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DISTRIBUTION OF PLATELET GLYCOPROTEINS AND PHOSPHOPROTEINS IN HYDROPHOBIC AND HYDROPHILIC PHASES IN TRITON X-114 PHASE PARTITION

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Platelets, either unlabelled, surface-labelled by the periodate NaB^3H_4 method or metabolically labelled with ^{32}P were solubilized in Triton X-114 and partitioned into aqueous and detergent phases. The phases were analysed by two-dimensional polyacrylamide gel electrophoresis followed by silver-staining, fluorography or indirect autoradiography. Each of the phases contains a distinct set of proteins. The surface-labelled glycoproteins partition into the hydrophobic phase with the notable exceptions of glycoproteins Ib and $GP_{17}^{5.8-6.5}$ and minor amounts of a few others. The phosphoproteins which undergo increased phosphorylation on platelet activation in general separate in the hydrophobic phase, while higher molecular weight phosphoproteins were principally in the hydrophilic phase. This method might be used as a first step in purifying many platelet components.

Introduction

Platelets play a vital role in haemostasis and in thrombosis and many of the receptors involved in these processes are glycoproteins of the plasma membrane. Several internal proteins are phosphorylated when platelets are activated and may be important mediators of the shape-change, secretion and contractile responses. Thus, improved methods for the isolation of these components and a better knowledge of their properties are essential for advances in structural and functional studies.

Recently, a simple method has been described for separating hydrophobic and hydrophilic proteins based upon phase-partitioning with Triton X-114 [1]. Briefly, the method is based upon solubilizing the protein in the non-ionic detergent Triton X-114, followed by heating the aqueous solution above the cloud point of Triton X-114 (about 30°C). The solution then separates into two phases, a Triton X-114 phase (present as fine droplets) containing the hydrophobic proteins and an aqueous phase containing the hydrophilic proteins. By centrifugation, the Triton X-114 phase, together with the hydrophobic proteins, can be recovered in a small volume, and the hydrophilic proteins are left in the aqueous phase.

This method has been applied to platelets [2] but the authors concentrated on the isolation of glycoproteins IIb and IIIa and have not studied the separation of the platelet proteins in detail.

In this study we have examined the distribution of platelet proteins separated by the Triton X-114 method by two-dimensional gel electrophoresis and silver-staining. We have used surface-labelling with the periodate NaB^3H_4 method [3,4] to identify surface glycoproteins and ^{32}P metabolic labelling [5] to identify phosphoproteins.

A provisional account of some of these results has appeared in abstract form [6].

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Abbreviation: Mes, 4-morpholineethanesulphonic acid.

Methods and Materials

Isolation of human blood platelets. Platelets were isolated from citrate-treated blood collected for the Central Laboratory of the Swiss Red Cross, within 20 h after collection [7]. The buffy coats were transferred into a buffered glucose solution to give platelet-rich plasma containing about 20 mM glucose, 12 mM sodium phosphate buffer and about $4 \cdot 10^9$ platelets per ml [8]. The platelets were isolated by centrifugation and were washed once with 12 mM sodium citrate/30 mM glucose/120 mM NaCl/10 mM EDTA buffer (pH 6.5) and twice with 10 mM Tris-HCl/150 mM NaCl/10 mM EDTA buffer (pH 7.4).

Surface-labelling by the periodate/[^{3}H]-NaBH$_4$ method. Platelets were isolated from platelet rich plasma, washed and surface-labelled by the periodate/[^{3}H]NaBH$_4$ method [3] as modified by Steiner et al. [4].

Metabolic labelling with ^{32}P. Platelets were isolated from platelet-rich plasma, suspended in 12 mM sodium citrate/30 mM glucose/120 mM NaCl/10 mM EDTA buffer (pH 6.5), centrifuged for 10 min at $1100 \times g$, then suspended in 15 mM 4-morpholineethanesulphonic acid (Mes, Sigma, St. Louis, USA)-HCl/15.5 mM glucose/140 mM NaCl/2 mM EDTA buffer (pH 6.7). The platelets were counted on a TOA platelet counter (TOA Medical Electronics Co., Japan) and diluted with the Mes/glucose/NaCl/EDTA buffer to $2 \cdot 10^9$ platelets/ml. Phosphorylation was performed by incubating sodium [^{32}P]orthophosphate (Amersham International, 1 mCi/ml) in sterile Mes/glucose/NaCl/EDTA buffer with 5 ml of platelet suspension at room temperature for 1 h with occasional shaking. The platelets were centrifuged for 10 min at $1100 \times g$ and washed twice with Mes buffer.

