Molecular cloning, over-expression, developmental regulation and immunolocalization of fragminP, a gelsolin-related actin-binding protein from *Physarum polycephalum* plasmodia

Davy T’Jampens1, Kris Meerschaert1, Bruno Constantin1, Juliet Bailey2, Lynnette J. Cook2, Veerle De Corte1, Hans De Mol1, Mark Goethals1, José Van Damme1, Joël Vandekerckhove1 and Jan Gettemans1, *1*

1Flanders Interuniversity Institute of Biotechnology, Department of Biochemistry, Universiteit Gent, Ledeganckstraat 35, B-9000 Gent, Belgium
2University of Leicester, Department of Genetics, University Road, Leicester LE1 7RH, UK

*Author for correspondence (e-mail: jan.gettemans@rug.ac.be)*

SUMMARY

FragminP is a Ca\(^{2+}\)-dependent protein of the gelsolin family. We screened a *Physarum polycephalum* cDNA library with polyclonal fragminP antibodies and isolated a cDNA clone of 1,104 bp encoding 368 amino acids of fragminP, revealing two consensus phosphatidylinositol 4,5 bisphosphate-binding motifs in the central part of the protein. The first methionine is modified by an acetyl group, and three amino acids were missing from the protein coded for by the cDNA clone. Full-length recombinant fragminP was generated by PCR, purified after over-expression from *Escherichia coli* and displayed identical properties to native *Physarum* fragminP. Northern blot analysis against RNA, isolated from cultures at various stages of development, indicated that fragminP is absent from amoebae and that expression is initiated at an early stage during apogamic development, in a similar way to that observed for the profilin genes. In situ immunolocalization of fragminP in *Physarum* microplasmodia revealed that the protein is localized predominantly at the plasma membrane, suggesting a role in the regulation of the subcortical actin meshwork. Our data indicate that we have isolated the plasmodium-specific fragminP cDNA (*frgP*) and suggest that, in each of its two vegetative cell types, *P. polycephalum* uses a different fragmin isoform that performs different functions.

Key words: Expression cloning, Fragmin, Actin-binding protein, Polyphosphoinositide, *Physarum polycephalum* development

INTRODUCTION

The acellular slime mould *Physarum polycephalum* is characterized by two distinctive growth phases: uninucleate amoebae and multinucleate plasmodia (Dee, 1987). Development of plasmodia by fusion of heterothallic amoebae (carrying compatible mating type) is a complex process that not only involves changes in cell morphology but is also accompanied by activation of a set of genes (Bailey, 1995), the products of which enable the plasmodium to display its characteristic behaviour. Not surprisingly, a considerable number of these genes encode cytoskeleton-associated proteins such as myosin (Kohama and Ebashi, 1986; Kohama et al., 1986) and profilin (Binette et al., 1990). Like higher eukaryotes, *Physarum* contains two genes that code for different isoforms of profilin, but unlike higher eukaryotes, these are expressed in a growth phase-dependent manner: amoebae express only profilinA whereas plasmodia express only profilinP (Binette et al., 1990). Interestingly, *P. polycephalum* is thought to express an amoeba-specific fragmin isoform (fragminA) and a plasmodial fragmin protein (fragminP), but the genetic basis for this dimorphism has not been elucidated (Uyeda et al., 1988). Fragmins A and P differ in molecular mass (40 kDa and 42 kDa, respectively) and the proteins are also immunologically distinct (Uyeda et al., 1988).

Plasmodial fragmin was originally isolated as a 42 kDa actin-binding protein that severs F-actin filaments (Hasegawa et al., 1980; Hinssen, 1981a,b). In addition it displays F-actin (+) end-capping and actin nucleating activity (Maruta and Isenberg, 1983; Gettemans et al., 1995). The actin subunit in the EGTA-resistant 1:1 actin-fragmin heterodimer is phosphorylated by the *P. polycephalum* actin-fragmin kinase (AFK) at residues Thr203 and Thr202 (Gettemans et al., 1992). AFK represents a novel type of protein kinase (Eichinger et al., 1996) and regulates the actin-binding properties of the actin-fragmin complex in two ways: upon phosphorylation, (1) the nucleating activity of the complex is inhibited, whereas (2) the F-actin capping activity of actin-fragmin becomes dependent on Ca\(^{2+}\) (Maruta et al., 1983; Gettemans et al., 1995).

FragminP, as a monomer or as a subunit of the actin-fragmin heterodimer, is also a substrate of casein kinase II-type enzymes from evolutionarily divergent species (De Corte et al., 1996a). *P. polycephalum* contains two variants of this kinase with a
molecular mass of 55 kDa and 150-160 kDa, respectively (De Corte et al., 1996a). Like other members of the gelsolin family, the actin nucleating and F-actin severing activities of plasmodial fragmin are inhibited by phosphatidylinositol 4,5 bisphosphate (PIP2) (Gettemans et al., 1995), thus pointing to a possible link between phosphoinositide-mediated signal transduction and microfilament organization, similar to that observed for profilin-actin (Lassing and Lindberg, 1985; Goldschmidt-Clermont et al., 1990) and gelsolin-actin interactions (Hartwig et al., 1995). Interestingly, De Corte et al. (1997) recently showed that PIP2 dramatically enhanced the phosphorylation of gelsolin, fragminP, CapG and profilin by pp60<sub>Src</sub>, thus pointing to a possible direct regulatory role of src kinase in the control of various actin-binding proteins.

