Evidence for the presence of tropomyosin in the cytoskeletons of ADP- and thrombin-stimulated blood platelets

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Stimulation of porcine platelets with ADP or thrombin and subsequent analyses of their cytoskeletons by SDS-polyacrylamide gel electrophoresis have shown the presence of a 30.5-kDa polypeptide in the cytoskeletons of activated as well as aggregated platelets. This polypeptide comigrates with pure porcine platelet tropomyosin in SDS gels, their mobilities being similarly and markedly decreased in the presence of 6 M urea. One-dimensional peptide mapping after limited proteolysis by *Staphylococcus aureus* protease gives the same pattern for pure tropomyosin and the 30.5-kDa polypeptide. This latter may thus be identified as the porcine platelet tropomyosin subunit, the role of which may not be solely structural.

Tropomyosin *Porcine platelet* *Cytoskeleton* *ADP stimulation* *Thrombin stimulation*

1. INTRODUCTION

Upon exposure to various stimuli, circulating blood platelets are triggered into activity and undergo dramatic morphological and ultrastructural changes that play an important role in hemostasis. Within seconds, their shape changes from smooth discoid to spheroid with several pseudopods and, if stimulation goes on, this activation normally leads to aggregation. Recently there has been increasing evidence for the involvement of contractile proteins in these platelet responses. Thus, one observes a significant increase in the polymerization of actin [1,2] which is already maximal with activation [2], and in the amounts of actin and myosin, associated with several other proteins, in the cytoskeletons of stimulated platelets [3].

In a comparative study of stimulations of porcine platelets with ADP and thrombin, we have also consistently observed an increase in the amounts of a 30.5-kDa polypeptide in the cytoskeletons of activated as well as aggregated platelets. A 31-kDa polypeptide has also been observed in human platelets stimulated by thrombin [4,5] and it has been suggested that it is equivalent in size to the monomer of platelet tropomyosin [5] without any other characterization.

Tropomyosin has been purified from human [6], equine [7] and porcine [8] platelets, but its role is as yet unknown. An exclusively structural role has been assigned to non-muscle tropomyosins [9] and its involvement in such a rapid and highly plastic response as platelet activation may raise some questions. Therefore, the nature of the 30.5-kDa polypeptide present in platelet cytoskeletons deserves further study.

Our results allow the identification of this polypeptide as porcine platelet tropomyosin subunit.

Abbreviations: PRP, platelet-rich plasma; PGE_1, prostaglandin E_1; PMSF, phenylmethylsulfonyl fluoride

2. MATERIALS AND METHODS

2.1. *Materials*

PGE_1, PMSF, human plasma thrombin (minimum 3000 NIH units/mg protein) were from Sigma, Dextran T_{10} from Pharmacia and ultrapure urea from Serlabo. Pure porcine platelet

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tropomyosin was a gift from Dr E. der Terrossian (Biologie Physico-Chimique, Université Paris Sud, Orsay).

2.2. *Platelet isolation*

Blood was collected directly from freshly killed pigs into citrate-phosphate-dextrose anticoagulant in plastic bottles and centrifuged at 280 × *g* and 25°C for 10 min. The supernatant PRP was made 7 ng/ml in PGE_1 and recentrifuged at 1900 × *g* for 20 min to remove contaminant erythrocytes.

Platelets were then isolated on Dextran T_{10} gradients by a modification of the method of [10]: the PRP was loaded on two layers of 25 and 10% Dextran in Hepes buffer (137 mM NaCl, 2.7 mM KCl, 3.2 mM NaH_2PO_4, 3.7 mM Hepes, 5.5 mM glucose; pH 7.4) containing 0.2 μg PGE_1. After 20 min centrifugation at 2357 × *g*, the platelets had banded at the interface of the two Dextran layers and were recovered and resuspended in Hepes buffer containing 4 mM $MgCl_2$, diluted with the same buffer to 2×10^8 platelets/ml and progressively warmed to 37°C for 30 min.

