PLATELET ACTIN: SUB-CELLULAR DISTRIBUTION AND ASSOCIATION WITH PROFILIN

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1. Introduction

Actin has been implicated in a variety of morphological changes associated with platelet function in haemostasis, including cell shape changes and microspike formation, secretion and clot retraction (reviewed [1]). The ordered and, in some circumstances, independent occurrence of these changes suggests control over both actomyosin ATPase activity and the organisation and location of both actin and myosin containing filaments. A particularly intriguing observation was that platelets contain a quantity of stable monomeric actin which is not polymerised under physiological conditions [2,3]. This actin might represent a storage pool able to form filaments following a specific physiological stimulus. The discovery of profilin, a small protein which binds to monomeric actin and stabilizes it [4], has prompted us to re-examine the unpolymerized actin of platelets to establish whether a similar protein is present. Our results show that platelets contain profilin at a molar ratio of 1:1 with the unpolymerized actin. In addition we have studied the quantitative distribution of actin in subcellular fractions, since platelets have been reported to contain actin specifically associated with plasma membranes [5] and secretory organelles [6]. The presence of two forms of actin has been demonstrated in a variety of cell types by isoelectric focussing [7] and we have analysed the distribution of these two forms, β and γ in the various platelet actin fractions under investigation.

2. Materials and methods

Pig blood was collected into anticoagulant containing 0.017 M trisodium citrate, 0.013 M citric acid and 0.4% dextrose (w/v) and 10^{-5} M adenosine (final concentrations) to inhibit platelet aggregation [8]. The platelets were collected by differential centrifugation [9] and washed with 0.14 M NaCl, 10 mM sodium phosphate, pH 6.0, 2 mM EDTA and 10^{-5} M adenosine (suspension buffer) to remove residual red cells and plasma proteins. 16 litres of blood yielded 5–7 g wet wt platelets. Washed platelets (5–15 g) were suspended in 10–20 ml suspension buffer and centrifuged through 30 ml of a 0–40% isotonic glycerol gradient [10]. The pellets could be stored at –20°C at this stage. Cells were lysed and fractionated as in [10]: platelets were suspended with thorough mixing in cold hypotonic buffer (10 mM Tris–HCl or 5 mM sodium phosphate pH 7.5, 0.25 M sucrose, 1 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethane sulphonyl fluoride added just before use) (lysis buffer), about 10 ml/5 g cells. This lysate was layered onto 2–5 ml 27% (w/v) sucrose in 14 ml tubes for a Beckman SW-40 rotor and centrifuged at 35 000 rev./min for 2 h. This yielded 4 fractions (fig.1): a clear supernatant 'A', a layer of membrane vesicles at the sucrose step interface 'B', a clear intermediate zone 'C' and a pellet of intracellular granules and organelles 'D'. Fractions were removed with a U-tipped pasteur pipette. The membrane fraction was washed several times with 10 ml lysis buffer to remove contaminants and the first wash was included with the soluble fraction 'A'. Centrifugation of fraction 'A' at $10^5 \times g$ for 3 h yields a gelatinous pellet of F-actin and a clear supernatant.

DNAase I inhibitor activity [11] was measured at 25°C by adding 3 ml 50 μg/ml calf thymus DNA in 125 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM $CaCl_2$ to 1 μg DNAase mixed with 5 μl sample and measuring the rate $\dot{A}_{260}$ increase.

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Protein concentrations were determined by the Folin method [12] using bovine serum albumin as a standard. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was carried out on slab gels using either a continuous Tris–bicine buffer [13] or a discontinuous Tris–glycine buffer system [14]. The latter system gives sharper bands although the molecular weight range is less broad but is the preferred method for gel densitometry. Isoelectric focussing gels were prepared in urea [15] in either 8.5 × 0.6 cm tubes or 10 × 17 cm flat bed slabs [16]. After electrophoresis, gels were washed and stained as in [16]. Peak areas on Coomassie brilliant blue stained gels were measured with a Joyce-Loebl densitometer for tube gels or a Camag scanning densitometer for slab gels.

