Human platelet P235: A high M_r protein which restricts the length of actin filaments

Nancy C. Collier and Kuan Wang†

Clayton Foundation Biochemical Institute and Department of Chemistry The University of Texas at Austin, Austin, TX 78712, USA

Received 12 May 1982

Human platelet P235 *Actin modulating protein* *Actin capping protein* *Actin polymerization* *Actin binding protein* *Platelet motility*

1. INTRODUCTION

We have reported the purification and properties of human platelet P235 – a new high M_r protein (a dimer of M_r = 235 000 subunit) which contributes ~ 3–8% of the total platelet protein. P235 is distinct from filamin, fibronectin, spectrin and myosin and is a facile endogenous protein substrate of the highly specific calcium-activated proteases [1]. Its function in the platelet remains unclear. We have speculated that P235 may be involved in the contractile activities of platelets, because the specific and selective proteolysis of P235 and filamin correlated with the loss of the platelets' ability to extend filopodia, a manifestation of actin-related contractility [2]. To pursue this idea, we have been investigating potential in vitro interactions of purified P235 with contractile proteins, in particular, with actin. We now demonstrate that substoichiometric amounts of P235, when present during actin polymerization, slow the rate of increase of viscosity and reduce the steady-state low shear viscosity value in a dose-dependent manner. Further studies by electron microscopy reveal that actin filaments are shorter when formed in the presence of P235, indicating that P235 restricts the length of actin filaments in vitro.

2. EXPERIMENTAL

Detailed experimental conditions are given in appropriate figure legends.

† To whom correspondence and reprint requests should be addressed

3. RESULTS

3.1. *An alternative procedure of P235 purification*

Human platelet P235 was purified by an alternative procedure in which the sequence of phosphocellulose and agarose gel filtration of the original method [1] was reversed. Briefly, this alternative procedure involves applying pool DEAE (~ 31 mg) from the Triton X-100 extract of 8.8 g washed platelets to a Biogel A-5m column equilibrated with 50 mM Tris–HCl, 3.0 mM EDTA, 0.1 mM dithiothreitol (pH 8.0) (buffer B). Peak fractions of P235 eluting between K_{av} = 0.56–0.67 were pooled and then applied to a phosphocellulose column (1.1 × 5.3 cm) in buffer B and eluted with a linear gradient of 40 ml each of buffer B and 0.5 M NaCl in buffer B. P235 (1.4 mg) eluted between 0.11 M and 0.2 M NaCl. This pool, consisting of ≥ 95% pure P235 and containing no detectable filamin and myosin (fig.1A), was substantially freer of residual protease activities than that obtained by the original method [1]. It should be emphasized that, although all results presented here were obtained with P235 prepared by the alternate procedure, similar results were obtained with P235 prepared by the original procedure (described in [1]).

3.2. *Effect of P235 on low shear viscosity of actin*

The first indication of interaction of P235 with actin was obtained in studies with the low shear viscometric method [3] which provides a crude yet rapid measure of the effectiveness of agents that may affect actin filament mass, length, flexibility and self-association. When gel-filtered G-actin was

