Isolation and characterization of human blood platelet gelsolin

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A 90-kDa protein–actin stable complex was purified from blood platelets by a short and efficient procedure giving at the same time actin used in polymerization assays. 90-kDa protein free of actin was prepared from the complex by 8 M ureau treatment and renaturation. By its molecular mass, immunological cross-reactivity with macrophage gelsolin and its effect on G- and F-actin the 90-kDa protein appears as the platelet gelsolin.

Blood platelet *Actin* *90-kDa protein* *gelsolin*

1. INTRODUCTION

In response to stimuli, blood platelets exhibit dramatic structural transformations. Upon activation, the discoidal cells round up, extend pseudopodia, aggregate and undergo an internal reorganization [1]. These events result from the activation and interaction of cytoskeletal proteins. Actin, one of the most abundant proteins in platelets, seems to be particularly involved in the shape changes and the internal movements during activation [2,3]. Indeed, platelet activation is accompanied by the polymerization of the major part of actin which is maintained in non-polymerizable state in intact platelets [4,5]. Moreover, it was also shown that calcium mediates the regulation of actin assembly in human platelet extracts [4].

Several groups have found that platelets contain a 90-kDa protein capable of modulating actin assembly. This protein had so far been isolated only as a complex with actin [6–9]. We have developed a rapid purification procedure which allows the isolation of a 90-kDa protein–actin stable complex. The 90-kDa protein free from actin was also obtained. We tried to gain an insight into the mechanism of the interaction with actin of pure 90-kDa protein and of its complex with actin.

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; ME, mercaptoethanol

2. MATERIALS AND METHODS

Human blood platelets (4–6 days old) were washed and stored as in [10].

Platelet actin was recovered from peak III of Sephadex G-150 column chromatography (fig.1) and purified as in [11]. Actin polymerization was followed by the increase of viscosity and of the absorbance at 232 nm [12]. Viscosity was measured at 25°C in a Cannon semimicroviscosimeter (size 100) with a buffer flow time of about 63 s.

The concentration of actin was determined using $E^{1\%}_{cm}$ at 290 nm of 6.3 for G-actin and of 6.6 for F-actin [13]. The concentration of the 90-kDa protein and of its complex with actin was determined as in [14].

DNase I was immobilized on Agarose according to [15].

Purification of the complex: 1.6 g of platelet actone powder [11] were extracted 30 min with 20 vols of 2 mM Tris–HCl, 0.2 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM ATP, 0.5 mM ME, 0.75 mM NaN_3 (pH 7.5). The crude extract was centrifuged for 2 h at 100000 × *g*. The supernatant

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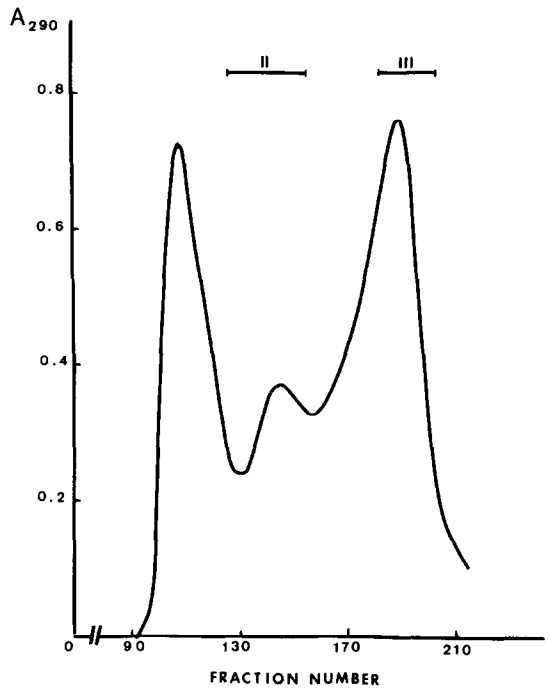


Fig.1. Gel filtration on Sephadex G-150 of platelet acetone powder extract. The column (4 × 92 cm) was eluted at a flow rate of 20 ml/h in 3-ml fractions. 90-kDa protein–actin complex from peak II and actin from peak III were separately pooled.

