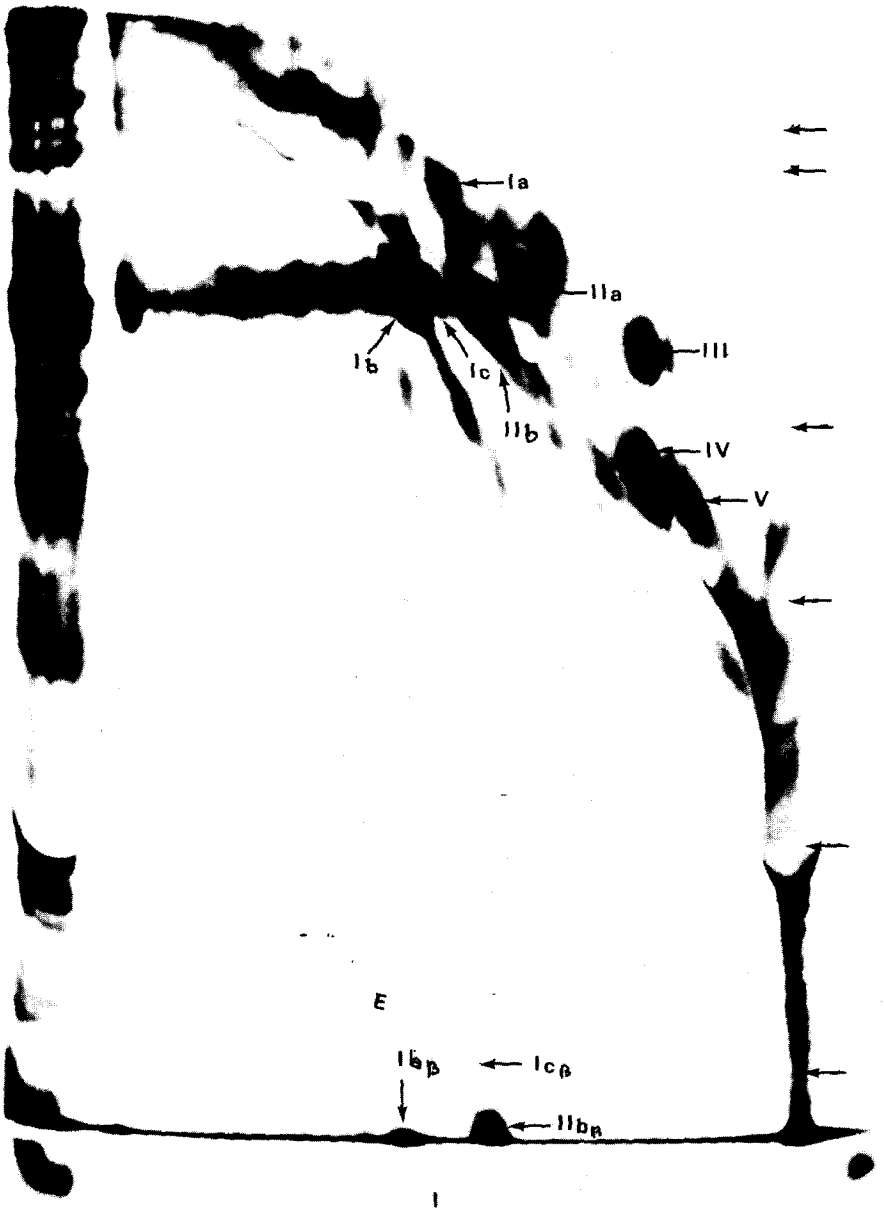

Fig. 6. (a) Coomassie blue staining patterns of two-dimensional non-reduced/reduced gels. The first dimension is from left to right. Components indicated are: (1) thrombospondin, (2) fibrinogen, (3) glycoprotein IIb, (4) glycoprotein III, (5) glycoprotein Ib, (6) glycoprotein Ic, (7) glycoprotein Ibβ (8) glycoprotein IIbβ, (9) actin. (b) Autoradiogram of borohydride-labelled platelets. The black ribbon to the left of Ib is artifactual. The arrows on the right indicate the molecular weight markers mentioned in Fig. 1.

teins Ibα, IIbα and Icα were lying beneath the diagonal as well as seven minor components. Glycoprotein IV was located exactly on the diagonal whereas Ia, IIa, III and four other components were lying above the diagonal.

In the area below 90 kdaltons, glycoprotein V was found slightly above the

diagonal. The front of the first dimension was at about 55 kdaltons. Evidence for labelling of thrombospondin and fibrinogen was only found in the lactoperoxidase-labelled sample. Such labelling was not seen with the iodosulfanilic acid and borohydride methods.

Discussion

A considerable advantage of the technique of O'Farrell [10] is that it combines high resolution with reproducibility. Because of this, proteins can be defined by their location in these gels and this has been used for the identification of single proteins produced by bacterial mutants [10]. In our hands, reproducible protein and radioactive labelling patterns were obtained, particularly for reduced samples. This may prove to be of great importance for unanimous and unambiguous classification and nomenclature.

