RECONSTITUTION OF Ca^{2+}-SENSITIVE GELATION OF ACTIN FILAMENTS WITH FILAMIN, CALDESMON AND CALMODULIN

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1. Introduction

The discovery of Ca^{2+}-activatable cyclic nucleotide phosphodiesterase [1] and the subsequent demonstration of a protein factor which confers Ca^{2+}-sensitivity upon this enzyme [2,3] coincided with the discovery of a protein activator of brain phosphodiesterase [4]. The identity of the 2 proteins as a Ca^{2+}-binding protein (calmodulin) was established subsequently [5].

The structural similarity of calmodulin and troponin Cs [6,7] suggests that both proteins may have stemmed from a common ancestral protein and may perform analogous functions as 4-domain calcium receptive proteins. However, surprisingly few studies have been done with regard to the interaction of calmodulin with components of the contractile or cytoskeletal system except for myosin light chain kinase. Calmodulin associates with skeletal muscle troponin components to form a soluble hybrid complex and neutralizes the inhibitory action of troponin I on the actomyosin ATPase activity [8]. A similar observation was made in [9]. We have purified, from erythrocytes [10,11], brain [12] and chicken gizzard smooth muscle [13,14], actin-related proteins that bind to calmodulin in the presence of Ca^{2+}. The calmodulin-binding protein from chicken gizzard, named caldesmon, interacted with calmodulin and F actin in the presence or absence, respectively, of Ca^{2+} and formations of the 2 species of protein complexes is regulated by $[Ca^{2+}]$ in a flip–flop fashion [14]. Here, this mechanism is extended to the control of the filamin-induced gelation of actin filaments: calmodulin–caldesmon system conferred Ca^{2+}-sensitivity upon the regulation of actin gel–sol transformation with purified filamin.

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate

2. Materials and methods

Caldesmon and filamin were purified from chicken gizzard smooth muscle as in [14] and [15], respectively. Preparation of chicken gizzard F actin was essentially as in [16]. After 2 cycles of polymerization and depolymerization, G actin solution was chromatographed on a Sephadex G-100 column to remove traces of contaminating proteins. At the final step, polymerization of actin was induced by adding KCl to 100 mM final conc. and F actin thus formed was collected by centrifugation. Calmodulin was prepared from bovine brain as in [17].

Filamin-induced gelation was assayed as follows: the reaction mixture contained, in 0.2 ml final vol., 5 mM Tris–HCl (pH 7.6), 100 mM KCl, 0.2 mM $MgCl_2$, 0.2 mM ATP, 0.1 mM dithiothreitol, either 0.2 mM $CaCl_2$ or 1 mM EGTA as indicated, 2.5 mg gizzard F actin/ml, 100 μg gizzard filamin/ml, caldesmon and calmodulin. Concentrations of caldesmon and calmodulin are indicated in the figures. The mixture was incubated for 30 min at 25°C, and the degree of gelation was determined by 2 different methods. Method 1 was adopted from [18], in which the degree of gelation was expressed by symbols (+) and (–); (+++), firm gel which could turnover easily without breaking the gel; (++), gel which showed no appreciable deformation when the tube containing it was brought to horizontal position; (+), gel which deformed but could resist tilting of the tube; (–), no gelation or gel easily broken down with slight disturbance. Assay tubes of 7.5 × 75 mm were used.

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Method 2 used low speed centrifugation as in [19,20]. The incubated mixture was centrifuged at 20 000 × *g* for 10 min at 25°C to precipitate the gelled pellet. The supernatant fluid was carefully aspirated and an aliquot from the pellet was assayed for protein as in [21]. Electrophoresis of proteins was done in 7.5% polyacrylamide gels in a buffer system consisting of 25 mM Tris–192 mM glycine (pH 8.3) and 0.1% SDS, which is a slight modification of the method in [22]. Proteins in the gels were stained with Coomassie brilliant blue R-250. Flow birefringence of protein solutions together with its extinction angle was determined in an Edsall-type rotating apparatus as in [23].

3. Results

In preliminary experiments to determine the flow birefringence of solutions of proteins, we found that the association of caldesmon with F actin does not alter the physical state of the actin filaments; i.e., gelation or severing did not occur. Moreover, addition of caldesmon did not alter the reannealing process of fragmented actin filaments that had been sonicated, indicating that caldesmon does not produce capping (or ending) of one end of the actin filament. Therefore, a possible role of caldesmon with regard to the control of the actin function is that it may intervene in the interaction of the actin filaments and other protein(s). A most likely candidate for the target protein in this connection is filamin (actin-binding protein) [24,25]. In the following experiments, this possibility was examined using the reconstituted system.