Using conventional protein chemical methods, Ampe and Vandekerckhove (1987) determined approximately 88% of the complete amino acid sequence of fragminP. However, to obtain a clear understanding of its function (i.e. PIP2-binding, enhancement of actin phosphorylation by the AFK, expression during development and subcellular localization), it was necessary to obtain the complete sequence of the fragminP coding region. We therefore screened the ML8A <i>P. polycephalum</i> cDNA library (Bailey et al., 1992) with fragminP antibodies and isolated a 1,104 bp fragminP cDNA clone. Using northern blot analysis we studied the expression of fragminP during development of amoebae into plasmodia, and in situ immunolocalization analysis was performed to gain insight into the subcellular distribution of fragminP in <i>Physarum</i> cells. Our data shed new light on the role of fragminP during <i>Physarum</i> development and strengthen the idea that plasmodial fragmin functions as an important regulator controlling the organization of the subcortical microfilament system of <i>Physarum</i> plasmodia.

**MATERIALS AND METHODS**

**Materials and proteins**

Pancreatic deoxyribonuclease I (DNase I) was obtained from Worthington (New Jersey, USA). The T7 Sequencing kit and lysozyme were from Pharmacia (Uppsala, Sweden). Bromo-chloro-indolyl phosphate (BCIP), nitro-blue tetrazolium chloride (NBT) and isopropyl β-D-thiogalactopyranoside were from Sigma (St Louis, MO). The Wizard plasmid purification kit and the pUC/M13 forward and reverse primers were from Promega (Madison, WI, USA). Nitrocellulose filters were purchased from Sartorius (Goettingen, FRG). Restriction enzymes were from New England Biolabs, Inc. (Beverly, MA, USA). Trypsin (sequencing grade) and T4 ligase were from Boehringer Corp. (Mannheim, FRG). [γ<sup>32</sub>P]-dATP was obtained from ICN (Costa Mesa, CA, USA). Alkaline phosphatase-conjugated goat anti-rabbit IgG was from Fluka (Buchs, Switzerland). The GeneClean kit was from Bio101 (Vista, CA, USA). Taq polymerase and goat serum were obtained from Gibco BRL (Gaithersburg, MD, USA). ProBlott was from Applied Biosystems (Foster City, USA). Tetramethylrhodamine-isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG was from Nordic Immunological laboratories (Tilburg, Netherlands).

**Cell culture**

The apogamic strain CL (Cooke and Dee, 1974) was used in this study. Amoebal stocks were cultured at 30°C as described by Blindt et al. (1986); at this temperature plasmodium formation is inhibited. Cultures of developing cells were incubated at 26°C, the permissive temperature for development. For each RNA sample, 100 dilute semi-defined medium (DSDM) plates were inoculated with 3-5×10<sup>5</sup> amoebal cysts and 0.1 ml SBS (standard bacterial suspension) and incubated at 26°C prior to RNA isolation (Blindt et al., 1986). The length of time at 26°C was different for each sample. The proportion of multinucleate cells present at the time of harvest was determined by counting the number of nuclei per cell in 500 cells by phase-contrast microscopy. The percentage of developing cells at the time of harvest was determined by replating a sample of the harvested cells on DSDM agar with SBS and incubating at 26°C. After 4 days, the plateable plasmodia and amoebal colonies were counted, and the percentage of plateable plasmodia determined; only cells committed to development at the time of replating give rise to plateable plasmodia.

CL microplasmodia were cultured axenically at 26°C in SDM liquid plus hematin (Blindt et al., 1986). RNA was isolated from 50 ml cultures inoculated the previous day with 5 ml of dense microplasmodial suspension. CL macroplasmodia were obtained by placing 0.1 ml of concentrated microplasmodial suspension on a double layer of sterile Oxoid Nufill filters placed on a metal grid in a 9 cm Petri dish filled with SDM. During the period of culture, the microplasmodia fused together and grew into a macroplasmodium. To avoid artifacts due to the synchronous cell cycle of plasmodia, several macroplasmodia were inoculated at different times and harvested simultaneously 1-2 days later.

**Growth and screening of the <i>P. polycephalum</i> cDNA library**

The ML8A <i>P. polycephalum</i> cDNA library (Bailey et al., 1992), was grown on 9 cm diameter Petri dishes with LB medium containing 50 μg/ml ampicillin and 15 μg/ml tetracyclin. Expression screening of the cDNA library was performed as described (Sambrook et al., 1989) with slight modifications. Briefly, colonies were induced by applying nitrocellulose filters soaked in 10 mM isopropyl β-D-thiogalactopyranoside (IPTG). After 3 hours further growing at 37°C, the colonies were lysed overnight at room temperature on the nitrocellulose replicas in lysis buffer (100 mM Tris-HCl, pH 7.8, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1.5% BSA, 1 μg/ml DNASE I and 40 μg/ml lysozyme). The nitrocellulose membranes were quenched in TNT buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% NP40) with 3% milk powder. Primary antibody (rabbit anti-fragmin antiserum) was incubated with the membranes at a dilution of 1:1,000 in TNT buffer-1% milk powder (overnight at 4°C). The secondary antibody (goat anti rabbit alkaline phosphatase-conjugated IgG) was used at a dilution of 1:1,000. Colonies were visualized with bromo-chloro-indolyl phosphate and nitro-blue tetrazolium chloride, dissolved in 100 mM NaHCO<sub>3</sub>, pH 9.8 and 2 mM MgCl<sub>2</sub>.

**Plasmid isolation and DNA sequencing**

Plasmid DNA was isolated with the Wizard MiniPreps Kit (Promega, Madison WI, USA). The complete nucleotide sequence of the fragmin clone was determined by the Sanger dye-deoxy chain termination method (Sanger et al., 1977), using the T7 Sequencing Kit (Pharmacia, Uppsala, Sweden) and by the method of primer-walking. For sequencing the minus and plus strands, pUC/M13 forward and reverse primers (Promega), respectively, were used in the first reaction. New primers (obtained from Eurogentec, Belgium) were designed for further sequencing until overlap between + and − strands was obtained. High resolution DNA polyacrylamide gelelectrophoresis (6% acrylamide gels) was carried out with a Bio-Rad SequenGen system according to the instructions of the manufacturer.

