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Amphiphilic and hydrophilic nature of sheep and human platelet phosphotyrosine phosphatase forms

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Abstract

To date, although at least 75 different PTPases (protein-tyrosine-phosphate-phosphohydrolase, EC 3.1.3.48) have been identified, those detected in platelets are rather scarce. Based on previous results from our laboratory, we investigated the existence of new PTPases in platelets. Triton X-114 phase partitioning of Triton X-100-solubilized human and sheep platelet membranes allowed PTPase to be recovered in the detergent-rich (40–35%, respectively) and -poor phases (60–65%, respectively). Sedimentation analyses of both phases from the sheep species revealed hydrophilic 6S and 3.7S, and amphiphilic 7.5S and 10.3S PTPase forms. Sedimentation analyses of human platelet membrane-associated or cytosolic PTPase revealed hydrophilic 6.7S and 4.3S, and amphiphilic 5.5S and 10.8S forms, or hydrophilic 4S, 5.9S and 6.9S forms, respectively. Western blot analysis using monoclonal antibodies (MoAb) against human PTP1B, PTP1C, PTP1D and RPTPα (mouse anti-human PTPase MoAbs) showed that RPTPα was not present in platelets and that the PTP1C type and PTP1D type (but probably not the PTP1B type) were expressed in sheep species. Immunoblots also revealed that all PTPases detected were mainly membrane-associated, with similar percentages of cellular distribution in both species. All PTPases were mainly recovered in the detergent-poor phases from the Triton X-114 phase partitioning, although PTP1D from human species was also significantly present (30%) in the detergent-rich phase. Additionally, all PTPases sedimented within the same PTPase peak in sucrose gradients (sedimentation coefficients around 4S). These findings indicate that amphiphilic and hydrophilic PTPases different from PTP1B, PTP1C, PTP1D or RPTPα, with higher sedimentation coefficients and with higher activity when *O*-phosphotyrosine or a synthetic peptide phosphorylated on tyrosine were used as substrates, are present in platelets.

Keywords: Protein tyrosine phosphatase; Amphiphilic form; Hydrophilic form; Platelet

1. Introduction

Protein tyrosine phosphorylation is an important process in the regulation of cell growth, differentiation, and proliferation [1] and has been implicated in most biochemical events that involve transmembrane signaling. Tyrosine phosphorylation is a dynamic

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme linked immunosorbent assay; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; MoAb, monoclonal antibody; PTKase, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; PVDF, polyvinylidene difluoride; SH2, Src homology 2

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and reversible process regulated by the activities of both protein tyrosine kinases (PTKases) and protein tyrosine phosphatases (PTPases). PTKases are not only found in proliferative and undifferentiated cells, but also in non-proliferative, terminally differentiated cells such as platelets [2,3], where numerous cytosolic PTKases have been described [4–7]. In fact, agonist-stimulated platelets show rapid changes in the tyrosine phosphorylation of many proteins [3,8]. Their role in the regulation of tyrosine phosphorylation has led phosphotyrosine phosphatases (PTPases) to become more appreciated as important regulators of cell function [9,10]. In this sense, many studies have demonstrated the presence of PTPases in different cells and tissues [11,12]. The family of tyrosine phosphatases is very diverse and multimembered. PTPases occur in soluble forms, membrane-associated forms, and receptor-like forms [13], and, in several cases, these distinct multienzyme forms can be separated chromatographically [14]. Non-transmembrane PTPases have N- or C-terminal domains that are important for enzymatic regulation and intracellular localization (i.e. PTP1B), or Src homology 2 (SH2) domains for protein-protein interactions (i.e. PTP1C).

To date, no receptor-type transmembrane PTPases have been found in platelets, although several other known PTPases have been described so far, including PTP1B (a substrate of calpain in thrombin-stimulated platelets) [15], PTP1C (also known as SHPTP1, HCP, SHP1, or PTP6N), an enzyme expressed predominantly in hematopoietic cells, and PTP1D (also known as SHPTP2, SHPTP3, PTP2C, or Syp), another non-transmembrane PTPase with two SH2 domains [16]. Additionally, a membrane-associated 53 kDa PTPase [17] and a 104–106 kDa non-transmembrane PTPase called MEG (also known as PTPH1), expressed by the human megakaryoblastic leukemia cell line MEG-01 [18] and that seems to be activated by proteolysis in human platelets [19], have also been described in this cell type. Nevertheless, it has been reported that in human platelets, most PTPase activity can be membrane bound [20], a phenomenon that we have also recently shown to occur for sheep species [21].

Recently, previous experiments from our laboratory employing sedimentation analysis of Triton X-100-solubilized sheep platelet membranes in sucrose gradients made up with Triton X-100 or Brij 97 led us to propose that sheep platelets contain different amphiphilic and hydrophilic forms of PTPases [21]. In this study, we corroborate and extend our observations to human platelet species, confirming that our results are not species specific. We also demonstrate that platelets have hydrophilic and amphiphilic PTPases displaying sedimentation coefficients that are clearly different from those PTPases already known to be present in platelets (PTP1B, PTP1C and PTP1D), enzyme forms that cosediment basically in the same place of the sucrose gradient.

