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Evidence for direct binding of vinculin to actin filaments

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The interaction of vinculin with actin filaments was investigated by methods which exclude interference by contaminating proteins which may occur in vinculin preparations. Vinculin which was blotted from SDS-polyacrylamide gels onto nitrocellulose, was stained specifically by fluorescently labeled polymeric actin (100 mM KCl, 2 mM $MgCl_2$). Vinculin which was purified from α-actinin and an actin polymerization-inhibiting protein (HA1), was found to be cosedimented with polymeric actin. Maximally one vinculin molecule was cosedimented per one hundred actin filament subunits. Half maximal binding of vinculin was observed at about 0.25 μM free vinculin. Vinculin could be replaced from actin by the addition of tropomyosin.

Actin; Vinculin; Tropomyosin; Staining; Fluorescence

1. INTRODUCTION

Vinculin has been reported to occur in the adhesion plaques where actin filament bundles are attached to cell membranes [1]. Because of the coincidence of vinculin with the ends of actin filament bundles it has been speculated that vinculin is an actin-binding protein which links actin filament bundles to cell membranes. Vinculin has been found to be cosedimented with actin filaments [2]. It has been reported that vinculin crosslinks or binds to the ends of actin filaments [2–5]. However, later it has turned out that at least some of these observations are to be attributed to contaminants occurring in vinculin preparations [6–9]. It has then been suggested that vinculin does not bind to actin and that other proteins (e.g. α-actinin) connect vinculin with actin filaments [8–10]. It is still a matter of controversy whether or not vinculin binds directly to actin. In this paper we investigated this question by applying methods which exclude interference by contaminating proteins.

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2. MATERIALS AND METHODS

2.1. *Preparation of the proteins*

Vinculin from chicken gizzard smooth muscle was prepared according to the method of Feramisco and Burridge [11] with the following modifications and additional purification steps. After DEAE-cellulose chromatography the vinculin-containing fractions were pooled and dialyzed overnight against buffer A (5 mM NaCl, 0.25 mM EGTA, 0.1 mM PMSF, 5 mM mercaptoethanol, 20 mM Tris/acetate, pH 7.6). The vinculin solution was concentrated by collecting on a DEAE-Sepharose CL-6B column and eluting with 200 mM NaCl in buffer A. Vinculin was further purified by chromatography on a Sepharose CL-6B column (1.5 × 100 cm, bed volume 175 cm^3) equilibrated with buffer B (200 mM NaCl, 3 mM NaN_3, 5 mM mercaptoethanol, 5 mM triethanolamine/HCl, pH 7.5) [3]. Protein concentrations were determined by the method of Lowry et al. [12].

Actin was prepared according to the procedure of Rees and Young [13]. Part of the protein was modified with *N*-ethylmaleimide at cysteine-374 and subsequently with 4-chloro-7-nitro-2-oxa-1,3-diazole at lysine-373 to produce fluorescently labeled actin (NBD-actin) [14,15]. The protein was applied to a Sephacryl S-200 column (2.5 × 90 cm) equilibrated with buffer C (0.5 mM ATP, 0.2 mM $CaCl_2$, 3 mM NaN_3, 5 mM triethanolamine/HCl, pH 7.5). The concentration of actin was determined photometrically at 290 nm by using an absorption coefficient of 24900 $M^{-1} \cdot cm^{-1}$ [16].

Tropomyosin was extracted from rabbit skeletal muscle [17] and purified by chromatography on hydroxyapatite [18]. The tropomyosin concentration was measured photometrically at 276 nm using an absorption coefficient of 24500 $M^{-1} \cdot cm^{-1}$ [19].

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2.2. *Cosedimentation*

The association of vinculin, tropomyosin and polymeric actin was investigated by cosedimentation [19,20]. Actin was polymerized in buffer C by addition of 2 mM $MgCl_2$ and 100 mM KCl. Actin filaments covered with tropomyosin were formed by combining monomeric actin and tropomyosin with 2 mM $MgCl_2$ and 100 mM KCl. After incubation for 30 min at 37°C various concentrations of vinculin were added. The samples were incubated for 30 min at 37°C and then centrifuged for 60 min at 100000 × *g* to sediment actin filaments and their associated proteins. Aliquots of the supernatants and of the pellets were applied to 7.5% SDS-polyacrylamide gels. The amounts of vinculin were estimated by photometric scans of Coomassie blue-stained gels at 595 nm. The scans were calibrated by vinculin standards.

