Interaction of smooth muscle calponin and desmin

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Abstract Interaction of smooth-muscle calponin and desmin was analyzed by means of ultracentrifugation, fluorescent spectroscopy and affinity chromatography. At low and intermediate ionic strength (30–50 mM NaCl) calponin is cosedimented with desmin with an apparent dissociation constant 3–15 μM and stoichiometry of 1 calponin/4–6 desmin. Calmodulin decreases the quantity of calponin bound to desmin. Increase of ionic strength up to 150 mM weakens calponin–desmin interaction, but even at this ionic strength part of calponin remains bound to desmin. Calponin increases the rate and extent of fluorescence quenching induced by polymerization of 5-iodoacetamidofluorescein-labeled desmin. Affinity chromatography data indicate that desmin-binding sites are located in the N-terminal 22 kDa fragment of calponin. Since calponin interacts with desmin with an affinity comparable with that of, e.g., tropomyosin and myosin we suppose that calponin–desmin interaction may be important for cytoskeleton organization.

Key words: Calponin; Desmin; Calmodulin; Intermediate filament

1. Introduction

Calponin is a widely distributed actin-binding protein [1–4]. Calponin inhibits actomyosin ATPase activity and its inhibitory activity is not dependent on the presence of tropomyosin [5,6]. There are few contradictory points on the functional role of calponin. On one hand, calponin has been deduced to play a role in the regulation of smooth-muscle contraction [2,5,7]. On the other hand, the low efficiency of calponin in inhibition of actomyosin ATPase [8,9] and its wide distribution in non-muscle tissues (such as brain, fibroblasts and platelets) [2,4,10] may indicate that calponin is not only involved in regulation of the actomyosin system but also has other functions.

Data on the intracellular location of calponin are controversial. Earlier published results indicate that calponin is located only on stress fibre bundles and is excluded from desmin-rich cytoskeleton domain [11]. Later the same authors found that calponin is distributed both in the contractile and cytoskeletal domains of the smooth-muscle cell [12]. These data agree with recently published results showing that calponin colocalizes with intermediate filaments in smooth muscle [13] and glial fibrillary acidic protein forming intermediate filaments in glial cells [4]. All these facts may indicate that, in addition to regulation of the actomyosin system, calponin may have an important cytoskeletal role. This paper concerns the interaction of calponin with desmin, the main intermediate filament protein of the smooth muscle.

2. Materials and methods

Calponin from duck gizzard and calmodulin from bovine brain were isolated by methods described earlier [1,14]. Limited chymotrypsinolysis of calponin was performed as described by Mezgueldi et al. [15]. Duck gizzard desmin was purified according to Geisler and Weber [16]. Duck gizzard desmin is very susceptible to proteolysis; therefore extraction of desmin by urea containing buffer was performed at 3°C instead of 37°C as recommended in the original method. After precipitation with ethanol and dialysis desmin was subjected to ion-exchange chromatography on Q-Sepharose, concentrated by ultrafiltration (up to 1–2 mg/ml), dialyzed against 10 mM Tris/acetate pH 8.3, containing 10 mM mercaptoethanol and stored for 5–7 days at 3°C. The yield of desmin thus obtained was 80 mg of protein from 100 g of duck gizzard. Duck gizzard desmin migrated either as a single band with an M_r of 55 kDa or as a closely separated doublet with an M_r of 55 and 53 kDa on SDS gel electrophoresis [17]. Both bands were stained by monoclonal anti-desmin antibodies (Amersham, RPN 1101). Limited chymotrypsinolysis of desmin was performed according to Kaufmann et al. [18] and the M_r of the first band appearing at a very short incubation time was equal to 53 kDa, thus indicating that the doublet observed in some desmin preparations was due to partial proteolytic degradation of desmin during purification.

