Identification and localization of myosin phosphatase in human platelets

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Type 1 (PP1) and type 2A (PP2A) phosphatase activity was measured in three subcellular fractions of human platelets. About 80 % of the activity was in the high-speed supernatant. Western blots showed that the catalytic subunit of PP1 (PP1c), including α- and δ-isoforms, was present in each fraction, but the level of the catalytic subunit of PP2A was very low in the low-speed pellet (cytoskeletal fraction). Various antibodies detected a subunit similar to the 130 kDa subunit (M130) of myosin phosphatase (MP) of smooth muscle in the low- and the high-speed pellets of human platelets. PP1c and associated proteins were isolated by microcystin-Sepharose. Many proteins were separated from each fraction, including myosin, actin and PP1c. M130 was separated only from the low-speed and the high-speed pellets. Kinase activities were detected in the unbound fractions, and fractions from the low- and high-speed pellets phosphorylated M130 and myosin respectively. Treatment of platelets with calyculin A increased the phosphorylation level of many proteins, including myosin heavy- and light-chains, and caused association of cytoskeletal proteins with the low-speed pellet. No marked change in the distribution of PP1c and M130 was detected. These results suggest that the MP in human platelets is composed of PP1c plus a subunit similar to M130 of the smooth muscle phosphatase.

INTRODUCTION

Platelet activation initiates a series of events that culminate in disruption of the cell and granule release [1]. The initial events involve a shape change that reflects alteration of the platelet cytoskeleton [2]. A major factor in this reorganization is thought to be phosphorylation of myosin [3] and its increased association with the cytoskeleton [4]. The level of myosin phosphorylation reflects the activities of two enzymes, i.e. myosin light-chain kinase (MLCK) and myosin light-chain phosphatase (MP). In contrast to MLCK, little is known about the identity or properties of MP, even though it plays a key role in platelet function.

To a large extent this study on platelet phosphatases was stimulated by recent information on smooth muscle MP. It was discovered that following agonist stimulation of smooth muscle, or on application of guanosine 5′-[γ-thio]triphosphate to α-toxin-permeabilized fibres, there was a shift in the Ca^{2+} sensitivity of myosin phosphorylation that reflected an inhibition of MP activity [5]. This was the first evidence that MP could be regulated and obviously represented a major component of the smooth muscle contractile mechanism. It is interesting that a similar effect may also have been detected in platelets. Hallam et al. [6] found that in the absence of external Ca^{2+}, platelet-activating factor and thrombin stimulated myosin phosphorylation even at relatively low internal Ca^{2+} concentrations. Although the mechanism underlying this effect was not identified one of the suggested possibilities was an increased Ca^{2+} sensitivity of myosin phosphorylation.

The MP holoenzyme is composed of three subunits [7–9], namely: the δ-isoform of the type 1 catalytic subunit (PP1cδ) [8], this is also referred to as the β-isoform [7]; a large non-catalytic subunit, referred to as M130 or M133 [8] or M110 [10]; and a smaller subunit, M20 or M21 [8,10]. It has been suggested that the two non-catalytic subunits may be target subunits and may also be regulatory [7]. Consistent with this idea was the finding that the holoenzyme exhibited higher activity towards phosphorylated myosin than the isolated catalytic subunit [8,9,11–13] and was also more effective in promoting relaxation in skinned fibres [9]. The mechanism of phosphatase inhibition has not been established. It may involve arachidonic acid [5] and/or phosphorylation of the large subunit [14,15]. Recently it was suggested that Rho-kinase could phosphorylate M130 and inhibit activity [16]. In addition, a mechanism involving protein kinase C has been proposed [17,18].

Previous studies have documented the presence of various types of phosphatase catalytic subunits in platelets [19,20]. It is thought that the catalytic subunits of protein phosphatase 1 (PP1c) and 2A (PP2Ac) translocate between subcellular fractions during platelet activation [21]. Studies with cell-permeant inhibitory toxins of PP1 and PP2A have established that phosphatase inhibition results in the inhibition of agonist-induced aggregation of platelets [20,22–25]. The phosphorylation pattern of the platelet proteins was also changed on phosphatase inhibition, and several proteins in the range 20–260 kDa were identified with increased phosphorylation levels [20,22,24–26]. These results suggest that PP1 and PP2A may play an important role in platelet activation. However, the regulatory proteins for these enzymes in platelets have not been identified.

The smooth muscle MP subunits are found in a wide variety of tissues [27], and it is possible that the MP is not confined to smooth muscle but also has a role in non-muscle cells. The goal of the present study was to identify and localize MP in human platelets. The current work provides evidence that the catalytic and the myosin-binding subunit (M130) of the smooth-muscle MP are present in platelets and are major phosphatase components of the platelet cytoskeleton. Data are also presented for the phosphorylation of M130 and, based on previous observations, this may constitute a regulatory mechanism in platelet function.