was concentrated in an Amicon ultrafiltration cell to about 40 mg/ml and loaded on the Sephadex G-150 column equilibrated with extraction buffer containing 0.05 mM EGTA (fig.1). The fractions in which 90-kDa protein was present were pooled and precipitated by 75% ammonium sulfate. The precipitate was resuspended in buffer A (20 mM Tris–HCL, 0.2 mM ATP, 0.5 mM ME, 0.2 mM PMSF; pH 7.8) containing 0.1 mM $MgCl_2$ and 1 mM EGTA. The protein solution was applied to an agarose–DNase I affinity column (1.7 × 2.6 cm) equilibrated with buffer A containing 5 mM $CaCl_2$. The column was washed with 6 vols of the same buffer and 3 vols of this buffer supplemented with 0.6 M NaCl. The complex was eluted from the column by 5 mM EGTA in buffer A containing 0.15 M NaCl. EGTA eluate was concentrated by dialysis against buffer B (10 mM Tris–HCl, 0.2 mM $MgCl_2$, 2 mM EGTA, 150 mM NaCl, 0.2 mM ATP, 0.5 mM ME, 0.75 mM NaN_3; pH 7.5) containing 15% polyethylene glycol 6000. To determine the molecular mass of the complex, this solution was loaded on Sephacryl S-300 column equilibrated with buffer B (fig.2).

Preparation of pure 90-kDa protein: about 5 mg of the purified complex in buffer C (10 mM Tris–HCl, 0.1 mM EDTA, 20 mM KCl, 0.5 mM ME, 0.75 mM NaN_3, 8 M urea; pH 7.5) containing 0.2 mM PMSF was loaded on a column of DEAE–Sepharose equilibrated with the same buffer. After washing with buffer C, a linear gradient between 20 and 300 mM KCl in the same buffer was applied. The fractions eluted between 185 and 205 mM KCl containing the major part of 90-kDa protein practically free from actin were pooled and assayed for renaturation. The best results were obtained by dialysis against 20 mM Tris–HCl (pH 7.5), containing 150 mM NaCl, 0.1 mM $CaCl_2$, 0.5 mM ME and 0.75 mM NaN_3.

Fractions corresponding to different steps of purification were analyzed by polyacrylamide gel electrophoresis (fig.3).

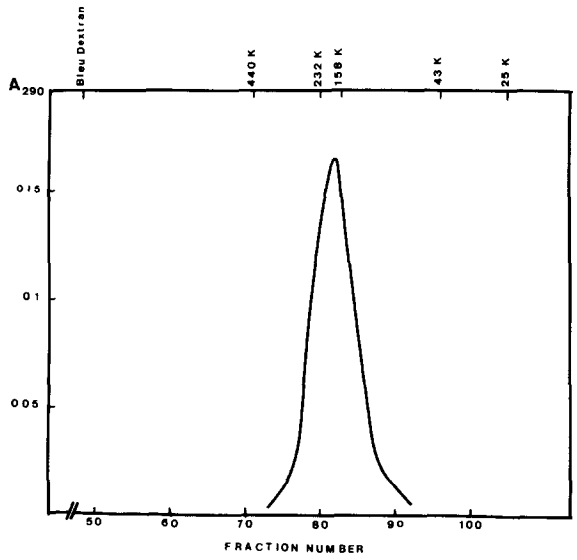


Fig.2. Gel filtration on Sephacryl S-300 of EGTA eluate. The column (1.6 × 74 cm) was eluted at a flow rate of 18 ml/h in 1-ml fractions. The column was calibrated using a Pharmacia M_r calibration kit: ferritin, 440000; catalase, 232000; aldolase, 158000; ovalbumin, 43000; chymotrypsinogen A, 25000.

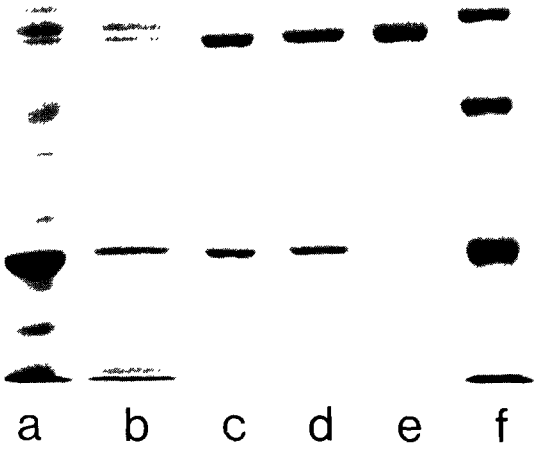


Fig.3. SDS-polyacrylamide gel electrophoresis of various purification steps of 90-kDa protein–actin complex and of isolated 90-kDa protein. Electrophoresis was performed as in [16] and gels were stained with Coomassie brilliant blue [17]. (a) Extract from acetone powder, (b) pooled complex fractions from Sephadex G-150, (c) pooled EGTA fractions from DNase I affinity column, (d) pooled complex fractions from Sephacryl S-300 chromatography column, (e) purified 90-kDa protein, (f) M_r standards: phosphorylase *b* (94000), albumin (67000), ovalbumin (43000), carbonic anhydrase (30000).