Ultracentrifugation was used for analyzing desmin–calponin interaction. A fixed quantity of calponin (2–5 μM) in 10 mM Tris/acetate, pH 8.3, containing 5 mM mercaptoethanol was titrated by increasing quantities of desmin (2–20 μM). Desmin polymerization was induced by addition of either 5 mM $CaCl_2$, 5 mM $MgCl_2$ or combined addition of divalent cations with NaCl (30–170 mM). After incubation for 15–20 min at 25°C the incubation mixture was subjected to ultracentrifugation (1 h, 105000×*g*). The protein composition of the initial mixture, supernatant and pellet was analyzed by SDS gel electrophoresis [17]. The stained gels were scanned on an LKB Ultrascan XL densitometer and the quantity of calponin bound to desmin or the quantity of calponin remaining unbound were plotted against total concentration of desmin in the incubation mixture.

Fluorescent spectroscopy was used successfully earlier for monitoring desmin polymerization and distinguishing polymerization from non-specific aggregation [19]. Duck gizzard desmin was modified by 5-iodoacetamidofluorescein as described [19]. The extent of desmin modification was equal to 0.15–0.25 mol fluorescent label per mol desmin. Labeled (20%) and unlabeled (80%) desmin were mixed together (total desmin concentration: 2.3 μM) and after addition of calponin (total concentration: 1.8 μM) dialyzed against 10 mM Tris/acetate, pH 8.3, containing 5 mM $MgCl_2$ with or without 50 mM NaCl. Dialysis was performed at 37°C and the intensity of fluorescence (excitation: 485; emission: 520 nm) was recorded on a Hitachi fluorescent spectrophotometer F-3000 every 15 min. Desmin polymerization is accompanied by quenching of fluorescence [19]. If calponin affects the time course of quenching of fluorescence we may conclude that calponin somehow affects desmin polymerization.

Affinity chromatography of calponin and its chymotryptic peptides on immobilized polymerized desmin was performed as follows. Desmin was polymerized for 2 h by dialysis against 10 mM Tris/HCl, pH 8.0, 5 mM $MgCl_2$, 50 mM NaCl and 0.1 mM PMSF at 37°C. Polymerized desmin was immobilized on BrCN-Sepharose according to the manufacturer's recommendation (Pharmacia). The affinity matrix containing about 0.5 mg of desmin was packed in the column (1.2×1.5 cm) and equilibrated by 10 mM Tris/HCl, pH 7.5, 1 mM mercaptoethanol and 0.1 mM PMSF. Intact calponin or its proteolyt-

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Abbreviations: IAF, 5-iodoacetamidofluorescein; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid

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ic fragments were loaded on the column and after 15 min incubation unbound proteins were eluted by 4–6 column volumes of the initial buffer. The proteins or peptides bound on the column were eluted by linear salt gradient (0–170 mM NaCl) in a total volume of 12 ml. Proteins in each sample were precipitated by 10% TCA and the protein composition was determined by SDS gel electrophoresis [17].

Protein concentration was determined either spectrophotometrically taking $A^{0.1\%}_{280}$ equal to 0.78 for calponin [20], 0.19 for calmodulin [21] and 0.55 for desmin [22] or by dye-binding assay [23] using bovine serum albumin as a standard.

3. Results

The method of ultracentrifugation was used for analyzing calponin–desmin interaction. At low ionic strength (10 mM Tris/acetate, pH 8.3) addition of 5 mM $CaCl_2$ induced desmin polymerization and desmin was found in the pellet after ultracentrifugation (Fig. 1). Under these conditions calponin was completely soluble and remained in the supernatant. At low ionic strength calponin tightly interacts with desmin and was coprecipitated with polymerized desmin (Fig. 1). The increase of ionic strength up to 150 mM NaCl significantly diminished the strength of interaction, but even at high ionic strength part of calponin remained bound to desmin and was detected in the pellet (Fig. 1). For determination of the apparent dissociation constant of the calponin–desmin complex we titrated a fixed amount of calponin (usually 2–5 μM) by desmin and plotted the quantity of calponin bound against the total concentration of desmin. At low ionic strength (10 mM Tris/acetate, pH 8.3, 5 mM $MgCl_2$ in the presence or absence of 0.1 mM $CaCl_2$) the apparent dissociation constant of the calponin–desmin complex determined from simple hyperbolic binding equation was equal to 2–3 μM and the stoichiometry of the complex was close to 1 : 1 (Fig. 2). At intermediate ionic strength (10 mM Tris/acetate, pH 8.3, 30–50 mM NaCl) the apparent dissociation constant increased up to 11–15 μM and the stoichiometry became equal to 1 mol calponin per 4–6 mol desmin. Thus, the stoichiometry of the calponin–desmin complex is similar to that of calponin–actin (1 calponin/2–4 mol target protein) and affinity of calponin to desmin is 10–100 times lower than that to actin [2,9,24,25]. Nevertheless, taking into account rather high content of both calponin and desmin in smooth muscle (≈130–150 μM for both proteins) [22,24], desmin–calponin interaction may be of physiological significance.

