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FILAMIN[1] INHIBITS ACTOMYOSIN ATPase ACTIVITY IN PLATELET

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SUMMARY: Filamin, an actin cross-linker protein, has been shown to exist in platelet. The role of this protein in the platelet has remained unclear. In this report, we show that filamin inhibits the actin-activated Mg^{2+}-ATPase activity of platelet myosin. The activation caused by platelet actin is inhibited by 50% at the molar ratio of filamin to actin of 1/50. Platelet tropomyosin, which we showed to enhance the ATPase activity, does not abolish the effect of filamin. The results support the view that filamin stabilizes the actin network in the resting platelet.

Platelet is a contractile cell mainly composed of actin (1-3).The mechanism of its contraction appears to be different from the sliding model proposed for skeletal muscle. The major difference lies in the fact that both actin and myosin are reorganized when the platelet is activated (4,5). The Ca^{2+}-calmodulin dependent kinase phosphorylates the light chain of myosin, converting myosin into an active form (6,7). Although actin comprizes the majority of protein in platelet, little is known about the way it is modified when the platelet is activated.

In an effort to clarify this point, we have been examining the possible modifiers of platelet actin. We reported that tropomyosin enhances the actomyosin ATPase activity in platelet (8,9). The result suggested that actin, phosphorylated myosin and tropomyosin are the motile elements in the activated platelet.

[1] In this report, we use the term "filamin" to indicate the 250K dalton actin cross-linker protein in platelet. Some authers call it "actin-binding protein in platelet", but this term is too general.

The abbreviations used are: EDTA, ethylendiaminetetraacetic acid ; EGTA, ethylene glycol bis-(beta-aminoethyl ether)-N'N'N'N'-tetraacetic acid.

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"Actin-binding protein" was first identified in rabbit alveolar macrophage by Stossel and Hartwig (10). The protein was later shown to be closely related to "filamin" found in various cells (11-13).Although it is established that filamin binds F-actin filaments to cross-link them (14,15), its physiological roles remain to be determined. We undertook the present experiments to determine the effect of filamin on the actomyosin ATPase activity in the presence or absence of tropomyosin.

In this report; (i) we describe a method for preparing both actin and filamin from the same porcine platelet source, and (ii) it is shown that filamin inhibits the actomyosin ATPase activity both in the presence and absence of tropomyosin.

This fact is compatible with the hypothesis that the primary role of filamin is to stabilize the network of actin in the resting platelet.

MATERIALS AND METHODS

(1) Preparation of proteins. Myosin and tropomyosin were purified from the porcine platelets as described earlier (8). Actin was purified from the porcine platelets by the method of Gordon et al. (16). We found that filamin is purified as a byproduct of this procedure. In the ion-exchange chromatography on DEAE-Sephacel, filamin was eluted at the concentration of KCl around 0.19M (Fig.1-a, lane 1). The 30-40% $(NH_4)_2SO_4$ fraction was then obtained (Fig.1-a, lane 2). This fraction was dialyzed against 100mM KCl, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol, and 20mM imidazole-HCl (pH 7.0)(solution A). It was then applied to Sepharose-4B gel filtration column and was eluted by the solution A. The fractions containing filamin were concentrated by ultrafiltration (Fig.1-a, lane 3).
(2) ATPase assay. The actin-activated Mg^{2+}-ATPase activity of platelet myosin was estimated by the method of Takeuchi and Tonomura (17). The standard assay medium contained 100mM KCl, 5mM $MgCl_2$ 1mM EGTA, 1mM EDTA, 10mM imidazole-HCl (pH 7.0), 2μg/ml of pyruvate kinase (Boeringer), 2mM phosphoenolpyruvate, and 1mM ATP. The reaction was started by the addition of phosphoenolpyruvate and ATP. The amount of pyruvate produced during the steady state (1 to 15 min.) at 37°C was measured.
(3) Falling-ball viscometry was performed by the method of MacLean-Fletcher and Pollard (18). The standard assay medium was; 100mM KCl, 5mM $MgCl_2$, 0.1mM $CaCl_2$, 0.5mM ATP, and 10mM imidazole-HCl (pH 7.0).
(4) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (19).
(5) Protein concentration was estimated by the method of Bradford using bovine serum albumin as a standard (20).