Abbreviations used: MLCK, myosin light-chain kinase; MP, myosin phosphatase; M130, M133, M110, isoforms of the large regulatory (myosin binding) subunit of smooth muscle myosin phosphatase; M20, M21, small regulatory subunit of smooth muscle myosin phosphatase; PP1c, catalytic subunit of type 1 protein phosphatase; PP2Ac, catalytic subunit of type 2A protein phosphatase; LC20, 20 kDa light-chain of smooth muscle myosin; P-LC20, phosphorylated form of the 20 kDa light-chain of smooth muscle myosin; MC, microcystin-LR; CL-A, calyculin A; DFP, diisopropyl fluorophosphate.

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EXPERIMENTAL

Materials

Chemicals and vendors were as follows: [γ-^{32}P]ATP (ICN Biomedicals or Hungarian Isotope Institute), microcystin-LR, calyculin-A (CL-A; Gibco), CNBr-activated Sepharose 4B (Pharmacia), 2-aminoethanthiol/HCl (Aldrich), horseradish peroxidase-labelled goat anti-rabbit, rabbit anti-chicken and goat anti-mouse immunoglobulins (Sigma), enhanced chemiluminescence reagent kit (Amersham). ^{32}P-Labelled phosphorylase *a* [28], ^{32}P-labelled 20 kDa light-chain of gizzard myosin [29] and microcystin-Sepharose (MC-Sepharose) [30] were prepared as described in the respective references.

Isolation of platelets

Venous blood, collected from healthy adult donors, was added to 0.1 vol. of acid citrate dextrose (85 mM trisodium citrate/111 mM D-glucose/71 mM citric acid, pH 6.5) and centrifuged at 650 ***g*** for 15 min. The platelet-rich plasma obtained as supernatant was centrifuged at 2000 ***g*** for 15 min. The platelet pellet was suspended and washed three times with a solution containing 150 mM NaCl, 10 mM Hepes (pH 7.6), 1 mM EDTA, 0.1 mM PMSF, 0.1 mM DFP, 0.1 mg/ml leupeptin and 1 mM benzamidine, then the washed pellet was suspended in Tyrode's buffer plus EGTA, i.e. 140 mM NaCl, 5 mM Hepes, 2 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 5.5 mM glucose, 1 mM EGTA, 0.36 mM sodium phosphate, 0.1 mM PMSF, 0.1 mM di-isopropyl fluorophosphate (DFP), 0.1 mg/ml leupeptin and 1 mM benzamidine (pH 7.4) at a concentration of about $(0.5–2) \times 10^9$ platelets/ml (termed as total platelets). All of the above operations were carried out at room temperature.

Radioactive labelling and CL-A treatment

Platelets were labelled with $^{32}P_i$ (0.5 mCi/ml) for 2 h and treated with CL-A (0.1 μM for 15 min) in the above Tyrode's buffer plus EGTA at 37 °C as described previously [25].

Isolation of subcellular fractions

Platelets were lysed by addition of an equal volume of ice-cold extraction buffer containing 100 mM Tris/HCl (pH 7.4), 10 mM EDTA, 2% Triton X-100, 0.1 mM PMSF, 0.1 mM DFP, 0.1 mg/ml leupeptin and 1 mM benzamidine. After 5 min on ice the extract was centrifuged at 10000 ***g*** for 5 min. The resulting pellet was rinsed with Tyrode's buffer plus EGTA and extraction buffer (1:1) and centrifuged again as above (low-speed pellet). The 10000 ***g*** supernatant was centrifuged at 100000 ***g*** for 3 h at 5 °C. The resulting pellet is termed the high-speed pellet and the supernatant is termed the high-speed supernatant.

Antibodies

Rabbit polyclonal antibody against a C-terminal peptide of PP1cα was purchased from Upstate Biotechnology Inc. (New York, NY, U.S.A.). A monoclonal antibody against M130 [14] and a rabbit polyclonal antibody against the N-terminal fragment of recombinant M130$^{1–674}$ [11] were generated as described in the respective references. Antipeptide antibodies against the N-terminal peptide (^{3}KAFTKELDQWIEQL^{16}) of PP2Ac and against an inner sequence of PP1c (^{179}DLQSMEQIRRIMRPTD^{194}) were raised in chickens using polylysine core-coupled peptides as antigens. The following anti-peptide antibodies were raised in rabbits using keyhole limpet haemocyanin-coupled peptides as antigens, against: the C-terminal peptide of PP1cδ (^{313}RPVTPPRTANPPKKR^{327}); the N-terminal region of M20 (^{6}TRSKEYTRSRKSQ^{18}); the leucine-zipper motifs of M20 (^{166}DNQRLKDENQALIR^{179}); and the N-terminal first 38 residue peptide (^{1}M–F^{38}) of M130. The antibodies were affinity purified from egg yolk extract or serum on CNBr-activated Sepharose 4B coupled with the antigen peptides. Polyclonal antibodies against the human lung- and brain-specific isoforms of M130 were raised in rabbits using recombinant proteins as antigens representing the C-terminal fragments of the isoforms [31]. The C-terminal fragments ^{858}W–N^{1019} and ^{671}Q–G^{851} of human lung and brain M130 were expressed as GST-fusion proteins respectively. cDNAs for these mutants were obtained by PCR amplification using the appropriate primers. The amplified fragments were subcloned into pCR^{TM}II vector, the cloned cDNAs were then excised from the vector by digestion with *Eco*RI and the DNA fragments were ligated into the pGEX4T vector. The constructs were used to transform competent *Escherichia coli* BL21 (DE3) cells for expression of the mutant proteins. The recombinant proteins from the cell lysate were purified by chromatography on ion-exchange (SP Sepharose FF, Mono-Q) as well as glutathione-Sepharose 4B columns, and the purified proteins were used to generate polyclonal antibodies in rabbits. These antibodies were pre-absorbed by glutathione S-transferase affinity columns, then the antibodies, specific for the M130 isoforms, were affinity purified using CNBr-activated Sepharose 4B coupled with the recombinant protein fragments.