All Sephadex columns were equilibrated and run at 4°C in 50 mM NaCl, 20 mM sodium phosphate pH 7.5, 0.2 mM ATP, 0.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM dithiothreitol and 2 mM NaN_3 unless otherwise specified. Viscosity measurements were made at 25°C in an Ostwald viscometer with a flow time for water of 80 s.

3. Results and discussion

3.1. *Distribution of actin in platelet subfractions*

Actin is the major protein of whole platelet lysates. The amount of actin in each lysate sample was determined by densitometry of polyacrylamide gels in sodium dodecyl sulphate, using 5–6 known actin amounts (0.5–5 μg) to construct a standard curve for each gel. The total protein loaded in each gel sample was calculated from the Folin reaction. Actin represents 21 ± 3% (n = 30) of platelet protein by this technique. This value is lower than the 30% reported in [17], but systematic errors might be introduced by either experimental method.

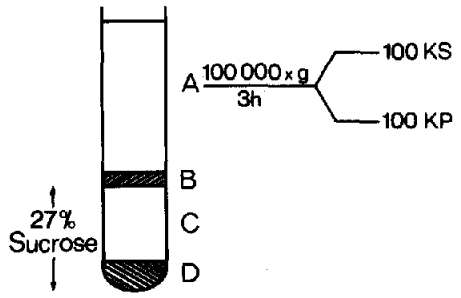

Fig.1. Fractionation of platelet lysates by centrifugation onto a sucrose step. 'A', soluble fraction; 'B', membranes; 'C', intermediate zone; 'D', pellet. 100 KS, 100 KP supernatant and pellet, respectively, from 100 000 × g centrifugation of 'A'.

The proportions of total protein and total actin in each of the 6 subfractions obtained as shown in fig.1 are presented in table 1. Although there was moderate variation between different batches of platelets, the following general points emerge: The major fractions 'A' and 'D' together with the 100 000 × g pellets and supernatant fractions from 'A' all contain about the same proportion of actin as the initial lysate. Most of this actin (54%) is found in the soluble fraction 'A', unattached to

Table 1
Distribution of actin in platelet subfractions

Subfraction	Total protein		Total actin	
	Percentage	Determinations	Percentage	Determinations
Lysate	100		100	
'A' Soluble	54 ± 8	9	54 ± 8	5
100 000 × g supernatant	38 ± 7	6	36 ± 4	4
100 000 × g pellet	13 ± 4	9	15 ± 3	5
'B' Membrane	1.1 ± 0.6	9	0.6 ± 0.2	5
'C' Intermediate	6 ± 2	6	8 ± 5	5
'D' Sucrose pellet	38 ± 7	6	37 ± 9	5

The proportion of actin in the original lysate was 21%; thus the major fractions 'A' and 'D' contain the same proportion of actin as in the original lysate. Details of the fractionation are given in fig.1

organelles or plasma membranes, and about 2/3 of this (36% total actin) is not sedimentable at 100 000 × *g* (table 1).

The membrane fraction is biochemically similar to plasma membranes from other sources [10]. These membranes contain only a very small proportion of the total cellular actin (< 1%). We have confirmed that this protein, identified initially as a band on polyacrylamide gel electrophoresis in sodium dodecyl sulphate, is actin by its specific retention on a DNAase I affinity column [16] and by isoelectric focussing. The amount of actin in membranes is very variable, although it usually forms a significantly lower proportion of the total protein than in the other fractions (table 1). Actin has been identified [5] which could not be dissociated from platelet membranes and which was suggested to form an integral part of the membrane.

The bulk of the remaining actin (37%) is in the pellet of cell organelles, granules and debris. Its properties have not been further analysed here, but specific association of microfilaments with platelet dense granules has been postulated [6].

Addition of 1 mM ATP to the lysis buffer does not affect the quantitative distribution of actin between the various subfractions. This suggests that the endogenous ATP concentration is sufficient to dissociate actomyosin during the fractionation, since more than 75% of platelet myosin is found in the soluble fraction 'A'.