2.3. *Activation and aggregation*

The suspended platelets were tested for activation and aggregation by ADP or thrombin. Measurements by the turbidimetric method [11] were made at 37°C in a Gilford spectrophotometer equipped with a variable speed stirrer (Spectro Stir 2445), an expanded scale recorder, a recorder offset and a thermoregulated sample compartment. Plastic cuvettes were used.

Activation was usually obtained by addition of ADP at 40 μM or thrombin at 0.4–0.6 units/ml, in the presence of 4 mM EGTA. Aggregation was obtained with ADP and 10–20 mM Ca^{2+} or thrombin and 2 mM Ca^{2+}.

2.4. *Cytoskeleton isolation*

Cytoskeletons from identical amounts of platelets, activated or aggregated under the optimal conditions thus defined, were obtained by lysis of these platelets with 1% Triton, 10 mM EGTA, 2 mM PMSF, 50 mM Tris (pH 7.4) and low-speed centrifugation of the lysate as in [3]. The pelleted cytoskeletons were washed twice by vortex mixing in the same lysis buffer (containing the same concentration of ADP, when stimulation was induced by this nucleotide).

2.5. *Analytical methods*

Washed cytoskeletons were dissolved in 4% SDS, 10% β-mercaptoethanol, 10 mM EGTA and incubated in a boiling water bath for 30 min. The samples were then readjusted to the same volume, and electrophoresed through slab gels according to [12], using a uniform or 5–25% concave gradient of acrylamide concentrations for the resolving gels.

Electrophoresis in urea was performed as in [13] with samples prepared in 3 M urea, stacking gels in 3 M and resolving gels in 6 M urea.

One-dimensional peptide maps of polypeptide bands isolated from the gels were obtained as in [14].

3. RESULTS AND DISCUSSION

SDS-polyacrylamide gel electrophoresis of the cytoskeletons of activated and aggregated porcine platelets revealed the appearance of new polypeptide bands or the increase in intensity of bands preexisting in the cytoskeleton of resting platelets. Particularly, whether stimulation was obtained with ADP or with thrombin, the increase in a polypeptide of higher mobility than actin was consistently observed (fig.1, 3A) and its apparent molecular mass was found to be 30.5 kDa (fig.2).

This polypeptide comigrates with pure porcine tropomyosin subunit. The mobilities of both polypeptides were markedly reduced in the presence of 6 M urea and became indistinguishable from the actin bands of the cytoskeletons (fig.3A,B).

The 30.5-kDa polypeptide and pure porcine tropomyosin bands were also isolated from SDS-polyacrylamide slab gels, submitted to limited proteolysis by *Staphylococcus aureus* V8 protease and reelectrophoresed. The proteolytic patterns given by these bands were similar, with a few polypeptides of low M_r, approximately between 20.1 and 14.4 kDa (fig.4).

The reported features strongly favor the identification of the 30.5-kDa polypeptide found in the cytoskeletons of activated or aggregated porcine platelets as a tropomyosin subunit.

Its molecular mass is similar to that reported for non-muscle tropomyosins, particularly platelet tropomyosins [6–8]. It comigrates with pure porcine tropomyosin in SDS-polyacrylamide gels and