2. Materials and methods

2.1. Preparation of platelets and isolation of crude membranes

Sheep platelets were isolated from the fresh blood of adult sheep (*Ovis aries* L.) sacrificed at a local slaughterhouse as described earlier [22] using 15 mM citric acid, 88 mM trisodium citrate, 16 mM NaH_2PO_4, 140 mM glucose, 2 mM adenine as anticoagulant. Sheep platelets were finally resuspended in buffer A (10 mM HEPES, pH 6.5, 0.2 mM EGTA, 5 mM KCl, 5.5 mM glucose and 145 mM NaCl) and washed twice in the same buffer. Human platelets were obtained from platelet transfusion packs obtained from the Transfusion Services of the University Hospital of Salamanca. Platelet concentrates were spun at $100 \times g$ for 20 min at 4°C to remove the majority of leukocyte and erythrocyte contaminants. Platelet-rich plasma was then centrifuged at 4°C at $2600 \times g$ for 10 min. The pellet was resuspended in buffer A, recentrifuged, and this step repeated twice. After washing, sheep or human platelets were resuspended in buffer A without NaCl but containing protease inhibitors (1 mM phenylmethylsulfonylfluoride and EDTA, 1 mg/ml pepstatin, leupeptin and aprotinin) to obtain homogenates and crude membranes as indicated previously [21].

2.2. Solubilization of membrane-bound PTPase

Crude membrane preparations were resuspended in buffer A and a stock solution of Triton X-100 (10% w/v) was added to obtain a final concentration

of 1% (w/v), keeping a detergent/protein ratio (w/w) of 7. Mixtures were shaken for 30 min at 4°C, diluted with buffer A to obtain a detergent concentration of 0.5% (w/v), and finally centrifuged at $105\,000 \times g$ for 1 h. The supernatant contained the detergent-solubilized PTPase.

2.3. Separation of amphiphilic and hydrophilic forms of PTPase

Triton X-100-solubilized sheep platelet membranes were subjected to phase partitioning with precondensed Triton X-114, as described elsewhere [23]. Amphiphilic enzyme molecules were recovered in the detergent-rich phase. To achieve phase separation in Triton X-100-containing samples, the procedure of Cánovas-Muñoz et al. [24] was used.

2.4. Sedimentation analysis

Triton X-100-solubilized platelet membranes and the cytosolic platelet fractions were layered onto 5–20% w/v continuous sucrose gradients made up in buffer A containing 0.5% Triton X-100 or 0.5% Brij 97 (w/v). The sucrose gradients employed for PTPase separation of the detergent-rich or -poor phases from the phase partitioning with Triton X-114 were also made up in buffer A but containing 0.25% Triton X-100 and 0.5% Triton X-114 (w/v). Ultracentrifugation was performed at $165\,000 \times g$ for 18 h. About 40 fractions (275 μl each) were collected from the bottom of each tube and assayed for PTPase activity and enzyme markers. Sedimentation coefficients were calculated according to Martin and Ames [25], using β-galactosidase ($16.0S_{20,w}$), catalase ($11.4S_{20,w}$), and alkaline phosphatase ($6.1S_{20,w}$) as standards.

2.5. Phosphotyrosine phosphatase activity determination

PTPase activity was assayed basically as indicated elsewhere [20,21]. The basic assay was run in a 40 μl volume containing 100 mM sodium acetate buffer, pH 6.0, 10 mM *O*-phosphotyrosine as substrate, 0.2% Triton X-100, 10 nM okadoic acid and 10 μl from the sucrose gradient fractions. After 15–25 min at 37°C, the reaction was stopped by the addition of 120 μl of 25% trichloroacetic acid followed by 40 μl of bovine serum albumin (BSA, 10 mg/ml). Precipitated protein was removed by centrifugation after incubation for 10 min on ice, and 130 μl of supernatant was used to measure the inorganic phosphate released according to Rouser et al. [26]. For substrate specificity determination, *O*-phosphothreonine and *O*-phosphoserine were used as substrates at the same concentration as that of *O*-phosphotyrosine. In some experiments, PTPase activity was determined with a PTPase Specific Enzyme Linked Immunosorbent Assay (ELISA) Kit (Boehringer Mannheim, Barcelona, Spain), employing as substrates the phosphopeptides corresponding to amino acids 1–17 of human gastrin, or the 53–65 C-terminal fragment of hirudin, phosphorylated on tyrosines 12 and 63, respectively, following the supplier's instructions.

The protein contents of different samples were determined by the method of Bradford [27], using BSA as standard.

2.6. Western blots

Molecular weight (mol. wt.) standards (Pharmacia Biotech AB, Uppsala, Sweden) and different samples from platelets or fractions from the sucrose gradients used in the sedimentation analyses in sample buffer with reducing agents (2% SDS, 5% glycerol, 0.003% bromophenol blue, 1% β-mercaptoethanol, and 125 mM Tris-HCl, pH 6.8) were electrophoresed on 10% SDS-polyacrylamide gels at a density of 5–160 μg proteins/well [28]. Samples from the sucrose gradients were obtained by collecting two to three fractions from around the different PTPase peaks and shoulders detected, as well as other fractions with no PTPase activity, thus ensuring that the whole gradient would be represented. Mixtures of fractions were appropriately condensed by speed-vacuum centrifugation before being subjected to electrophoresis. Proteins were then electrophoretically transferred (4°C for 1 h at a constant voltage of 60 V using 25 mM Tris-HCl, 190 mM glycine, 20% methanol) to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Madrid, Spain) and the transfer efficiency was sometimes examined by staining with 0.1% Ponceau S. Membranes were washed three times (10 min/wash) with buffer B (10 mM Tris-HCl, 100 mM NaCl, 0.1% Tween 20, pH 7.5) and then blocked

with 5% Nestlé instant non-fat dry milk in buffer B (blocking solution) for 2 h at room temperature. They were then incubated overnight at room temperature with the appropriate primary monoclonal antibodies (MoAb) conveniently diluted in the blocking solution (anti-PTP1B, 1/2000; anti-PTP1C, 1/500; anti-PTP1D, 1/2500; anti-RPTPα, 1/2000; v/v; Transduction Laboratories, Lexington, KY, USA). Following this, they were washed three times with buffer B at 10 min/wash, and then incubated for 1 h with a 1/5000 dilution of a horseradish peroxidase-linked goat antimouse antibody (Transduction Laboratories) in blocking solution. The second antibody was replaced by fresh buffer B and the membranes washed three times for 10 min with the same buffer. The blots were visualized by chemiluminescence using the Western View chemiluminescent substrate system for horseradish peroxidase according to the supplier's instructions (Transduction Laboratories). Membranes were exposed for different times, depending on the sample and primary antibody used, to Hyperfilm-ECL (Amersham, Little Chalfont, UK), which was finally developed with Kodak GBX developer and replenisher (Sigma, St. Louis, MO, USA).