Fig.1 shows the SDS–polyacrylamide gel electrophoresis of purified proteins (caldesmon, filamin, actin). These proteins were shown to be free from the contaminating proteins. The caldesmon M_r was estimated on SDS gels to be ~150 000 [13,14]. Here, using a buffer system for the SDS gels slightly modified from [13,14], we obtained a doublet of 150 000 and 147 000 M_r polypeptide bands for caldesmon. Since the M_r of the native protein was estimated to be ~300 000 [13], caldesmon appears to be a heterodimer composed of 2 subunits.

In fig.2, gel formation of actin filaments by purified filamin was determined by 2 different methods (section 2). The results obtained by 2 methods agreed with each other. The molar ratio of filamin to actin monomers was 1:290. Under this condition, a firm

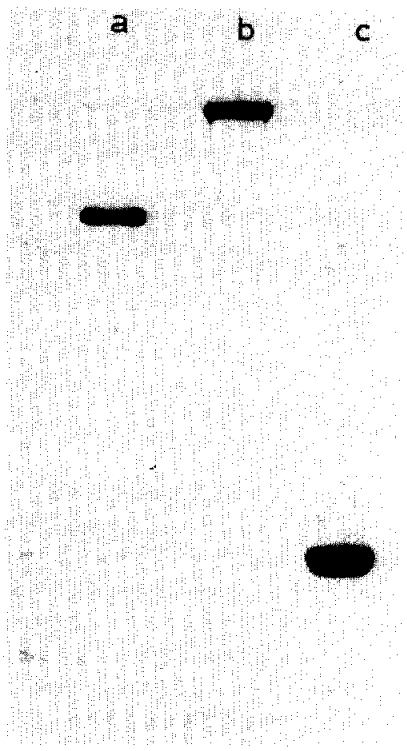

Fig.1. 7.5% SDS–polyacrylamide gel electrophoresis of purified proteins: (a) caldesmon; (b) chicken gizzard filamin; (c) chicken gizzard actin.

gel was formed at 25°C. Addition of increasing concentrations of caldesmon to this mixture caused a dose-dependent inhibition of the gelation. The inhibition was insensitive to [Ca^{2+}], the result being consistent with our observation [14] that the interaction of

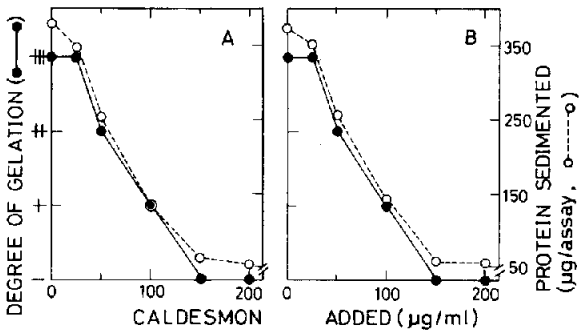

Fig.2. Effect of caldesmon on gelation of actin filaments by filamin. The reaction mixture contained, in 0.2 ml total vol., 2.5 mg F actin/ml, 100 μg filamin/ml, various concentrations of caldesmon as indicated in the figure, and either 0.2 mM $CaCl_2$ (A) or 1 mM EGTA (B). For other conditions see section 2.

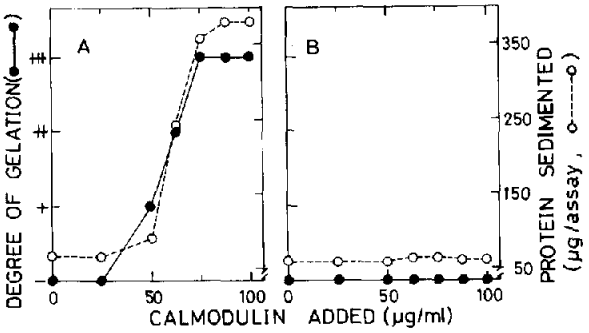

Fig.3. Effects of Ca^{2+} and calmodulin on the inhibition by caldesmon of filamin-induced gelation of actin filaments. The reaction mixture contained, in 0.2 ml total vol., 2.5 mg F actin/ml, 100 μg filamin/ml, 150 μg caldesmon/ml, either 0.2 mM $CaCl_2$ (A) or 1 mM EGTA (B), and various concentrations of calmodulin as indicated in the figure.

caldesmon and F actin is not affected by the presence and absence of Ca^{2+} in the medium (fig.2). The molar ratio of filamin dimer to caldesmon heterodimer was 1:2.5 when gelation was maximally inhibited. This caldesmon-dependent inhibition of the gelation can be overcome by the addition of calmodulin in a dose-dependent manner (fig.3). This effect of calmodulin was dependent on the presence of Ca^{2+} in the medium. The molar ratio of calmodulin to caldesmon heterodimer was 9:1 when the inhibition was completely overcome and the firm gel was restored.

