C-terminally deleted fragments of 40-kDa earthworm actin modulator still show gelsolin activities

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Abstract C- and N-terminally truncated fragments of earthworm gelsolin were constructed, cloned and expressed in *Escherichia coli*. G-actin-binding properties of these fragments and their influences on the polymeric state of actin were investigated. A construct lacking a large part of the third segment [E(1–295)] supports actin nucleation similar to the complete protein and shows reduced actin fragmentation property, but is no longer Ca^{2+}-sensitive in its activity. The first and the second segments (E1 and E2) each contain one actin-binding site. In contrast to human gelsolin, E1 in combination with a short N-terminal region of E2 is not sufficient for the F-actin-severing activity of the protein.

Key words: Gelsolin; Actin-binding protein; Actin fragmentation; Actin nucleation; Earthworm muscle

1. Introduction

Actin is involved in several basic functions of the organism such as cell division, cell motility or muscle contraction. The importance of controlling the polymeric state of actin is reflected by the large number of actin-binding proteins [1,2]. One group of these are the members of the gelsolin family, also termed actin-modulating proteins [3]. They occur in both muscle and non-muscle cells and show basically the same functions in vitro regardless of their different sources. They influence the polymeric state of actin in a Ca^{2+}-dependent manner by promoting the nucleation of G-actin to F-actin on the one hand, and by actively severing actin filaments on the other. There are two classes of actin-modulating proteins with molecular masses of 80 and 40 kDa, respectively. Human gelsolin as the most prominent representative of the 80-kDa class consists of six segments with repeated sequence motifs [4], whereas the small gelsolin-related proteins severin and fragmin from *Dictyostelium discoideum* and *Physarum polycephalum*, respectively, are half as large having only three such segments. Vertebrate gelsolin occurs in two splice variants encoded by a single gene [5]. Cytoplasmic gelsolin stays in the cells, whereas plasma gelsolin is exported into the extracellular space and into the bloodstream. The earthworm actin modulator (EWAM) from the annelid *Lumbricus terrestris* was the first 40-kDa protein of the gelsolin family isolated from invertebrate muscle tissue and is so far the only small gelsolin that occurs in at least two different isoforms [6]. EWAM shows the above-mentioned gelsolin properties and binds two molecules of G-actin in a highly cooperative manner [6]. One isoform has been cloned and its sequence determined. EWAM shows more than 40% sequence identity to severin and 33% sequence identity to the N-terminal half of human plasma gelsolin [7]. To investigate the functional properties of different parts of EWAM we have constructed and expressed C- and N-terminally deleted mutants of the protein. We analyzed the complete EWAM (E1–3), one construct consisting of the first EWAM segment plus 27 amino acids [E(1–185)], another containing the first two EWAM segments plus 27 amino acids [E(1–295)], and a third one lacking the first EWAM segment (E2–3) (Fig. 1) with respect to complex formation with G-actin, F-actin severing and the promotion of actin nucleation.

2. Materials and methods

2.1. Cloning of EWAM and its truncated derivatives

The pBluescript vector, into which the complete cDNA of earthworm gelsolin has been cloned previously [7], was used as template in PCR amplifications to create the DNA for the whole EWAM as well as for its truncated mutants. All sense oligonucleotides contained a recognition site for the restriction endonuclease *Mlu*I, whereas all antisense primers contained a *Sal*I site. PCR products were cloned into the *Mlu*I and *Sal*I restriction sites of the pET-23W1 vector, a pET-23a vector (Novagen), whose multiple cloning site has been altered [8]. Because of this cloning strategy all expressed proteins had three extra amino acids (M-T-R) at their N-terminus and two (V-D) at their C-terminus. The two C-terminal amino acids are followed by six histidines (His tag) and the E-E-F motif (Immuno tag).