Electrophoresis and Western blotting

SDS/PAGE was performed using the conditions of Laemmli [32] on gradient gels of 7.5–20% polyacrylamide. The low- and high-speed pellets of the platelet lysate prepared from 50–200 ml of blood were directly dissolved in 50–200 μl of SDS sample buffer and boiled. To the total platelet and the high-speed supernatant 6 × SDS sample buffer was added and the solutions were boiled. Volumes of 5–40 μl of these solutions were applied to SDS/PAGE. The gels were stained and subjected to autoradiography or blotted on to a nitrocellulose membrane (0.25 μm pore size). For immunodetection of PP1c and PP2Ac in the platelet subcellular fractions, 5–10 μl of SDS/PAGE samples, prepared as described above, were electrophoresed on an SDS/12%-PAGE mini-gel (0.75 mm thick). Immunoblotting from gradient gels was carried out as described [33] at 5 °C for 16 h at 25 mA. Proteins from mini-gels were transferred for 90 min at 250 mA using a Bio-Ice cooling unit in the blotting apparatus to avoid overheating. For blocking of the membrane, 5% non-fat dried milk was used, and as a carrier 1% non-fat dried milk was used, both in TBS containing 0.05% Tween 20. After blocking, the membranes were washed and exposed to the primary antibodies for 90 min at room temperature. For detection of primary antibodies on Western blots, peroxidase-coupled secondary antibodies were used with enhanced chemiluminescence reagent.

Assays

Protein phosphatase was assayed at 30 °C with [^{32}P]phosphorylase (10 μM) or ^{32}P-labelled myosin light-chain (5 μM) as described [29]. For the assay of phosphatase activity, the low- and high-speed pellets were suspended in 10 mM Tris/HCl (pH 7.4), 0.1 mM EGTA 0.25 mM dithiothreitol, 0.1 mM PMSF, 0.1 mM DFP, 0.1 mg/ml leupeptin and 1 mM benzamidine (buffer A) and further diluted in buffer A + 1 mg/ml BSA. The high-speed supernatant was also diluted in buffer A + 1 mg/ml BSA. One unit of the protein phosphatase activity releases 1 μmol of P_i from the phosphosubstrate per min at

30 °C. Protein concentrations were determined according to the method of Bradford [34] using BSA as standard.

MC-Sepharose chromatography

The low- and high-speed pellets of platelet lysate were extracted with 0.8 ml of buffer A plus 0.6 M NaCl. After centrifugation (10000 ***g*** for 2 min), the supernatants from the low-speed and the high-speed pellets were added to 0.2 ml of MC-Sepharose respectively. The high-speed supernatant (10 ml) was added directly to the 0.2 ml of MC-Sepharose. The mixtures were gently shaken for 60 min, then centrifuged. Comparison of the phosphatase activities before and after exposure to the MC-Sepharose established that more than 95% of the phosphatase activity was bound to the matrix. The MC-Sepharose was washed with buffer A plus 1 M NaCl, then with buffer A alone and centrifuged. The 0.2 ml of MC-Sepharose was boiled in an equal volume of SDS sample buffer and the bound proteins were analysed by SDS/PAGE and Western blotting. For phosphorylation of the MC-Sepharose-bound proteins the supernatants after interaction of each fraction (extract of low- and high-speed pellets and high-speed supernatant) with MC-Sepharose were saved and the MC-Sepharose-bound proteins were isolated as described above. The MC-Sepharose obtained from each fraction was divided into two 50 μl portions and incubated in 0.1 ml of buffer A in the presence of 0.3 mM [γ-^{32}P]ATP (4000 c.p.m./pmol)/5 mM $MgCl_2$, or in the presence of 0.3 mM [γ-^{32}P]ATP (4000 c.p.m./pmol)/5 mM $MgCl_2$, and the supernatant of the respective fraction obtained after exposure to MC-Sepharose. Incubations were carried out for 15 min at 37 °C, then the MC-Sepharose matrix was isolated by centrifugation and washed with buffer A plus 1 M NaCl and with buffer A as described above. The MC-Sepharose was boiled in SDS sample buffer and the proteins were electrophoresed on SDS/PAGE and subjected to autoradiography.