**Construction of the full-length fragminP cDNA clone**

The seven nucleotides that were missing at the 5’ end of the fragP cDNA clone were deduced by reverse translation of the NH2-terminal amino acid sequence of <i>P. polycephalum</i> fragminP and inserted in the cDNA clone using the polymerase chain reaction (PCR). For this purpose we made use of a unique NcoI restriction site (bp 186-191). The pBlue-
from E. coli fragminP. was used as a source for the production and purification of recombinant that already contained the pSCM26 plasmid (Mertens et al., 1995a; Tilling et al., 1996) and harbours the T7 polymerase gene. This strain NdeI and NotI and ligated into the pLT10T3 expression vector (Mertens et al., 1995b) that was treated with the same restriction enzymes. The fragminP ATG codon represents the authentic start codon. A sample of the mixture was transformed into competent MC1061 λ cells, plated onto LB medium supplied with 50 μg/ml ampicillin and grown at 28°C. This pLT10T3-fragP plasmid was then transformed into E. coli MC1061 that already contained the pSCM26 plasmid (Mertens et al., 1995a; Eichinger et al., 1996) and harbours the T7 polymerase gene. This strain was used as a source for the production and purification of recombinant fragminP.

Over-expression and purification of recombinant fragminP from E. coli

Trial assays indicated that induction of fragminP with IPTG resulted in very high expression levels, but a considerable amount of fragminP was found in the pellet fraction. We therefore chose to grow the cells at 28°C without induction.

The MC1061-pSCM26-pLT10T3-fragP strain was grown overnight at 28°C in LB medium with 50 μg/ml ampicillin and 25 μg/ml kanamycin. This culture was diluted 1:10-1:20 in the same medium and further grown for 6-8 hours. Cells were harvested, resuspended in 2 volumes TDA buffer (10 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.02% Na3VO4) with 1 mM phenylmethylsulfonyl fluoride (PMSF) and subsequently lysed with a French Press. The lysate was centrifuged for 1 hour at 50,000 g and the clear supernatant was applied onto a DEAE-cellulose ion exchange column (2.5 cm × 15 cm), equilibrated with the same buffer. Adsorbed proteins were eluted with a 0-0.3 M linear gradient of NaCl in TDA buffer and 4 ml fractions were collected. Samples were analyzed on 10% SDS mini-slab gels and fractions containing fragminP were pooled, dialyzed against TDA buffer, pH 8.0 and applied onto a mono Q HR 5/5 column, connected to an FPLC system (Pharmacia, Uppsala, Sweden). Adsorbed proteins were eluted with the same NaCl gradient as for the DEAE column (20 ml total volume). FragminP eluted at 90 mM NaCl. The fractions were concentrated in a Speed Vac Concentrator (Savant Instruments, Farmingdale, NY) and dialyzed against a solution containing 60% glycerol, 50 mM Tris-HCl, pH 7.5, 1 mM DTT and 0.02 % Na3VO4. This preparation was essentially free of contaminating proteins.

Southern and northern hybridization

RNA isolation

Cultures of amoebae and developing cells were washed off agar plates with ice-cold water and rinsed twice with ice-cold water. Microplasmodia were pelleted and washed as above. Macroplasmodia were grown as described above and scraped directly into the guanidine lysis buffer. Total RNA was isolated from all cell types using the method of Chomczynski and Sacchi (1987), with modifications by Puisant and Houdebin (1990).

Northern blotting

Northern blots were run with 10 μg of total RNA in 1.1% agarose gels using the method of Murray et al. (1994). Transfer to Hybond-N membrane followed the method of Sambrook et al. (1989). The RNA was fixed to the membranes by exposure to UV irradiation prior to hybridization.

Isolation of genomic DNA

Genomic DNA was isolated using a method modified from that of Burland and Pallotta (1985). Briefly, 3 ml packed volume of exponentially growing CL microplasmodia was spun down, washed once in sterile water, and resuspended in 40 ml ice-cold NHS (nuclear homogenization solution: 250 mM sucrose, 0.1% Triton X-100, 15 mM CaCl2, 10 mM Tris-HCl, pH 7.5; stored frozen in 100 ml fractions). The microplasmodia were homogenized in a Waring blender using ice-cold cups. A sample of the homogenized material was examined microscopically to ensure that complete homogenization had occurred without disruption of nuclei. Any poorly homogenized material was removed by filtration through a milk filter. The volume of the filtrate was made to 35 ml with NHS, 10 ml of Percoll was added and mixed by inversion. This mixture was centrifuged at 4°C in a Sorvall RT600 rotor at 2,500 rpm. The supernatant was removed, the pellet resuspended in 45 ml NHS and centrifuged again. This washing was repeated once more. The nuclear pellet was resuspended in the residual liquid and kept on ice. Nuclear lysis buffer (100 mM EDTA, 0.5% Triton X-100, 25 mM Tris-HCl, pH 8.0) was mixed with one twentieth volume of proteinase K stock (2 mg/ml in water, stored at −20°C) and 4 ml of the mixture was added to the resuspended nuclei. After incubation at 70°C for 15 minutes, the mixture was cooled to 37°C and, after addition of the same amount of proteinase K, incubated at 37°C overnight. Next day, RNaseA was added to 1 mg/ml (stock solution: 100 mg/ml in 10 mM potassium acetate, pH 5.2, heated to 80°C for 10 minutes and stored at −20°C) and incubated for a further 2 hours at 37°C. The DNA was extracted once each with equal volumes of Tris-saturated phenol, phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). It was then precipitated by addition of one fifteenth volume 3 M sodium acetate and two volumes of ethanol. The pellet was washed twice in 5 ml of 70% ethanol, air dried and resuspended in 500 μl TE. The concentration was determined by optical density reading using a spectrophotometer.

Southern blotting

DNA was digested to completion and electrophoresed in 0.8% agarose gels prior to blotting using the method of Sambrook et al. (1989). The DNA was transferred to Hybond-N membranes and fixed by exposure to UV irradiation.