2.2. Expression and purification of EWAM mutants

LB-Miller medium containing 100 μg/ml ampicillin, 34 μg/ml chloramphenicol and 2% glucose was inoculated with transformed *Escherichia coli* BL21 (DE 23) pLysS bacteria and shaken for 2 h at 37°C. The culture was grown to logarithmic stage at room temperature, induced with 0.05 mM IPTG and expressed for a further 3 h at the same temperature. All subsequent steps were carried out at 4°C. Cells were sedimented at 4000×*g* for 10 min and frozen. Bacterial pellets were suspended in 30 ml buffer A (50 mM KH_2PO_4 pH 8.0, 500 mM KCl, 0.2% Tween 20, 5 mM β-mercaptoethanol, 1 mM $PhMeSO_2F$, 10 μg/ml trypsin inhibitor [Sigma No. T9253]) containing 10 μg/ml lysozyme and then sonicated. Cell debris was pelleted for 20 min at 30 000×*g*. The supernatant was loaded onto a Ni-column previously equilibrated with buffer A. After washing the column with buffer A and buffer B (same as buffer A, but pH 6.0) protein was eluted with buffer C (500 mM imidazole/HCl pH 7.6, 1 mM EGTA, 0.1 mM $PhMeSO_2F$). Protein-containing fractions were dialyzed for 15 h against buffer D (10 mM imidazole/HCl pH 7.6, 1 mM EGTA, 0.1 mM $PhMeSO_2F$) and stored in liquid nitrogen. To finally separate

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Abbreviations: EWAM, earthworm actin modulator or earthworm gelsolin; E1–3, the three repeating segments of EWAM; S1–3, the three repeating segments of severin; G1–6, the six repeating segments of human plasma gelsolin; IAEDANS, *N*-(iodoacetyl)-*N*-1-sulfo-5-naphthylethylenediamine; IPTG, isopropyl-β-D-thiogalactoside; PI-actin, G-actin labeled with *N*-(1-pyrenyl) iodoacetamide; $PhMeSO_2F$, phenylmethanesulfonyl fluoride

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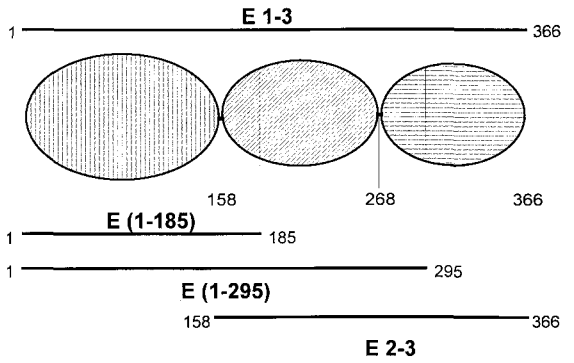

Fig. 1. Schematic representation of EWAM constructs.

EWAM fragments from contaminations an anion exchange column (MonoQ HR 5/5, Pharmacia) in 50 mM Tris-HCl pH 7.6, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM EGTA was performed. For protein elution a 10–300 mM KCl gradient was used.

2.3. Protein estimation

Protein concentration was calculated from absorbance at 280 nm based on the contents of tyrosine and tryptophan of each construct [9]. Values for EWAM constructs at $A_{280} = 1.0\ cm^{-1}$ are: E1–3 = 16 μM, E(1–295) = 19 μM, E2–3 = 41.4 μM, E(1–185) = 23.9 μM. The value for actin at $A_{290} = 1.0\ cm^{-1}$ is 37.4 μM. The calculated values of molecular masses are as follows: E1–3: 43287 Da; E(1–295): 35552 Da; E(1–185): 23047 Da; E2–3: 23278 Da.

2.4. Preparation and labeling of actin

Actin from rabbit skeletal muscle was purified from acetone-dried muscle powder as described by Spudich and Watt [10]. Fluorescence labeling of G-actin with *N*-(iodoacetyl)-*N*-1-sulfo-5-naphthylethylenediamine (IAEDANS) and *N*-(1-pyrenyl) iodoacetamide was performed as described previously [11,12].

