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Calponin binds G-actin and F-actin with similar affinity

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Abstract **Calponins are actin-binding proteins that are implicated in the regulation of actomyosin. Calponin binds filamentous actin (F-actin) through two distinct sites ABS1 and ABS2, with an affinity in the low micromolar range. We report that smooth muscle calponin binds monomeric actin with a similar affinity (K_d of 0.15 μM). We show that the arrangement of binding is similar to that of F-actin by a number of criteria, most notably that the distance between Cys273 on calponin and Cys374 of actin is 29 Å when measured by fluorescent resonance energy transfer, the same distance as previously reported for F-actin.**

Keywords: Actin; Cytoskeleton; Calponin; Cell signalling

1. Introduction

Calponins (CH) are a small group of widely expressed actin-binding proteins [1,2]. Three vertebrate calponin isoforms have been identified: the basic, h1, the neutral, h2 and the acidic isoform, h3. Calponins h1 and h2 are expressed in smooth muscle cells whereas h3 is expressed in smooth muscle and in non-muscle cells, particularly neurons [3]. Calponins are well characterized components of the smooth muscle thin filament where they are suggested to regulate interactions between actin and myosin II. Calponins also interact with a number of other thin filament regulatory proteins (reviewed [2]), through the ''regulatory region'' [4]. This short region (145–182) contains binding sites for actin [5], and gelsolin [6] in addition to sites for tropomyosin, S100B, calmodulin and caltropin (reviewed [7]). There is likely to be competition between the various binding partners for this small site as there are so many potential binding proteins. Furthermore, the binding of several proteins to calponin is known to be determined by the phosphorylation of sites within the regulatory region [2].

In addition to their established cytoskeletal structural/regulatory functions, calponins are considered as being potential players in signal transduction. For example, calponin may function as an adaptor protein connecting the protein kinase C (PKC) cascade to the extracellular regulated kinase (ERK) cascade [8] as calponin binds ERK through the CH domain [9] and is a substrate for PKC [10]. Upon stimulation with agents that activate PKC, calponin translocates from the cytoskeleton to the plasma-membrane [11], possibly as a complex with PKC-α [12]. Phosphorylation of calponin by PKC at Thr184 [13], in the regulatory region, reduces the affinity for actin [10] and so calponin is thought to leave the actin cytoskeleton and bind the membrane through direct interactions with phospholipids, particularly negative charged lipids, such as phosphatidylserine and phosphatidylinositol [14].

Calponin is only one of many proteins that bind actin and these proteins tend to either bind F-actin or G-actin. A few, such as gelsolin, bind both and we now report that calponin too is a member of this group since it binds G-actin with a similar affinity to that previously reported for F-actin. Binding of G-actin to the regulatory region is likely to influence binding to other partners and so may constitute another level of modulation over calponin's function.

2. Materials and methods

2.1. Proteins and peptides

Actin was prepared from rabbit muscle acetone powder as previously described [15] and stored in buffer G (2 mM Tris [pH 7.5], 0.1 mM $CaCl_2$, 0.1 mM ATP). Basic calponin (calponin h1) was isolated from fresh chicken gizzards, as reported previously [1,4]. Calponin was specifically labeled at Cys273 with fluorescent reagents (acrylodan or 1,5-IAEDANS) as previously described [16]. The labeling ratios were estimated spectroscopically using the following extinction coefficients: acrylodan = 16 400 M^{-1} cm^{-1} at 387 nm and 1,5-IAEDANS = 6200 M^{-1} cm^{-1} at 335 nm. The labeling stoichiometry was 0.50 for acrylodan-calponin and 0.80 for AEDANS-calponin. Calponin was cleaved by chymotrypsin at a substrate weight ratio of 1/1000 as previously described [4]. Briefly, the resulting two fragments of 22 and 13 kDa were purified by affinity chromatography on a calmodulin-Sepharose column. The 22 kDa N-terminal fragment was eluted by 1 mM EGTA, while the 13 kDa C-terminal fragment was not absorbed on the resin [4].