2.3. *Electrophoresis and staining with NBD-actin*

SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn [21] or Laemmli [22]. The gels were stained with Coomassie blue. When NBD-actin was used for staining the protein bands were blotted electrophoretically onto nitrocellulose sheets (BA 85, Schleicher and Schuell) [23]. The nitrocellulose sheets were incubated for 1 h with a staining solution containing 20 μM polymeric actin (10% NBD-actin, 90% unlabeled actin polymerized by addition of 2 mM $MgCl_2$ and 100 mM KCl) and 3% bovine serum albumin at room temperature under slow shaking. The stained nitrocellulose sheets were washed twice for 10 min with 20 mM NaCl, 0.1% Tween-20 and 5 mM triethanolamine/HCl, pH 7.5. The location of the protein bands stained with polymeric actin was visualized by UV-irradiation (284 nm). Photographs were taken using a Polaroid 4 × 5 Land Film T55.

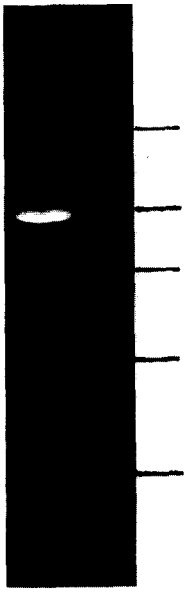

Fig.1. Actin stain of vinculin. Vinculin (25 μg) was blotted from a 7% SDS-polyacrylamide gel onto nitrocellulose, stained with fluorescently labeled polymeric actin and visualized by fluorescence. Bars to the right, location of the molecular mass standard proteins: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (92 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa).

3. RESULTS

Vinculin was identified as an actin-binding protein by staining with actin of vinculin blotted electrophoretically onto nitrocellulose sheets. Binding of polymeric anion to vinculin could be detected by fluorescence (fig.1). The blotted vinculin could not be stained by monomeric actin. In control experiments other actin-binding proteins were tested for staining by polymeric actin. Gelsolin, α-actinin and tropomyosin were found not to be stained when tested by the same assay. However, tropomyosin and α-actinin which was bound to nitrocellulose without preceding SDS electrophoresis, were stained by polymeric actin. Vinculin blotted onto nitrocellulose was stained both with and without preceding SDS electrophoresis. Evidently, many actin-binding proteins lose their ability to bind actin by irreversible denaturation during SDS electrophoresis. In order to investigate unspecific staining proteins which are known not to bind actin, such as myosin in the presence of ATP, β-galactosidase, phosphorylase, bovine serum albumin and ovalbumin, were tested for staining. These proteins did not bind polymeric actin. These results demonstrate by overlay assays that not all actin-binding proteins can be identified but that only some actin-binding proteins can be detected.

Cosedimentation was used as a second method for investigation of binding of vinculin to actin. According to SDS electrophoresis (fig.3, lane 5) vinculin used for the sedimentation experiments was free of α-actinin which has been reported to link vinculin to actin filaments [5,10]. Vinculin was also tested for contamination by the actin polymerization-inhibiting protein (HA1) which occurs in crude vinculin preparations [6–8]. Vinculin was separated from the actin polymerization-inhibiting activity by chromatography on the Sepharose CL-6B column. Polymeric actin (20 μM) was combined with various concentrations of vinculin (0.1–1 μM). Following sedimentation of polymeric actin the pellets and supernatants were analyzed by SDS-PAGE. The results of a typical experiment are displayed in fig.2. Part of

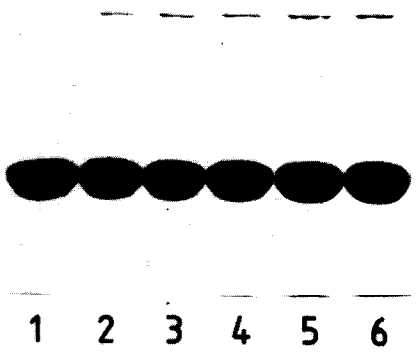

Fig.2. SDS-polyacrylamide gel (7%) of vinculin (upper band) cosedimented with 20 μM polymeric actin (lower band). Total vinculin concentrations: 1, 0.1 μM; 2, 0.2 μM; 3, 0.4 μM; 4, 0.6 μM; 5, 0.8 μM; 6, 1.0 μM.

the vinculin was cosedimented with polymeric actin.