2.5. Gel electrophoresis

Electrophoresis on polyacrylamide gels in the presence of sodium dodecylsulfate (SDS/PAGE) was carried out as described by Laemmli [13]. Gels were stained and destained according to Matsudaira and Burgess [14]. Non-denaturing PAGE was performed after Safer [15] with slight modifications. EWAM fragments and labeled actin were mixed on ice in various molar ratios in 10 mM Tris pH 8.0, 0.2 mM ATP, 0.2 mM dithiothreitol and 0.5 mM $CaCl_2$ 1 h prior to running. In order to study simultaneously the formation of the complexes in the presence of Ca^{2+} and their dissociation upon removal of Ca^{2+}, two gels, a control and a test gel, were run in parallel. The control gel was run under standard conditions with 0.1 mM Ca^{2+} in the gel and in the electrode buffer. In the test gel, Ca^{2+} was omitted from the electrode buffer of the anode buffer chamber. After 20 min, when both gels showed the formation of exactly the same two fluorescent bands of the EWAM/actin complex and the excess unbound G-actin, the cathode buffer of the test gel was replaced by a buffer containing 4 mM EGTA. Electrophoresis was completed under the same conditions as before (20 mA constant current, 4°C and darkness). The successful dissociation of actin monomers from the complex was additionally controlled using vertebrate gelsolin, from which one G-actin dissociates from the 1:2 complex under these conditions and crustacean gelsolin, which forms a quaternary complex with G-actin and loses all three actin monomers when switching from Ca^{2+} to EGTA [16].

2.6. Fluorimetric nucleation and fragmentation assays

The time-dependent influences of earthworm gelsolin on the polymeric state of actin were determined in a spectrofluorimeter (Kontron SFM 23). Wavelengths for excitation and emission were 408 and 365 nm respectively. Data were analyzed using GOLD-HPLC Analysis software (Beckman, version 4.01) and Excel (Microsoft, version 7.0). All measurements were performed in 1-ml cuvettes at 25°C.

For studying the fragmentation of F-actin by EWAM a dilution-induced depolymerization assay was used. 9.5 μM G-actin containing 10% PI-actin was polymerized overnight on ice in F-buffer (10 mM imidazole pH 7.5, 1 mM ATP, 0.3 mM $CaCl_2$, 100 mM KCl, 2 mM $MgCl_2$) in the presence of 32 nM E1–3 to create precapped filaments. In the absence or in the presence of various amounts of EWAM or its fragments the actin was diluted in F-buffer to 0.4 μM, which is below the critical concentration of the free pointed filament end [17]. Under these conditions the decrease of fluorescence is proportional to the number of pointed ends [18].

The nucleation assays contained 7 μM unlabeled G-actin and 0.5 μM PI-actin in 10 mM Tris pH 8.0 and 1 mM $CaCl_2$ or 4 mM EGTA. Polymerization was initiated by adding $MgCl_2$ and NaCl to final concentrations of 1 mM and 100 mM, respectively, followed by EWAM fragments in various molar ratios to actin.

3. Results

3.1. Expression and purification of mutants

Our isolation procedure led to 2–20 mg of virtually pure fragments E(1–185), E(1–295), E2–3 and E1–3 (Fig. 2). It is shown that E1–3 expressed by *E. coli* and EWAM isoform 1 prepared from earthworm have virtually the same mobility in the SDS-PAGE. Also there are no differences when comparing these two proteins in functional assays.

3.2. Actin binding

Analyzing 1:2 premixes of E1–3 or E(1–295) with G-actin by non-denaturing PAGE shows the building of a single complex for both fragments (Fig. 3). Premixing with three G-actins leads to a strong fluorescence of non-bound G-actin in both cases. With varying EWAM/actin ratios there is always only one complex band showing the high cooperativity of the binding of the two actins. The additional faint band of lower mobility in the case of E(1–295) could not be explained. In summary, both E1–3 and E(1–295) form 1:2 complexes with G-actin. The fragment E(1–185) shows only very little fluorescence of an actin complex, indicating that this segment binds one G-actin very weakly. The signal is not enhanced when the amount of actin in the premix is increased. On the other hand, there is a fluorescence signal of non-bound actin even at ratios as high as 1:0.25. E2–3 did not show any complex formation with G-actin in the non-denaturing PAGE. In addition it did not show any F-actin binding in cosedimentation assays (data not shown). Both fragments, E1–3 and