2.7. Analysis of spots on the blots

Film images were scanned using the Adobe Photoshop program (2.5.1. version) on a Macintosh computer connected to Scanjet 3C Hewlett Packard scanner (DeskScan II 2.2 alias program). Images were then analyzed with the MacBas v 2.5. program. Films were also scanned by densitometry with a Shimadzu CS-9000 densitometer at a wavelength of 595 nm. Particular PTPases revealed by blotting were estimated quantitatively and data expressed as percentages. For quantitative estimation, PTPases in different fractions – (i) platelet membranes versus platelet cytoplasm, and (ii) PTPases in the detergent-poor phase versus the detergent-rich phase of the Triton X-114 phase partitioning – were compared. For this purpose, equal amounts of proteins were loaded into the two wells corresponding to the two fractions to be compared, and the integrated density or the densitometric value of a PTPase spot was multiplied by the total amount of proteins within each fraction. Then, relative percentages were calculated, considering the sum of the values obtained for each pair of fractions (membranes/cytoplasm and detergent-poor/-rich phases) as 100%. Differences of less than 3% were observed using the two methods employed for quantitative estimation of the spots.

3. Results and discussion

It is now clear that in platelets an exquisite coordination of several transduction pathways due to protein kinases and protein phosphatases regulates platelet activation. All the available data also point to an integrated and coordinated interaction between serine/threonine and phosphotyrosine phosphatases for platelets to be fully functional.

3.1. Amphiphilic and hydrophilic nature of sheep and human platelet PTPase

The amphiphilic or hydrophilic nature of a membrane-bound protein once solubilized with a detergent is an empirical observation that can be achieved after phase partitioning of the detergent. Not all membrane-bound proteins displays necessarily amphiphilic characteristics after detergent solubilization. Triton X-114 phase partitioning is used in this kind of study because it affords a detergent-rich phase when warmed to temperatures above its cloud point (20°C), where micellar aggregates carrying amphiphilic proteins are formed and come out of solution [29]. This method could therefore be employed as the basis for a preparative separation technique, and indeed has been used to study the distribution of membrane platelet proteins [30].

Triton X-114 phase partitioning of Triton X-100-solubilized platelet membranes revealed that the relative percentages of PTPase activities in the detergent-rich and detergent-poor phases are similar for sheep [21] and human platelets (this work), with values of 35 and 40, and 65 and 60% for the detergent-rich and -poor phases in sheep and human platelet species, respectively (data not shown). The presence of platelet PTPases in the detergent-rich and detergent-poor phases can be explained by regarding the hydrophilic or amphiphilic nature of PTPases in two different ways: (i) there is a significant amount of enzymes (that are located in the detergent-rich phase)

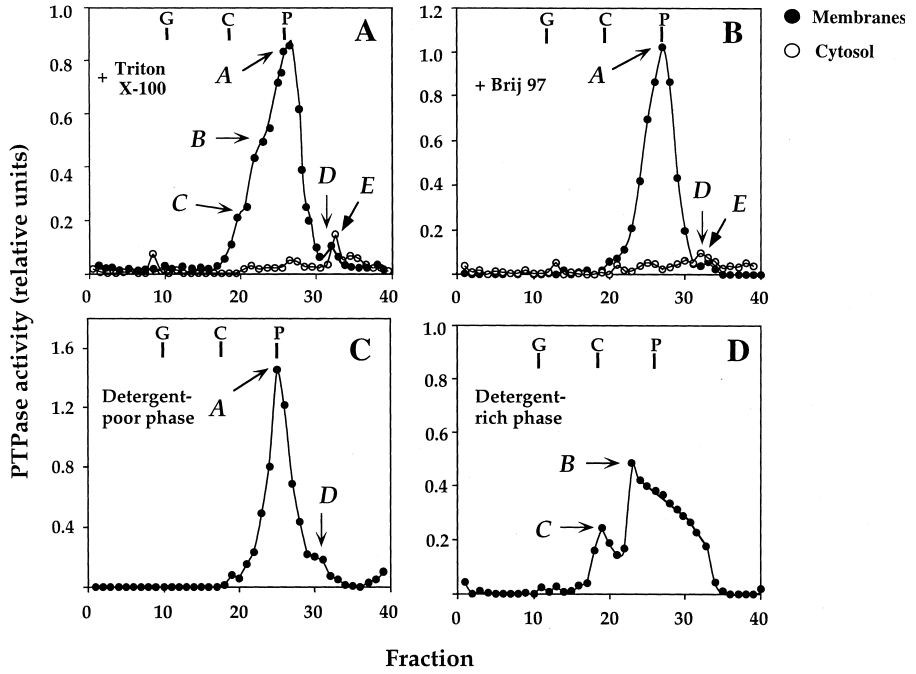

Fig. 1. Sedimentation profiles of PTPase in crude membranes, cytosol or the phases from Triton X-114 phase partitioning from sheep platelets. Crude membranes were separated from the cytosol by centrifugation and then extracted with 1% Triton X-100, as indicated in Section 2. Samples of Triton X-100-solubilized membranes were subjected to phase partition with Triton X-114 as indicated in Section 2. The Triton-X100 supernatant and the cytosolic fraction (A,B) or the detergent-poor (C) and -rich (D) fractions from the Triton X-114 phase partitioning were analyzed by centrifugation on linear sucrose gradients (5–20%, w/v) made up in 10 mM HEPES, pH 6.5, 0.2 mM EGTA, 5 mM KCl, 5.5 mM glucose, 145 mM NaCl, containing 0.5% (w/v) Triton X-100 (A), 0.5% (w/v) Brij 97 (B) or 0.25% Triton X-100 plus 0.5% Triton X-114 (w/v) (C,D). Enzyme markers were β-galactosidase (16.0S, G), catalase (11.4S, C) and alkaline phosphatase (6.1S, P). PTPase activity was assayed using *O*-phosphotyrosine as substrate. Gradient with Triton X-100 (A): *A* (6S), *B* (7.5S), *C* (10.3S), *D* (3.7S), *E* (3.5S). The profiles shown are the results from a representative experiment repeated six times.