RESULTS

The subcellular fractions from the platelet lysate were assayed for phosphatase activity using ^{32}P-labelled phosphorylase and ^{32}P-labelled myosin light-chain (P-LC20) as substrates (Table 1). Most of the total activity for both substrates was detected in the high-speed supernatant. In the two pellet fractions the most marked difference was in the proportion of the activities found in the low-speed pellet, in which phosphatase activity was higher toward P-LC20 compared with phosphorylase *a*. Assays carried out under conditions where the activity of PP2A would be inhibited (1 nM okadaic acid) resulted in inhibition by 6–10%, 15–20% and 25–30% for the low-speed pellet, high-speed pellet and high-speed supernatant respectively.

Table 1 Distribution of phosphorylase and P-LC20 phosphatase activities in the subcellular fractions of resting platelets

Phosphatase activity of the platelet subcellular fractions was determined as described in the Experimental section.

	Platelet fractions		
Phosphatase activity	Low-speed pellet	High-speed pellet	High-speed supernatant
Phosphorylase phosphatase			
m-Units in fraction*	0.11 ± 0.012	0.79 ± 0.11	5.05 ± 0.19
Percentage of total†	1.8	13.3	84.9
P-LC20 phosphatase			
m-Units in fraction	0.4 ± 0.06	0.3 ± 0.05	2.9 ± 0.35
Percentage of total	11.1	8.3	80.6
P-LC20/phosphorylase phosphatase ratio	3.72	0.37	0.54

* Mean ± S.E.M. for four determinations.
† The sum of the activities in the subcellular fractions was taken as 100%.

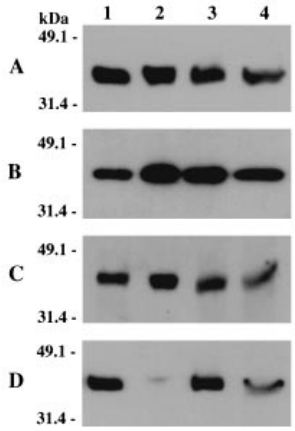

Figure 1 Identification and subcellular distribution of PP1c and PP2Ac by Western blotting in human platelets

Lanes: 1, total platelet (40 μg of protein from 2.4×10^7 platelets); 2, low-speed pellet (30 μg of protein from 3.3×10^7 platelets); 3, high-speed pellet (40 μg of protein from 3.1×10^7 platelets); 4, high-speed supernatant (15 μg of protein from 1.3×10^7 platelets). (**A**) Anti-PP1c against a C-terminal peptide of PP1cα; (**B**) anti-PP1c against a C-terminal peptide of PP1cδ; (**C**) anti-PP1c against an inner sequence common to the distinct isoforms; and (**D**) anti-PP2Ac against an N-terminal peptide of PP2Ac. 31.4 and 49.1 kDa refer to the position of molecular-mass markers.

The distribution of PP1 and PP2A catalytic subunits in these fractions was examined by Western blots using various antibodies. The four antibodies used were: specific for PP1cα; specific for PP1cδ; reactive to all PP1c isoforms; and specific for PP2Ac. For each antibody samples in four lanes were analysed: total platelet homogenate, low-speed pellet, high-speed pellet and high-speed supernatant (Figure 1). PP1c, both α- and δ-isoforms, was found in each fraction and there did not appear to be a preferential distribution. However, the distribution of PP2Ac was distinct in that it was extremely low in the low-speed pellet. Thus from these results it is apparent that only PP1c was associated with the low-speed pellet (i.e. the cytoskeletal fraction). The bands obtained by Western blotting were compared by scanning densitometry, and an approximate percentage distribution of PP1c and PP2Ac in the subcellular fractions was calculated and normalized to the same number of platelets. The results obtained indicated that the low-speed pellet, high-speed pellet and high-speed supernatant contained about 27%, 21% and 52% of total PP1c respectively. The percentage distribution of PP2Ac was 5%, 28% and 67% in the low-speed pellet, high-speed pellet and high-speed supernatant respectively. A similar distribution of PP1c and PP2Ac, also based on scanned Western blots, was found by Toyoda et al. [21]. It should be pointed out, however, that the percentage distribution of the protein phosphatase catalytic subunits in the subcellular fractions obtained by Western blots are distinct from that of the percentage distribution of phosphatase activities obtained with either P-LC20 or phosphorylase *a* as substrate (Table 1). This difference suggests that the association of various regulatory proteins with PP1c and PP2Ac could alter phosphatase activity towards the