Synthesis of probes and hybridization (northern and Southern blots)

The cDNA insert was digested from the vector with EcoRI and NotI, purified and used as probe. Radiolabelled DNA probes were synthesized by the method of Feinberg and Vogelstein (1983). For these probes, prehybridization and hybridization were carried out at 65°C using the method of Church and Gilbert (1984). Stringency washes were carried out at 65°C in 0.2-0.4 M Na2HPO4, pH 7.5 with 0.1% SDS. Filters were exposed at −70°C for 5-14 days with an intensifying screen.

Protein chemical methods and mass spectrometry

Fragmin19 (De Corte et al., 1996b) was cleaved with trypsin for 4 hours at 37°C. The resulting peptides were separated on a microbore reversed-phase HPLC system and peptide profiles were analyzed by electrospray ionization-mass spectrometry as described elsewhere (De Corte et al., 1996a). Amino acid sequence analysis was performed on a 477A model pulsed liquid-phase sequenator equipped with a 120 A phenylthiohydantoin amino acid analyzer (Applied Biosystems Inc., USA). Matrix-assisted laser desorption ionization reflection time of
flight-mass spectrometry (MALDI-RETOF-MS) and post-source decay (PSD) mode analysis of peptides were performed as described elsewhere (K. Gevaert et al., submitted for publication). Chemical modification of the NH₂-terminal fragminP peptide with 2-iminothiolane and iodoacetamide was performed as described by Bartlett and Bush (1994).

**Immunofluorescence of microplasmodia**

Microplasmodia of *P. polycephalum* grown in axenic cultures were pretreated and fixed as described previously (Kukulies et al., 1985), with some modifications. Microplasmodia were first repeatedly washed by gentle agitation in 1 mM Ca(H₂PO₄)₂ solution containing 10 mM glucose, pH 6.3, in order to remove the thick extracellular slime layer. Freshly washed microplasmodia were then allowed to settle for 1 hour onto coverslips coated with 0.1% poly-L-lysine. Various fixation and extraction solutions were tested to improve the staining conditions. After washing three times in Tris-buffered saline (TBS, 10 mM Tris-HCl, pH 7.4, 170 mM NaCl), the samples were fixed at room temperature for 20 minutes in a solution of 3% paraformaldehyde in TBS. The fixed cells were then washed in TBS and permeabilized by 1% Triton X-100 in TBS for 30 minutes at room temperature. After washing in TBS (3× 5 minutes), cells were incubated for 30 minutes at 37°C in the presence of affinity-purified rabbit anti-fragminP polyclonal antibodies (Gettemans et al., 1995), diluted 1:100 in TBS and supplemented with 1% BSA and 2% goat serum (Gibco BRL). After washing 3× 10 minutes in TBS, cells were subsequently incubated for 30 minutes at 37°C with tetramethylrhodamine-isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG, diluted 1:200 in TBS/1% BSA/2% goat serum. For F-actin staining, FITC-phalloidin was used at a final concentration of 1 µg/ml. After washing, samples were mounted on a glass slide with Vectashield mounting medium (Vector Laboratories). Slides were observed with an inverted microscope (Axiovert 135 M, Zeiss), equipped with 20× and 40× Plan-Neofluar objectives, a HBO 50W mercury short-arc lamp, and a 560/590 nm filter combination. The fragmin and F-actin distribution were studied using a system for laser scanning confocal microscopy (Zeiss LSM 410), based on an inverted microscope (Zeiss axiovert 100), equipped with 40× and 100× Neofluar oil immersion objectives. Samples were excited with the visible lines (488 nm and 543 nm) of an argon laser beam, and emissions were collected via a photomultiplier tube through a band pass filter (515-525 nm) for green FITC fluorescence or a band pass filter (590 nm-610 nm) for red TRITC fluorescence.

**Miscellaneous**

SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) was performed on 10% or 15% polyacrylamide mini slab gels according to Matsudaira and Burgess (1978). Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard. Protein sequence alignments were done using the GCG program.

Labelling of actin with pyrene-iodoacetamide (Brenner and Korn, 1983) and F-actin severing assays were performed as described (Gettemans et al., 1995). Recombinant actin-fragmin kinase was purified from *E. coli* as described (Eichinger et al., 1996).

**RESULTS**

**Isolation and sequence of the fragminP cDNA clone**

Approximately 13,000 colonies of the *P. polycephalum* cDNA library were screened with polyclonal anti-fragminP antibodies for expression of recombinant fragminP. Using this approach we specifically selected for fragminP cDNAs that were cloned in the correct reading frame. The library was originally designed to identify genes involved in the transition from amoeba to plasmodium, and was therefore constructed from a developing cell population, consisting of 45% uninucleate committed cells, 5% multinucleate cells and 50% amoebeae (Bailey et al., 1992).

We isolated three positive colonies putatively encoding fragminP. One of these contained an insert of 1.17 kb following *EcoRI*/*NarI* digestion of the plBluescript II KS vector and agarose gel electrophoresis (data not shown). Since fragminP was thought to contain approximately 400 amino acid residues (Ampe and Vandekerckhove, 1987), we estimated the size of the cDNA coding region at 1.2 kb. The insert was sequenced by the method of primer walking. The nucleotide and deduced amino acid sequences are presented in Fig. 1. The cDNA insert comprises a 1,171 bp open reading frame with 1,104 bp coding for 368 amino acids of fragminP. Between the TAA stop codon and the poly(A)+ tail there is a 46 nucleotides long 3' untranslated sequence. This clone is not full length, as can be deduced from the lack of an ATG initiation codon. However, when the molecular mass of this 368-residue segment was calculated (40,632.5 Da) and compared with the experimentally determined mass of native plasmodial fragmin (41,080 Da; De Corte et al., 1996a), it was obvious that only a few amino acids were missing at the NH₂ terminus (Fig. 1). At this stage it appeared therefore more convenient to determine the amino acid sequence at the extreme NH₂ terminus of fragminP by protein chemical means.