that are strongly membrane associated, thus displaying amphiphilic properties, along with other enzymes (that are located in both phases) that are bound to the membrane peripherally or not so tightly [31]; (ii) no platelet PTPases display strong amphiphilic characteristics, therefore being located in both phases. Recently, we have proposed that sheep platelets contain different amphiphilic and hydrophilic forms of PTPases [21]. To gain further insight into the nature of the amphiphilic and hydrophilic forms of platelet PTPase, we extend our observation to human platelet species, performing sedimentation analyses in sucrose gradients containing Triton X-100 or Brij 97, because amphiphilic molecules have hydrophobic interactions that allow them to bind to non-denatured detergent micelles (such as Triton X-100) and their sedimentation coefficient is modified in the presence of detergent [32]. These experiments, and sedimentation analyses of the detergent-rich and -poor phases from the Triton X-114 phase partitioning, are shown in Figs. 1–3

The first set of experiments were designed to gain further insight into the nature of the amphiphilic and hydrophilic forms of platelet PTPase previously detected by us in sheep [21]. Accordingly, we first subjected the detergent-poor and -rich phases from the Triton X-114 phase partitioning of Triton X-100-solubilized platelet membranes to sedimentation analysis using sucrose gradients. Fig. 1A shows that, similarly to our previous results [21], sedimentation analysis of the cytosolic enzyme in gradients with Triton X-100 afforded profiles with a consistent peak of 3.2S (peak E in the figure); these remained unmodified in gradients with Brij 97 (Fig. 1B). In Fig. 1A it may also be seen that sedimentation analysis of the membrane-associated Triton X-100-solubilized PTPase disclosed profiles with one major peak of 6S, a minor peak of 3.7S, and shoulders at

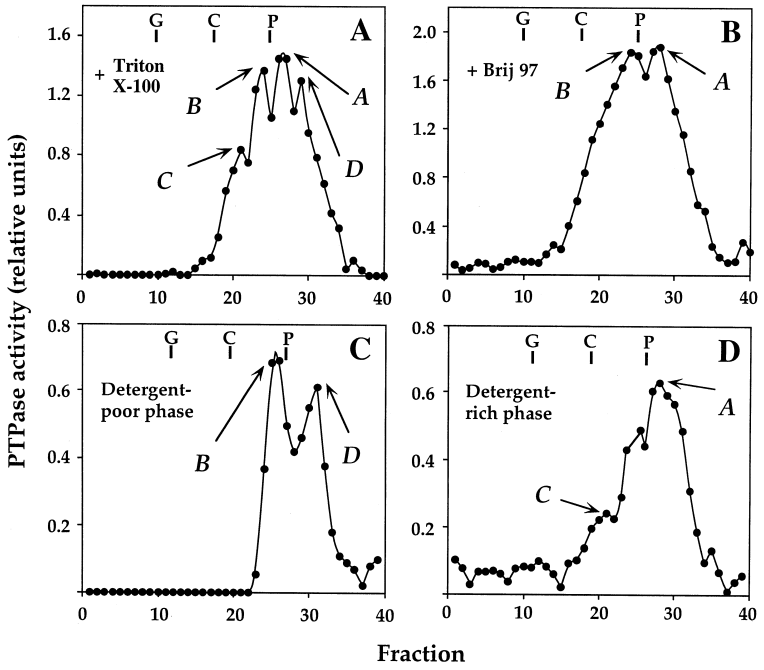

Fig. 2. Sedimentation profiles of PTPase in crude membranes or the phases from Triton X-114 phase partitioning from human platelets. Membrane protein extraction with Triton X-100, Triton X-114 phase partitioning, and sucrose gradient analysis were as indicated in the legend to Fig. 1. Enzyme markers were also as indicated in the legend to Fig. 1. PTPase activity was assayed using *O*-phosphotyrosine as substrate. Gradient with Triton X-100 (A): *A* (5.5S), *B* (6.7S), *C* (10.8S), *D* (4.3S). The profiles shown are the results from a representative experiment repeated six times.

7.5S and 10.3S (peaks A and D, and shoulders B and C in the figure, respectively). In gradients with Brij 97 (Fig. 1B), the sedimentation values of peaks A and D remained unmodified, whereas the shoulders seen in Fig. 1A disappeared. This kind of behavior suggests that peaks A and D represent hydrophilic species and that shoulders B and C correspond to amphiphilic species of PTPase. Confirming this, Fig. 1C and D show the sedimentation analyses of phases from a Triton X-114 phase partitioning of the Triton X-100-solubilized proteins. In Fig. 1C, corresponding to the detergent-poor phase, it may be seen that only the peaks at 6S (peak A) and 3.7S (peak D) were present, whereas shoulders at 7.5S (shoulder B) and 10.3S (shoulder C) were only detected in the gradient of the detergent-rich phase (Fig. 1D).

