Cytoskeletons of ADP- and thrombin-stimulated blood platelets

Presence of a caldesmon-like protein, α-actinin and gelsolin at different steps of the stimulation

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Comparative analyses of the cytoskeletons of resting and stimulated platelets point out the involvement of a 79 kDa polypeptide in the activation step and its increased incorporation during aggregation. It appears as a doublet and cross-reacts with an antibody to chicken gizzard caldesmon, whereas no 150 kDa immunoreactive form was detected. α-Actinin and gelsolin were detected only in the aggregation step.

Caldesmon *α-Actinin* *Gelsolin* *Cytoskeleton* *Platelet stimulation*

1. INTRODUCTION

Blood platelets display a remarkable reactivity to various stimuli and a high plasticity in their responses. This makes them a choice material for the study of cellular dynamics.

Many of the changes observed appear to be mediated by actin microfilaments and associated proteins [1]. Isolation of the filaments at low centrifugal forces after platelet lysis by 1% Triton X-100, followed by comparative analyses of the cytoskeletons thus obtained is a useful approach for the identification of the proteins involved in these processes and sometimes for the discrimination of different steps in platelet stimulation.

By this procedure we have previously identified tropomyosin in the platelet cytoskeletons during activation as well as aggregation, which suggests a possible dynamic role for this protein in platelets [2].

We now present evidence for the presence of a caldesmon-like protein with a molecular mass of 79 kDa also throughout activation and aggregation, whereas α-actinin and gelsolin are observed only during aggregation.

2. MATERIALS AND METHODS

2.1. *Materials*

Human plasma thrombin (minimum 3000 NIH units/mg protein) was from Sigma. Human platelet α-actinin was a gift from Dr F. Landon (Biochimie Cellulaire, Collège de France, Paris); human platelet gelsolin was prepared as previously described [3]. Antibodies raised in rabbits against chicken gizzard caldesmon and bovine plasma gelsolin were gifts from Dr T. Hirabayashi (University of Tsukuba, Japan) and Dr M. Coué (Biochimie Cellulaire, Collège de France, Paris), respectively; antibodies against human platelet α-actinin were prepared as described in [4]. Peroxidase conjugated anti-rabbit IgG was from Institut Pasteur Production (Marnes, France).

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2.2. *Methods*

Porcine platelets were isolated as described [2], removal of contaminant red cells from the platelet rich plasma being achieved by centrifugation at 190 × *g*. Platelet activation and aggregation, turbidimetric measurements, cytoskeleton isolation and SDS-polyacrylamide gel electrophoresis were also performed as described [2].

Densitometry of Coomassie blue-stained cytoskeletons on slab gels was carried out on a Vernon PHI 5 (Paris, France). Platelet actin was used as a standard. Different aliquots of cytoskeleton solutions were electrophoresed for actin and for 79 kDa polypeptide determinations as the ratio 79 kDa/actin is quite low.

For immunoblotting analyses the cytoskeletal proteins were electrophoretically separated on 10% slab gels before transfer to nitrocellulose sheets. The primary antibodies were rabbit anti-chicken gizzard caldesmon or anti-human platelet α-actinin or anti-plasma gelsolin and the secondary antibody was peroxidase-labelled anti-rabbit IgG.