Calponin interacts with different Ca-binding proteins and the dissociation constant of calponin–calmodulin complex lies in micromolar range [2,24,26]. We analyzed the effect of calmodulin on calponin–desmin interaction. At intermediate ionic strength (30 mM NaCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$) 5-fold molar excess of calmodulin over calponin induces a 1.5–2 time decrease in the amount of calponin bound to polymerized desmin. This effect of calmodulin on calponin–desmin interaction is similar to the effects of calmodulin on calponin–actin [2,24] and calponin–myosin [27] complexes described earlier.

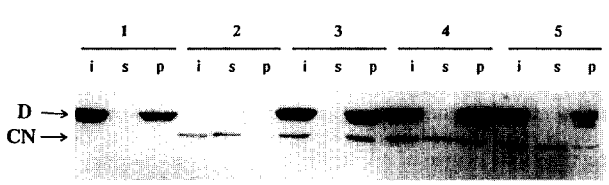

Fig. 1. Interaction of calponin and desmin revealed by ultracentrifugation. Protein composition of initial sample (i), supernatant (s) and pellet (p) after ultracentrifugation of isolated desmin (15 μM) (1), isolated calponin (5 μM) (2) or their mixture (3–5). Proteins were incubated in 10 mM Tris/acetate, pH 8.3, and desmin polymerization was induced by addition of $CaCl_2$ up to the final concentration of 5 mM. Samples 4 and 5 contained 50 and 150 mM NaCl, respectively. Position of desmin (D) and calponin (CN) are marked by arrows.

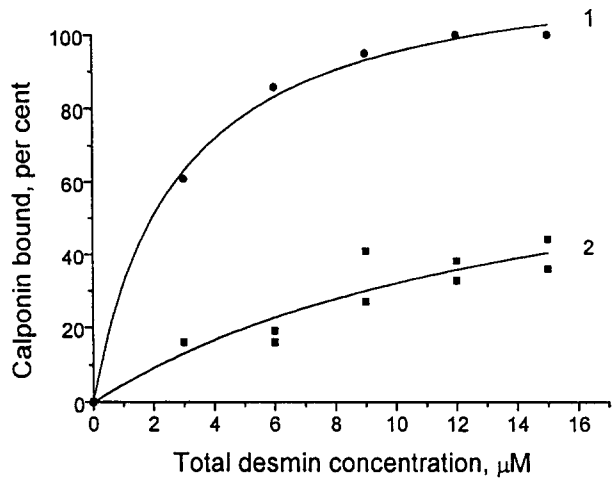

Fig. 2. Titration of calponin by desmin. Calponin (5 μM) was mixed with desmin and polymerization was induced by addition of 5 mM $MgCl_2$ in the absence (1) or presence (2) of 50 mM NaCl.