3. RESULTS

3.1. *Isolation of 90-kDa protein–actin complex and of free 90-kDa protein*

From 24 units platelet concentrate we obtained about 3.3 mg 90-kDa protein–actin complex. The progress of purification was evaluated by following the increase of inhibitory activity of actin polymerization according to [18]. The most efficient step was the DNase I treatment which allows 10-fold purification. A 50% inhibition of polymerization at the steady state was obtained when 0.018 μM purified complex was added to 10 μM G-actin.

Analysis by gel electrophoresis in SDS of the final product shows (fig.3) two bands of polypeptides, one at 90 kDa and the other, corresponding to actin, at 42 kDa. According to densitometry of the Coomassie-stained gels the molar ratio of the two proteins is one 90-kDa protein to about 1.7 actins. This ratio was confirmed by the estimation of the molecular mass of the complex (fig.2). The complex is not dissociable in EGTA even under conditions of high ionic strength (gel filtration in 0.8 M KCl). It was confirmed that antibodies against macrophage gelsolin cross-react with 90-kDa component of the complex (not shown).

By treatment in 8 M urea and separation on DEAE–Sepharose the 90-kDa protein was obtained practically free of actin (fig.3). After renaturation pure protein recovers about 90% activity it had formerly in the complex.

3.2. *Effect of platelet 90-kDa protein and of its complex with actin on the polymerization of platelet actin*

To study the polymerization of actin by following the changes in specific viscosity, the polymerization was initiated by 0.76 mM $MgCl_2$; these conditions favor the sigmoid kinetics of the control actin (fig.4a). In the presence of the complex, the lag time is abolished, the initial rate of polymerization increases and the final level of viscosity decreases. At a molar complex to actin ratio of 1:165, the final viscosity is reduced by 70%. Similar effects of the complex on actin polymerization can be observed by following the changes of absorbance at 232 nm (not shown). In the absence of free calcium (fig.4b), i.e., in 2 mM EGTA, the final viscosity is also reduced but this effect is smaller as compared to that in the presence of calcium. There is also a different effect on the initial rate of polymerization which decreases with addition of increasing amounts of free complex. To explain these results it is necessary to keep in mind that in the presence of EGTA a spontaneous nucleation takes place so that the additional nucleation by the complex seems cancelled. The decrease of the initial rate of polymerization and of final viscosity shows that even in the absence of free calcium, the complex is still capable of interacting with actin, probably by capping the fast growing end of nuclei and thus slowing down the elongation.

Pure 90-kDa protein exerts the same effects on actin polymerization as does its complex with actin.