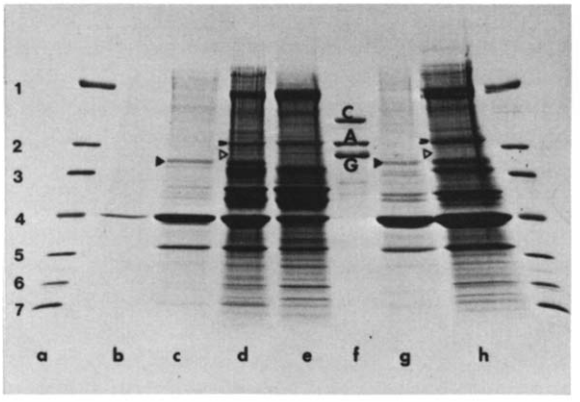

Fig.1. SDS-polyacrylamide gel electrophoresis of Triton-insoluble cytoskeletons from resting and stimulated platelets. Lanes: (a) M_r standards – (1) myosin heavy chain (200000), (2) phosphorylase *b* (94000), (3) bovine serum albumin (67000), (4) ovalbumin (43000), (5) carbonic anhydrase (30000), (6) trypsin inhibitor (20100), (7) α-lactalbumin (14400) (from Pharmacia electrophoresis calibration kit, except for myosin heavy chain); (b) resting platelets; (c) ADP-activated platelets (40 μM ADP + 4 mM EGTA); (d) ADP aggregated platelets (40 μM ADP + 380 μg/ml fibrinogen + 2 mM Ca^{2+}); (e) as in (d) except that Ca^{2+} was 10 mM; (f) control chicken gizzard caldesmon (C), α-actinin (A) and gelsolin (G); (g) thrombin-activated platelets (0.4 U/ml thrombin + 4 mM EGTA); (h) thrombin-aggregated platelets (0.4 U/ml thrombin + 2 mM Ca^{2+}); (►, ≻, ▷) 79 kDa caldesmon-like protein, α-actinin and gelsolin in the cytoskeletons. A concave 5–25% gradient of polyacrylamide was used for the resolving gel.

3. RESULTS

3.1. *Caldesmon-like protein*

Comparison of the cytoskeletons from resting, activated and aggregated platelets consistently revealed, as early as in the activation step, the appearance, among others, of a new polypeptide, the molecular mass of which was found to be 79 kDa (fig.1 lanes c,d,g,h). In the highly resolvent polyacrylamide gradient gel system, it appears as a doublet (fig.1). It cross-reacts with an antibody raised against chicken gizzard caldesmon, which stains no other polypeptide band in the cytoskeletons (fig.2, lanes c,d,g,h).

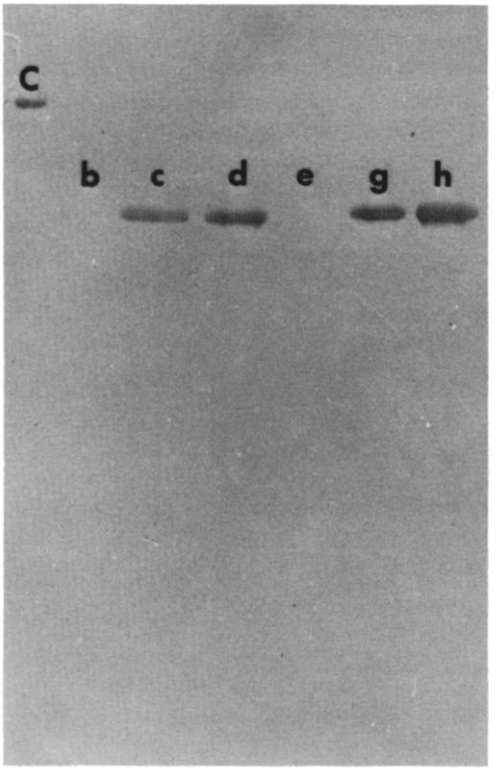

Fig.2. Immunoblot of platelet cytoskeletons with anti-chicken gizzard caldesmon IgG. (C) Chicken gizzard caldesmon; (b–h) platelet cytoskeletons lettered as in fig.1.

This 79 kDa polypeptide was observed whether stimulation was induced by ADP or thrombin. Aggregation with ADP is usually obtained in the presence of 350–400 μg/ml fibrinogen. As the high concentration of this protein, which binds to platelets during aggregation, obscured the polypeptide patterns of the cytoskeletons we have also attempted to induce aggregation with ADP without fibrinogen by increasing the calcium concentration to 10 mM. The 79 kDa band was no longer observable in the cytoskeletons thus obtained (not shown); this effect of calcium was confirmed when aggregation was achieved with ADP and fibrinogen (figs 1e and 2e) or with thrombin (not shown) in the presence of the same concentration of calcium.