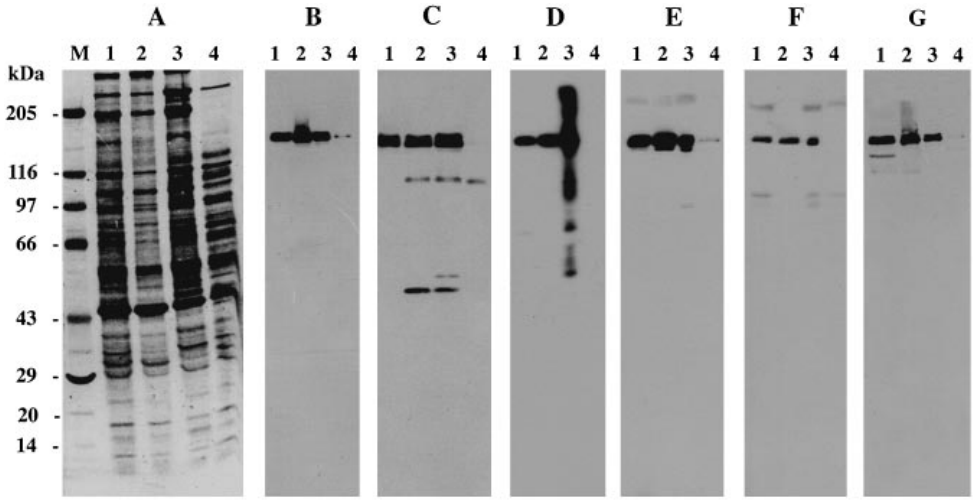

Figure 2 Identification of the myosin-binding subunit of smooth-muscle phosphatase in human platelets

Lanes: M, molecular-mass standards; 1, total platelet (60 μg of protein from 3.6×10^7 platelets); 2, low-speed pellet (45 μg of protein from 5×10^7 platelets); 3, high-speed pellet (63 μg of protein from 5×10^7 platelets); 4, high-speed supernatant (48 μg of protein from 4.2×10^7 platelets). (**A**) Protein stain with Naphthol Blue Black; (**B**) anti-M130 against the N-terminal part of the recombinant protein ($M130^{1-674}$); (**C**) monoclonal anti-M130; (**D**) anti-M130 specific for an N-terminal peptide ($M130^{1-38}$); (**E**) anti-M130 specific for the isoform predominantly expressed in human lung; (**F**) anti-M130 specific for the isoform predominantly expressed in human brain; (**G**) anti-M20 against the leucine-zipper region of the protein (also recognizes M130 isoforms containing the same leucine-zipper sequence).

two substrates. Other phosphatases (e.g. protein phosphatase 2B or 2C) were not examined.

The observation that the cytoskeleton fraction possessed higher activity towards P-LC20 and that it also contained PP1c raised the question of whether this reflected the presence of the large non-catalytic subunit (i.e. similar to M130) of a smooth muscle-type phosphatase. Several antibodies (see the Experimental section) were used to screen for the presence of M130. SDS gel patterns for the platelet homogenate and for three subcellular fractions are shown in Figure 2(A), and Western blots using six antibodies directed towards M130 are shown in Figures 2(B)–2(F). All of the antibodies demonstrated the presence of M130 in both the low- and high-speed pellets and essentially its absence from the high-speed supernatant. For Figure 2(B) a polyclonal antibody to the N-terminal two thirds of M130 was used. This gave a simple banding pattern. A monoclonal antibody was used in Figure 2(C) and its epitope is between Ser-304 and Ala-374 of the gizzard M130 sequence. The lower-molecular-mass bands were proteolytic products of M130 and these were seen frequently. In Figure 2(D) a polyclonal antibody to the N-terminal sequence residues 1–38 (synthetic peptide) was used. For the low-speed pellet a single band was detected. However, the high-speed pellet gave a complex banding pattern. The reason for the apparent multiple cross-reactivity is not known, but it was a consistent observation. One possible explanation is that other PP1c-binding proteins were concentrated in the high-speed pellet. The sequence 1–38 is known to contain a four amino acid repeat that is thought to be required for interaction with PP1c and is found in many PP1c-binding proteins [35,36]. Figures 2(E) and 2(F) show results using antibodies to two isoforms of M130 originating from separate genes, these are termed the lung and brain isoforms. The former is found in smooth muscle and many other tissues (including lung) and is the prevalent form of M130. The latter is thought to be expressed only in heart and brain (M. Ito, unpublished work). It is interesting that both isoforms were detected in platelets (Figures 2E and 2F), but the smooth muscle isoform was the major species. The conclusion that the two isoforms represent the products of different genes must be confirmed by additional experiments. The antibody used in Figure 2(G) was directed towards the leucine-zipper repeats (see the Experimental section) found in the C-terminal region of M20 and also in the C-terminal half of the rat M110 isoform. This anti-leucine-zipper antibody recognized only M130 and this was confined to the two pellet fractions (Figure 2G). A subunit corresponding to the gizzard M20 was not detected in human platelets. This conclusion is based on the lack of cross-reaction of platelet proteins at 20 kDa with the anti-leucine-zipper antibody (Figure 2G) and also with an antibody generated against the N-terminal sequence of M20 (results not shown). Another point to be made from these results is that the M130 isoform(s) present in human platelets also contain the leucine-zipper motifs, and in this regard are distinct from the chicken gizzard isoforms [8] but similar to the rat M110 isoform [10].