Platelet actin contains two isoelectric components, β and γ [7]. We have compared the relative amounts of the two forms in the platelet subfractions and in actin purified from the 100 000 × *g* pellet (F-actin) and supernatant (unpolymerized actin) (fig.2). No marked differences were observed between the different actin fractions (table 2). This indicates that the organization of actin within platelets is effected by mechanisms which do not discriminate between β and γ forms.

3.2. *Unpolymerized actin is stabilized by profilin*

The unpolymerized actin present in the 100 000 × *g* supernatant fraction (fig.1) has been separated from higher molecular weight proteins on Sephadex G-150 and further purified by ammonium sulphate fractionation. Addition of ammonium sulphate to 33% saturation precipitated pure actin,

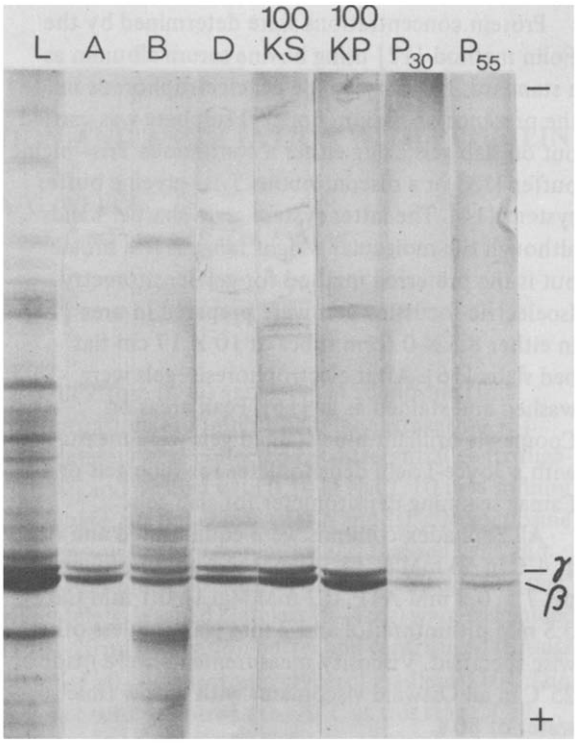

Fig.2. Isoelectric focussing in tube gels of platelet subfractions. β, γ are actin subforms. 'L', cell lysate; 'A', soluble fraction; 'B', membranes; 'D', pellet; 100 KS, 100 KP supernatant and pellet, respectively, from 100 000 × *g* centrifugation of 'A'; P_{30}, partially purified actin, 0–30% ammonium sulphate fraction, i.e., contains only uncomplexed actin; P_{55}, 30–55% ammonium sulphate fraction, i.e., profilactin.

which polymerized in the presence of 0.5 mM ATP, 2 mM $MgCl_2$ and 50 mM NaCl (fig.3). A further actin-containing precipitate was obtained in the 33–55% ammonium sulphate saturation fraction, but this did not polymerize under the conditions given above. Gel electrophoresis showed this actin to contain an additional component of about 15 000 mol. wt (fig.3), in a molar ratio of 1.0 ± 0.1 (n = 14) by gel densitometry (assuming equal staining intensity for this polypeptide and actin). This low molecular weight protein resembles 'profilin' isolated from spleen and other sources [4]; it co-migrates with thymus profilin on polyacrylamide gels in the presence of sodium dodecyl sulphate and on isoelectric focussing. When this non-polymerizable actin obtained from ammonium sulphate fractiona-

Table 2
Distribution of β and γ components of actin in the platelet subfractions

Subfraction	β:γ ratio		
	Mean	SD	Determinations
Lysate	1.5	0.2	3
'A' Soluble	1.8	0.2	7
'B' Membrane	1.7	0.3	6
'D' Sucrose pellet	1.8	0.1	4
100 000 × *g* supernatant	1.7	0.3	6
100 000 × *g* pellet	1.6	0.3	4
pure F-actin[a]	1.7	0.2	6
pure profilactin[b]	1.4	0.1	3
All determinations	1.6	0.2	39