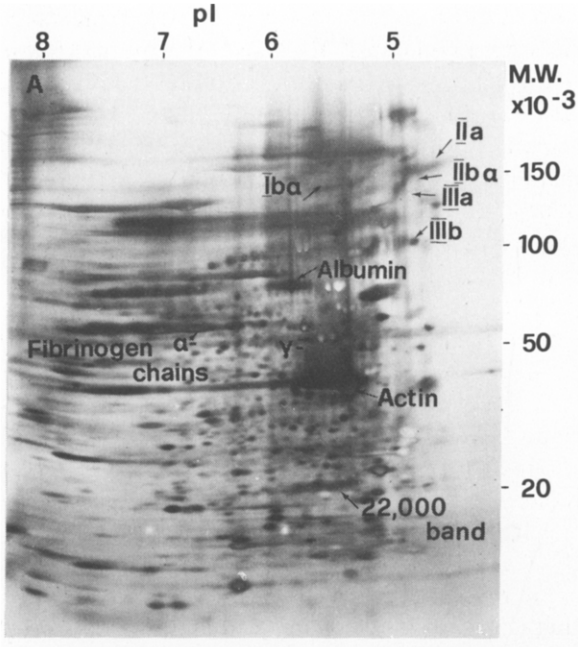
Triton X-114 phase separation. Platelets (unlabelled, ^{3}H-labelled or ^{32}P-labelled) were solubilized in 1% Triton X-114/10 mM Tris-HCl/154 mM NaCl/20 mM EDTA/2 mM phenylmethylsulphonyl fluoride/100 $\mu g \cdot ml^{-1}$ leupeptin (Sigma) for 30 min at 0°C (on ice) (1 ml/10^9 platelets). The solution was then centrifuged for 30 min at $100\,000 \times g$ at 0°C and the supernatant was used for phase separation. The supernatant could also be stored at −25°C but on thawing developed a fibrin-like precipitate which could be pelleted by brief centrifugation ($1100 \times g$, 5 min). Removal of this precipitate did not affect the results. Phase partition was carried out essentially as described by Bordier [1]. Briefly, the Triton X-114 supernatant (2 ml) was layered on to 3 ml of 6% sucrose/10 mM Tris-HCl/154 mM NaCl/0.06% Triton X-114/1 mM EDTA solution in a 10 ml polystyrene tube. The tube was warmed to 37°C for 5 min and then centrifuged for 10 min at $1100 \times g$. The upper layer was carefully removed, mixed with a further 2 ml of 1% Triton X-114/10 mM Tris-HCl/154 mM NaCl/20 mM EDTA at 0°C and then layered back onto the sucrose cushion. After warming to 37°C for 5 min and centrifuging for 10 min at $1100 \times g$ the upper layer was collected as the aqueous phase and the combined pellet as the Triton X-114 phase.

Polyacrylamide gel electrophoresis. Two-dimensional polyacrylamide gel electrophoresis (isoelectric focusing, 5–20% gradient polyacrylamide gel electrophoresis) was performed as described earlier [9] except that 10–50 μg protein was loaded on each gel. Reduced conditions were used throughout. The gels were silver-stained by the method of Sammons et al. [10] or were prepared for fluorography [11] or indirect autoradiography [12].

Results

Comparison of the silver-stained two-dimensional polyacrylamide gel electrophoresis results obtained with whole platelets, Triton X-114 phase and aqueous phase (Fig. 1A, B and C, respectively) shows clearly that the Triton X-114 phase and the aqueous phase contain quite distinct sets of proteins and that most of these can also be recognised in the gel of the whole platelets. The Triton X-114 phase gel shows heavy staining of about six spots in the major glycoprotein region and a heavily stained band at a molecular weight of about 22000. This phase also contains some actin. There are many other minor spots which cannot yet be identified but which are evidently hydrophobic components.