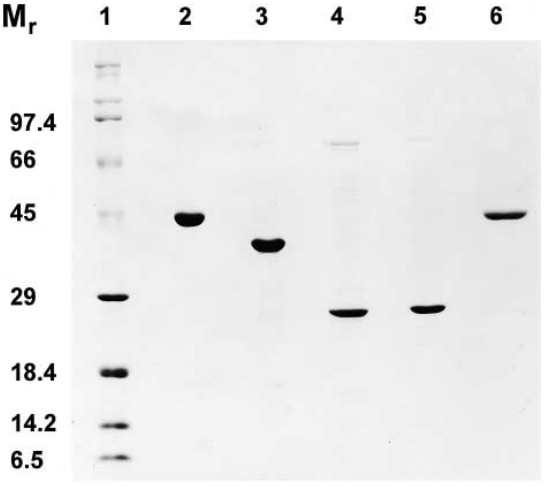

Fig. 2. SDS-PAGE of purified EWAM fragments. Relative molecular masses of marker proteins (lane 1) are given; recombinant E1–3 (lane 2) and EWAM isoform 1 purified from earthworm body wall muscle (lane 6) have the same apparent molecular mass of about 42 kDa; lane 3: E(1–295); lane 4: E2–3; lane 5: E(1–185).

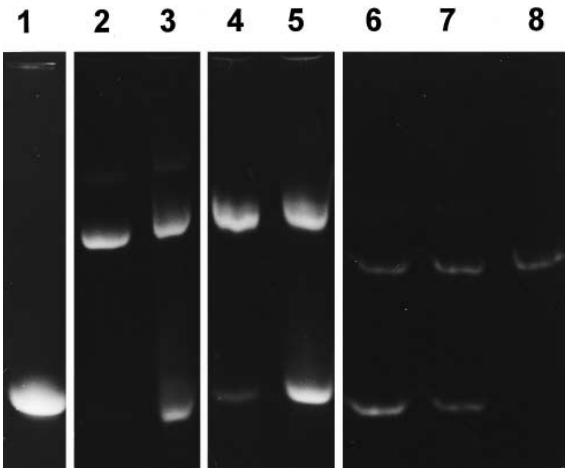

Fig. 3. Non-denaturing PAGE under Ca^{2+} conditions. Lane 1: Fluorescence signal of unbound G-actin as control; lanes 2 and 3: E1–3 and actin in molar ratios of 1:2 and 1:3; lanes 4 and 5: E(1–295) and actin in molar ratios of 1:2 and 1:3; for both E(1–295) and E1–3 there is no significant amount of unbound G-actin visible in the case of the 1:2 ratio; lanes 6, 7 and 8: E(1–185) and actin in molar ratios of 1:0.5, 1:0.25 and 1:0.1; only in the latter case free actin is no longer visible.

E(1–295) show a similar behavior when switching from Ca^{2+} to EGTA conditions (Fig. 4). After the switch one actin is lost from the preformed ternary complex and appears as an additional fluorescence band of non-bound actin running about half-way between the bands of the complex and the first free G-actin.

3.3. Fragmentation properties of EWAM and its mutants

In our fragmentation assay E1–3 and the shorter E(1–295) both show F-actin-severing activity. Analyzing different EWAM/actin ratios revealed that E1–3 severs F-actin about three times more efficiently than E(1–295) (Fig. 5). Whereas the fragmentation activity of E1–3 is strictly Ca^{2+}-dependent, E(1–295) severs F-actin also in the absence of Ca^{2+}. E(1–185), though containing 27 amino acids of the second segment,

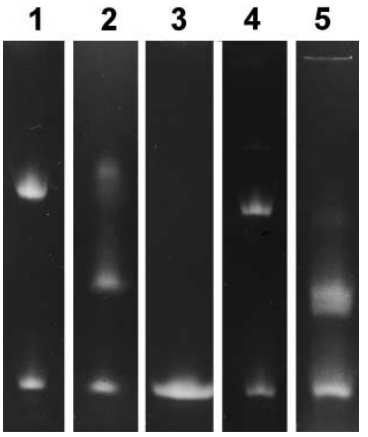

Fig. 4. Non-denaturing PAGE with switch from Ca^{2+} to EGTA conditions. Lane 1: E1–3 and actin in a 1:3 ratio in Ca^{2+} showing the 1:2 complex and unbound excess G-actin; lane 2: same, after the switch to EGTA conditions; lane 3: uncomplexed G-actin as control; lane 4: E(1–295) and actin in a 1:3 ratio in Ca^{2+}; lane 5: same, after the switch to EGTA conditions.