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

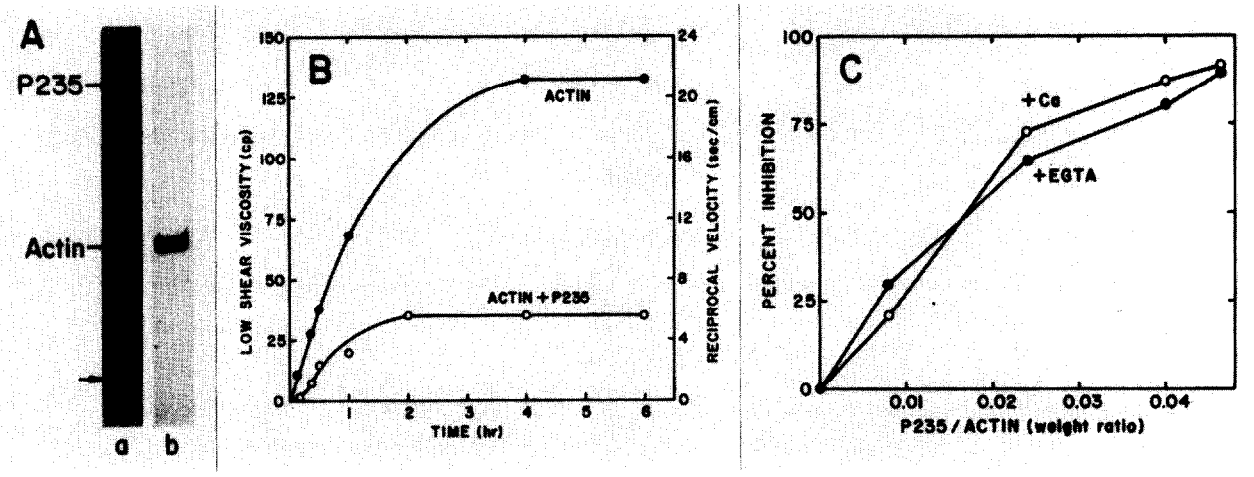

Fig.1. (A) Purity of protein preparations. SDS–polyacrylamide gel (3–12% gradient slab) analysis of: (a) P235 (~ 1 μg) purified by the alternative method as in section 3; (b) rabbit skeletal actin (~ 3 μg), purified according to [14] and gel-filtered as in [15]. Arrow denotes position of the tracking dye.

(B,C) Effect of P235 on the low shear viscosity of actin. (B) Time course: Gel-filtered G-actin (540 μg) was incubated at room temperature (10 min) in 1 ml of 10 mM imidazole, 0.1 mM ATP (pH 7.2) in the absence (•——•) or presence (○——○) of P235 (50 μg). KCl and $MgCl_2$ were added to 150 mM and 2.9 mM, respectively, to induce actin polymerization and the samples were immediately drawn into 100 μl glass capillary tubes. The low shear viscosity of the samples was measured at a 30° angle in separate tubes at 10 min, 20 min, 30 min, 1 h, 2 h, 4 h and 6 h after addition of salt by a falling ball method as in [6]. The reciprocal velocity (the fall time divided by the distance the ball travels) was converted to centipoise as in [6]. Note the decrease in viscosity of actin in the presence of P235 throughout the time course and the significant reduction of viscosity at steady state (2–4 h after addition of salt). (C) Dose dependence: Gel-filtered G-actin (250 μg) was incubated at room temperature (10 min) in 0.15 ml 10 mM imidazole, 0.1 mM ATP (pH 7.2) with various amounts of P235: 0 μg, 2 μg, 6 μg, 10 μg and 11.6 μg in the presence of either 10 μM $CaCl_2$ (○——○) or 0.1 mM EGTA (•——•). Actin was induced to polymerize by addition of salt as above (1B). The low shear viscosities of the mixtures were measured after samples of actin alone had reached steady state viscosity (~ 2 h).

induced by salt to polymerize in the presence of substoichiometric amounts of P235, the rate of increase of low shear viscosity of the mixture throughout the course of the experiment was significantly diminished compared to that of pure actin (fig.1B). Furthermore, the low shear viscosity at steady state was greatly reduced compared to that of pure actin. Such an inhibitory effect was dose-dependent. When increasing amounts of P235 were present, the steady state viscosity was progressively lowered. For example, at a weight ratio of P235-actin of 1:100, a 20% reduction of steady state

→

Fig.2. Morphological analysis of P235–actin mixtures. Gel filtered G-actin (24 μg) was incubated at room temperature (10 min) in 0.1 ml 5 mM Tris–HCl, 0.5 mM β-mercaptoethanol, 0.2 mM ATP, 0.2 mM $CaCl_2$ (pH 8.0) in the absence (A) or presence (B) of P235 (2.4 μg). KCl and $MgCl_2$ were added to 150 mM and 2.9 mM, respectively, to induce actin polymerization. After ~ 2 h the mixtures were diluted 5-fold (to 50–100 μg/ml protein) with 150 mM KCl and 2.9 mM $MgCl_2$ in the above buffer and immediately stained for electron microscopy. Aliquots (10 μl) were gently placed onto grids (400 mesh) coated with a glow discharged carbon film. After 30 s, excess sqlution was removed by a piece of filter paper and the grids were washed twice (30 s each) with the dilution buffer and then stained with 1% (w/v) aqueous uranyl acetate. The grids were viewed on an JEOL 100 CX electron microscope operated at 80 kV. Micrographs were taken at 16 000–33 000 × magnification: (A) actin polymerized in teh absence of P235; (B) actin polymerized in the presence of P235; (C) P235 sample; (D) selected areas of 2B enlarged to show morphological details of P235–actin mixtures (Note the sharp breaks and bends (→) and the sharp curvature at the ends (▲) of some actin filaments polymerized in the presence of P235); (E) laterally aligned filaments seen in 2B were enlarged to show presence of globular material (→) at crossover points of filaments; bars = 0.1 μm.