It is known that addition of divalent cations to the desmin solution may cause not only polymerization but aggregation of desmin [19]. Measurement of the fluorescence of IAF-labeled desmin provides a reliable approach for following real polymerization of desmin [19]. IAF-labeled desmin in the presence or absence of calponin was dialyzed against polymerization buffer: 10 mM Tris/acetate, pH 8.3, 5 mM $MgCl_2$ with or without 50 mM NaCl. The intensity of fluorescence was measured each 15 min and the time course of fluorescence quenching in the presence and absence of calponin was compared (Fig. 3). Addition of calponin increased the rate of fluorescence quenching indicating that calponin may increase the rate of desmin polymerization and promote intermediate filament assembly. This conclusion agrees with our preliminary electron microscopy data. We found that dialysis of unmodified desmin against the buffer containing 5 mM $MgCl_2$ and 50 mM NaCl produced short filaments. Under these conditions addition of calponin seems to increase the length of filaments and formation of a net. At high ionic strength (5 mM $MgCl_2$, 170 mM NaCl) calponin seems to induce bundling of intermediate filaments. All the data presented indicate that under certain conditions calponin may interact with desmin. In order to identify the sites of calponin involved in the interaction with desmin we used affinity chromatography.

Intact calponin binds to immobilized polymerized desmin and is eluted from the affinity column at about 50–60 mM NaCl. Interaction of calponin with immobilized desmin is similar to that of calponin with tropomyosin and caldesmon [28]. In all these cases calponin was eluted from the affinity matrix at about 70–100 mM of the salt and interaction of calponin with desmin is comparable with two other thin-filament-associated proteins. Limited chymotrypsinolysis of calponin results in production of long 22 kDa N-terminal and short 13 kDa C-terminal fragments [15] (Fig. 4). When the

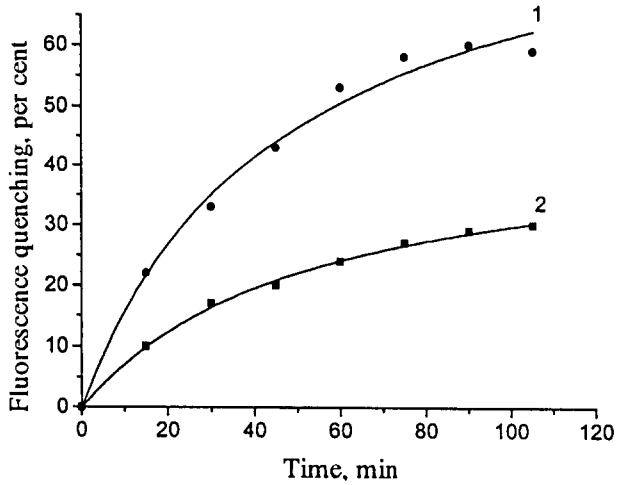

Fig. 3. Polymerization of IAF-desmin (2.3 μM) in the presence (1) or absence (2) of calponin (1.8 μM). Solution of desmin was dialyzed against 10 mM Tris/acetate, pH 8.3, 5 mM $MgCl_2$ containing 50 mM NaCl at 37°C and fluorescence intensity was measured each 15 min. The percentage of fluorescence quenching is plotted against time of dialysis.

mixture of chymotryptic peptides of calponin was loaded on the column of immobilized desmin the C-terminal 13 kDa fragment was detected in the flow through (Fig. 4). The N-terminal 22 kDa fragment together with traces of non-hydrolyzed calponin were tightly bound to the column and eluted by stepwise increase of ionic strength (Fig. 4). The 22 kDa fragment was heavily contaminated by peptides with molecular weights between 22 and 13 kDa which seems to be the breakdown products of the N-terminal part of calponin. These peptides were absent from the initial sample loaded on the gel. We assume that interaction of desmin with the N-terminal peptide of calponin induces its conformational changes which increase the susceptibility of calponin fragment to proteolysis. A similar effect was earlier observed during affinity chromatography of calponin on immobilized tropomyosin [28]. The data presented indicate that the putative desmin-binding site is located in the N-terminal 22 kDa fragment of calponin.