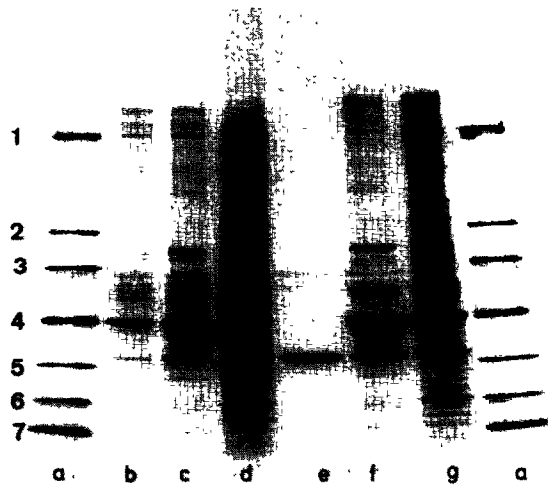

Fig.1. SDS-polyacrylamide gel electrophoresis of Triton-insoluble cytoskeletons from resting and stimulated platelets. Lanes: (a) M_r standards – 1, myosin heavy chain (200000), 2, phosphorylase *b* (94000), 3, bovine serum albumin (67000), 4, ovalbumin (43000), 5, carbonic anhydrase (30000), 6, trypsin inhibitor (20100), 7, α-lactalbumin (14400) (from Pharmacia electrophoresis calibration kit, except for myosin heavy chain); (b) resting platelets; (c) ADP-activated platelets; (d) ADP-aggregated platelets; (e) pure porcine tropomyosin; (f) thrombin-activated platelets; (g) thrombin-aggregated platelets. A 5–25% concave gradient of acrylamide was used for the resolving gel.

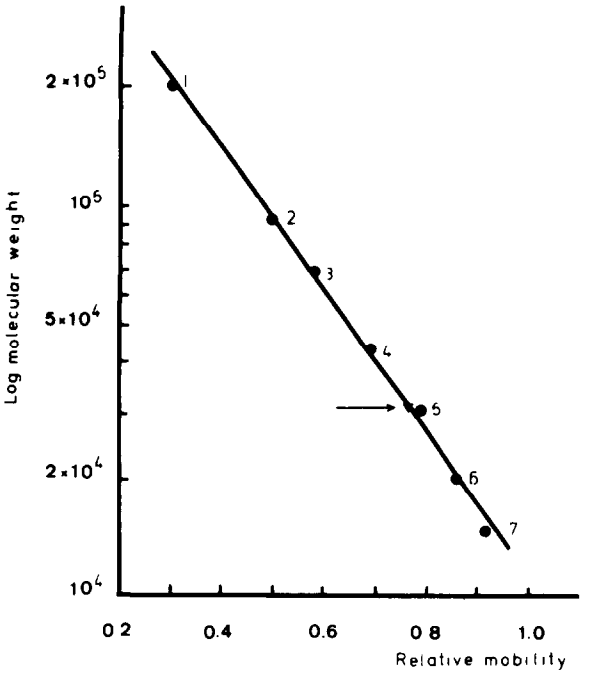

Fig.2. M_r determination from 5–25% gradient gels. (1–7) M_r standards as indicated in fig.1. (▼) 30.5 kDa and pure porcine tropomyosin.

undergoes an identical sharp decrease in mobility in the presence of high urea concentrations, a property associated almost exclusively with the tropomyosin molecule [13]. Furthermore, limited proteolysis of both 30.5-kDa polypeptide and tropomyosin subunit gave similar electrophoretic patterns. There are very few differences between those obtained with 1 and 50 ng protease for both polypeptides and this is also in keeping with the known periodic and conservative structure of tropomyosins.

From immunofluorescence studies, tropomyosin has long been shown to associate with actin cables in stress fibers of non-muscle cells and an exclusively structural role has been envisaged for this protein in this class of cell [9]. Besides, the Ca^{2+} regulation of actomyosin–ATPase and contractility, assumed by tropomyosin and its complexes with the troponin molecules in skeletal muscle, is effected mainly by phosphorylation of the myosin light chain in platelets [15]. However, the appearance of tropomyosin in cytoskeletons obtained from such a rapid phenomenon as platelet activation, which is induced in a few seconds, may reveal some involvement of this protein in the dynamic functioning of platelet contractile proteins. Recent papers have shown the existence of tropomyosin bound to the erythrocyte membrane [16,17] and even in the ruffling lamellae of normal and transformed cells [18], and some role in the regulation of actin interactions has been suggested by the authors. Moreover, an immunofluorescence study on platelets' spreading on a surface, a process which causes shape changes similar to those occurring in platelet activation, has shown a strong decoration of the pseudopods in the dendritic and late intermediate stages by antibodies to tropomyosin [19]. The role of tropomyosin may thus be more complex than expected. Work is currently underway to study further the interactions of tropomyosin with other proteins detected in the cytoskeletons of stimulated platelets.