As it might be possible that vinculin was contained in the volume of sedimented actin without

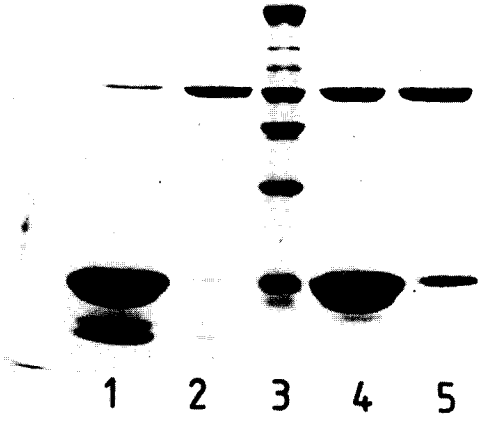

Fig.3. SDS-polyacrylamide gel (7%) of vinculin (total concentration, 1 μM) cosedimented with 20 μM polymeric actin in the presence or absence of 3 μM tropomyosin. Lanes: 1, pellet in the presence of tropomyosin; 2, supernatant in the presence of tropomyosin; 4, pellet in the absence of tropomyosin; 5, supernatant in the absence of tropomyosin; 3, molecular mass standard proteins: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (92 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa).

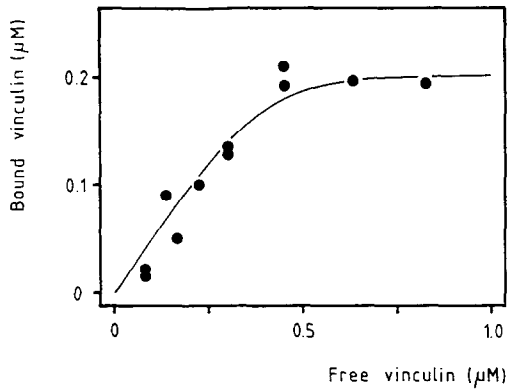

Fig.4. Plot of the concentration of vinculin bound to 20 μM polymeric actin versus concentration of free vinculin. Vinculin concentrations were determined by photometric scanning of Coomassie blue-stained polyacrylamide gels of pellets and supernatants of vinculin-actin solutions.

binding to actin filaments, we searched for other actin-binding proteins which could displace vinculin from actin by competitive binding. Tropomyosin was found to inhibit cosedimentation of vinculin with actin filaments (fig.3). According to photometric scanning of Coomassie blue-stained gels about 10-fold less vinculin is cosedimented with actin filaments in the presence of tropomyosin than in its absence. Thus, vinculin appears to bind specifically to polymeric actin and to compete with tropomyosin for binding to actin.

The amount of actin-bound vinculin was quantitatively estimated by photometric scanning. In fig.4 a plot of bound vinculin versus free vinculin is depicted. Maximally 0.2 μM vinculin were bound by 20 μM polymeric actin. This corresponds to a stoichiometric ratio of about one vinculin molecule per 100 actin filament subunits. Half saturation was reached at a concentration of 0.25 μM free vinculin suggesting that the equilibrium constant for dissociation of vinculin from actin filaments is in the range of 10^{-7} M.

4. DISCUSSION

Several reports have appeared in which it has been suggested that vinculin does not associate with actin [9,25]. The interaction between vinculin and actin has probably been overlooked because only one vinculin molecule is bound per 100 actin filament subunits. Isenberg et al. [2] have found

that one vinculin molecule may be bound even per 10 actin filament subunits. Vinculin has been reported to increase the low shear viscosity of polymeric actin [6,24,25]. This effect has been shown to be reversed by the addition of tropomyosin [25]. Unfortunately, viscosity experiments cannot be interpreted in terms of the mechanism of the interaction between vinculin and actin. In this paper we found that vinculin can be replaced from actin by tropomyosin. Tropomyosin binds both along actin filaments and near the ends of actin filaments as recently demonstrated by the ability of tropomyosin to retard association of actin monomers with the ends of actin filaments [26]. Thus, based on the observation that vinculin and tropomyosin compete for binding to actin, no decision can be made whether vinculin binds near the ends to actin filaments or along actin filaments. During aggregation of platelets vinculin has been found to be incorporated into the cytoskeleton at a vinculin to actin ratio of 1:100 [27]. These in vivo experiments are in good agreement with the results of our in vitro study.

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