Contamination by other blood cells was avoided by discarding part of the platelet button. The influence of proteolysis was minimized by boiling the platelets in the presence of 1% SDS with either 5% β-mercaptoethanol or 30 mM *N*-ethylmaleimide. Radioactively labelled secreted platelet proteins were sometimes observed in lactoperoxidase-labelled cells. Secretion was probably enhanced by H_2O_2 formation [15] but centrifugation may also induce platelet release [16]. This may also play a role in other labelling techniques. Some indications for this were found in iodosulfanilic acid labelling but not with borohydride labelling. The secreted proteins were thrombospondin and fibrinogen. They apparently stick to platelets after labelling. A washing step with 0.5% bovine serum albumin reduced this, but did not entirely abolish it. Major contamination with intracellular labelled proteins is unlikely because all three procedures show similar results though the periodate-borohydride procedure only labels sialic acid residues of sugar chains. All three procedures have also been utilized with a variety of other cells with very little labelling of intracellular proteins. Cell fractionation studies after iodosulfanilic acid labelling (not shown) showed no labelled protein characteristic of the cytosol fraction.

Utilizing the non-reduced/reduced gel system, Phillips and Agin [2] were able to identify seven glycoproteins. We found a similar pattern to that described by these authors, but the utilization of higher labelling rates and different labels enabled us to identify 18 proteins in the same region (for a discussion see below). In one-dimensional gels, Phillips and Agin [2], Mosher et al. [4], McGregor et al. [14], Cooper et al. [7] and Apitz-Castro et al. [18] have recently identified a whole new range of glycoproteins. All these studies were performed on reduced samples which gave improved resolution and reproducibility. These data are compared with ours on two-dimensional gels of reduced samples in Table I. Values for molecular weights should be viewed with caution because these data may vary considerably for glycoproteins depending on the techniques used.

The identification of the major glycoproteins numbered I—IX by Phillips and Agin [2] is relatively easy. Most of these proteins have also been found by other investigators (Table I). Table II shows the spots in the reduced and non-reduced gels representing these glycoproteins.

TABLE I

PATTERN OF REDUCED ECTOPROTEINS DESCRIBED IN THIS PAPER AS COMPARED TO DATA OBTAINED IN ONE-DIMENSIONAL SYSTEMS IN THE LITERATURE

The numbers for each region refer to those used in Fig. 3. Where given, values are expressed in kdaltons. HMW, high molecular weight ectoprotein.

Reference	Region I							Region II									
	1	2	3	4	5/6	7/8	9	10	11	12	13	14	15	16/17	19	18	20/21
This paper					230	200								105			85
	360	300	280	260	230	200	185	175	155	150	135	130	122	105	97	93	
McGregor et al. [14]				250	225	215	197	165		148		133	119	107			93
McGregor et al. [17]			268	241	222	200	192	148		138		130	114	108			93
Apitz-Castro et al. [18]	HMW	310		250	218		190	160					117	106			95
Mosher et al. [4]						190		145		132			122	112			90
						210		165									
Phillips and Agin [3]	three bands							Ia		Ibα			IIbα	III			IV

Reference	Region III						Region IV				
	22	23/a	24	25/26	28	27	29	30	31	32	33	34/35
This paper		76		65								29
	78	75	67	65	62	58	48	41	37	36	32	29
McGregor et al. [14]	84			67		59		47		36		
McGregor et al. [17]	77			70		64		48	42	36		
Apitz-Castro et al. [18]	89	79		68		54		45		39		
Mosher et al. [4]	85					57		42			34	26/22
Phillips and Agin [3]	V			VI		VII		VIII		IX		

TABLE II

IDENTIFICATION OF MAJOR GLYCOPROTEINS

Glycoprotein	No. in reduced gel (Fig. 3)	No. in non-reduced gel (Fig. 5)
Ia	10	11
Ib	12	8
Ic	13	10
IIb	14	12
IIc	15	13
III	16	16
IV	20	16
V	22	17 Thrombin substrate
VI	26	22
VII	27	23
VIII	30	24 HLA*-complex?
IX	33	26
Ibβ		
Icβ	35	—
IIbβ		

* HLA, human leukocyte antigen.

The identification of glycoprotein IIc is tentative. It is sited at the acidic side of glycoprotein IIb in reduced gels and might then represent glycoprotein IIa. In contrast, however, it does not change in apparent molecular weight on reduction. A glycoprotein with similar characteristics was termed IIc by Clemetson et al. [9]. They suggest that IIa coincides with Ib in the reduced system.

The high molecular weight glycoproteins are similar in the reduced and non-reduced states (though the molecular weight may increase slightly (Fig. 7)). Proteins 6 and 8 of Fig. 3 were not observed in the non-reduced system. One of these may represent thrombospondin, which as mentioned may stick to the platelet after release and become labelled in lactoperoxidase labelling.

The possibility exists that several of the high molecular weight glycoproteins may represent aggregates of lower molecular weight species. Evidence for similarity in peptide maps of a 210 kdalton protein and glycoprotein I complex with a molecular weight of 150 000 was recently presented by Nachman et al. [19].

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