It is known that the PP1c binds to MC-Sepharose and this has been used to analyse PP1c and its associated subunits [37]. The following procedure, therefore, was used to investigate PP1c and associated proteins in the subcellular fractions of the platelet. The phosphatase was extracted from the pellets at high ionic strength and applied to MC-Sepharose (see the Experimental section). The high-speed supernatant at high ionic strength was also applied to MC-Sepharose. The proteins bound to MC-Sepharose were analysed by SDS/PAGE and silver staining (Figure 3A). Several bands were observed for each fraction. Although most of the components were not identified, likely candidates include the cytoskeletal proteins: actin, corresponding to a major band at 43 kDa; myosin heavy-chain at $\sim$ 200 kDa; and a high-molecular-mass component ($>$ 200 kDa), either actin-binding protein or talin. Some of the components in each fraction are common, e.g. the putative actin and myosin, but there are differences in the banding patterns. These results suggest that PP1c in each fraction is associated, directly or indirectly, with several other proteins, and the cytoskeletal proteins appeared to be major components, at least in the two pellet fractions. The presence of PP1c and

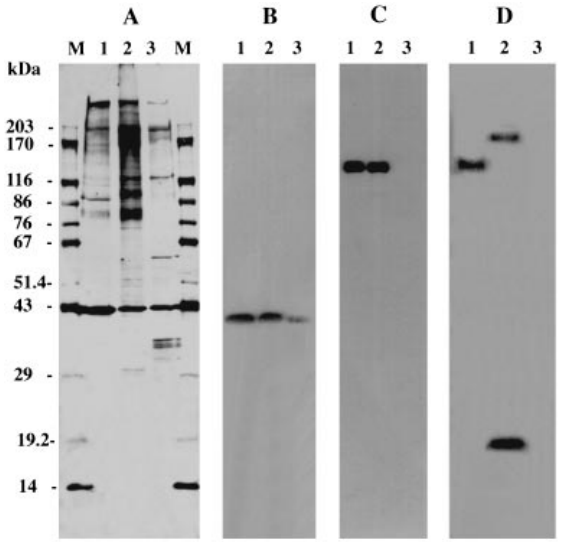

Figure 3 Binding of platelet PP1c and M130 to MC-Sepharose

The proteins solubilized from the low-speed (lanes 1) and high-speed pellet (lanes 2) in the presence of 0.6 M NaCl, and the high-speed supernatant (lanes 3), were exposed to MC-Sepharose. The MC-Sepharose-bound and unbound proteins were separated by centrifugation as described in the Experimental section. The MC-Sepharose-bound proteins were subjected to electrophoresis on 7.5–20% gradient gel followed by silver staining (**A**), or transferred to nitrocellulose membrane and exposed to antibody to PP1cδ (anti-PP1c) (**B**), or exposed to antibody to rM130$^{1-674}$ (anti-M130) (**C**), or subjected to phosphorylation (**D**) in the presence of 0.3 mM [γ-^{32}P]ATP, 5 mM Mg^{2+} and the MC-Sepharose unbound protein fraction for 15 min at 37 °C. At the end of incubation MC-Sepharose was treated as described in the Experimental section, then subjected to electrophoresis followed by autoradiography. A slight phosphorylation of a 20 kDa protein was visible in the absence of the MC-Sepharose unbound fraction only in the high-speed pellet, and no phosphorylation was detected in any of the fractions in the presence of 8 mM EDTA (results not shown). Lane M, molecular-mass markers.

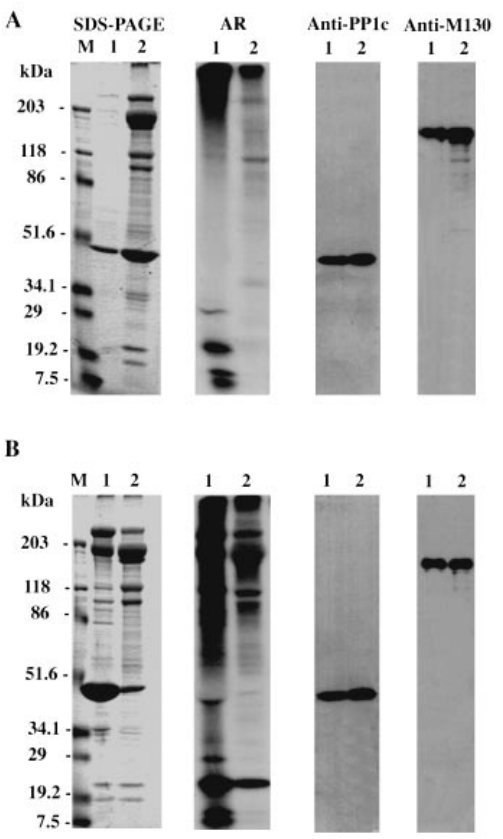

Figure 4 Effect of CL-A on the phosphorylation and translocation of platelet proteins in the cytoskeletal and membrane skeleton fractions

