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ASP-56, a new actin sequestering protein from pig platelets with homology to CAP, an adenylate cyclase-associated protein from yeast

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A new 56 kDa actin-binding protein (ASP-56) was isolated from pig platelet lysate. In falling ball viscosimetry it caused a reduction in viscosity that could be attributed to a decrease in the concentration of polymeric actin. Fluorescence measurements with NBD-labelled actin showed reduction of polymeric actin, too. These results could be explained by sequestering of actin in a non-polymerizable 1:1 ASP-56/actin complex. Sequencing of about 20 tryptic peptides of ASP-56 and comparison with known sequences revealed about 60% homology to the adenylate cyclase-associated protein (CAP) from yeast.

Actin-sequestering protein; Actin; Adenylate cyclase-associated protein

1. INTRODUCTION

The actin cytoskeleton of platelets is known to undergo a dramatic change in organisation during activation [1]. In resting platelets 40–60% of the actin are monomeric [2]. At the end of the activation cascade, however, most of the actin is filamentous. A number of proteins have been isolated which seem to play an active part in the organisation of actin filaments [3]. Among these proteins are profilin and thymosin β4, which sequester actin molecules in a non-polymerizable 1:1 complex [4,5], and gelsolin, which acts as a nucleus for actin polymerization in a calcium-dependent manner [6]. In this paper we report the isolation of a new actin binding protein, ASP-56, from pig platelets, and its characterization by actin-binding assays and partial sequence analysis.

2. MATERIALS AND METHODS

2.1. *Isolation of ASP-56*

Isolation of platelets from fresh pig blood was performed according to [7] with the minor modification that a fixed angle rotor was used to prepare platelet rich plasma.

About 12 g of platelets was resuspended in 30 ml of 10 mM Tris, pH 8, 70 mM KCl, 0.1 mM EDTA. Protease inhibitors were added to a final concentration of 2.5 μg/ml leupeptin, 2 U/ml aprotonin and 0.1 mM PMSF. A double volume of ice-cold lysis buffer, containing 10 mM Tris, pH 8, 0.1 mM EDTA, 200 mg/ml digitonin, 10 mM EGTA was added. The suspension was gently mixed, left on ice for 10 min and then centrifuged at 100,000 × *g* for 1 h. The supernatant was applied to a DE-52 column (2 × 40 cm) equilibrated with 10 mM Tris, pH 8, 30 mM KCl, 0.1 mM EDTA. The column was washed with one column volume of the same buffer, followed by a gradient from 0.03–0.3 M KCl (total volume 500 ml). The protein eluted as a complex with actin at 100 mM KCl. Pooled fractions were dialysed against 50 mM Tris, pH 8, 0.1 mM EDTA, 0.02% NaN_3 and applied to a P11 column (1 × 20 cm) equilibrated with the same buffer. ASP-56/actin complex, like most of the smaller and less basic proteins, did not bind to the column. The flowthrough was collected and dialysed against 0.6 M KCl, 0.1 mM EDTA, 10 mM Tris, pH 8, 0.1 mM DTT, 0.1 mM PMSF, 1 mM benzamidine, 0.02% NaN_3. The proteins were then concentrated with an amicon filtration cell (YM 30 membrane) and applied to a CL-6B gelfiltration column (2 × 90 cm) equilibrated with the same buffer. ASP-56/actin complex eluted before the bulk protein. Pooled fractions were dialysed against 10 mM phosphate buffer (PB), pH 7.5, 0.1 mM EDTA, 0.02% NaN_3 and applied to a hydroxyapatite column (1 × 10 cm) equilibrated with the same buffer. The column was washed with one column volume. Actin was separated from ASP-56 by elution with 3 M urea, 10 mM PB, pH 7.5, 0.1 mM EDTA for 6 h. ASP-56 was eluted with a gradient from 10 mM to 0.5 M PB in 3 M urea (total volume 400 ml). ASP-56 eluted at 0.2 M PB and was then dialysed extensively against 100 mM KCl, 0.1 mM EDTA, 10 mM Tris, pH 8, 1 mM benzamidine, 0.1 mM PMSF, 0.02% NaN_3 and 0.1 mM DTT. The yield was around 0.5 mg from 12 g platelets.

2.2. *Isolation of other proteins*

Actin was prepared according to [8]. Part of the preparation was labeled with NBD-Cl according to [9]. Gelsolin was isolated from pig platelets according to [10].