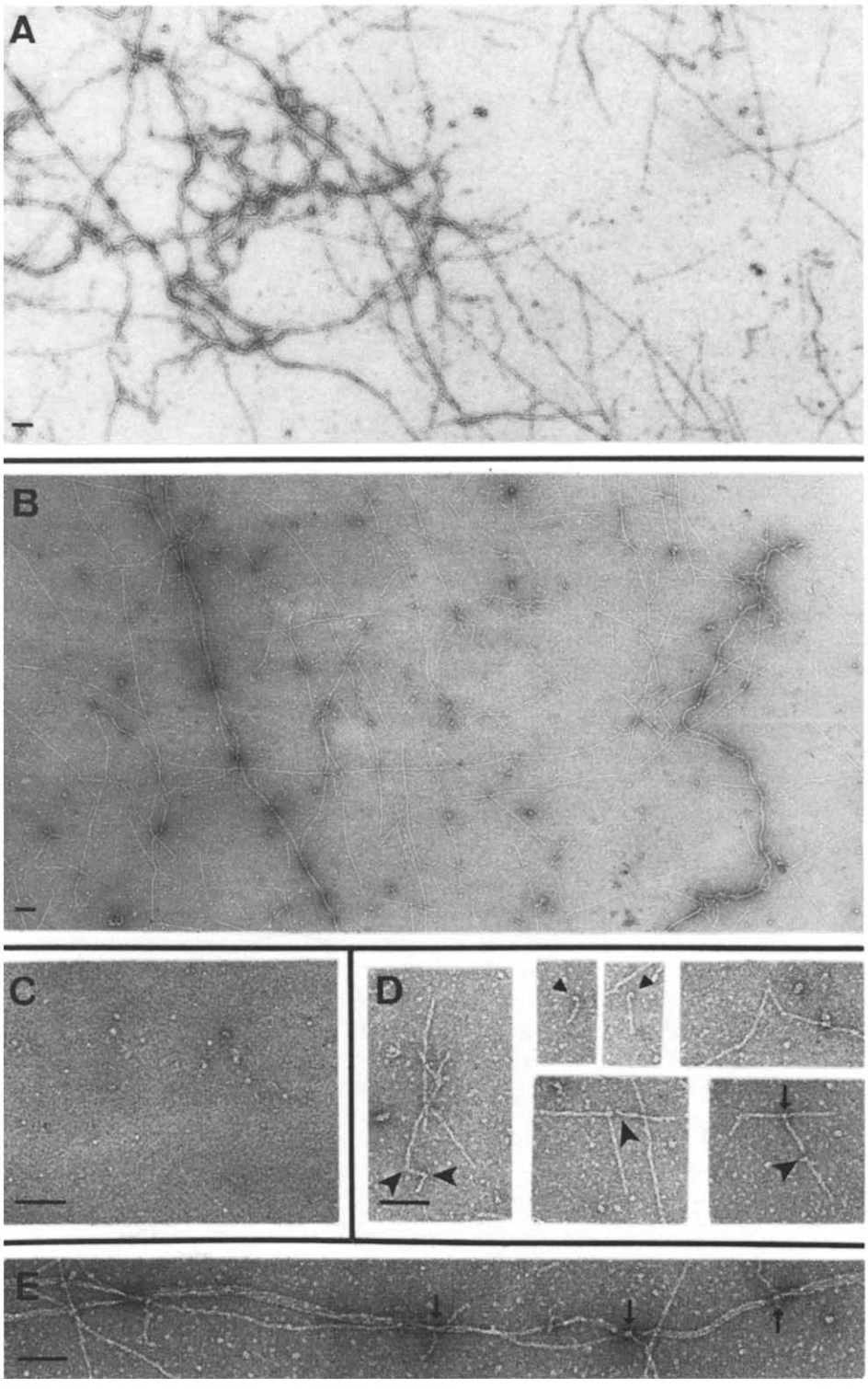

Fig.2

viscosity was detected. A ratio of 1:10 caused the viscosity to drop by > 80% (fig.1C). The latter weight ratio, corresponding to a molar ratio of 1:100 (P235) dimer to actin protomer), approximates the ratio of P235 to actin in platelet cytoplasm [1]. The addition of either 0.1 mM EGTA or 10 μM $CaCl_2$, which yielded 10^{-8} M or 9×10^{-6} M of Ca^{2+}, respectively, in the assay media, gave essentially the same reduction in the steady state viscosity (fig.1C), suggesting that Ca^{2+} near physiological concentrations are not required for the inhibitory effect of P235. However, the inhibitory effect of P235 was apparent only when it was present prior to actin polymerization; incubation of P235 with preformed actin filaments led to no significant changes in steady state viscosity even after prolonged incubation ($\leq$ 6 h).