Platelets were labelled with $^{32}P_i$ and incubated for 15 min without (**A**) or in the presence of 0.1 μM C-LA (**B**). Cytoskeletal (lanes 1) and membrane skeleton (lanes 2) fractions were isolated by centrifugation of the platelet lysate at 10000 ***g*** and 100000 ***g*** respectively. The pellets obtained from 2×10^7 platelets were subjected to electrophoresis on 7.5–20% gradient gels and analysed by Commassie Brilliant Blue staining (SDS/PAGE), autoradiography (AR) and Western blotting with antibodies specific for PP1cδ (anti-PP1c) or rM130$^{1-674}$ (anti-M130). Lane M, molecular-mass markers.

M130 was established by Western blots. PP1c was detected in each fraction (Figure 3B) and M130 was found only in the low- and high-speed pellets (Figure 3C). To check for non-specific binding the Sepharose matrix was subjected to the coupling conditions, but in the absence of microcystin, and then treated with each fraction. Silver staining of the gel after SDS/PAGE (results not shown) revealed binding of actin to this control Sepharose matrix from each fraction (although the staining intensity was less than in case of MC-Sepharose). A faint band at 200 kDa, possibly corresponding to myosin heavy-chain, also appeared from the low- and the high-speed pellets. However, no significant amount of phosphatase was absorbed by the control resin, and consistent with this, Western blots could not detect either M130 or PP1c in these non-specifically bound fractions. A recent hypothesis, developed mostly from studies with smooth muscle, is that phosphorylation of M130 may be a mechanism to regulate phosphatase activity [14–16]. To assess whether the PP1c-associated proteins could be phosphorylated and to establish if the MC-Sepharose matrix provided a suitable experimental medium, phosphorylation assays were carried out. MC-Sepharose containing the bound proteins from each subcellular fraction was incubated with [γ-^{32}P]ATP and Mg^{2+} in the absence or presence of the unbound components, e.g. the supernatant after sedimentation of the MC-Sepharose, and subjected to autoradiography. Phosphorylation was detected only when the unbound fraction was incubated with the MC-Sepharose complex (Figure 3D). For the low-speed pellet a single band was detected corresponding to M130. In the high-speed pellet two components were phosphorylated and these were probably myosin heavy-chain and LC20. When the unbound fraction from the low-speed pellet was incubated with the MC-Sepharose complex obtained from the high-speed pellet only M130 was phosphorylated (results not shown). Incubation of the low-speed MC-Sepharose complex with the unbound fraction of the high-speed pellet resulted in phosphorylation of the 200 kDa and 20 kDa proteins. Thus it is apparent that the myosin kinase(s) and the M130 kinase are separated and found in the high-speed and low-speed pellets respectively. It should also be pointed out that the phosphorylation assays were carried out in the absence of Ca^{2+}.

Kurisaki et al. [25] showed that exposure of platelets to cell-permeant phosphatase inhibitors (CL-A and tautomycin) caused a partial translocation of myosin to the cytoskeletal fraction. This occurred in the absence of an increase in the intracellular Ca^{2+} concentration. Platelet M130 was present in both the low- and the high-speed pellet fractions, and it was of interest to determine if increased phosphorylation levels, achieved by treatment with CL-A, caused a translocation of M130 to the cytoskeletal fraction. Low- and high-speed pellets were prepared from ^{32}P-labelled platelets under resting conditions (Figure 4A)

or following exposure to CL-A (Figure 4B). These fractions were analysed by Western blotting with antibodies to PP1c and M130, and by autoradiography. Only a few proteins were phosphorylated in resting platelets, including a high-molecular-mass component and the putative LC20. CL-A treatment increased the overall level of protein phosphorylation. Many proteins were phosphorylated in both fractions (Figure 4B) and, as suggested earlier [25], may include talin, myosin heavy-chain and LC-20. Several other phosphorylated components, in the range 60–200 kDa, were unidentified and these could include M130. As shown in Figure 4(B), treatment with CL-A caused a marked translocation of cytoskeletal proteins (including myosin and actin) into the low-speed pellet fraction. However, the M130 band was present in both of the pellet fractions and a marked shift in the distribution of M130 (or PP1cδ) did not occur. Partial translocation of M130 and PP1c would not have been detected in this system.

