Archives of Biochemistry and Biophysics 536 (2013) 38–45

Contents lists available at SciVerse ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Isoforms of gelsolin from lobster striated muscles differ in Calcium-dependence

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ARTICLE INFO

Article history:
Received 10 February 2013
and in revised form 7 May 2013
Available online 23 May 2013

Keywords:
Actin filaments
Actin-binding proteins
Gelsolin
Isoforms
Lobster striated muscle
Calcium-dependence

ABSTRACT

Two distinct isoforms of the Ca-dependent actin filament severing protein gelsolin were identified in cross-striated muscles of the American lobster. The variants (termed LG1 and LG2) differ by an extension of 18 AA at the C-terminus of LG1, and by two substitutions at AA735 and AA736, the two C-terminal amino acids of LG2. Functional comparison of the isolated and purified proteins revealed gelsolin-typical properties for both with differences in Ca^{2+}-sensitivity, LG2 being activated at significant lower Ca-concentration than LG1: Half maximal activation for both filament severing and G-actin binding was $\sim 4 \times 10^{-7}$ M Ca^{2+} for LG2 vs. $\sim 2 \times 10^{-6}$ M Ca^{2+} for LG1. This indicates a differential activation for the two isoproteins in vivo where they are present in almost equal amounts in the muscle cell. Structure prediction modeling on the basis of the known structure of mammalian gelsolin shows that LG2 lacks the C-terminal alpha-helix which is involved in contact formation between domains G6 and G2. In both mammalian gelsolin and LG1, this "latch bridge" is assumed to play a critical role in Ca^{2+}-activation by keeping gelsolin in a closed, inactive conformation at low [Ca^{2+}]. In LG2, the reduced contact between G6 and G2 may be responsible for its activation at low Ca^{2+}-concentration.

Introduction

Gelsolin and related proteins form[1] a diverse superfamily of actin-binding proteins (for reviews see [1,2]). Severing of actin filaments, capping of the barbed filament end, nucleation of actin polymerization and calcium-dependence of interaction with actin are usually regarded as the most characteristic properties of gelsolin-like proteins. These proteins have a modular structure which contain up to six "gelsolin"-domains [3,4]. Several gelsolin-related proteins have functional properties varying from the typical properties, i.e. actin bundling (villin [5]), capping without severing, or the lack of Ca^{2+}-dependence (CapG [6]). On the other hand, some proteins which qualify as "gelsolin-like" with regard to their functions differ structurally from gelsolin since they contain only 3 or 4 typical gelsolin modules. Especially in invertebrates, i.e. slime molds [7], nematodes [8], annelids [9] and echinoderms [10], these proteins are widely distributed. The six module type is predominantly found in vertebrates but also in tunicates [11] insects [12,13] and crustaceans [14,15].

The diversity of gelsolin–related proteins has been extensively investigated, but little information exists about closely related isoforms of gelsolin within one organism, and their possible functional relevance. Mammalians contain cytoplasmic and plasma gelsolin differing by an N-terminal extension [3] in plasma gelsolin. A third variant of gelsolin was found in rat oligodendrocytes [16]. Examples of invertebrate organisms containing isoforms of gelsolin-like proteins are the annelid *Lumbricus terrestris* where at least three isoforms with significant differences in sequence exist [17,18], and the slime mold *Physarum polycephalum* with two isoforms of the protein fragmin [19].

Striated muscles of the lobster contain six-module gelsolin with considerable structural and functional homology to mammalian gelsolin [15]. It fragments actin filaments, promotes nucleation of actin polymerization, and is activated by Ca^{2+}. As in other invertebrate muscles, gelsolin is present in significantly higher concentrations than in mammalian skeletal muscle. In this study we have identified two distinct gelsolin isoforms (LG1 and LG2) in cross-striated muscles of the lobster (*Homarus americanus*), and describe their isolation as well as structural and functional characterization. They show clear differences with regard to their Ca^{2+}-dependence of actin binding: LG2 is activated at significantly lower Ca^{2+} – concentrations than LG1. The only difference in sequence is a modification of the C-terminus of LG2. This apparently eliminates the contact between the domains 2 and 6 that is present in both

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[1] *Abbreviation used:* EDC, 1-ethyl-3 [3-(dimethylamino)propyl] carbodiimide

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http://dx.doi.org/10.1016/j.abb.2013.05.005

mammalian gelsolin and LG1, and keeps the molecule in a closed conformation under Ca^{2+}-free conditions. The missing contact in LG2 apparently leads to a more open conformation which requires less Ca^{2+} for activation.

Material and methods

Purification of gelsolin isoforms

Gelsolins from cross-striated muscles of *H. americanus* were purified by a method described in [14] with modifications. Muscle tissue was minced and homogenized with 5 vol. of extraction medium (20 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 20 mM imidazole and 1 mM phenyl-methyl-sulfonylfluoride, pH 6.8). After centrifugation, the extract was fractionated by differential ammonium sulfate precipitation, the fraction between 35% and 50% saturation was used for preparation of LG2, and the fraction from 50 to 80% saturation was used for LG1. Both fractions were subjected to the following purification steps: (1) ion exchange chromatography on TMAE-Fractogel EMD (Merck, Germany) anion exchanger with a gradient from 0.15 to 0.45 M KCl for elution; (2) gel filtration on Ultrogel ACA 44 (Pall Corp., U.S.); (3) Anion exchange chromatography on monoQ 5/5 HR (GE Healthcare, Munich). 1 mM EGTA was included in all solutions during purification. The activity of the actin modulators in various column fractions was monitored by Oswald viscometry as described below.