In the polyacrylamide gel electropherogram of the aqueous phase the principal stained spot is actin but several other components are enriched compared to whole platelets. These include fibrin-



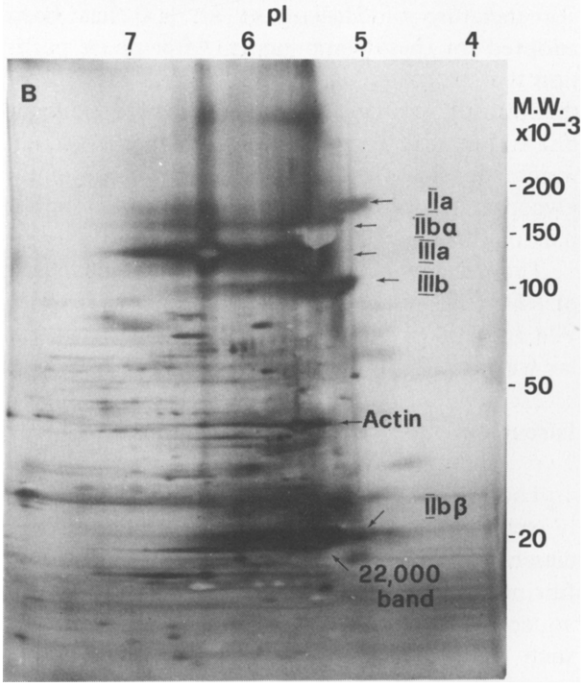
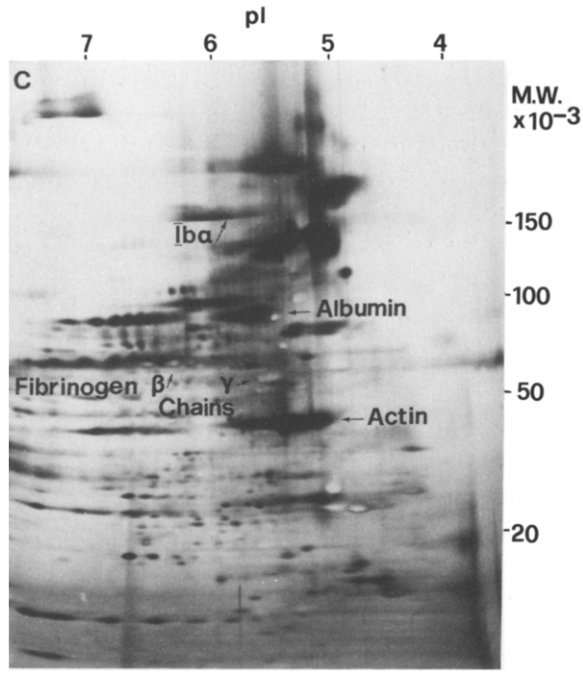


Fig. 1. Two-dimensional polyacrylamide gel electrophoretic separations of platelet proteins silver-stained by the Sammons et al. [10] method. (A), whole platelets; (B), Triton X-114 phase from phase-partition of platelet proteins; (C), aqueous phase from Triton X-114 phase-partition of platelet proteins.



ogen, serum albumin and a high molecular component which is probably myosin heavy chain. The identity of these components was established by comparison with previous results [9,13] and also by the characteristic colours given by the Sammons silver-staining method [10] e.g., orange for fibrinogen chains.

Surface-labelling with periodate NaB^3H_4 gives gel patterns which are simpler to interpret than the silver-staining patterns, since only the plasma membrane glycoproteins are visible. Fig. 2A shows the pattern obtained by labelling whole platelets with the nomenclature indicated. Fig. 2B shows the fluorogram obtained with the two-dimensional gel of the Triton X-114 phase. Most of the glycoproteins present in whole platelets are present but there are some noticable absences, in particular the α and β subunits of glycoproteins Ib and $GP_{17}^{5.8-6.5}$. Fig. 2C shows the fluorogram of the two-dimensional gel of the aqueous phase. The principal spot is glycoprotein Ibα; Ibβ and $GP_{17}^{5.8-6.5}$ are also present. There are, however, some other glycoproteins detectable, one in the position of IIbα and a further minor glycoprotein which is probably glycoprotein IIa. There are also faint spots corresponding to glycoproteins IIbβ, VIa, VIb and glycocalicin.