4. Discussion

It is well known that calponin interacts with actin with high affinity (K_d $5 \cdot 10^{-8}$–$2 \cdot 10^{-7}$ M) [2,24,25]. Therefore starting investigation of calponin with desmin we placed stringent requirements upon the purity of desmin. Extraction of insoluble residue of smooth muscle by KI provides solubilization of the major part of myosin and actin. Duplicate extraction by KI followed by alcohol precipitation and ion-exchange chromatography provides desmin preparations which were completely free of actin [16]. Even on heavily overloaded gels we could not detect actin and only minor contamination with molecular weights between 55 and 48 kDa were sometimes observed on the gel. All these bands were stained by monoclonal antibodies to desmin which indicate that they are proteolytic products of desmin.

At low and intermediate ionic strength calponin interacts with desmin with a K_d of 3–15 μM. The affinity of calponin to desmin is significantly lower than that to actin but is comparable with that to myosin [27] and tropomyosin [28]. The interaction of calponin with desmin depends on ionic strength but, even at physiological ionic strength (150 mM salt), part of calponin remained bound to polymerized desmin (Fig. 1). In this respect interaction of calponin with desmin seems to be similar to interaction of calponin with myosin and tropomyosin.

Fluorescent spectroscopy data indicate that under certain conditions calponin may affect the process of desmin polymerization. Our semiquantitative fluorescence data may mean that calponin somehow decreases the critical concentration of desmin or promotes intermediate filament assembly. One can speculate that this is due to the interaction of highly basic calponin with acidic desmin. Electrostatic interaction of calponin with desmin may also cause aggregation of intermediate filaments in the presence of calponin.

Desmin-binding site is located in the N-terminal 22 kDa chymotryptic peptide of calponin. This peptide contains actin, tropomyosin and calmodulin-binding sites of calponin [2,15,24,28]. Close separation of calmodulin- and desmin-binding sites of calponin explains the effect of calmodulin on calponin–desmin interaction and may provide for regulation of this interaction by Ca^{2+}.

Physiological significance of calponin–desmin interaction is unclear. High concentration of actin is smooth muscle (> 800 mM) [24] and its high affinity will cause competition for the interaction of calponin with any other contractile proteins. At the same time many actin-binding proteins will compete with calponin for interaction with actin. Moreover, high concentration of desmin (70–140 μM) [16,22] and calponin (≈ 150 μM) [24] as well as their strong interaction with K_d in the micromolar range will provide for calponin–desmin interaction. The morphological data indicate that in smooth muscle [12,13] and cerebellar neurons [4] calponin is located close to the intermediate filaments. This makes calponin–desmin interaction even more probable.

The data presented together with recently published results on calponin–phospholipid interaction [29–31] and location of

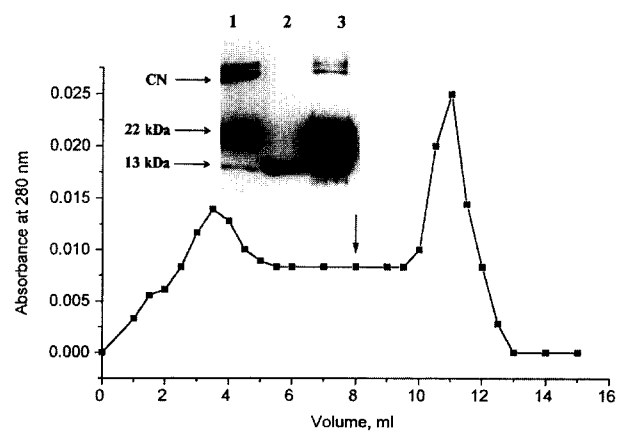

Fig. 4. Affinity chromatography of chymotryptic peptides of calponin on immobilized polymerized desmin. The mixture of chymotryptic peptides of calponin obtained after limited proteolysis [15] was loaded on the affinity matrix. Peptides bound to desmin were eluted by 170 mM NaCl (marked by ↓). The protein composition of initial sample (1), break through (2) and fraction bound to affinity matrix (3) was analyzed by SDS-gel electrophoresis and is shown in the insert. Positions of calponin (CN) and its 22 kDa N-terminal and 13 kDa C-terminal fragments are marked by →.

calponin in dense plaques and dense bodies [12] point to the important role of calponin in cytoskeleton organization.

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