RESULTS

As shown in Fig.2-a, platelet actin (Fig.1, lane 6) activates the Mg^{2+}-ATPase activity of platelet myosin (Fig.1, lane 4). The extent of the activation is virtually the same as that by skeletal actin and is dependent on the

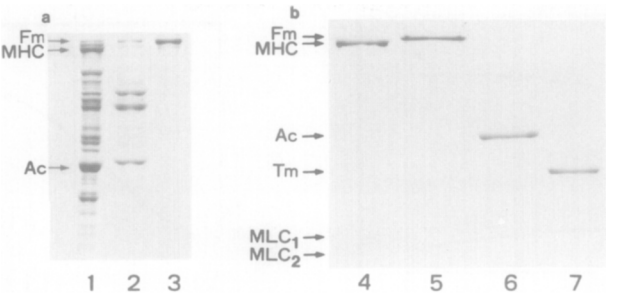
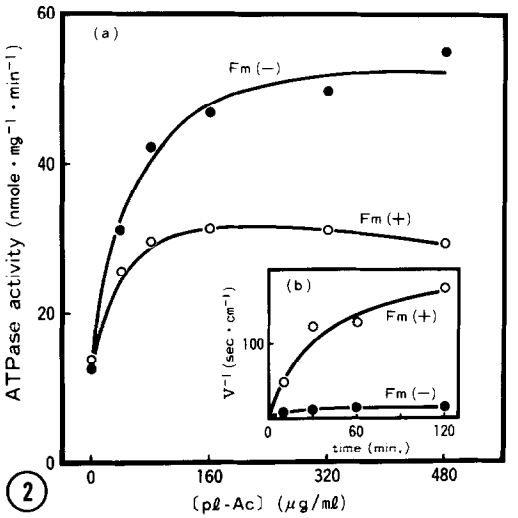


Fig.1: (a); Purification of platelet filamin.
1. The combined DEAE fractions. 2. The 30-40% $(NH_4)_2SO_4$ fraction.
3. The fractions after Sepharose 4B gel filtration.
(b); Proteins used in the experiments.
4. Platelet myosin. 5. Platelet filamin. 6. Platelet actin.
7. Platelet tropomyosin.
MHC; Myosin Heavy Chain. MLC; Myosin light chain. Ac; Actin. Tm; Tropomyosin. Fm; Filamin. The (a) 3-10%, or (b) 3-12.5% discontinuous density gradient SDS-PAGE was carried out as in the method.

state of the phosphorylation of the 20K dalton light chain of myosin (data not shown). Platelet filamin purified by our method (Fig.1, lane 5) markedly increases the viscosity of platelet actin, as already reported by others (Fig.2-b). Electron micrography shows the typical cross linkage between the actin filaments (data not presented).

When platelet filamin is added to the reconstituted actomyosin, it inhibits the ATPase activity (Fig.2-a). The maximum activation by actin is reduced more than by 50% at the concentration of filamin of 50μg/ml (molar ratio of filamin to actin of 1/50). The effect is virtually the same when skeletal actin is used instead of platelet actin (data not shown). Filamin does not affect the level of the phosphorylation of the myosin light chain (data not presented). Therefore, filamin inhibits the ATPase activity by modifying F-actin.

Previously, we found that tropomyosin enhnces actomyosin ATPase activity in platelet (8). This was the first indication that the actomyosin ATPase activity is modified through an actin-linked mechanism in non-muscle cells. It is of interest to see if filamin inhibits the activity in the presence of tropomyosin. The result of such an experiment is shown in Fig.3. As shown in Fig.3-a, platelet tropomyosin (Fig.1, lane 7) enhances the ATPase activity