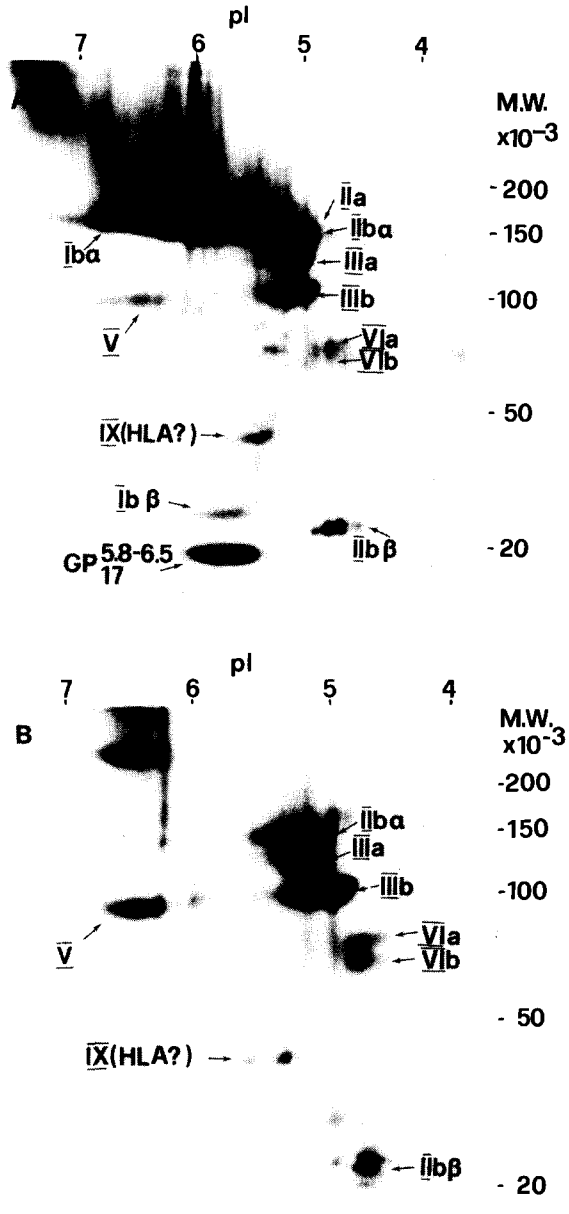


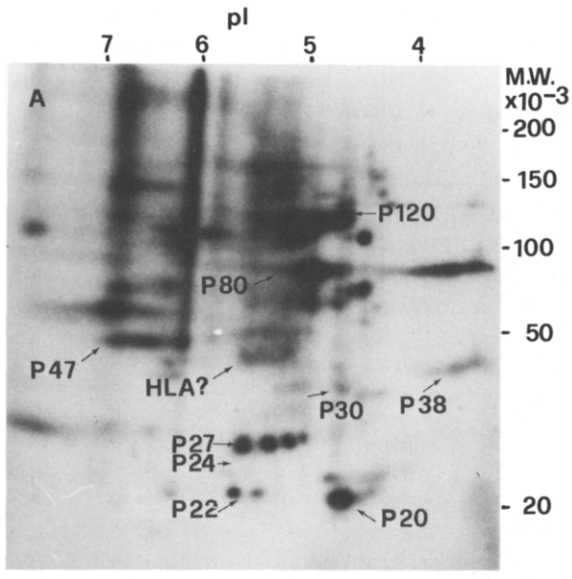
Fig. 2. Fluorograms of two-dimensional polyacrylamide gel electrophoretic separations of platelet membrane glycoproteins labelled by the periodate NaB^3H_4 method. (A), whole platelets; (B), Triton X-114 phase from phase-partition of platelet proteins; (C), aqueous phase from Triton X-114 phase-partition of platelet proteins.