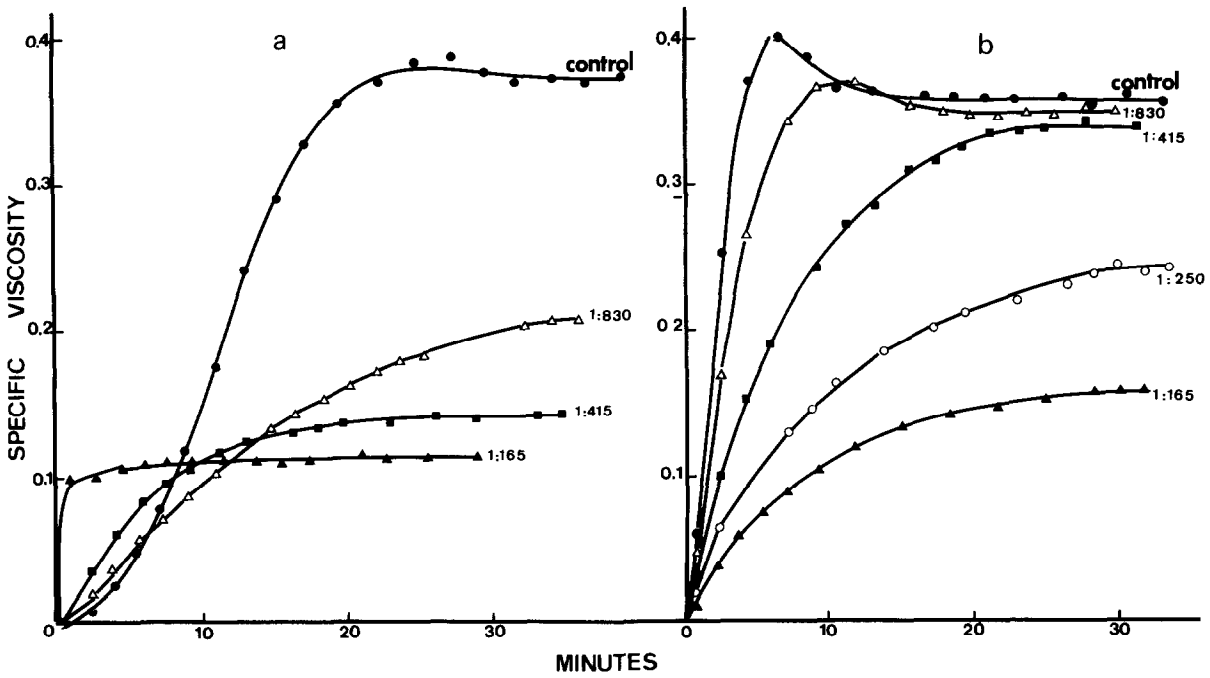
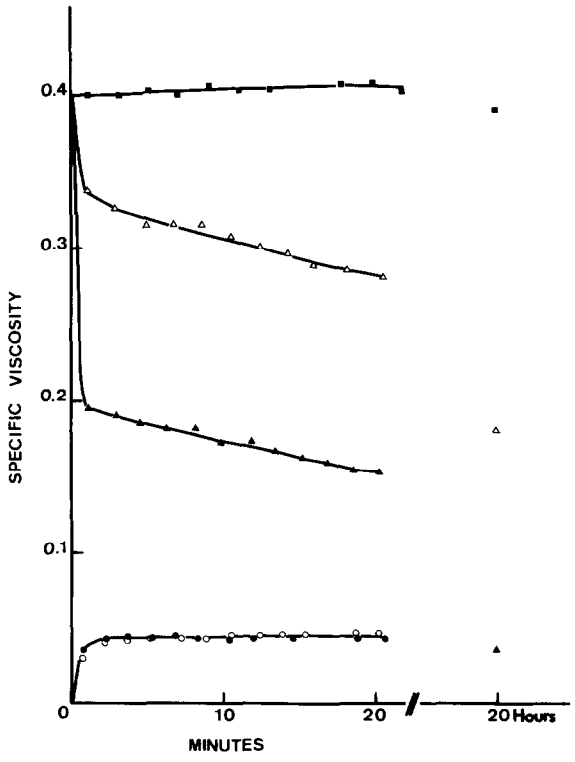


Fig.4. Viscometric analysis of the effect of 90-kDa protein–actin complex on the time course of platelet G-actin polymerization. The assays contained 9.5 μM actin in 2 mM Tris–HCl, 0.1 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM ME, 0.75 mM NaN_3 (pH 7.5) without (a) or with (b) 2 mM EGTA. The polymerization was initiated by the addition of 0.76 mM $MgCl_2$. Molar complex to actin ratios are indicated on each curve.



3.3. *Effect of platelet 90-kDa protein and of its complex on preformed actin filaments*

The addition of pure 90-kDa protein to F-actin induced a rapid drop of viscosity followed by a slow equilibration leading to the same steady-state viscosity as that obtained when 90-kDa protein was added to G-actin before the initiation of polymerization (fig.5).

Under the same conditions the addition of the complex to F-actin led to a small initial drop of viscosity followed by a slow equilibration. At

Fig.5. Effect of platelet 90-kDa protein and of its complex on preformed filaments of platelet actin. G-actin in 2 mM Tris–HCl, 0.1 mM $MgCl_2$, 2 μM EGTA, 0.2 mM ATP, 0.5 mM ME, 0.75 mM NaN_3 (pH 7.5) was polymerized by the addition of 2 mM $MgCl_2$. Both complex and purified 90-kDa protein were added to 9.5 μM F-actin in a molar ratio 1:20. F-Actin control (■—■), F-actin with complex (△—△) and with 90-kDa protein (▲—▲). Polymerization of G-actin in the presence of complex (○—○) and of 90-kDa protein (●—●).

equilibrium this effect was much weaker than that with G-actin (fig.5).