2.2. Fluorescence studies

Fluorescence experiments were performed at 21 °C using a LS 50 Perkin–Elmer Luminescence spectrometer. Spectra for IAEDANS-labeled proteins were obtained with the excitation wavelength set at

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Abbreviations: CH, calponin homology domain; ELISA, enzyme-linked immunosorbant sandwich assay; PKC, protein kinase C; ERK, extracellular regulated kinase

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340 nm and fluorescence changes deduced from the corresponding area of the emission spectra between 470 and 490 nm. In the particular case of acrylodan labeled calponin, the area of the emission spectrum was determined for wavelengths higher than 497 nm (wavelength corresponding to the maximum of fluorescence for calponin alone) that is, between 500 and 530 nm.

The parameters K_d (apparent dissociation constant) and A_{max} (maximum effect) were calculated by non-linear fitting of the experimental data points (means of at least triplicate measurements) by using the following equation:

$$\Delta F = 1/2A_{max} * [E]^{-1}(([E] + [L] + K_d) - (([E] + [L] + K_d)^2 - 4[E]^*[L])^{0.5}) \quad (1)$$

In Eq. (1), $[E]$ is the concentration of the fluorescent protein or peptide and L the ligand concentration. Non-linear fitting was performed by using the Curve Fit software developed by K. Raner (Mt. Waverley, Vict., Australia). Additional details on the different experimental conditions are given in the figure legends.

Fluorescence energy transfer measurements were performed as previously described [16,17] to estimate the distance between AEDANS on Cys273 of calponin, acting as donor, and acetamidofluorescein on Cys374 of actin, acting as acceptor, within the G-actin–calponin complex. As we observed changes in the environment of the donor (around the Cys273 of calponin) in the presence of G-actin, the following relation was used. Enhancement upon donor excitation in the acceptor excitation spectrum resulting from energy transfer was used to calculate the efficiency

$$E = (F_{340}/F_{490} - A_{340}/A_{490}) * A_{490}/D_{340} \quad (2)$$

In Eq. (2), F_{340} and F_{490} are the intensities of the excitation spectrum, A_{340} and A_{490} the molar extinction coefficients of fluorescein at 340 and 490 nm, respectively and D_{340} the extinction coefficients of dansyl at 340 nm.

The distance, R between the fluorophores was calculated using the equation

$$R = R_0(E^{-1} - 1)^{1/6}\text{Å} \quad (3)$$

In Eq. (3), R_0 is the Förster's critical distance corresponding to a transfer efficiency of 50%. For the present donor–acceptor pair, the R_0 value was 25.7 Å as calculated using the quantum yield of the donor equal to 0.011 and $J = 1.69 \times 10^{-13}$ M^{-1} cm^3.

2.3. Immunological techniques

Enzyme-linked immunosorbant sandwich assay (ELISA), was used to monitor interaction between coated calponin fragments and biotinylated actin. Fragments (5 μg/ml) in 50 mM $NaHCO_3/Na_2CO_3$, pH 9.5, were immobilized on plastic microtiter wells. The plate was then saturated with 0.5% gelatin, 3% gelatin hydrolyzate in 140 mM NaCl, 50 mM Tris buffer, pH 7.5. Binding was monitored at 405 nm using alkaline phosphatase labeled streptavidin (dilution 1/1000). Similarly, plates were coated with G-actin and calponin binding was monitored using anti-calponin antibodies [4] and alkaline phosphatase coupled anti-IgG. Control assays were carried out in wells saturated with gelatin and gelatin hydrolysate used alone. Each assay was conducted in triplicate and the mean value plotted after subtraction of nonspecific absorption. The results were analyzed by non linear fitting (Curve Fit software developed by Raner) using the following equation:

$$A = A_{max} * [L]/(K_d + [L]) \quad (4)$$

In Eq. (4), A is the absorbance at 405 nm and L the ligand concentration. Additional details on the different experimental conditions are given in the figure legends.