**Determination of the amino acid sequence of the blocked NH₂ terminus of fragminP by MALDI-RETOF-mass spectrometry**

The sequencing strategy starts from the knowledge that conventional sequencing of a CNBr-cleavage fragment (Ampe and Vandekerckhove, 1987), covering the NH₂-terminal region of fragminP, overlaps with the deduced cDNA sequence, but contains a NH₂-terminal Gln-Lys extension. Taking into account the specificity of the CNBr cleavage procedure used, we could thus postulate a Met-Gln-Lys sequence at the amino terminus of fragminP. The overall mass of fragminP, including this tripeptide, differed by only 43 Da from the experimentally determined value. The difference corresponds exactly with the mass of an acetyl group, suggesting the sequence Ac-M-Q-K at the extreme NH₂-terminal primary structure of fragminP. This was verified by isolating the corresponding tryptic tripeptide by reversed-phase HPLC in combination with on-line electrospray ionization-mass spectrometry (ESI-MS; results not shown). Since Gln and Lys have a nearly identical mass, their exact order in this peptide could not be ascertained. We therefore determined the amino acid sequence of the peptide by a combination of MALDI-TOF and MALDI-RETOF mass spectrometry (K. Gevaert et al., submitted for publication) following modification of the lysine residue with 2-iminothiolane and iodoacetamide, which results in a mass increase of 158 Da. The Ac-M-Q-K M+H⁺ parent ion has a monoisotopic mass of 448.6 Da (Fig. 2A) and amino acid sequence analysis confirmed that it was blocked (not shown). The modified M'+H⁺ parent ion displays a monoisotopic mass of 606.7 Da (Fig. 2B). Both the unmodified and modified parent peptides were subsequently fragmented by laser-induced ionization and analyzed by MALDI-reflection mode of flight mass spectrometry. A variety of ions are thus generated, each corresponding to a particular ionized peptide fragment. The mass spectra obtained are shown in Fig. 2C-D. The peaks with masses of 258.1 and 275.1 Da...
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represent the Gln-Lys b- and Gln-Lys c-ions, respectively, whereas fragments of 130.1 and 174.1 Da match with the Lys z- and Ac-Met b-ions (Fig. 2C). Significantly, both spectra contain a peak with a monoisotopic mass of 302 Da. This single positively charged peptide fragment can only correspond with the sequence Ac-Met-Gln, because the mass of this peptide remains the same before and after modification. In the putative sequence Ac-Met-Lys, the mass would increase with 158 Da after modification, and this was not observed. The peak with a monoisotopic mass of 305 Da in Fig. 2D represents the modified Lys residue (147+158 Da). In the unmodified peptide, this amino acid is identified as a peak with a monoisotopic mass of 147 Da (Fig. 2C). We therefore concluded that the NH2-terminal sequence of fragminP corresponds to Ac-Met-Gln-Lys and that only three amino acids were missing from the cDNA clone.

The amino acid sequence of fragminP and delineation of fragmin19, a proteolytic fragment that covers the NH2-terminal half of fragminP

The predicted molecular mass of the cloned fragminP, including the missing start Met, Gln and Lys residues, was 41,080 Da and this value is in agreement with the value that was previously determined experimentally by ESI-mass spectrometry using native fragminP (De Corté et al., 1996a). Thus fragminP consists of 371 amino acids. An alignment of the deduced amino acid sequence of fragminP with D. discoideum severin is shown in Fig. 3. The overall homology with severin (66% identity) and the NH2-terminal half of gelsolin (57% identity) is rather high, pointing to similar functions.

Fig. 1. Nucleotide and deduced amino acid sequence of the fragP cDNA clone. The 1,171 bp insert is flanked by the EcoRI linker (5’ end) and a NotI restriction site at the 3’ end (underlined). The fragminP cDNA starts at the arrow (these nucleotides are designated As). Nucleotides at the 5’ end represented in bold were deduced by reverse translation after determination of the NH2-terminal sequence of fragminP by mass spectrometry. The TAA stopcodon is indicated by an asterisk and the putative polyadenylation signal is boxed. Amino acids are represented in the three letter code. This sequence is available from GenBank under accession number U70047.
The deduced amino acid sequence also connects the NH2-terminal half with the COOH-terminal half by filling a gap of 11 residues, TRLLHLKGKKH (Fig. 3, boxed region) that were previously not encompassed (Ampe and Vandekerckhove, 1987). It constitutes a highly basic and hydrophobic region. Interestingly, this new sequence revealed a PIP2-binding motif (RLLHLKGKK), very similar to the consensus sequences of other actin-binding proteins of the gelsolin family (Yu et al., 1992; Janmey et al., 1992). The identification of a second PIP2-binding site that conforms more closely to the canonical PIP2-binding properties of fragminP by PIP2 (Gettemans et al., 1995).

In some preparations of the Physarum EGTA-resistant 1:1 actin-fragmin complex we noticed the presence of a dimer consisting of actin and a 20 kDa polypeptide, immunologically related to fragminP (De Corte et al., 1996b). Amino acid sequencing and molecular mass measurements identified the 20 kDa component as the NH2-terminal half of fragminP, covering amino acids 1-168. Its exact mass of 19,123 Da was determined by ESI-MS and we therefore call it fragmin19. The COOH terminus of fragmin19 is located within the internal peptide of 11 amino acids that also contains the PIP2 binding consensus motif, and illustrates why it was previously not possible to correctly delineate the size of fragmin19. Because fragmin19 is not detected in the crude cytosol of Physarum microplasmodia we assume that it originates by proteolysis in the course of the isolation of the actin-fragmin complex.