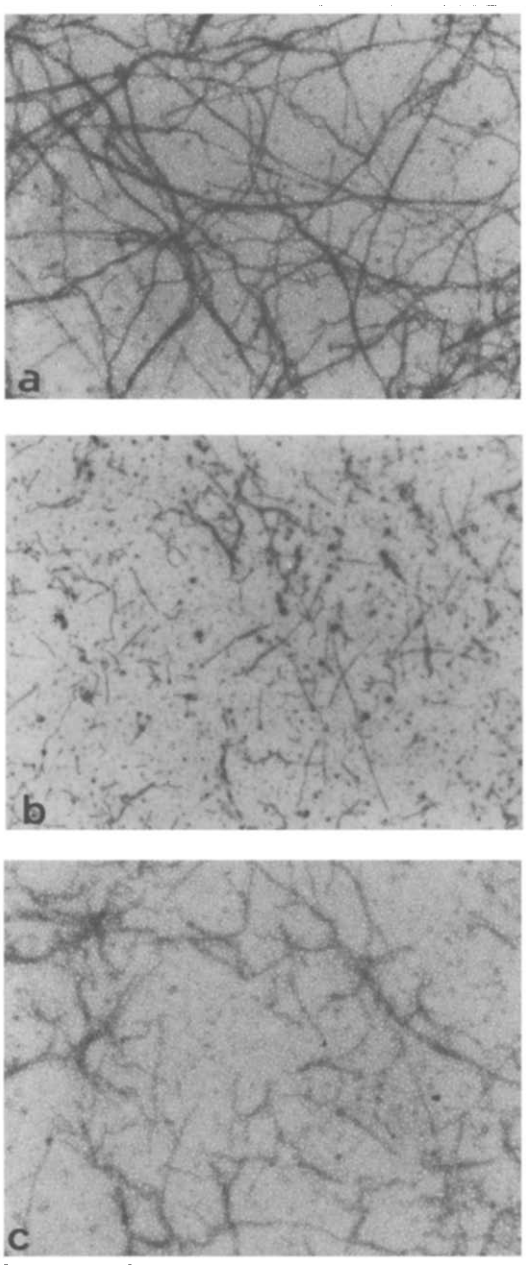


Fig.6. Electron micrographs of negatively stained samples of 4.8 μM actin filaments. F-Actin alone (a), after 1 min contact with purified 90-kDa protein (b) or with 90-kDa protein–actin complex (c). The molar ratio of F-actin to 90-kDa protein or to its complex was 20:1. Experimental conditions as in fig.5. Magnification × 20000.

The complex and the pure 90-kDa protein interacted with F-actin both in the presence and in the absence of calcium.

F-Actin mixed with 90-kDa protein under the same conditions as those used for viscometric assays was viewed in an electron microscope. Compared to the control actin filaments appeared appreciably shorter (fig.6a,b). When a molar equivalent of the complex was used instead of pure 90-kDa protein no significant shortening of the filaments was observed (fig.6c).

4. DISCUSSION

The 90-kDa protein from platelets is a modulator of actin polymerization. The free protein and its stable complex with actin accelerate the nucleation step and reduce the extent of actin polymerization at the steady state. The decrease of the final viscosity is primarily due to the formation of more numerous but shorter filaments as confirmed by electron microscopy (not shown). The reduction of the steady-state absorbance at 232 nm signifies that the total amount of F-actin is diminished. This is in favour of the capping of 'barbed' fast growing ends of actin filaments since the critical concentration of actin at 'pointed' ends is higher [19].

The effect of the complex on preformed actin filaments differs from that of pure 90-kDa protein. The complex resembles the AM-protein (fragmin) heterodimer [18] or the villin–actin complex [20], all of them having little ability to depolymerize F-actin. Fragmin and 90-kDa protein form stable, EGTA non-dissociable, urea-dissociable complexes with actin, having a low calcium sensitivity in their effects on actin assembly [21].

Pure 90-kDa protein has a more powerful capacity than the complex to decrease the viscosity of F-actin, probably by fragmenting actin filaments (fig.5). In this respect, its activity resembles those of fragmin, villin, severin and some gelsolins [21–26]. With respect to its molecular mass, immunological cross-reactivity with macrophage gelsolin and its effect on G- and F-actin, 90-kDa protein is closely related to the family of gelsolins [25–28].

The lack of calcium sensitivity of platelet gelsolin liberated from its complex with actin was also reported in [29]. This property which differen-

tiates platelet gelsolin from other gelsolins could be due to some modifications during platelet stimulation. It now seems necessary to determine whether gelsolin isolated from unstimulated fresh platelets is calcium sensitive. This point is now under investigation in our group.

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