The results from similar experiments to those described above but using human platelets are shown in Fig. 2. Sedimentation analysis of Triton X-100-solubilized PTPase disclosed profiles with peaks of 5.5S (5.5 ± 0.2S, $n = 6$), 6.7S (6.7 ± 0.1S, $n = 6$), 4.3S (4.3 ± 0.3S, $n = 6$) and 10.8S (10.8 ± 0.01S, $n = 6$), which (by analogy to the forms observed in sheep species, see Fig. 1A) are referred to as peaks A, B, D, and C, respectively, in the figure. In gradients with Brij 97 (Fig. 2B), only two peaks are visible: peaks A and B, with 4.8 ± 0.2S and 6.7 ± 0.1S ($n = 6$), respectively. Thus, the sedimentation coefficient of peak B remained unmodified in the presence of Brij 97 and can therefore be said to represent a hydrophilic species of PTPase. By contrast, the sedimentation coefficients of peaks A and C seem to shift to lower values (shifted to the right); accordingly, peak C would appear below the main peak B, and peak A would mask peak D. In this regard, both peaks A and C would represent amphiphilic species of PTPase.

Nevertheless, with these experiments it was not possible to determine the exact nature of the form at 4.3S (peak D). To corroborate these observations and to gain further insight into the nature of the form at 4.3S, we next performed sedimentation analysis of phases from a Triton X-114 phase partitioning of the Triton X-100-solubilized proteins. Fig. 2C, corresponding to the detergent-poor phase, shows the presence of the peaks at 6.7S (peak B) and 4.3S

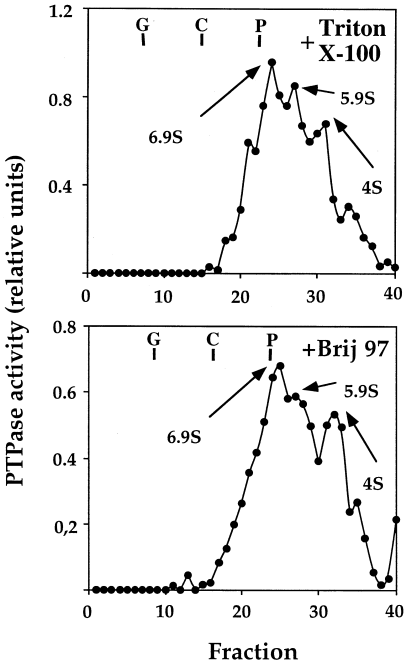

Fig. 3. Sedimentation profile of PTPase in human platelet cytosol. After centrifugation of platelet homogenates, the cytosolic fraction was analyzed by sucrose gradient centrifugation as indicated in the legend to Fig. 1. Enzyme markers were also as indicated in the legend to Fig. 1. PTPase activity was assayed using *O*-phosphotyrosine as substrate. The profiles shown are the results from a representative experiment repeated six times.

(peak D), whereas peaks at 5.5S (peak A) and 10.8S (peak C) were only detected in the gradient of the detergent-rich phase (Fig. 2D). This experiment confirmed that the peak at 4.3S (peak D) does represent a hydrophilic species of PTPase.

As can be seen in Fig. 3, sedimentation analysis of the cytosolic enzyme in gradients with Triton X-100 afforded profiles with three consistent peaks of 4S (4.03 ± 0.3S, $n = 6$), 5.9S (5.9 ± 0.15S, $n = 6$) and 6.9S (6.9 ± 0.01S, $n = 6$). In gradients with Brij 97, the sedimentation values of these peaks remained basically unchanged, indicating that they have hydrophilic properties.

It should be emphasized that no protein phosphatase activity could be determined in the homogenates, crude membranes, cytosols or fractions from the detergent gradients of sheep or human platelets when *O*-phosphothreonine or *O*-phosphoserine were used as substrates rather than *O*-phosphotyrosine (data not shown). This corroborates the specificity of the enzyme reaction against phosphorylated amino acid *O*-phosphotyrosine and supports the view that we are not dealing with dual-specificity phosphatases.

3.2. Platelet PTPase sedimentation profile by a tyrosine phosphatase-specific assay

Since it has been described that *O*-phosphotyrosine may be a poor substrate for some PTPases, we decided to repeat some of our PTPase determinations using a synthetic peptide phosphorylated on tyrosine as substrate. Fig. 4 shows the sedimentation analysis of Triton X-100-solubilized sheep platelet membranes when a tyrosine phosphatase-specific ELISA assay kit was employed to measure PTPase activity. In this case, a synthetic oligopeptide corresponding to amino acids 1–17 of human gastrin, phosphorylated on tyrosine 12, was used as enzyme substrate rather than *O*-phosphotyrosine. It can be seen that the profile of the PTPase is quite similar to that depicted in Fig. 1A, with peaks of sedimentation coefficients identical to those observed when *O*-phosphotyrosine was used as substrate. Thus, a main peak of 6.5S, a peak of 10S, and a shoulder of 7.7S (peaks A and C and shoulder B in Fig. 4, respectively) could be distinguished. The small peak at 3.7S in Fig. 1A now seems to be formed by two different peaks of 4.5S and 3S (peak D in Fig. 4). Basically the same profile was observed when a different oligopeptide corresponding to the hirudin 53–

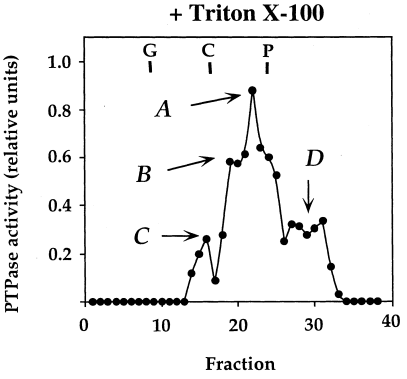

Fig. 4. Sedimentation profile of PTPase in sheep platelet crude membranes. Experimental procedures were as described in the legend to Fig. 1, but PTPase activity was determined using a PTPase-specific ELISA assay kit as indicated in Section 2. *A* (6.5S), *B* (7.7S), *C* (10S), *D* (4.5S+3S). The profile shown is the result from a representative experiment repeated four times.