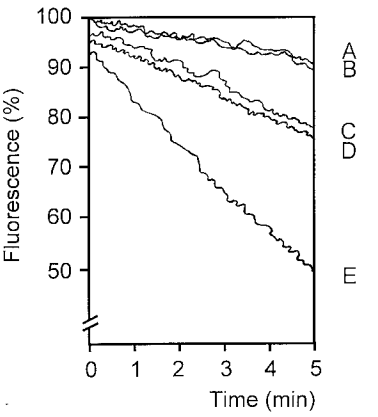

Fig. 5. Severing activity by EWAM and its fragments measured by the dilution-induced depolymerization assay. Fluorescence of F-actin was taken as 100% and that of G-actin as 0. F-actin control (A) and E(1–185) in a molar ratio of 1:5 to actin (B) are similiar in the first 5 min after dilution. E(1–295) is shown in a 1:10 ratio to actin (C) and E1–3 as 1:30 (D) and 1:10 (E).

shows no effect in this assay. To measure the severing activity we also used a second approach analyzing a dose-dependent decrease of the steady state viscosity of F-actin (data not shown). Comparable results were obtained for E1–3 and E(1–295). For E(1–185) a slight decrease was observed, which may be due to low affinity binding of E(1–185) to the barbed ends of the filaments.

3.4. Nucleation properties of EWAM and its mutants

Both constructs, E1–3 and E(1–295), are able to promote actin polymerization, E(1–295) even in the absence of Ca^{2+}. At low EWAM/actin ratios E1–3 is about 10-fold more effective than E(1–295). However, at higher EWAM/actin ratios it is seen that E(1–295) eliminates the lag phase in the actin polymerization process more efficiently than E1–3. E(1–185) has only a small effect on the polymerization of actin (Fig. 6).

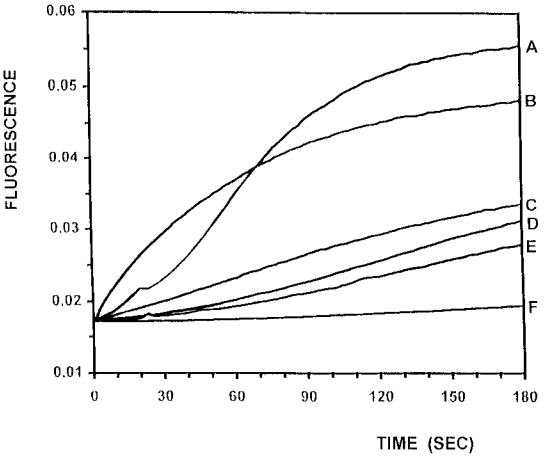

Fig. 6. Time-dependent polymerization of F-actin under the influence of EWAM fragments. (F) Actin control. At low molar ratios to actin E1–3 apparently promotes actin polymerization about 10-fold faster than E(1–295) and 100-fold faster than E(1–185), as the polymerization curves are parallel for the 1:1000 ratio of E1–3 (D), the 1:100 ratio of E(1–295) (C) and the 1:10 ratio of E(1–185) (E). Comparing a 1:100 ratio of E1–3 to actin (A) and a 1:10 ratio of E(1–295) to actin (B) it becomes obvious that E(1–295) overcomes the lag phase of polymerization more efficiently than E1–3.