2.3. *Analytical methods*

Actin concentration was determined photometrically with an absorption coefficient of 0.64 for 1 mg/ml at 290 nm [11]. The concentrations of ASP-56 and gelsolin were determined by the method of [12].

Falling-ball viscosimetry was done according to [13]. 100 μl glass capillaries (Brand) were filled with actin and ASP-56 or gelsolin under polymerization conditions (80 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.5 mM ATP, 0.2 mM DTT, 10 mM Tris, pH 7.5. After 0.5 of polymerization capillaries were tilted (30°) and a stainless steel ball

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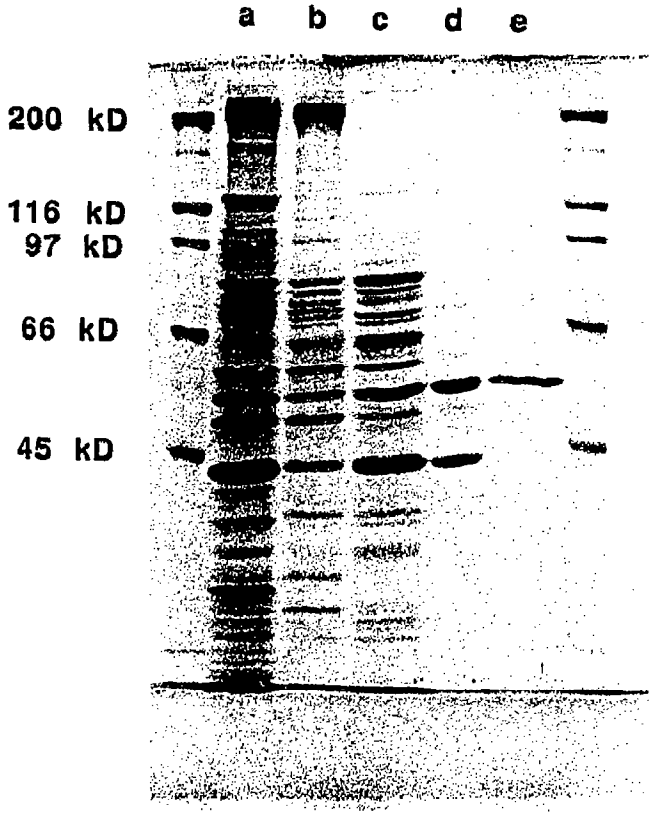

Fig. 1. Isolation of ASP-56 documented by SDS-PAGE. (Lane a) supernatant after centrifugation of platelet lysate; (lane b) pooled fraction after DE-52 chromatography; (lane c) protein not bound to a P11 column; (lane d) pooled fractions after CL-6B gelfiltration; (lane e) isolated protein after hydroxyapatite chromatography.

(New England Miniature Ball Co., Norfolk, USA) was run through the capillary. The run was measured by light barriers.

Fluorescence measurements were done with a Spex 1681 0.22 m Double Spectrometer operated by a Fast PC. One-tenth NBD-actin was mixed with unlabeled actin. Polymerization was induced by 80 mM KCl, 2 mM $MgCl_2$, 0,2 mM $CaCl_2$, 0.5 mM ATP, 0.2 mM DTT, 10 mM Tris, pH 7.5. Fluorescence excitation wavelength was 480 nm and emission wavelength was 540 nm. The portion of polymeric actin was calculated directly from the increase of fluorescence intensity [9]. Binding of ASP-56 to actin did not change the fluorescence of the NBD label.

SDS-polyacrylamide mini slab gel electrophoresis was performed according to [14]. Molecular mass markers were from Bio-Rad (Munich, Germany). The gels were stained with Coomassie blue R-250.

2.4. *Cleavage of ASP-56, isolation of peptides and sequence analysis*

The lyophilized protein was dissolved in 0.1 M Tris–HCl, pH 8.5, containing 6 M guanidine hydrochloride, reduced with dithiothreitol (final concentration 2 mM) under nitrogen for 6 h at room temperature and carboxymethylated with iodoacetic acid (final concentration 10 mM) under nitrogen at room temperature overnight. The carboxymethylated protein was precipitated with 10 vols. ethanol overnight at −18°C. The precipitate was solubilized in a small volume of 8 M urea and diluted with 0.2 M ammonium hydrogen carbonate to 1 M urea. The dissolved protein was then cleaved with 2 μg trypsin (TPCK-treated, Worthington) overnight at 23°C. The reaction mixture was acidified to pH 2 with trifluoroacetic acid and the peptides were