Densitometry showed that the 79 kDa polypeptide/actin ratios were increased about 2-fold in the cytoskeletons of aggregated platelets as compared to those of activated platelets. This means that besides an increase of the actin content in these cytoskeletons there was also a net increase in incorporation of the 79 kDa polypeptide.

3.2. *α-Actinin*

Coomassie blue staining of the cytoskeletons revealed that a polypeptide with the same mobility and hence the same molecular mass (105 kDa) as pure human platelet α-actinin was observed only in the cytoskeletons of aggregated platelets (fig.1d,h). It also cross-reacted strongly with affinity-purified anti-human platelet α-actinin (fig.3). This sensitive reaction detected traces of α-actinin in resting and activated platelet cytoskeletons, but both Coomassie blue and immunostainings indicated that the content of this protein is by far larger than what could be expected from a general increase in cytoskeletal proteins (and particularly actin) in itself. These results thus

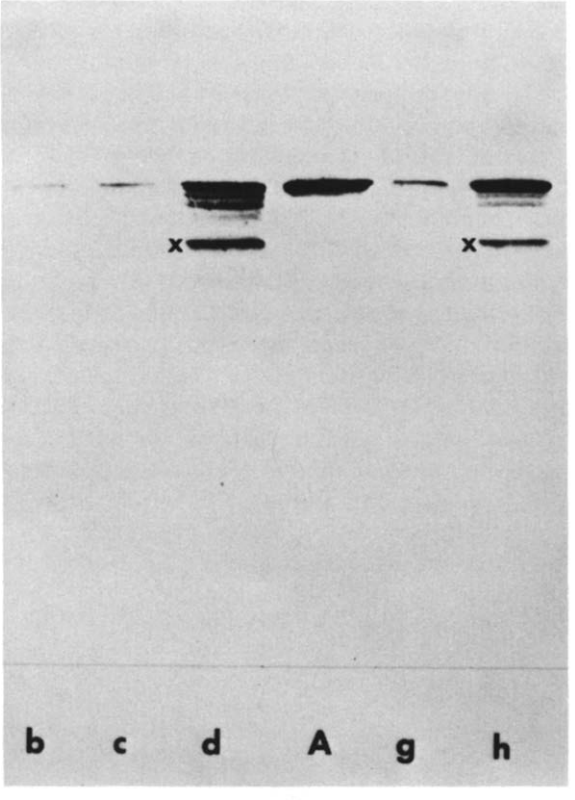

Fig.3. Immunoblot of platelet cytoskeletons with anti-human platelet α-actinin IgG. (A) α-Actinin *aa* isoform; (b–h) platelet cytoskeletons lettered as in fig.1; (x) cleavage product from α-actinin appearing after boiling samples of this product [5].

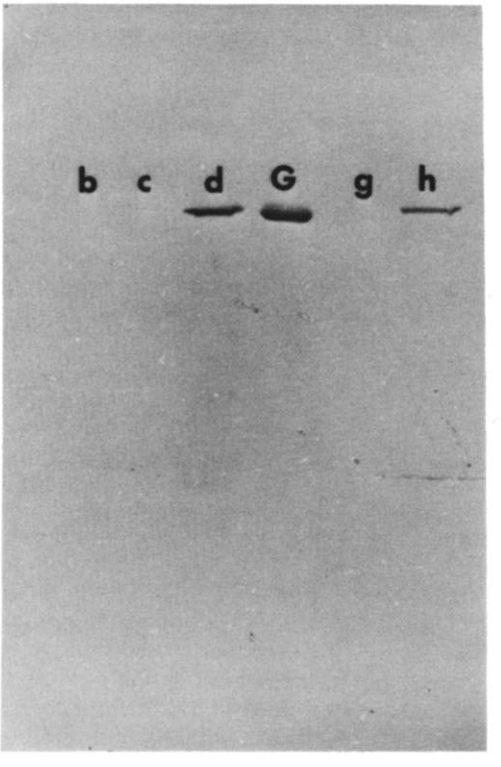

Fig.4. Immunoblot of platelet cytoskeletons with anti-plasma gelsolin IgG. (G) gelsolin; (b–h) platelet cytoskeletons lettered as in fig.1.

clearly favour the involvement of α-actinin only in the aggregation step, whether the inducer was thrombin or ADP.