DISCUSSION

PP1 appears to represent the major phosphatase activity in human platelets (considering PP1 plus PP2A as total phosphatase activity under the assay conditions used). PP1c and PP2Ac showed distinct subcellular distribution, since PP1c was present in each fraction while PP2Ac was apparently absent from the low-speed pellet (cytoskeletal fraction). A fraction of PP1c may be complexed with the large, non-catalytic subunit of smooth muscle MP (M130) in the platelet cytoskeleton and in the high-speed pellet. Interaction of smooth muscle M130 with PP1c was shown to stimulate P-LC20 phosphatase activity and to suppress phosphorylase phosphatase activity [7,12,13]. The P-LC20/phosphorylase phosphatase activity ratio was low in the high-speed pellet and supernatant. In these fractions both PP1c and PP2Ac are present. The M130 is associated with the high-speed pellet but it may be complexed with only part of the total PP1c in this fraction. The 10-fold higher P-LC20/phosphorylase phosphatase activity ratio in the cytoskeletal fraction ($> 90\%$ of PP1-like activity) could be due to a higher extent of association of PP1c with M130 and the absence of PP2A. The data given above suggest that the major myosin phosphatase in the platelet cytoskeleton is PP1 and that it includes a regulatory subunit similar to the M130/133 in smooth muscle MP.

The structure of platelet M130 is not known, but some information can be obtained from the use of various antibodies. Cross-reaction of the antibodies with smooth muscle and platelet M130 isoforms implies similarity of the epitopes. For example, it is expected that the platelet M130 isoform contains a sequence similar to the N-terminal smooth muscle M130 sequence, i.e. residues 1–38. This region binds to PP1c [12,13] and contains the conserved four-residues sequence (Arg/Lys-Val/Ile-Xaa-Phe) found in most PP1c-binding proteins [35,36]. It has also been shown for smooth muscle M130 that the N-terminal peptide of 38 amino acids and the second half of the ankyrin repeat region may be important in determining relative substrate specificity towards phosphorylase *a* and P-LC20 [12,13]. In addition, platelet M130 contains leucine-zipper motifs that are present in the rat M110 [10] but absent from the chicken gizzard M130/M133 isoforms [8].

The smooth-muscle M130 is known to bind to myosin [11,38], therefore, co-localization of MP with myosin should be considered. In the absence of ATP, M130 binds to both phosphorylated and dephosphorylated myosin [8,9]. However, under physiological conditions and in the presence of ATP, M130 binds only to phosphorylated smooth-muscle myosin [11]. Thus, it is assumed that MP is partitioned between the phosphorylated myosin (in thick filaments) and the cytosol, particularly under relaxing conditions. Studies on the localization of M130 in various cells show a localization with stress fibres and also in the cytosol [39,40], and these observations are consistent with binding only to phosphorylated myosin. However, a membrane localization of M130 has also been found: Inagaki et al. [39] observed in MDCK cells that the M130 was located at the cell–cell adhesion sites that formed at confluence. Thus, while it is known that myosin is an important target for M130, alternative or complementary targets may exist. It is interesting that in resting platelets M130 was not found in the cytoplasm; this indicates an altered binding pattern of M130 compared with other cells examined. A difference in either platelet M130 and/or myosin could result in stronger binding, or additional ligands may be involved.

The results obtained by isolation of platelet MP with MC-Sepharose indicate that PP1c in each subcellular fraction was associated with other proteins. It is known that PP1c binds to M130 [13] and that M130 binds to myosin [11,38] and possibly other proteins. Thus various linkages are built up to form a complex of proteins that may reflect the intracellular assembly of PP1c and M130. Most of the proteins in each fraction were not identified, although actin and myosin were probably common constituents. Polanowska-Grabowska et al. [41] found that immunoprecipitation from non-adherent platelets with antibodies against the 70 and 90 kDa heat-shock proteins yielded eight phosphoproteins that included PP1c and M130. Adhesion of the platelets resulted in dephosphorylation and dissociation of several of these components. In another study Campos et al. [37] used biotinylated microcystin to identify 29 proteins from skeletal muscle that bound to PP1c. Thus, a complex pattern of proteins attached to MC-Sepharose was not unexpected, and the challenge is to identify each component and to characterize its interactions in the hope that these reflect assemblies *in vivo*.

The complexes bound to MC-Sepharose did not contain kinase activity, which was detected in the unbound fraction. Extraction of the pellet fractions at high ionic strength removed kinase activity and this was also found with the smooth muscle MP holoenzyme [15]. It is surprising that despite overlap of some protein components in the two pellet fractions, e.g. actin, myosin and M130, the kinase(s) were distinct. The kinase(s) from the low-speed pellet phosphorylated M130 and that from the high-speed pellet phosphorylated myosin. Thus, the two fractions represent convenient starting points for the identification of the kinases involved. Several possibilities should be considered. For the myosin light-chain phosphorylation the candidates are MLCK (even though the assays were carried out in the absence of Ca^{2+}), protein kinase C, Rho kinase [42] and the p21-activated protein kinase [43]. For the heavy-chain kinase, candidates include protein kinase C [44] and casein kinase-2 [45,46]. With respect to protein kinase C it is possible (based on work done with smooth muscle) that an atypical isoform is involved and the ζ-isoform should be considered [47]. Similarly, the identity of the M130 kinase is not known. Obviously an important objective for future research is to determine if phosphorylation of M130 constitutes a regulatory mechanism in platelets and, if so, to identify the components involved.

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