Figure 2a

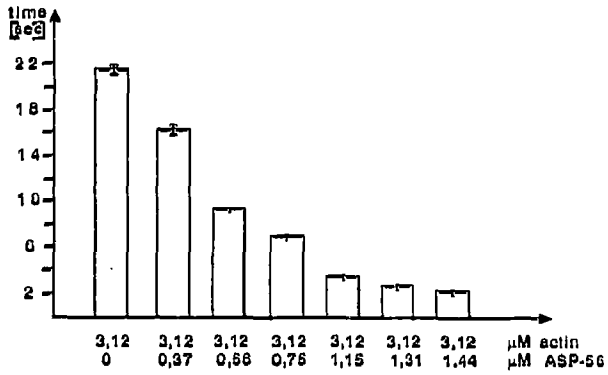

Figure 2b

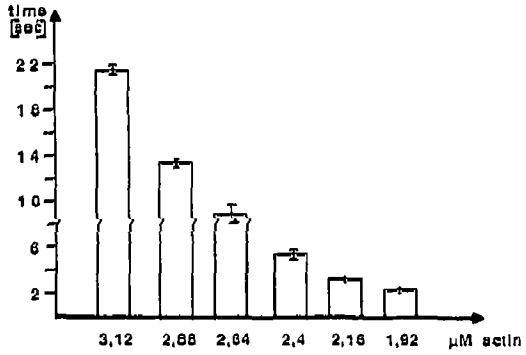

Figure 2c

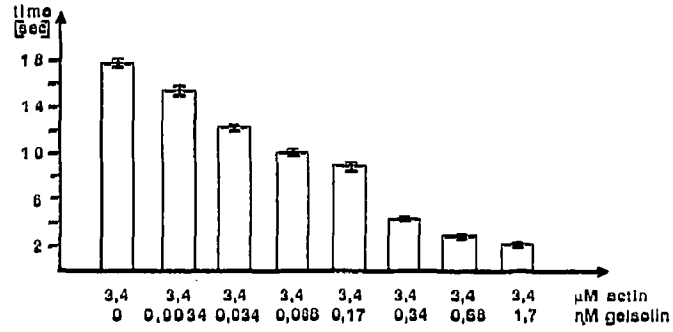

Fig. 2. (a) Histogram of the run times of falling-ball viscosimetry of polymerized actin with different concentrations of ASP-56. Polymerization conditions are described in Materials and Methods. The bars indicate the standard deviation of three parallel measurements. (b) Histogram of the run times of the ball through actin networks of different concentrations of actin. (c) Falling-ball viscosimetry of actin with various concentrations of gelsolin.

separated by reversed phase chromatography on a C18 column (Vydac, 4.6 × 250 mm) using 0.1% trifluoroacetic acid (solvent A) and 70% acetonitrile in 0.1% trifluoroacetic acid (solvent B) with a gradient from 0–60% B in 160 min and from 60–95% B in 40 min. The flow rate was 0.25 ml/min. The peptides were sequenced with a gas-liquid phase sequencer (Applied Biosystems, models 470A and 473A) following the manufacturer's instructions. Sequence comparisons were done with the FASTP programme [15] using the Martinsried Institute for Protein Sequences (MIPS) data collection.