These viscosity data are insufficient to define the molecular mechanism of P235–actin interaction. A decrease of low shear viscosity could be caused by a reduction of filament length, a reduction in filament mass, a modulation of filament–filament interaction such as an increase in lateral association of filaments or a dissociation of three-dimensional filament lattices or a combination of these factors. To clarify the mechanism of viscosity reduction, we examined the morphology of the P235–actin mixtures by electron microscopy after polymerization.

3.3. *Effect of P235 on actin filament length and morphology*

In the absence of P235, actin filaments appear as long, thin and smooth filaments (at the present resolution) which are frequently tangled and occasionally laterally associated (fig.2A). The presence of P235 during actin polymerization led to the formation of shorter and straighter filaments (fig.2B). Histograms of the length distribution of actin filaments in the absence and presence of P235 are shown in fig.3. In the presence of P235 (1:10 (w/w), P235 to actin), most of the actin filaments were 0.1–0.2 μm long. Actin filaments longer than 0.5 μm were rarely observed in the presence of P235 (fig.3). In contrast, in the absence of P235, > 30% of the filaments are longer than 1 μm. P235 alone, processed and stained identically in parallel experiments, appeared as a heterogeneous mixture of globular beads of 4–15 nm diam., the majority measuring 8 nm (fig.2C). Since the predominant

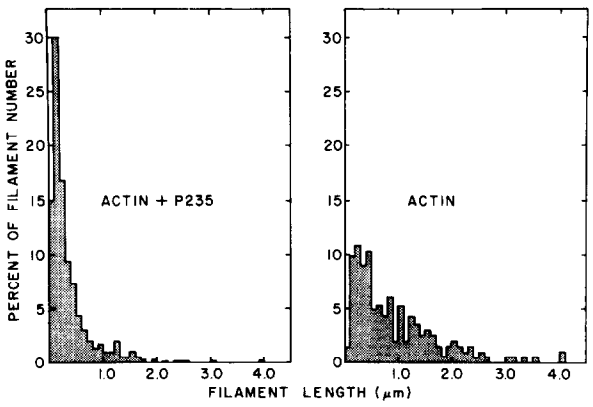

Fig.3. Effect of P235 on the length distribution of actin. Length distribution of actin filaments formed in the presence (left) and absence (right) of P235 (P235:actin (w/w) = 1:10), as in fig.2, was measured from electron micrographs with a Hewlett-Packard 9815A calculator equipped with a 98134A option 064 Interface Digitizer and plotted; ~ 400 filaments were measured for each histogram.

size is very similar to the Stokes' radius of the P235 dimer (6.7 nm, see [1]), it is likely that the 8 nm beads correspond to the P235 dimer and other sizes correspond to dissociation and aggregation products. No filaments were observed in the P235 sample; therefore, the short filaments present in the P235–actin mixture are indeed shortened actin filaments. Because the size range of P235 is similar to the diameter of actin filaments, it is difficult to detect P235 morphologically. However, on closer examination some short actin filaments appear to have a slightly enlarged end that is absent in pure actin filaments (fig.2D). This may be the binding of P235 to filament ends. Furthermore, larger globular material (9–10 nm) was occasionally seen attached to the cross-over points of laterally-aligned long actin filaments, as if these globules are joining the filaments together (fig.2E). Since these globules are in the size range of aggregates of P235 dimers, it is possible that P235 aggregates may bind to and crosslink actin filaments to form side-to-side connections.

Our electron microscopy results therefore suggest that the reduction of low shear viscosity of actin results from, at least in part, the shortening of actin filaments. The mechanism by which P235 restricts

the length of actin filaments is unclear. The morphological evidence suggests that P235 may bind to the ends and also the sides of actin filaments. It is therefore possible that P235 may behave as an actin-binding protein which either promotes actin nuclei formation or severs preformed filaments. It should be noted that we have detected no significant effect of P235 on the low shear viscosity of preformed actin filaments. It is possible that P235 may bind, but not sever, actin filaments under the assay conditions.