65 C-terminal fragment phosphorylated on tyrosine 63 was used as substrate (data not shown). When the PTPase ELISA assay kit was employed in the sedimentation analysis of Triton X-100-solubilized membrane proteins from the human platelet species, a profile quite similar to that shown in Fig. 2A was observed (data not shown). The results in Fig. 4 clearly indicate not only that we were detecting most forms of platelet PTPases, but also that, under our experimental conditions, *O*-phosphotyrosine is not a poor substrate in comparison to several peptides phosphorylated on tyrosine, meaning that *O*-phosphotyrosine can be a good and inexpensive alternative when many PTPase determinations need to be done, at least in the case of platelets.

The profiles shown in Figs. 1–4 remained unchanged when the amount of NaCl present in the sucrose gradients was increased up to 1 M (data not shown).

The sedimentation experiments described so far clearly demonstrated that in both sheep and human platelet species there are amphiphilic and hydrophilic membrane-bound PTPases. The results support the view that in platelets there are PTPases that are strongly membrane bound, together with others that may be associated with the membrane peripherally, or at least not very strongly membrane associated.

3.3. Identification of PTPases in human and sheep platelets by Western blot

Since the sedimentation analyses had indicated the presence of multiple PTPases in platelets, we then tried to assign some of the peaks or shoulders detected in the gradients to some PTPases known to be present in platelets (intracellular PTP1B, PTP1C and PTP1D), as well as a receptor-like PTPase, RPTPα (which although particularly abundant in brain, has been reported to be widely expressed, although hitherto unreported in platelets). The availability of commercial antibodies against these PTPases prompted us to carry out Western blot assays on platelet cell fractions (crude membranes and cytoplasm), detergent-rich and -poor phases and fractions from the sedimentation gradients. The most significant data from the blots indicate that: (i) RPTPα does not seem to be present in platelets; (ii) all intracellular PTPases studied are located around one of the peaks of the membrane-bound or cytosolic protein gradients; (iii) PTP1B does not seem to be expressed in sheep platelets.

3.3.1. Studies with sheep platelets

The first experiments indicated that anti-PTP1B MoAb did not recognize any PTP1B-type form in sheep platelet species, whereas PTP1C-type and PTP1D-type forms were well recognized by the appropriate MoAb (Fig. 5). Data from blots indicate that: (i) PTP1C type and PTP1D type had approximate molecular masses of 68 kDa and 72 kDa, respectively; (ii) quantitative analysis of the spots afforded values of $76 \pm 4\%$ and $21 \pm 2\%$ (PTP1C type), and $48 \pm 4\%$ and $49 \pm 4\%$ (PTP1D type) in the membranes and the cytoplasm, and $92 \pm 3\%$ and $7 \pm 1\%$ (PTP1C type), and $91 \pm 3\%$ and $8 \pm 1\%$ (PTP1D type) in the detergent-poor and -rich phases, respectively ($n = 5$, representative blots not shown); (iii) membrane-associated forms were located in the sedimentation gradients (Fig. 1) around peak D, and the cytoplasmic forms around peak E (Fig. 5). Thus, membrane-associated sheep platelet PTP1C-type and PTP1D-type forms have hydrophilic characteristics, because they are mainly located in the detergent-poor phase of the Triton X-114 phase partitioning.

3.3.2. Studies with human platelets

We then designed similar experiments to study the presence of PTP1B, PTP1C and PTP1D in human platelet samples. Data from blots indicate that: (i) PTP1B, PTP1C and PTP1D had molecular masses of approx. 50 kDa, 68 kDa and 72 kDa, respectively; (ii) quantitative analysis of the spots afforded values of $89 \pm 2\%$ and $9 \pm 1\%$ (PTP1B), $72 \pm 3\%$ and $25 \pm 2\%$ (PTP1C), and $64 \pm 3\%$ and $33 \pm 3\%$ (PTP1D) in the membranes and the cytoplasm, and $92 \pm 3\%$ and $5 \pm 1\%$ (PTP1B), $91 \pm 2\%$ and $8 \pm 1\%$ (PTP1C), and $65 \pm 4\%$ and $31 \pm 4\%$ (PTP1D) in the detergent-poor and -rich phases, respectively ($n = 5$, representative blots not shown); (iii) PTP1B, PTP1C and PTP1D were located in the sedimentation gradients (Figs. 2 and 3) around peak D (membrane-associated forms) and the peak at 4S (cytoplasmic forms) (Fig. 6). Thus, membrane-associated human platelet PTP1B and PTP1C are hydrophilic in nature because they