3.3. *Gelsolin*

In the cytoskeletons of aggregated platelets, a polypeptide having the same mobility as human platelet gelsolin is also observable, although the colour intensity of this band is weaker than that of α-actinin (fig.1d,h). This polypeptide cross-reacts with an antibody raised against plasma gelsolin (fig.4d,h).

4. DISCUSSION

Our results suggest that a 79 kDa caldesmon-like polypeptide is involved in the formation of the cytoskeletons of activated as well as aggregated platelets. Kakiuchi et al. [6] have found that human platelet caldesmon was a doublet of 147–150 kDa, similar to chicken gizzard caldesmon. However, these authors indicated later on [7] that traces of a 77 kDa polypeptide reactive to 150 kDa caldesmon antibody was also present. This caldesmon form is more widely distributed than caldesmon$_{150}$ in a variety of cells and tissue types [7]. Bretscher and Lynch [8] also reported two classes of immunoreactive forms, 120–140 and 71–77 kDa, of caldesmon in various cultured cells. All these authors detected immunostaining of these proteins along stress fibers and in the leading edge of motile cultured cells. It is thus not surprising that a 79 kDa caldesmon-like polypeptide has been found as a component of stimulated platelet cytoskeletons. Its disappearance from the cytoskeletons obtained in the presence of high concentration of exogenous calcium is also in keeping with the calcium-calmodulin dependence of its interaction with F-actin [6,9] and/or susceptibility to proteases [9,10]. These results on a 79 kDa caldesmon-like protein in platelet cytoskeletons are thus in agreement with those of Dingus et al. [11].

Its appearance as soon as platelets are activated and its increased incorporation into the cytoskeletons during aggregation suggests it may play an important role in the dynamic processes of platelet stimulation. This may be a yet unknown one. It may also be the actin-linked regulation of actin myosin interaction as described in chicken gizzard smooth muscle [12] and bovine adrenal medulla [13]. The presence in the cytoskeletons of all the factors required for such a process, actin and myosin [14], tropomyosin [2,15], calmodulin [16] and the 79 kDa caldesmon-like protein is quite compatible with such a hypothesis. The detailed mechanism of this regulation, however, remains to be elucidated.

Another problem to be solved is the discrepancy between the molecular masses determined for platelet caldesmon. We were not able to detect immunologically any other reactive species besides the 79 kDa form, even in the Triton-soluble extract of resting platelets. Whether this latter exists as such or comes from an as yet undetectable 150 kDa form in porcine platelets, in a proteolytic step before isolation, is still uncertain.

Work is currently underway to investigate further these aspects of caldesmon biochemistry.

α-Actinin has been recognized in cytoskeletons of stimulated platelets by its molecular mass, which ranges from 95 to 105 kDa with various researchers [17–19]. Nachmias et al. identified it in cytoskeletons of resting and activated platelets both by its molecular mass and immunoreactivity to α-actinin antibodies [18,20,21], but its presence in these cytoskeletons is probably due to platelet isolation at low temperature: electron microscopy has shown that this procedure leads to very irregularly shaped platelets and microaggregates linked to cold stimulation [22]. Our results are in agreement with those of Rotman et al. [23] based on molecular mass and clearly point out the involvement of α-actinin in the aggregation step. This is compatible with the highly organized structure of microfilaments in aggregated platelets [1,24,25] and the cross-linking action of α-actinin on microfilaments [26]. Nevertheless, there is a discrepancy with the in vitro inhibition of this action by calcium [19,27] and this remained unexplained.

The present identification of gelsolin in the cytoskeletons of aggregated platelets lends support to the hypothesis that this actin binding protein is involved in the later stage of aggregation: its calcium-dependent severing effect [28] is thought to take part in the regulation of the microfilament reorganization when platelet aggregates condense and fibrin clots retract [1].

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