Over-expression and characterization of intact recombinant fragminP

To obtain full-length recombinant fragminP, the three missing amino acids at the NH2 terminus (M-Q-K) were added by PCR (see Materials and Methods). We purified recombinant fragminP from E. coli MC1061, transformed with pSCM26 (Mertens et al., 1995a) and the pLT10T3-frgP expression vector, to homogeneity by a two-step procedure. The purity of the final preparation is shown in Fig. 4A and the identity of the protein was confirmed after determination of the NH2-terminal amino acid sequence by automated Edman degradation (data not shown). Approximately 1 mg of fragminP was purified from 62 mg of crude bacterial extract.

The biological activities of recombinant fragminP were assayed by two criteria: F-actin severing and its ability to

![Fig. 2. Determination of the fragminP amino-terminal sequence by MALDI-RETOF analysis. Mass/charge spectrum of the blocked NH2-terminal fragminP peptide before (A) and after (B) modification with 2-iminothiolane and iodoacetamide. Notice the relative increase of 158 Da after modification. (C,D) Post-source decay spectra of the unmodified (C) and modified (D) fragminP peptide. The peak with a monoisotopic mass of 302 Da (small arrow) is present in both C and D and is consequently unaffected by modification. The mass of this peak corresponds to the sequence Ac-Met-Gln. The peak with a monoisotopic mass of 147 Da (arrowhead, C) represents the lysine residue (amino acid 3) and shifts to 305 Da (arrowhead, D) after modification. m/z, mass/charge. a.i., absolute intensity.](attachment:image)
enhance actin phosphorylation in the 1:1 EGTA-resistant actin-framin complex by the AFK. F-actin severing proteins introduce new free (−) ends resulting in the depolymerization of F-actin in a manner proportional to the number of free filament ends. At a concentration of 25 nM, recombinant framinP severed F-actin filaments as efficiently as native framinP, shown by the decrease in fluorescence intensity of a pyrene-labeled F-actin solution (Fig. 4B). This activity was abolished by preincubation of the protein with PIP2 (Fig. 4B).

As a second proof for biological activity, recombinant framinP was preincubated with muscle actin in the presence of micromolar amounts of Ca2+ and the mixture was subsequently added to the actin-framin kinase in the presence of \( \gamma -32 \text{P} \text{ATP/Mg}^2+ \) and EGTA. A strong phosphorylation of actin was observed (Fig. 4C, lanes 2-5), indicating that framinP is able to stimulate actin phosphorylation by the
kinase through formation of a stoichiometric complex with actin. No actin phosphorylation was noticed in the absence of fragminP (Fig. 4C, lane 1).

Transcriptional regulation of the fragminP gene during development of P. polycephalum amoebae into plasmodia

Genomic DNA from the haploid apogamic strain CL was digested, Southern blotted and hybridized against a 32P-labelled fragminP cDNA probe. HindIII and KpnI digestion both resulted in two major hybridizing fragments of 7.0/1.6 kb and 9.4/1.8 kb, respectively, whereas digestion with PstI resulted in two hybridizing fragments of 3.5 and 2.0 kb (data not shown). HindIII and PstI both cut at a unique site in the coding region of the fragminP cDNA insert, in agreement with two hybridizing fragments. Since KpnI does not cut within the known cDNA but results in two hybridizing fragments, there must be at least one intron in the coding region of fragminP. Additional faint bands were also visible: a 6.6 kb band for HindIII and a 15 kb band for KpnI. The origin of these bands was clarified by the results of the northern hybridization.

For northern analysis we screened RNA that was isolated from the apogamic P. polycephalum CL strain. The cellular and molecular events of apogamic development have been well characterized (Bailey et al., 1987, 1990; Bailey, 1995) and these strains therefore provide a good source of material for the study of genes involved in plasmodium development in P. polycephalum. The fragminP cDNA hybridized to a mRNA with a size of approximately 1.2 kb (Fig. 5A, upper panel). Interestingly, no signal was detected in amoebae (Fig. 5A, lower panel).

Fig. 5. Developmental regulation of fragminP. (A) The figure shows a northern blot of total RNA isolated from the following cultures of the apogamic strain CL: A, amoebae; Mi, microplasmodia; Ma, macroplasmodia; 1-56%, cultures containing cells that were developing into plasmodia. The figure indicates the percentage of developing cells in each sample. fragminP is a 1.2 kb mRNA encoding fragminP that is expressed in plasmodia but not amoebae (top panel). mRNA size markers (Promega) are shown on the left. The middle and lower panels show the same RNA samples probed with two constitutively expressed genes: actin and a cDNA D6/16A, coding for an unknown gene. The slight variations in the signals are due to differences in expression levels and transfer efficiency onto the membrane rather than to differences in the amount of RNA loaded (10 µg per lane). (B) FragminP is not present in amoebae. A western blot is shown with cytosolic proteins from amoebae (a) or microplasmodia (Mi), probed with fragminP antiserum (left panel) or affinity-purified fragminP antibodies (right panel). 5 µg crude extract was applied. Lane c, purified recombinant fragminP (control). Molecular mass markers are indicated.

Fig. 6. Phase-contrast and epifluorescence analysis of fragminP in microplasmodia. (A) Phase-contrast image of a microplasmodial segment. (B) Epifluorescence staining pattern of fragminP from the same segment as in A. Note the intense staining along the plasma membrane and diffuse cytoplasmic staining. Circular structures are visible in the center of the segment. Scale bar applies to A and B.
upper panel, lane 1). As the percentage of developing cells in the population increased, so did the amount of fragminP mRNA (Fig. 5A, upper panel, lanes 2-10). The highest level of expression was detected in microplasmodia; macroplasmodia also expressed fragminP, but at a somewhat lower level (Fig. 5A, upper panel, lanes 11-12, respectively). Two constitutively expressed genes, actin and a clone D6/16A coding for an unknown gene (Bailey et al., unpublished observations), were used as controls. The expression levels of the corresponding mRNAs remained essentially the same throughout development (Fig. 5A, lower panels). Western blots on crude extracts of amœbae or microplasmodia using fragminP antisera or affinity-purified antibodies confirmed the data from the northern blot: no fragminP was detected in amœbae (Fig. 5B).