Figure 3a

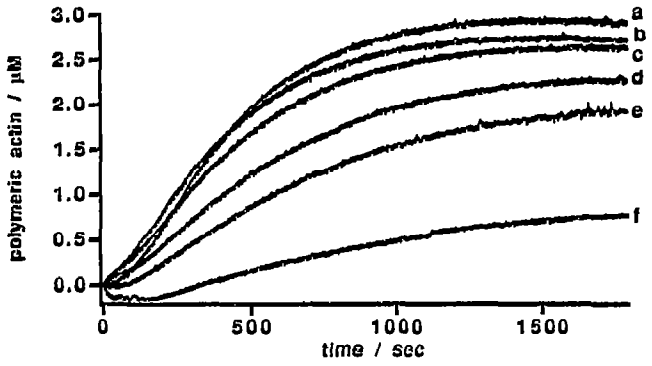

Figure 3b

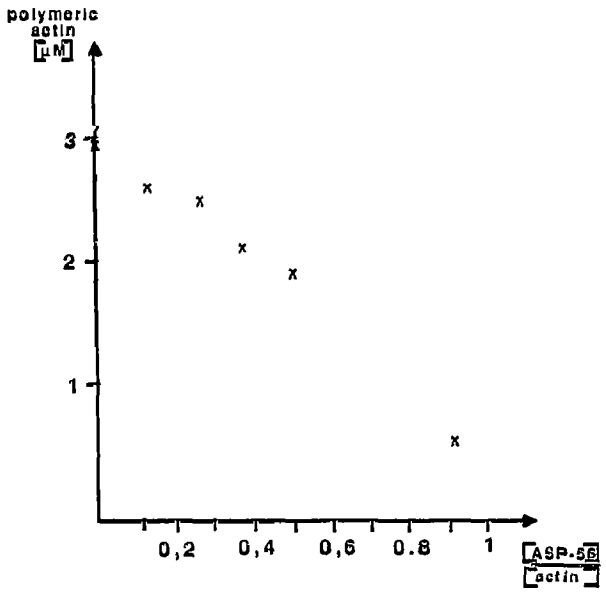

Fig. 3. (a) Increase of polymeric actin during the polymerization time as measured with NBD-actin. (Lines a–f) 3 μM actin under polymerisation conditions with different concentrations of ASP-56; (a) 0 μM ASP-56; (b) 0.37 μM; (c) 0.74 μM; (d) 1.11 μM; (e) 1.48 μM; (f) 2.74 μM. (b) Plot of polymeric actin after 0.5 h polymerization against the ratio of ASP-56 to actin. Data were derived from the upper diagram.

3. RESULTS AND DISCUSSION

ASP-56 was purified from pig platelets by a combination of ion exchange, size exclusion and hydroxyapatite chromatography (Fig. 1). Since ASP-56 co-purified with actin until treated with 3 M urea, we tested its in vitro binding activity by falling ball viscosimetry (Fig. 2a). A decrease in viscosity with increasing amounts of the isolated protein added to 3 μM actin was observed. In a control experiment the viscosity of decreasing concentrations of actin was measured (Fig. 2b). When the concentration of ASP-56 was subtracted from the total actin concentration, both histograms became similar. This suggested that ASP-56 decreases the concentration of polymerized actin by sequestering actin in a non-polymerizeable complex.

Gelsolin, another actin filament severing and nucleating protein, showed a much higher activity in this assay than ASP-56. At a ratio of one gelsolin to 2,000 actin molecules a substantial decrease in viscosity could be observed (Fig. 2c). This can be attributed to the nucleating activity of gelsolin. In comparison, the ASP-56 seems to sequester actin monomers and has no nucleating activity [16].

In a second assay, the actin sequestering activity of this protein was tested with actin labeled by a fluorescent dye. Fig. 3a shows the effect of ASP-56 on the time-course of actin polymerization. The time-course of polymerization revealed an initial lag phase. This provides additional evidence that ASP-56 has no-nucleating activity [17].

In Fig. 3b the concentration of polymerized actin (0.5 h polymerization time) is plotted against the ratio of ASP-56 to actin. With increasing amounts of ASP-56 *the portion of polymeric actin decreases. The plot shows* a linear decrease of polymerized actin with increasing concentrations of ASP-56. At a ratio of one ASP-56 molecule per one actin molecule polymerization is nearly completely inhibited. Taken together these results demonstrate that ASP-56 is an actin sequestering protein which binds actin in a 1:1 stoichiometry.

In order to test whether the protein was already known from other tissues or species, it was partially sequenced. Since the N-terminus was found to be

Table 1

Sequences of the peptides in single letter code

T2a	(X G P V A K
T2b	(V L K
T3	(A)Q S G P V R
T4	(E Y K D V D K
T5a	(T)H K N P A(D)K
T5b	(E)I G G D V L(K)
T6	(K W R
T7a	(H V A D D(E)K
T7b	(Q F K Y
T8	(H A E(M)V H T G L K
T9	(X A S K P G I S P S P K P V T K
T11	(L E A V S H A S D T H Y G Y G D S A(A K)
T12	(I N S I T V D N Y K
T14	(X I W N G Q K
T15	(S V N S T L Q I K
T16	(X G P K P F S A S K P G I S P S X K P V
T18	(N S L D C E I V S A K
T20/21	(E L S G L P S G P S A G S G P P P(P)
T24	(V P X I S I N K X D G R H I Y L S K
T25	(X E N X E N V S N L V I D(D)T E L K
T26/27	(S A L F A Q I N Q G E S I T H A L K
T28/30	(S(S)E M N V L I P(T)E G G D F N E F P V P E Q X K

Uncertain assignments are in brackets. X = unidentified residues. Numbering refers to Fig. 4.