4. DISCUSSION

4.1. *Actin-modulating proteins*

The behavior of P235 on actin was highly reminiscent of those of a group of recently described actin-modulating proteins. These proteins regulate actin polymerization by either restricting the filament length and/or depolymerizing actin filaments (reviews [4,5]). However, a meaningful comparison has to await studies in progress on the effect of P235 on each stage of the actin polymerization process. P235 differs from these actin-modulating proteins in its M_r-value and subunit structure (a dimer of M_r 235 000 subunit). By comparison, most, if not all, of these proteins are monomers $< 95\,000$ M_r. It is conceivable that the large size of P235 may reflect the potential complexity of its interaction.

4.2. *Implication in platelet cytoarchitecture*

Irrespective of the molecular mechanism of P235–actin interaction, the observation that P235 restricts and thus regulates the length of filaments during actin polymerization may be highly significant in understanding the architecture and dynamics of platelet cytoplasm. There is now ample evidence indicating that a major portion of actin in unactivated resting platelets exists as a non-filamentous precursor of microfilaments and that rapid polymerization and assembly of non-filamentous actin accompanies the activation and shape change of platelets (e.g., [6–8]). The ionic conditions in the cytoplasm favor the polymerization of actin, so there must be factors which interact with non-filamentous actin (i.e., actin monomer or actin nuclei) to prevent actin polymerization or to restrict the filaments to a very limited length. Indeed, 2 such platelet proteins have been described:

(i) Profilin, a M_r = 12 000–14 000 protein which inhibits actin polymerization [9,10];
(ii) A M_r = 90 000–95 000 protein which restricts the length of actin filaments in the presence of μM Ca^{2+} levels [11–13].

Our results suggest that P235 may perform similar or complementary functions in platelet physiology by restricting the length of actin filaments. Consistent with this idea are the observations that P235 is a major platelet protein, yet is not detected in the Triton X-100, EDTA resistant platelet cytoskeleton [1]. Although elucidation of the exact role(s) of P235 in platelet function awaits further studies, P235 may function in the maintenance and regulation of the delicate, dynamic transitions of actin structures in the platelet cytoplasm.

ACKNOWLEDGEMENTS

We thank Dr R. Slocum for his generous assistance with electron microscopy and the Cell Research Institute of the University of Texas at Austin for the use of microscopy facilities. This work was supported in part by United States Public Service grant AM 20270, NIH Training Grant CA 09182 and a grant from the American Heart Association, Texas Affiliate, Inc. to K.W.

REFERENCES

[1] Collier, N.C. and Wang, K. (1982) J. Biol. Chem. in press.
[2] Nachmias, V.T., Sullender, J.S. and Fallon, J.R. (1979) Blood 53, 63–72.
[3] MacLean-Fletcher, S. and Pollard, T.D. (1980) J. Cell Biol. 85, 414–428.
[4] Pollard, T.D. and Craig, S.W. (1982) Trends Biochem. Sci. 6, 55–58.
[5] Craig, S.W. and Pollard, T.D (1982) Trends Biochem. Sci. 7, 88–92.
[6] Nachmias, V.T. (1980) J. Cell Biol. 86, 795–802.
[7] Jennings, L.K., Fox, J.E.B., Edwards, H.H. and Phillips, D.R. (1981) J. Biol. Chem. 256, 6927–6932.
[8] Carlsson, L., Markey, F., Blikstad, I., Persson, T. and Lindberg, U. (1979) Proc. Natl. Acad. Sci. USA 76, 6376–6380.
[9] Markey, F., Lindberg, U. and Eriksson, L. (1978) FEBS Lett. 88, 75–79.
[10] Mockrin, S.C. and Korn, E.D. (1980) Biochemistry 19, 5359–5362.

[11] Wang, L.-L. and Bryan, J. (1981) Cell 25, 637–649.
[12] Grumet, M. and Lin, S. (1980) Cell 21, 439–444.
[13] Lin, S., Wilkins, J.A., Cribbs, D.H., Grumet, M. and Lin, D.C. (1982) Cold Spring Harbor Symp. Quant. Biol. in press.
[14] Spudich, J.A. and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871.
[15] MacLean-Fletcher, S. and Pollard, T.D. (1980) Biochem. Biophys. Res. Commun. 96, 18–27.