A second mRNA of ±2.5 kb was also detected by the fragminP probe and this RNA is present only in fully developed micro- and macroplasmodia (Fig. 5B, lanes 11 and 12). It is unlikely that the cross-reaction was due to non-specific hybridization because this particular RNA seems also to be developmentally regulated. Furthermore, several weaker bands were also visible in Southern blots of genomic DNA (data not shown) and may be derived from the gene coding for this particular protein. Because fragminP is expressed predominantly in *P. polycephalum* plasmodia we term the gene coding for this protein as *frgP* and we propose to call the as yet uncloned amoebal fragmin gene *frgA*. The gene coding for the unknown fragminP-related protein (mRNA of 2.5 kb) is designated *frgR*.

**FragminP is located at the plasma membrane of microplasmodia**

We have previously demonstrated that fragminP, like other members of the gelsolin family, specifically interacts with the phospholipid PIP2 (Gettemans et al., 1995). To investigate the role of fragminP in *P. polycephalum* plasmodia, we studied its subcellular localization by staining with fragminP antibodies and analyzed the resulting pattern by epifluorescence and confocal microscopy. Although microplasmodia are morphologically heterogenous, a consistent staining pattern was found. Epifluorescence showed that fragminP stains predominantly along the rim of the cells, whilst a diffuse cytoplasmic staining was also observed (Fig. 6B). However, in view of the irregular and complex morphology of microplasmodia, the cytoplasmic staining of fragminP could reflect peripheral staining but in a different focal plane. This possibility was investigated by confocal microscopy. Fig. 7A illustrates that fragminP localizes almost exclusively at the plasma membrane in confocal sections of plasmodia (stage II microplasmodia, 100-300 µm). This is particularly visible in a transverse section (Fig. 7B). In control experiments where either preimmune serum or the secondary antibody was used, we did not observe staining. Nor was staining observed in the nuclei, arguing against phosphorylation-mediated shuttling of fragminP to and from the nucleus as was found for CapG (Onada and Yin, 1993). This suggests that fragminP phosphorylation by casein kinase II (De Corte et al., 1996a) is probably related to another, as yet unidentified, mechanism. In larger microplasmodia (stage III, 300-1,000 µm) that were double-stained for F-actin and fragminP, fragminP did not co-localize with cytoplasmic fibrillar F-actin (Fig. 7D). FragminP staining was observed predominantly in small surface extensions resembling lobopodia. These structures are visible in epifluorescence images as circular spots, seemingly present in the cytoplasm but actually rising out of the focal plane. A similar overall plasma membrane staining pattern was found in small, spherical (stage I) microplasmodia (not shown). Taking into consideration the interaction between fragmin and PIP2 in vitro, the general staining pattern may suggest that fragminP is anchored to the plasma membrane lipid bilayer through interaction with PIP2. Alternatively, fragminP may be associated with the cortical microfilament system, but it certainly does not seem to interact with cytoplasmic fibrillar actin filaments.

**DISCUSSION**

We have isolated a cDNA clone encoding fragminP, an actin-binding protein from *Physarum* that is structurally and functionally homologous to vertebrate gelsolin, a well-known regulator of cytoskeletal organization in cells (Weeds and Maciver, 1993). Only seven nucleotides of the cDNA clone were lacking at the 5' end, and this enabled us to predict the 5' end by reverse translation of the Met-Gln-Lys sequence. The reverse translated nucleotide sequence at the 5' end of the
fragminP cDNA clone is represented in bold in Fig. 1. This prediction was used for construction of the full-length frgP cDNA clone by PCR. Purified recombinant fragminP displayed actin-binding properties identical to native fragminP.

Three amino acid differences were identified between the deduced sequence and the partial amino acid sequence determined previously (Ampe and Vandekerckhove, 1987). The substitutions were verified by peptide sequences of fragmin19, a truncated form of native fragminP. This showed that the cDNA deduced sequence was correct. We therefore ascribe the three amino acid changes to allelic differences between strains of different genetic background. Such changes are not exceptional, as allelic actin and tubulin restriction-length polymorphisms have been demonstrated previously in *Physarum polycephalum* (Schedl et al., 1984; Schedl and Dove, 1982). Apart from these observations, Southern blotting with genomic DNA indicated that fragminP is a single copy gene.

No cross-hybridization was observed in the mRNA of fragminA (expected size approximately 1.0-1.1 kb), even at low stringency conditions. FragminA and fragminP probably share amino-acid sequence homologies that are not reflected at the DNA level. Similarly, profilinA and profilinP from *Physarum* do not cross-hybridize at the DNA level, although both proteins are 66% homologous (Binette et al., 1990). Our results therefore indicate that fragminA and fragminP are coded by different genes.

The finding that *Physarum polycephalum* expresses two isoforms of fragmin is significant but not a singular observation in this organism. *Physarum* contains five actin genes at four loci, *arda-E* (Hamelin et al., 1988; Gonzalves-y-Merchand and Cox, 1988; Nader et al., 1986; Adam et al., 1991). Only *ardB* and *ardC* are abundant and constitutively expressed at all stages of development (Monteiro and Cox, 1986; Hamelin et al., 1988).