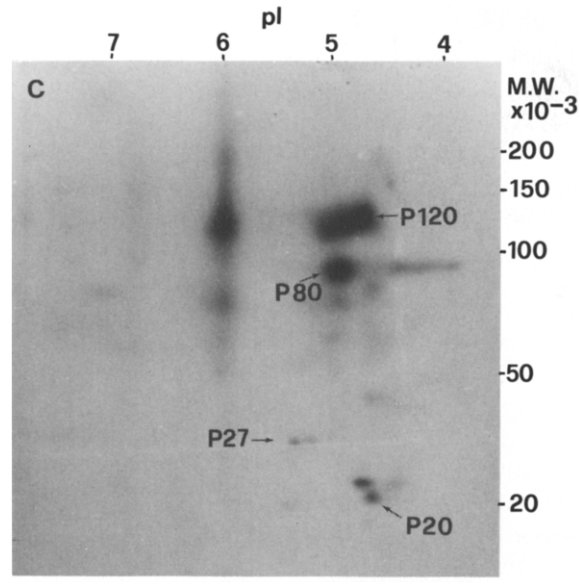
Metabolic labelling with ^{32}P gave a pattern with whole platelets (Fig. 3A) which has some superficial similarity to that obtained with surface-labelling (Fig. 2A). Only a few of the phosphoproteins have been characterized and in the figures the nomenclature of Haslam et al. [14] has been adopted for these components. After phase partition the similarity is no longer seen, as the bulk of the phosphoproteins are found in the aqueous phase (Fig. 3C). This includes the higher molecular weight phosphoproteins which come in the major glycoprotein region of the gel but also a minor part of P27 and P20 (myosin light chain).

The detergent phase (Fig. 3B) contained most of P47, P27 and P20 (myosin light chain) but also P24 and P30 and minor amounts of higher molecular weight phosphoproteins.

Discussion

The Triton X-114 phase-partition method clearly separates platelet proteins into two discrete classes. As expected, hydrophilic proteins such as fibrinogen, albumin and actin are found in the aqueous phase, while known hydrophobic proteins such as the membrane glycoproteins are found principally in the detergent phase. There are, however, some exceptions; some actin is found in the detergent phase and some of the glycoproteins, notably glycoprotein Ib, are found in the aqueous phase. Newman et al. [2] used Triton X-114 to separate platelet proteins and reported that glyco-





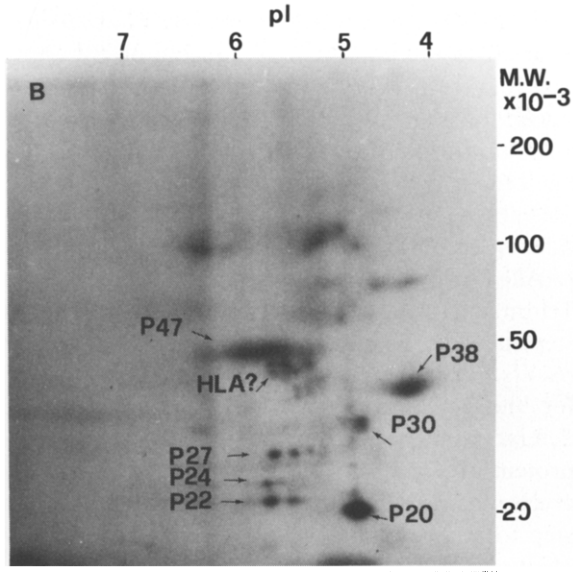


Fig. 3. Indirect autoradiograms of two-dimensional gel electrophoretic separations of platelet phosphoproteins metabolically labelled with ^{32}P. (A), whole platelets; (B), Triton X-114 phase from phase-partition of platelet proteins; (C), aqueous phase from Triton X-114 phase-partition of platelet proteins.

proteins IIb/IIIa and proteins with molecular weights of 77 500 and 22 000 on one-dimensional gel electrophoresis were enriched in the detergent phase. Our results confirm the presence of glycoprotein IIb/IIIa in the Triton phase and indicate that the 77.5 kDa component may well be glycoprotein IIIb and glycoprotein V. The 22 kDa component is markedly enriched in the Triton phase compared to the whole platelet extract (Fig. 1A, B) but is not surface-labelled as a glycoprotein (compare Figs. 1B and 2B). It may therefore be present in the platelet membrane on the cytoplasmic face. Triton X-114 is a non-denaturing detergent and does not dissociate protein complexes. It is well known [15,16] that glycoproteins IIb/IIIa associate with the platelet cytoskeleton when platelets are activated and, thus, actin may be brought into the hydrophobic phase via a complex with glycoproteins IIb/IIIa.