4. Discussion

The consistency changes of cytoplasm, brought about by gel–sol transformations of actin filaments, correlate with specific cellular functions including shape change, locomotion and phagocytosis [26]. A high M_r (dimer M_r 250 000) actin-binding protein (filamin) that can crosslink actin filaments to form a gel was isolated from chicken gizzard smooth muscle [15,24] and rabbit alveolar macrophages [20,25]. About 80% of the recovered actin-gelling activity in macrophage extracts co-isolated with this protein [20]. Besides the above 2 tissues, this protein has been shown in a variety of tissues that include kidney [24], liver [24], brain [24], leucocytes [27], platelets [28], fibroblasts [28] and vas deferens [28]. By immunofluorescent staining, filamin antibody has been localized in cellular structures known to contain actin filaments, i.e., during interphase, stress fibers [29,30], membrane ruffles [29,30] and microspikes [29,30] and, during cytokinesis, cleavage furrow [30]. Thus, filamin is intimately implicated in the function of actin filaments in the cell. In spite of these findings, the interaction of actin and filamin in vitro has not been affected by Ca^{2+} [20].

Having discovered in chicken gizzard smooth muscle an actin-binding protein (caldesmon) whose binding to actin filaments is controlled by its Ca^{2+}-dependent interaction with calmodulin in a flip–flop fashion [13,14], we predicted that the caldesmon–calmodulin system could confer Ca^{2+}-sensitivity upon gel–sol transformations of actin filaments in the presence of filamin. Here, this prediction is experimentally verified. Actin gelation by filamin became sensitive to Ca^{2+} when caldesmon and calmodulin were included: in the absence of Ca^{2+} (+EGTA), caldesmon interacts with F actin thus inhibiting the interaction between the F actin and filamin whereas, in the presence of Ca^{2+}, a Ca^{2+}-dependent binding of calmodulin to caldesmon abolishes the interaction of caldesmon and F actin thus making available F actin for the interaction with filamin (fig.4). The caldesmon-induced inhibition of the interaction of F actin and filamin may be explained by the competition of caldesmon and filamin for the same binding site on F actin. An alternative explanation is that the binding of caldesmon to F actin may lead to an allosteric change of the F actin molecule to a form incapable of interacting with filamin. Clearly, further work is needed for the direct evidence for these possibilities.

There are other proteins that can confer Ca^{2+} sensitivity on gelation of F actin by filamin. Thus, gelsolin (M_r 91 000) from macrophages [31], villin (M_r 95 000) from intestinal epithelial cell microvilli [32], and fragmin (M_r 43 000) from *Physarum polycephalum* [33] have been isolated. However, their mode of action on the actin gelation is distinctly different from that of caldesmon–calmodulin system:

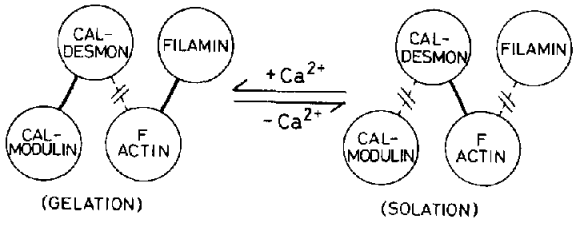

Fig.4. Regulation of filamin-induced gelation of actin filaments by a Ca^{2+}- and calmodulin-dependent flip–flop switch mechanism. For explanation, see text.

(1) In the presence of Ca^{2+}, these proteins inhibit filamin-induced F actin gelation by reducing the average filament length and increasing the actin filament number [34–36]. This is caused by the Ca^{2+}-dependent increase in the rates of nucleation of actin assembly and of severing of preformed actin filaments together with one end of the resulting filament being capped. In contrast, caldesmon did not alter the physical state of the actin filaments.

(2) The regulation of gelation by these proteins does not require the participation of calmodulin. In contrast, the present investigation dealt with a calmodulin-dependent control of actin gelation.

Thus, this report provides the first successful reconstitution of a Ca^{2+}-sensitive gelation of actin filaments in which calmodulin is implicated as a Ca^{2+}-sensitizing factor of the system.

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