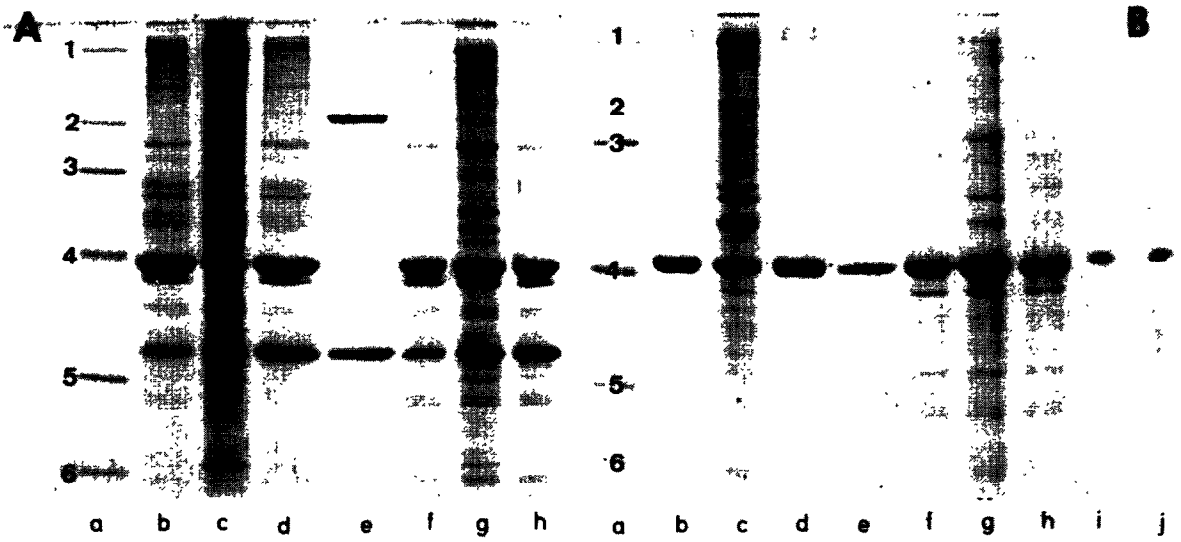

Fig.3. SDS-polyacrylamide gel electrophoresis on 10% acrylamide of cytoskeletons from stimulated platelets. (A) Without urea; (B) in the presence of 6 M urea. Lanes: (a) M_r standards numbered as in fig.1; (b) ADP-activated platelets; (c) ADP-aggregated platelets; (d) ADP-activated platelets + pure porcine tropomyosin; (e) tropomyosin (lower band in A); (f) thrombin-activated platelets; (g) thrombin-aggregated platelets; (h) thrombin-activated platelets + pure tropomyosin; (i,j) 30.5-kDa bands isolated from thrombin and ADP-aggregated platelets, respectively, and reelectrophoresed.

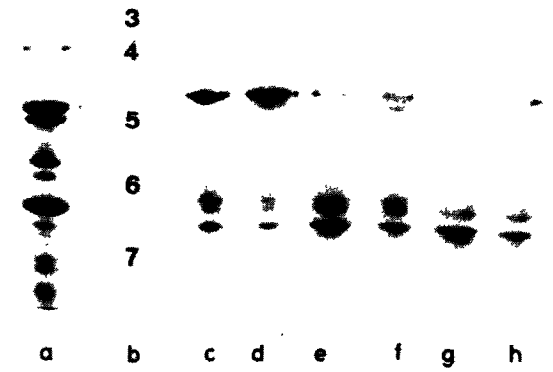

Fig.4. Comparison of partial proteolyses during reelectrophoresis of 30.5-kDa and tropomyosin polypeptides. A 15% acrylamide resolving gel was used. Lanes: (a) 42-kDa polypeptide (actin) (as a control of protease activity); (b) M_r standards as in fig.1; (c,e,g) porcine tropomyosin; (d,f,h) 30.5-kDa polypeptide. Amounts of protease used: 1 ng (c,d), 5 ng (e,f), 50 ng (a,g,h).

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