4. Discussion

Human gelsolin has three actin-binding sites [19]. Its first segment (G1) contains a high affinity monomer-binding site [20]. A second calcium-sensitive monomer-binding site is located in G4 [21], whereas G2 contains a filament-binding site. Complete vertebrate gelsolin forms a ternary complex with two G-actin monomers [22]. Structural information about gelsolin segments was obtained by the crystallization of G1 [23], and segment 1 of villin [24], another protein of the 80-kDa class, and by NMR studies on S2, the second severin segment [25]. A common structure for all segments of gelsolins is assumed [26]. Based on the conserved amino acid sequence of segment 1 in EWAM and other members of the gelsolin family a monomer-binding site is predicted in E1 [7].

In human gelsolin segment 2 plays a crucial role in both severing and nucleation, whereas G1 is only involved in F-actin fragmentation [27]. The minimum unit required for severing is G1 plus the first 11 amino acids of G2. Thus, the actin-binding site of G2, which has to serve as monomer-binding site in nucleation and as filament-binding site in fragmentation, is suggested to be located at the N-terminus of the second segment [27]. Though human gelsolin and the 40-kDa protein severin differ with respect to the amino acid sequence of this region, the second actin-binding site of severin was assumed to be also located at the N-terminus of the second segment, namely amino acids 4–12 of S2 [25]. Therefore, it was of special interest to investigate the N-terminal part of the second EWAM segment (E2) as a critical region for actin binding, especially because amino acids 5–12 of segment 2 are 100% identical between severin and EWAM [7]. However, our construct E(1–185), which consists of the first 27 amino acids of segment 2 in addition to segment 1, does not bind two G-actin molecules. E(1–185) binds just one actin monomer with low affinity. The N-terminal region of E2 either does not contain the complete binding site for the second G-actin or this part of the construct is not folded in the right way. The usually observed high cooperativity between the two monomer-binding sites of EWAM might be the reason that the actin binding of E1 is very weak in this construct.

When both actin-binding sites of EWAM are present, as in the construct E(1–295), we observe the cooperation in the binding of two G-actin molecules in non-denaturing PAGE and see considerable severing and nucleation activity in fluorimetric assays. The main functional difference of E(1–295) with respect to E1–3 is the loss of the Ca^{2+} sensitivity for the processes of severing and nucleation. We conclude that a region within the third segment of EWAM confers Ca^{2+} regulation on the gelsolin.

Regarding the actin-binding properties of segments 2 and 3, EWAM is different from severin. A construct comprising segments 2 and 3 of severin (S2–3) shows F-actin-bundling activity [28]. Furthermore, S2 reveals binding to F-actin, but not to actin monomers [28]. The mutant E2–3, which we constructed for comparison, does not bind actin, presumably again due to the requirement of cooperativity between the two monomer-binding sites of EWAM. The possibility of two actin-binding sites within segments 2 and 3 of EWAM can be excluded. In contrast to severin, which has been described to have three potential actin-binding sites [28], complete EWAM has only two actin-binding sites, one in E1, the other in E2. An involvement of the N-terminal region of E3 in binding the second actin can be excluded, as a shorter EWAM mutant consisting of segments 1 and 2 without the additional amino acids of E3 shows the same functional behavior as E(1–295).

Our results suggest that EWAM has two actin-binding sites, a monomer-binding site in E1, and a second actin-binding site in E2, which like the actin-binding site of G2 serves for both severing and nucleation. In contrast to that, S2 of severin is only involved in severing. Nucleation by severin is assumed to be promoted by binding two actin monomers to S1 and S3 [28]. Regarding the second segment and its role in severing and nucleation, the annelid protein EWAM is functionally more closely related to human gelsolin than to severin from *Dictyostelium discoideum*. Despite the strong structural homology between E1 and G1 [7] there is an important difference between these two segments, as E1 in contrast to G1 is also involved in nucleation. It remains unclear why the C-terminally deleted constructs G1–2 and G1–3, which contain one monomer-binding site in G1 comparable to that of E1, and a filament-binding site in G2 that like the filament-binding site of E2 also binds monomers, do not show any nucleation activity. We are currently investigating why the binding of two monomers promotes G-actin nucleation in the case of E1–2, but not in the case of G1–2.

In contrast to human gelsolin and severin, EWAM uses the same directly adjacent segments for both the binding of monomers in the process of nucleation and filament severing.

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