[a] Actin purified from 100 000 × *g* pellet by Sepharose 4B gel filtration in the presence of KI [18]

[b] Profilactin purified from 100 000 × *g* supernatant by Sephadex G-150 gel filtration, ammonium sulphate fractionation and DEAE-cellulose chromatography [19]

The β and γ components were separated by isoelectric focussing in the pH 5–8 range and densitometry was carried out as in section 2

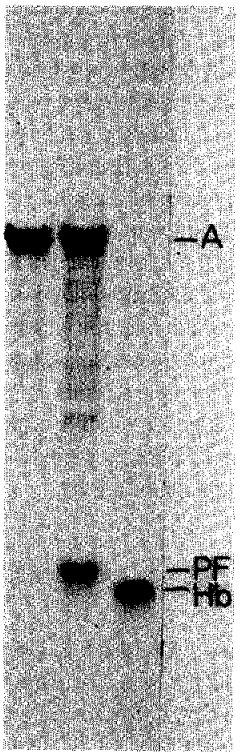

Fig.3. 7.5% polyacrylamide–sodium dodecyl sulphate slab gel, Tris–bicine buffer system of (left) platelet actin, (centre) platelet profilactin, (right) haemoglobin marker.

tion was further purified on DEAE-cellulose in the presence of ATP [19], some of the 15 000 mol. wt protein was isolated in the breakthrough, while the remainder was eluted together with actin using a salt gradient. Spleen profilin does not bind to DEAE-cellulose [4] and therefore we would expect any uncomplexed profilin to be eluted in the breakthrough. These results suggest that the unpolymerized actin of platelets is complexed with a protein a analogous to spleen profilin in a 1:1 molar ratio. This complex will be termed 'profilactin' by analogy with [4]. In order to confirm that this complex can be dissociated into profilin and native actin which is capable of polymerization, some platelet 'profilactin', isolated on Sephadex G-100 was incubated in 1 mM ATP, 2 mM $MgCl_2$, 50 mM NaCl, pH 7.5, containing 10 mM EGTA, conditions known to cause dissociation of spleen profilactin (J. Kendrick-Jones and R. Jakes, personal communication). A 20-fold increase in specific viscosity was observed under these conditions, consistent with actin polymerization.

Platelet profilactin is not completely stable to biochemical manipulations and purification on Sephadex G-100 yields a small amount of free profilin at higher elution volumes than the profilactin complex. This profilin has been shown to behave like spleen profilin in preventing the polymerization of rabbit G-actin and promoting inhibition of DNAase I activity. Incubation of 13 μM rabbit G-actin with 8 μM platelet profilin under polymerizing conditions (0.5 mM ATP, 2 mM $MgCl_2$, 50 mM NaCl, pH 8.0) gave specific viscosity values between 10% and 15% of those obtained when G-actin was polymerized in the absence of profilin. In a further experiment where 8 μM platelet profilin was mixed with 10 μM platelet G-actin and incubated for 1 h under actin polymerizing conditions, we found 80% inhibition of DNAase I activity as compared with less than 30% inhibition for a control experiment where 10 μM platelet actin was incubated without the profilin.

We conclude that the unpolymerized actin of platelets contains profilin, similar to that isolated from

spleen [4] in an equimolar complex with the G-actin. The profilin:actin molar ratio in platelet lysates is 0.31 ± 0.07 ($n = 8$) as determined by gel densitometry. This is approximately sufficient to account for all the unpolymerized actin in crude lysates (36 ± 4%) (table 1). The profilin:actin molar ratio in the 100 000 × g supernatant fraction is 0.8 ± 0.2 ($n = 6$), not significantly different from the equimolar ratios obtained for purified complex. By formation of this complex a pool of unpolymerized actin may be maintained at a concentration in the cells that is well above the critical concentration for actin polymerization [20]. Further experiments are in progress to establish whether any of this unpolymerized actin is utilised in microfilament formation during processes involved in platelet stimulation.

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