Although microfilaments in amoebae and plasmodia are assembled from the same actin protomers, these *Physarum* cell types differ profoundly in organization of microfilaments (i.e. amoebae do not contain thick cytoplasmic actin fibrils), mode of locomotion and use of microfilaments (i.e. cytokinesis – or lack of it in plasmodia). Consequently, it is reasonable to conjecture that the (re)organization of microfilaments during *Physarum* development must be the result of the interaction with actin-binding proteins. This hypothesis is strengthened by the observation that several other actin-binding proteins from *Physarum* are developmentally regulated, including profilin (Binette et al., 1990) and the LAV3-4 and LAV3-5 cDNAs, encoding alpha-actinin and an actin-bundling protein, respectively (St-Pierre et al., 1993). Based on our and previous observations (Uyeda et al., 1988), it is very likely that fragminA and fragminP also play a distinct role in the remodeling of microfilaments. Furthermore, myosin heavy and light chains and tubulin are also expressed in a cell-specific manner (Burland et al., 1983; Kohama and Ebashi, 1986; Kohama et al., 1986).

The tubulin isoforms participate in the different mitotic spindles that are formed in amoebae versus plasmodia (Solnica-Krezel et al., 1991).

FragminP expression starts early in development, at a time when apogamic amoebae acquire characteristics that are typical for plasmodia such as switching from open to closed mitosis. During development the cells undergo an extended cell cycle that takes about two and a half times as long to complete as a normal cell cycle. During this cell cycle, the amoebae reach a ‘point of no return’, at which stage they become committed to development. It is in the second half of this extended cell cycle that fragminP (this paper), profilinP (Binette et al., 1990; J. Bailey, unpublished observations) and the myosin heavy and light chain proteins (Kohama and Ebashi, 1986; Uyeda and Kohama, 1987) are first expressed. Several lines of evidence indicate that the initiation of fragminP expression runs parallel with the developmental switch from amoeba to plasmodium. For instance, tubulin genes alter their pattern of expression during the second half of the extended cell cycle (Solnica-Krezel et al., 1990; 1991; Bailey et al., 1990). In addition, normal developing cells acquire the ability to ingest amoebae (a trait characteristic of plasmodia) in the second half of the extended cell cycle and also change their mode of locomotion. Finally, during the long cell cycle many other cell-type specific genes modify their pattern of expression (Sweeney et al., 1987). These observations further indicate that the timing of frgP expression coincides with the time when the cell begins to modify its structural organization and behaviour and gradually gains characteristics typical for plasmodia. Since the fragminP and profilinP mRNA are first detectable at the same time, we predict that the fragminA mRNA would disappear at the same time as profilinA mRNA, which starts to decline in abundance at the 20% developing cells level (J. Bailey, unpublished observations).

*Physarum* plasmodia contain an extensive cortical actin cytoskeleton, resembling the erythrocyte membrane actin skeleton. This membrane-associated network differs in its three-dimensional organization from the cytoplasmic actin scaffold that is more fibrillar in nature. In amoebae, F-actin stains mostly in patch-like structures, coinciding with relatively large pseudopods and finger-like small filopodia (Pagh and Adelman, 1988). In microplasmodia, F-actin typically stains the many invaginations that result in a two- to fivefold increase in cell surface, allowing efficient and quick response to variations in the external environment. FragminP did not co-localize with the fibrillar cytoplasmic actin network, in contrast to what was found for macroplasmodia (Naib-Majani et al., 1983; Osborn et al., 1983). Based on our in situ localization studies, fragminP could control assembly-disassembly of microfilaments in the cortical area of the cell and since this system is thought to be the basis for motive force generation (Brix et al., 1987) it could be involved in cell motility.

FragminP is the most recent member of the gelsolin family for which the cDNA sequence and amino acid sequence have been determined. At micromolar [Ca\(^{2+}\)] it forms an actin-fragmin trimer, indicating that fragminP contains two actin-binding sites. In contrast, fragmin19 only makes up a 1:1 complex with actin (De Corte et al., 1996b), suggesting that the second Ca\(^{2+}\)-dependent actin-binding site is located beyond Leu169 in the COOH-terminal half of the molecule. FragminP also contains two PIP\(_2\)-binding motifs. The first motif was previously postulated at residues 142 through 149 (Gettemans et al., 1995). As in severin (Eichinger et al., 1991; Eichinger and Schleicher, 1992) and CapG (Yu et al., 1990), this motif is less tightly conserved than that of gelsolin. Residues 167RLHLKGKK175, deduced from the frgP cDNA clone, constitute the second PIP\(_2\)-binding consensus motif of fragminP and this sequence is in fact more similar to villin than to gelsolin (Arpin et al., 1988).
FragminP, fragmin19 and fragmin 60 (Furuhashi and Hatano, 1989) can each form a dimeric complex with actin that is phosphorylated in the actin subunit by the *P. polypecephalum* actin-fragmin kinase (AFK) (Gettemans et al., 1992; De Corte et al., 1996b; Furuhashi et al., 1992). The interaction between fragminA and actin may also give rise to a substrate for the AFK, but this remains an open question. The actin-fragmin kinase is remarkable because of its unusual structure (Eichinger et al., 1996). It does not contain conserved residues commonly found in the majority of kinases and the COOH-terminal half of the AFK is made up almost entirely of six so-called *kelch* repeats (Xue and Cooley, 1993), a structural motif found in proteins from diverse species such as *Limulus* scruin (Way et al., 1995) and the human Host Cell Factor (Wilson et al., 1993). Apart from this, the function of fragminP in phosphorylation of actin by the AFK can now be studied at the molecular level by designing truncation mutants of the *frgP* cDNA clone and analyzing the ability of the recombinant deletion mutants to activate phosphorylation of actin after complex formation. This approach should lead to a better understanding of the structural requirements necessary to make up a protein complex that is recognized by the AFK. A more thorough assessment of the function of fragminP in vivo can now be performed by disrupting the gene in apogamic amoebae using established methods (Burland and Pallotta, 1995) and examining whether the cells develop normally or which structures are being affected during development. Since, other genes and proteins from amoebae and plasmodia were found to cross-react with *frgP* at the mRNA or protein level, the *frgP* cDNA clone may therefore serve as a stepping stone towards the isolation and characterization of these genes and determining their role in modelling the actin cytoskeleton in *Physarum polypecephalum*.

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