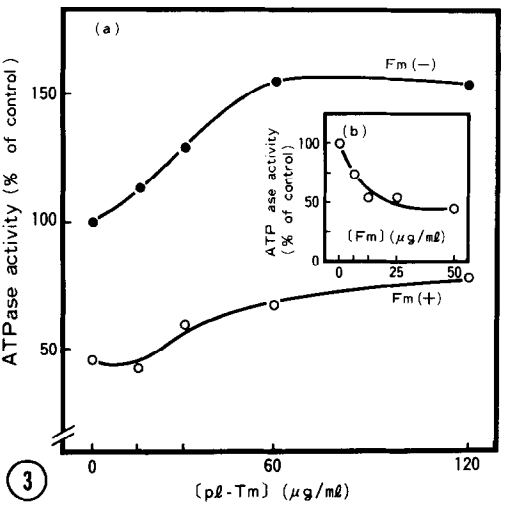


Fig.2: (a); The effect of filamin on the actomyosin ATPase activity.
The ATPase activity was measured as in the method.
Platelet myosin; 200ug/ml. Platelet actin; 0-480ug/ml. Platelet filamin; 50ug/ml.
(b); The effect of filamin on the viscocity of platelet actin.
The time course of the changes in the viscocity after the initiation of polymerization was measured as in the methods. The viscocity is expressed as a reciprocal of the velocity of the falling ball.
Platelet actin; 600ug/ml. Platelet filamin (Fm); 50ug/ml.
Closed circle; in the absence of filamin. Open circle; in the presence of filamin.

Fig.3: (a); The effect of filamin on the ATPase activity in the presence or absence of tropomyosin.
Platelet myosin; 200ug/ml. Platelet actin; 200ug/ml. Platelet filamin (Fm); 50ug/ml. Platelet tropomyosin (Tm); 0-120ug/ml.
(b); The effect of filamin on the actomyosin ATPase activity as a function of the concentration of filamin.
Platelet myosin; 200ug/ml. Platelet actin; 200ug/ml. Platelet fialmin; 0-50ug/ml.
The actin-activated ATPase activity in the absence of filamin is taken as 100%.
Closed circle; in the absence of filamin.
Open circle; in the presence of filamin.

of reconstituted platelet actomyosin. The effect levels off at the molar ratio of tropomyosin to actin of one third. When filamin is added to this system, the control activity in the absence of tropomyosin is inhibited by 55%(Fig.3 b). Tropomyosin enhances the ATPase activity in the presence of filamin,too (open circle; Fig.3-a). However, it does not restore the activity so much as to abolish the effect of filamin. The data indicate that the inhibition of the ATPase activity by filamin is independent of the effect of tropomyosin and that tropomyosin does not compete with filamin to abolish the effect of

the latter. This effect of tropomyosin is similar when arterial tropomyosin is substituted for platelet tropomyosin (data not shown).

These effect of filamin are not altered when Ca^{2+} is added to the assay medium (data not presented).

DISCUSSION

Filamin is known to interact with F-actin to cross-link the individual filaments (10-15). The cross-linkage is the basis for the gelation phenomena observed when filamin is added to the solution of F-actin. The physiological role of filamin, however, is not clear. The crucial point is whether filamin stabilizes or mobilizes actomyosin. Stendahl and Stossel reported that actin-binding protein from macrophage reduced the concentration of myosin to contract F-actin (21). Based on these data, they proposed that filamin amplifies the contraction of actomyosin. The data presented in this report are against their hypothesis. On the contrary, the data indicate that filamin affects F-actin in such a way as to prevent the interaction between actin and myosin. It should be pointed out that the above authors used reconstructed skeletal actomyosin to show the effect of the actin-binding protein from macrophage.

Many proteins have been found to associate with F-actin in non-muscle cells (for review, ref.22). To our knowledge, however, no such protein has been shown to inhibit the actomyosin ATPase activity. In smooth muscle, Ngai and Walsh reported that caldesmon inhibits actomyosin ATPase activity (23). We found that gizzard caldesmon inhibits the ATPase activity of platelet actomyosin (unpublished observation). It is interesting to point out that; (i) caldesmon binds F-actin to cross-link it (24),and (ii) caldesmon inhibits the effect of filamin in the gelation of F-actin (25). It appears that filamin and caldesmon are functionally related. Sobue et al. proposed that the inhibitory effect of caldesmon is released by Ca^{2+}-calmodulin (26). Ngai and Walsh proposed that the inhibitory effect is abolished when caldesmon is phosphorylated by a specific kinase in the presence of Ca^{2+} (23).

If the inhibitory effect of filamin is physiological, there may be a mechanism by which the inhibition is removed when the platelet is activated. Filamin is shown to be a preferred substrate for Ca^{2+}-activated neutral protease

(calpain) in the platelet (27). This fact suggests that the inhibition by filamin is released when calpain degrades it. This suggestion is supported by an recent report that filamin is rapidly degraded by calpain after the activation of the platelet (28).

We postulate, therefore, that filamin stabilizes the actin network in the resting platelet (static, or cytoskeletal role). The activation of the platelet may lead calpain to break down filamin,releasing actin and tropomyosin to fully activate the phosphorylated myosin.

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