Purification of muscle actin

Actin from rabbit skeletal muscle was prepared according to [20] with an additional gel filtration step on Sephadex G-150 [21]. G-actin in G-buffer (0.2 mM ATP, 0.1 mM $CaCl_2$, 0.4 mM beta-mercaptoethanol, 1 mM NaN_3; 5 mM Tris–HCl, pH 8.2) was stored on ice and used within 1 week. For fluorometric measurements, N-(1-Pyrenyl)-iodoacetamide was coupled to actin according to [22] and stored lyophilized at −80 °C until use. Protein concentrations were determined by the biuret method.

SDS–PAGE and immunoblotting

SDS–PAGE was carried out using the Laemmli buffer system [23] in slab gels containing 12.5% polyacrylamide. To calculate the apparent molar masses of the separated proteins a set of standard proteins with 43, 67, 94, 116 and 205 kDa was used. For immunoblot analysis the proteins were transferred onto nitrocellulose by semidry electroblotting. The blots were transiently stained with Ponceau S to monitor transfer efficiency, then washed with Tris-buffered saline, and incubated for 2 h with a polyclonal antibody [15] directed against lobster gelsolin. The antibody detected both isoforms of gelsolin. A secondary alkaline phosphatase-conjugated goat anti rabbit antibody was used for immune detection. For the analysis of LG isoform distribution in various muscles, muscle pieces were dissected from the muscles in different regions ($n = 3$) immediately after the lobster was sacrificed. The samples were weighed, homogenized in 20 mM imidazole pH 7.0 with an Ultra Turrax (Ika, Germany), heated in SDS-sample buffer for 2 min and immediately subjected to SDS–PAGE and immunoblotting.

Ostwald viscometry

Gelsolin activities were determined by measuring the reduction of F-actin viscosity either after salt-induced polymerization of rabbit skeletal muscle G-actin in the presence of gelsolin (which reflected the nucleating activity on actin polymerization) or after the addition of gelsolin to pre-assembled actin filaments (reflecting the actin filament severing activity of the respective gelsolin). The conditions were: 24 μM actin, 100 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 10 mM imidazole pH 7.4 and 0.24 μM gelsolin – in the case of purified gelsolin – or 20–100 μl of the respective column fractions. The total volume of the assays was 1 ml. High shear viscosity was measured using Ostwald viscometers (Canon-Manning, PA, USA) with an outflow for water of 27 s at 25 °C. The relative gelsolin activity was calculated as percent reduction of the specific viscosity of F-actin. Measurements of the steady state viscosity of actin after severing were taken 5 min. after addition of gelsolin.

Fluorometry

The nucleation activity of gelsolin on actin polymerization was determined by fluorometry using pyrene-actin [22], with continous fluorescence monitoring on a spectrofluorometer (Shimadzu, RF-5001 PC). 10 μM G-actin containing 10% pyrene-actin was mixed with various concentrations of the lobster gelsolin isoforms or pig smooth muscle gelsolin in a final assay volume of 2 ml. All samples contained 1 mM ATP, 10 mM imidazole/HCl pH 7.4 and 1 mM of the respective Ca^{2+}/EGTA buffers to adjust [Ca^{2+}] to the desired concentrations. The polymerization was initiated by addition of KCl to a final concentration of 70 mM. All measurements were made at 25 °C. Excitation wavelength was 365 nm, emission was measured at 407 nm. To determine the relative nucleation activity of gelsolin at different Ca^{2+}-concentrations, the slopes of the linear part of the polymerization curves were determined and divided by the values obtained for actin without gelsolin. The increase in polymerization rate induced by gelsolin represented the nucleation activity. For comparison, the values were normalized taking the value at the highest Ca^{2+} concentration as 100% to obtain the relative nucleation activity for the respective gelsolin.

Calcium/EGTA buffers

[Ca^{2+}] in Ca^{2+}/EGTA mixtures was calculated using the program EQCAL (Biosoft, Cambridge, UK) with consideration of the concentrations of Mg^{2+}, ATP and pH. The respective stability constants included with the program were used. The following [Ca^{2+}] were used in various experimental approaches: (1) 7.8×10^{-8} M; (2) 1.4×10^{-7} M; (3) 3.7×10^{-7} M; (4) 5.4×10^{-7} M; (5) 7.8×10^{-7} M; (6) 1.3×10^{-6} M; (7) 5.3×10^{-6} M; (8) 9.8×10^{-6} M; (9) 3.4×10^{-5} M; (10) 1.1×10^{-4} M Ca^{2+}.

Chemical cross-linking

Chemical cross-linking was performed with the zero length cross-linker EDC (1-Ethyl-3 [3-(dimethylamino)propyl] carbodiimide) to analyze the formation of complexes between gelsolin and G-actin. The reaction was allowed to proceed for 10–90 min. Cross-linked proteins were detected by SDS–PAGE and corresponding immunoblots.

Cosedimentation

Abdominal *Extensor* muscle strips were freshly dissected and homogenized in Ca^{2+}/EGTA buffers. After ultracentrifugation for 45 min at 120,000*g*, both the supernatants and resuspended pellets were subjected to SDS–PAGE and subsequent immunoblotting with anti-gelsolin.