2.4. Analytical methods

Protein concentrations were determined by UV absorbency using a Varian MS100 spectrophotometer. A value A1%/277 = 11.3 was used for calponin determination [4]. Extinction coefficients were calculated by tryptophan, tyrosine, and cysteine content. Calponin concentration was also estimated by the Bradford assay.

3. Results

3.1. Interaction of calponin with monomeric actin

The interaction of calponin with F-actin is well documented. At low ionic strength, a stochiometry of 1:1 associated with an apparent K_d in the μM range was previously reported [18,16]. In contrast, we show for the first time that calponin interacts with G-actin with approximately equal affinity. Calponin was coated on plastic and increasing concentrations of biotinylated G-actin were added to the well of the microtiter plates. G-actin

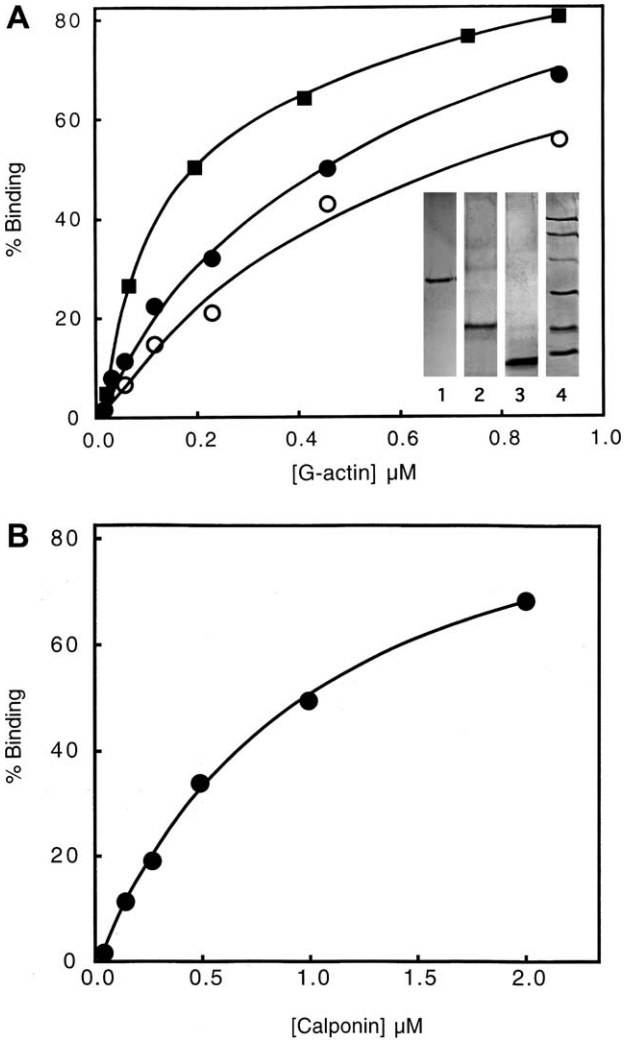

Fig. 1. Interaction of calponin and its fragments (13 and 22 kDa) with G-actin monitored by ELISA. A). Increasing amount of biotinylated G-actin (0–0.9 μM) was incubated with plastic coated calponin (full squares), 13 kDa (full circles) or 22 kDa (empty circles) in 30 mM Tris, $CaCl_2$ 0.1 mM, ATP 0.04 mM, pH 7.5. The binding was detected on the microtiter plate by the biotin-streptavidin system (see Section 2). Inset, digestion of calponin by chymotrypsin at a substrate weight ratio of 1/1000 [4]. Analysis of the purified fragments on 12.5% SDS PAGE : Calponin (5 μg) (lane 1). Calponin N-terminal fragment (22 kDa) (2.5 μg) (lane 2) and Calponin C-terminal fragment (4 μg) (13 kDa) (lane3). Molecular weight marker (lane 4) are phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). (B) G-actin (at 0.3 μg/ml) was immobilised on ELISA plates and challenged with various calponin concentrations between 0 and 2 μM as in (A).