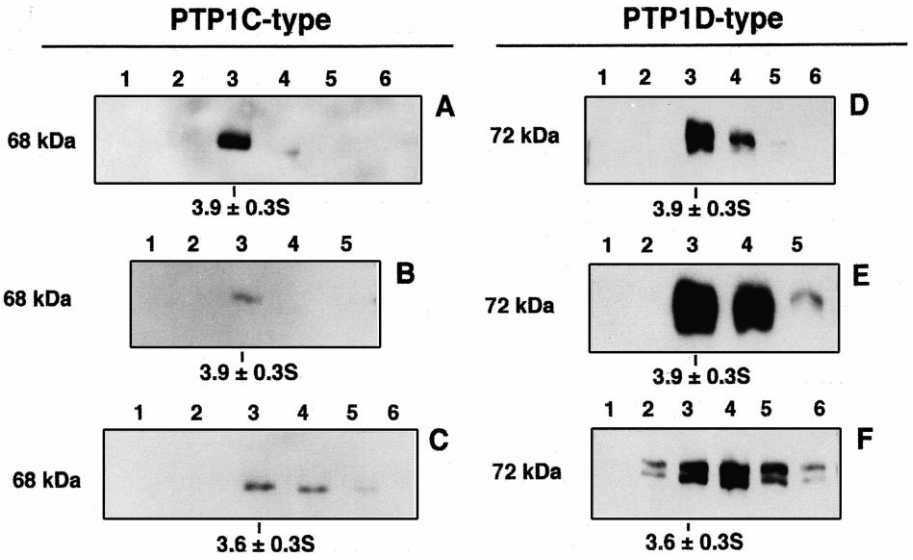

Fig. 5. Western blot analysis of PTP1C type and PTP1D type in sheep platelet samples. Platelet samples were applied to SDS-PAGE followed by immunoblots using mouse anti-human PTP1C or PTP1D MoAb as described in Section 2. (A,B,C) PTP1C type in the Triton X-100-solubilized membrane protein sucrose gradient (Fig. 1A), the detergent-poor phase from Triton X-114 phase partitioning (Fig. 1C) and the sucrose gradient from the cytosol (Fig. 1A), respectively. Each lane in A and B contained 30 μg proteins and in C 40 μg proteins. Lanes: 1, 2 ± 0.2S fractions; 2, 2.7 ± 0.3S fractions (A,B) and 2.5 ± 0.3S fraction (C); 3, peak D (3.9 ± 0.3S) (A,B) and peak E (3.6 ± 0.5S) (C); 4, peak A (6.2 ± 0.2S) (A,B) and 5.4 ± 0.1S fraction (C); 5, shoulder B (8 ± 0.5S) (A), 10.9 ± 0.3S fraction (B) and 6.1 ± 0.3S fraction (C); 6, shoulder C (10.4 ± 0.6S) (A) and 7.2 ± 0.4S (C). (D,E,F) PTP1D type in the same gradients as PTP1C type. Each lane in D and E contained 5 μg proteins and in F 6 μg proteins. Lanes as in A, B and C, respectively. Sedimentation coefficients are means ± S.E.M. of the sedimentation coefficients corresponding to the pooled fractions loaded in each lane.

are mainly located in the detergent-poor phases, but a significant amount of membrane-associated human platelet PTP1D has amphiphilic characteristics because more than 30% of the enzyme was recovered in the detergent-reach phase of the Triton X-114 phase partitioning.

Finally, RPTPα does not seem to be expressed in human or sheep platelet species as indicated by immunoblotting (data not shown).

Consistent with the way in which PTP1B is believed to be regulated and intracellularly located [15,16], we were unable to find any PTP1B signals in the cytoskeleton of resting human platelets (data not shown). Additionally, the fact that human platelet PTP1B was mainly recovered in the detergent-poor phase of the Triton X-114 phase partition indicated that the C-terminal hydrophobic domain for endoplasmic reticulum insertion is insufficient for conferring PTP1B an amphiphilic nature, in agreement with its location in the hydrophilic peak D from the Triton X-100-solubilized platelet crude membrane protein sucrose gradients (Fig. 2A and 6A). However, it is interesting that we were unable to detect any PTP1B-type enzyme in sheep species. Although it is possible that our antibody might not have recognized sheep platelet PTP1B, considering that the two other similar MoAbs were from the same supplier, and since we clearly detected PTP1C-type and PTP1D-type enzymes in the sheep species, and because the immunogen included the C-terminal non-catalytic region for enzyme targeting and regulation, it is tempting to speculate that PTP1B is not expressed in sheep platelets, at least in appreciable amounts. If this is indeed the case, one might conjecture that the potential function of PTP1B in dephosphorylation events in platelet physiology would be carried out by other PTPases in sheep platelet species, suggesting that some of the functions of specific PTPases might be carried out by different enzyme forms, depending on the particular platelet species.

In agreement with previous observations [33,34], PTP1C was seen to be located not only in the cytoplasmic fraction but also in the membrane fraction of resting platelets. PTP1C has been reported to be associated, at least after activation, with the cytoskele-