There has been some controversy about which glycoproteins are integral membrane components and which are peripheral with claims that glycoprotein Ic [17] and glycoprotein V [18] are peripheral and easily removed from the platelet surface. The results here show that glycoprotein V is clearly hydrophobic. Glycoprotein Ic is not strongly labelled and is difficult to distinguish under reducing electrophoresis conditions. However, comparison with polyacrylamide gels run under non-reducing conditions (not shown) indicate that glycoprotein Ic is also found in the detergent phase. Glycoprotein Ib, which is normally a hy-

drophobic integral membrane component [19,20], is found in the aqueous phase as was also noted by Solum et al. [21]. This may possibly be due to either the very high sugar content of glycoprotein Ib [22,23] or to formation of complexes between glycoprotein Ib and other platelet components [24].

The other glycoproteins found in the aqueous phase may also represent complexes with hydrophilic components or possibly with glycoprotein Ib itself. An example of the latter is $GP_{17}^{5.8-6.5}$ which is known to complex with glycoprotein Ib [25,26] and is also found in the aqueous phase.

Little is known of the properties of the phosphoproteins with the exception of P47 which has recently been isolated [27,28] and P20 which has been shown to be myosin light chain [29]. The degree of phosphorylation of P47, P27, P24 and P20 change on platelet activation [14] and the subcellular distribution of the phosphoproteins has been determined [30]. It is interesting that P47 was found almost exclusively in the cytosol while P24 and P22 were enriched in the particulate fractions. The distribution of P20 was not determined, since it was largely dephosphorylated under the fractionation conditions used, but a futher phosphoprotein with a molecular weight of about 34000 was found completely in the particulate fractions. This may be the P38 indicated in Figs. 3A and B. P27 was not detected in the above mentioned study [30].

A phosphoprotein corresponding to P22 was found in isolated platelet membrane vesicles [31]. It has been suggested that phosphorylation of P22 is linked to active transport of Ca^{2+} out of the platelet cytosol [13,30].

The results obtained have shown that most of the phosphoproteins found in the particulate fractions from platelets show hydrophobic properties in the Triton X-114 phase separation. P47, which was found in the supernatant fraction [27,30], is also hydrophobic in the Triton X-114 separation, however, it was previously shown that it binds to phenyl-Sepharose, and this was used as a purification step [27].

The phosphorylation of myosin light chain (P20) has been shown to be related to an increase in myosin ATPase activity. P27 was shown to undergo rapid phosphorylation/dephosphorylation when platelets were activated [32]. Most of P20 was found in the Triton X-114 phase while P27 was present in both phases. It is interesting to note that many of the phosphoproteins thought to be involved in regulatory functions (P47, P24, P22, P20) show hydrophobic properties.

Sixma et al. [17] suggested that a protein just in front of actin might be the heavy chains of the HLA antigens. This protein which we have called glycoprotein IX [33], partitions into the Triton X-114 phase (Fig. 2B) and is also phosphorylated (Fig. 3A and B). It showed the typical heterogeneity found with HLA heavy chains which are also known to be phosphorylated [34].

The results show that the Triton X-114 phase-partition method can be applied to complex systems such as whole cells and that it gives a reproducible separation into two classes of proteins. In general the separation into hydrophobic and hydrophilic proteins follows the properties of these components and may be used as a criterion where these are not yet known. There are, however, exceptions to this rule such as the case of glycoprotein Ib, which may be due to the extremely acid nature of part of the molecule or else to the formation of very tight complexes. In either case the Triton X-114 method gives valuable information about the solubility properties of such molecules in an undenatured state.

As already shown by Newman et al. [2], the Triton X-114 phase provided a useful purification step in the isolation of glycoprotein IIb/IIIa. Our results indicate that it provides a similar first step for the isolation of many glycoproteins in the detergent phase while, in the aqueous phase, glycoprotein Ib is separated from most of the other glycoproteins. It also provides a useful separation step for many of the phosphoproteins.

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