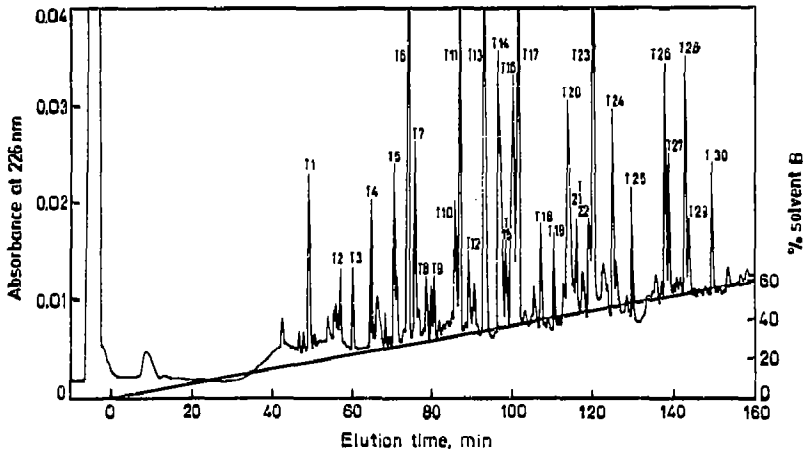

Fig. 4. Tryptic peptides of ASP-56 separated by reversed phase chromatography.

blocked, the denatured, reduced and carboxymethylated protein was cleaved with trypsin and the resulting peptides were separated by reversed phase chromatography (Fig. 4). The sequences of the proteolytic fragments chosen for sequence analysis are shown in Table I. A search for homologous sequences in the MIPS database with the FASTP programme showed a high percentage of identical amino acids between the long ASP-56 peptides T24 and T28 and a recently sequenced yeast protein CAP 70 [18], which had been shown to be an adenylate cyclase-associated regulatory protein. Since there was no other homology to a known protein we tried to align the other peptides to the CAP sequence (Fig. 5). Most of the ASP-56 peptides could in fact be aligned with some confidence: only few very short ones

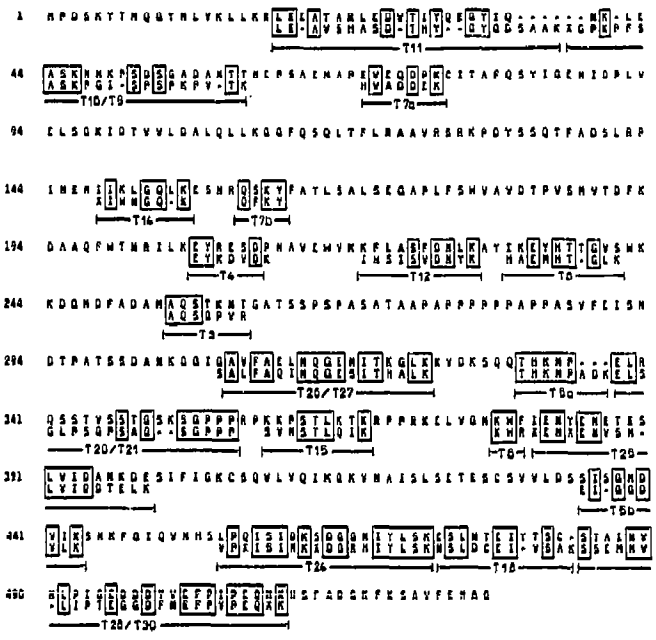

Fig. 5. Comparison of the amino acid sequences of the tryptic peptides of ASP-56 with CAP from *Saccharomyces cervisiae*.

did not match to the yeast sequence. Introducing a few limited gaps, we obtained 62% identical residues for the alignment shown in Fig. 6. Considering the evolutionary distance between yeast and pig we believe that these proteins are related.

The homology of this actin-binding protein to the yeast adenylate cyclase-associated protein raises the question of whether ASP-56, too, influences adenylate cyclase activity. This is currently under investigation. Field et al. [18] showed that yeast mutants lacking CAP also expressed phenotypes which seemed to be unrelated to effects due to the adenylate cyclase system. They found a swollen cell morphology, which could be due to cytoskeleton rearrangements. They also observed unusually large yeast cells hinting probably at some disturbance of the budding process, which is also known to depend on the cytoskeleton [19]. Therefore we have to consider the possibility that ASP-56, which has been isolated as actin-binding protein from pig platelets, is also acting on the adenylate cyclase pathway, while the yeast homologue, discovered as adenylate cyclase-binding protein may also act on the yeast cytoskeleton, possibly via actin binding.

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