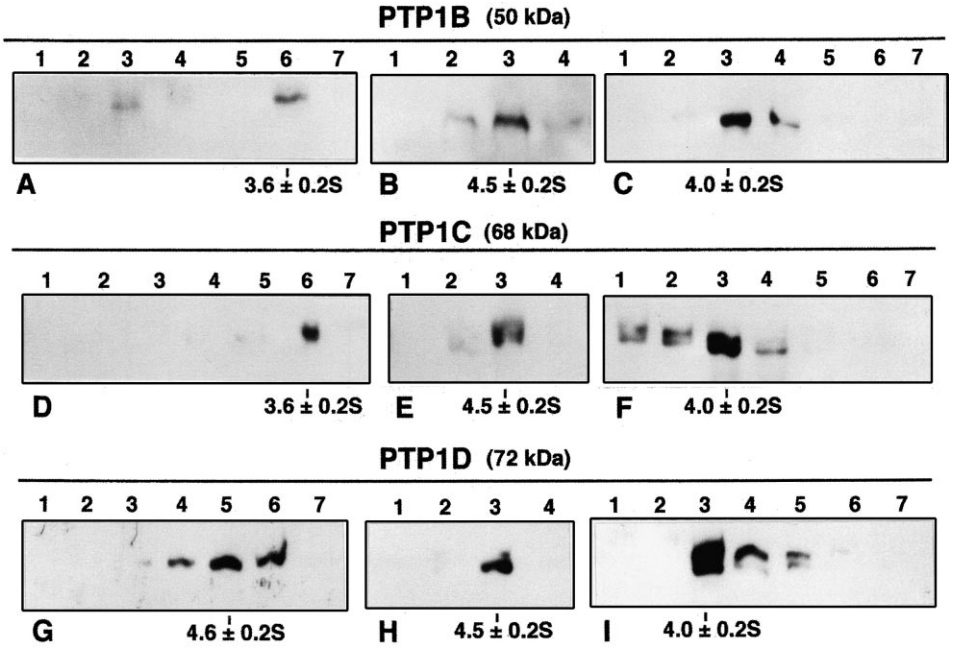

Fig. 6. Western blot analysis of PTP1B, PTP1C and PTP1D in human platelet samples. Platelet samples were applied to SDS-PAGE followed by immunoblots using mouse anti-human PTP1B, PTP1C and PTP1D MoAbs as described in Section 2. (A,B,C) PTP1B in the Triton X-100-solubilized membrane protein sucrose gradient (Fig. 2A), the detergent-poor phase from Triton X-114 phase partitioning (Fig. 2C) and the sucrose gradient from the cytosol (Fig. 3), respectively. Each lane in A, B and C contained 80, 100 and 20 μg proteins, respectively. (A) Lanes: 1, 12.8 ± 0.3S fraction; 2, shoulder C (10.4 ± 0.2S); 3, peak B (6.7 ± 0.2S); 4, peak A (5.7 ± 0.1S); 5, peak D (4.6 ± 0.2S); 6, end of peak D (3.6 ± 0.2S); 7, 2.1 ± 0.1S fraction. (B) Lanes: 1, (10.9 ± 0.5S); 2, peak B (6.4 ± 0.2S); 3, peak D (4.5 ± 0.2S); 4, 1.8 ± 0.2S fraction. (C) Lanes: 1, 1.1 ± 0.2S fraction; 2, 3.1 ± 0.2S fraction; 3, 4 ± 0.2S fraction; 4, 6.1 ± 0.2S fraction; 5, 7.4 ± 0.2S fraction; 6, 10.9 ± 0.2S fraction; 7, 13.8 ± 0.4S fraction. (D,E,F) PTP1C in the same gradients as PTP1B. Each lane in D contained 30 μg proteins and in E and F 20 μg proteins. Lanes as in A, B and C, respectively. (G,H,I) PTP1D in the same gradients as PTP1B. Each lane contained 10 μg proteins. Lanes as in A, B and C, respectively. Sedimentation coefficients are means ± S.E.M. of the sedimentation coefficients corresponding to the pooled fractions loaded in each lane.

ton and the membrane skeleton of platelets [16,33], probably through its two SH2 domains to phosphotyrosyl proteins located within those cellular structures. Nevertheless, Triton X-100-solubilized platelet crude membrane proteins should not contain cytoskeletal proteins, which are resistant to Triton X-100 solubilization. Considering that cytoplasmic PTP1C would become membrane associated by protein-protein interactions through its SH2 domains, it is possible that PTP1C relocates to the membranes during platelet fractionation, thus allowing its recovery in the detergent extract after the Triton X-100 solubilization step. Consistent with an ionic interaction of PTP1C with other membrane proteins, extractions of platelet crude membranes with 0.5 M NaCl buffered solutions afforded the appearance of PTP1C in the soluble saline fraction as determined by immunoblotting (data not shown). Additionally, the hydrophilic nature of PTP1C is in accordance with its soluble nature and originally cytoplasmic location. This observation is still consistent with our previous results indicating that PTPase solubilization from sheep platelet membranes required the presence of detergent and that it was not possible to extract PTPase activity by increasing the ionic strength of the incubation medium or by freeze-thawing cycles [21], simply because PTP1C activity represents a very small percentage of total membrane-associated PTPase activity as indicates its location in the sedimentation gradients.

Human platelet PTP1D was the only PTPase studied that was significantly recovered in the detergent-rich phase of the Triton X-114 phase partition experiments (30%). As already indicated, some peripherally membrane-associated enzymes are distributed between both phases of Triton X-114 phase

partition [31]. It is interesting that in sheep platelet species, PTP1D was not located in the detergent-rich phase. This, along with the observation that human platelet PTP1D from the detergent-rich phase has a sedimentation coefficient lower (3.6 ± 0.2S, data not shown) than that shown by the PTP1D located in the detergent-poor phase (4.5 ± 0.2S, Fig. 5C), could indicate the existence of some specific structural difference between human platelet PTP1D from both phases. Experiments are currently being designed to test this hypothesis. One of the most significant features with respect to PTP1D is the observation that the blot signals were always stronger in sheep than in human platelets, even though the antibody was raised against the human species. Accordingly, it is possible that PTP1D could be expressed in higher amounts in sheep platelets, which must be independent of any genetic activation factor since platelets are anucleate cells. The meaning of this increased expression is unknown, but significantly, as already mentioned, we were unable to detect any PTP1B-type form in the sheep species.

Finally, to date no receptor-type PTPase has been described in platelets, CD45 [35] and RPTPα (this work) seem not to be present in this kind of blood cell.

In conclusion, all the data shown in this work clearly demonstrate that PTPases that do not correspond to PTP1B, PTP1C or PTP1D, PTPases described until now in platelets along with PTPMEG [18], must coexist in this blood cell. The unknown PTPase forms have the higher PTPase activity when *O*-phosphotyrosine or a synthetic peptide phosphorylated on tyrosine were used as substrates and the highest sedimentation coefficients, probably indicating that they are proteins with higher molecular weights. Particularly interesting is the finding that some of these forms are amphiphilic in nature. Considering that a C-terminal hydrophobic domain would be insufficient for a protein to display amphiphilic characteristics (as seen to occur in the case of PTP1B in this work), the possibility of PTPases with more hydrophobic domains gains credibility. This hydrophobicity would not only depend on transmembrane domains, indicating that some type of unknown receptor-type PTPase might be present in platelets, but also on some type of lipid modification since this type of posttranslational modification, as well as the existence of lipid-binding domains, has already been reported for some PTPases [36–38].

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