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The F-actin capping proteins of *Physarum polycephalum*: cap42(a) is very similar, if not identical, to fragmin and is structurally and functionally very homologous to gelsolin; cap42(b) is *Physarum* actin

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We have carried out a primary structure analysis of the F-actin capping proteins of *Physarum polycephalum*. Cap42(b) was completely sequenced and was found to be identical with *Physarum* actin. Approximately 88% of the sequence of cap42(a) was determined. Cap42(a) and fragmin were found to be identical by amino acid composition, isoelectric point, mol. wt, elution time on reversed-phase chromatography and amino acid sequence of their tryptic peptides. The available sequence of cap42(a) is >36% homologous with the NH_2-terminal 42-kd domain of human gelsolin. A highly homologous region of 16 amino acids is also shared between cap42(a), gelsolin and the *Acanthamoeba* profilins. Cap42(a) binds two actin molecules in a similar way to gelsolin suggesting a mechanism of F-actin modulation that has been conserved during evolution.

Key words: actin-binding domains/cap42(a+b)/F-actin capping/fragmin/gelsolin

Introduction

Eukaryotic cells express a variety of actin-binding proteins regulating the assembly of the microfilament system (for recent reviews see Stossel *et al.*, 1985; Pollard and Cooper, 1986). Among these, proteins which bind to the barbed end and block further growth of the actin filaments at this end, have been intensively investigated. One class of these proteins is the gelsolin/villin group, so far only found in vertebrates. These proteins have mol. wts of ~90 kd; they have been purified from rabbit macrophages (gelsolin) (Yin and Stossel, 1979), chicken intestinal microvilli (villin; Bretscher and Weber, 1980; Glenney *et al.*, 1980) and human plasma (actin depolymerizing factor or brevin; Chaponnier *et al.*, 1979; Norberg *et al.*, 1979; Harris and Schwartz, 1981). All these proteins share Ca^{2+}-dependent actin-modulating activities. They cap and fragment F-actin and nucleate actin filament formation (Mooseker *et al.*, 1980; Yin and Stossel, 1980; Harris and Schwartz, 1981; Glenney *et al.*, 1981; Janmey *et al.*, 1985).

A second group contains proteins with similar properties but a lower mol. wt (45 kd); these are found in more diverged species, e.g. bovine thyroid (Tawata *et al.*, 1983), rabbit alveolar macrophages (Southwick and DiNubile, 1986), bovine brain (Hurny and Wnuk, 1986), sea urchin egg (Ohnuma and Mabuchi, 1986), *Dictyostelium* (severin; Brown *et al.*, 1982) and *Physarum* plasmodia. So far, proteins of the latter organism are the only members of this class which have been studied in some detail. Fragmin was isolated as a 1:1 complex with actin, from which it was separated either by selective cleavage of actin (Hasegawa *et al.*, 1980) or by ion exchange chromatography in the presence of 8 M urea (Hinssen, 1981a). The other *Physarum* F-actin capping proteins are the subunits of the cap42(a+b) complex (Maruta and Isenberg, 1983; 1984).

These proteins were reported to be non-polymerizable regulatory variants of actin encoded by genes derived from a common ancestral actin gene (Maruta *et al.*, 1984). These conclusions were based on immunological data, one-dimensional peptide mapping, affinity labelling experiments using nucleotide triphosphates and functional studies (Maruta and Isenberg, 1984; Maruta *et al.*, 1984). They form the basis for a hypothesis of F-actin modulation by actin variants. The F-actin treadmilling hypothesis (Wegner, 1976) proposes that at steady-state, new actin monomers are added to the + (barbed) end of the filament, while at the same time others dissociate from the − (pointed) end. In such a system, actin variants, of which one actin-binding site is modified, will bind to the filament through their intact binding site. Once occupying the barbed end, they will block further addition of 'normal' actin monomers and thus function as terminator or capping proteins.

The real mechanism is probably more complicated since fragmin and cap42(a), but not cap42(b) display a Ca^{2+}-dependent activity (Hasegawa *et al.*, 1980; Hinssen, 1981b; Maruta and Isenberg, 1983; 1984), which is not present in the normal actin–actin protomer interaction. This hypothesis also does not explain satisfactorily the F-actin severing activity of fragmin (Hinssen, 1981b). In addition, cap42(a) and cap42(b) form a tight complex which caps actin filaments irrespective of whether Ca^{2+} is present or not (Maruta *et al.*, 1983). Interestingly, the cap42(b) subunit can be phosphorylated by an endogenous kinase imposing Ca^{2+}-dependency on both the capping activity of the cap42(a+b) complex and the cap42(b) subunit (Maruta *et al.*, 1983; Maruta and Isenberg, 1984). No severing activity was attributed to either the cap42(a+b) complex or to the subunits, or to the phosphorylated forms (Maruta *et al.*, 1984).

To investigate further the suggested structural relationship between *Physarum* actin, cap42(a), cap42(b) and fragmin, to identify the expected 'mutated' actin-binding sites and to learn more about the complex structural–functional aspects of these proteins, a detailed protein-chemical analysis was performed. The results are discussed in terms of the actin mutant F-actin capping hypothesis and in terms of homology with gelsolin and profilin.

Results

Characterization of cap42(a), cap42(b) and fragmin

The three *Physarum* actin-binding proteins, cap42(a), cap42(b) and fragmin, were compared with each other and with *Physarum* actin by several criteria. (i) Reversed-phase HPLC dissociates the cap42(a+b) complex into cap42(a) and a more hydrophobic cap42(b) (Figure 1A). In the same chromatographic system, fragmin elutes at the same position as cap42(a) (Figure 1B), while *Physarum* actin elutes identically to cap42(b) (Figure 1C). (ii) Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of the HPLC-purified components shows that cap42(a) does not separate from fragmin (Figure 1D and E), while *Physarum* ac-

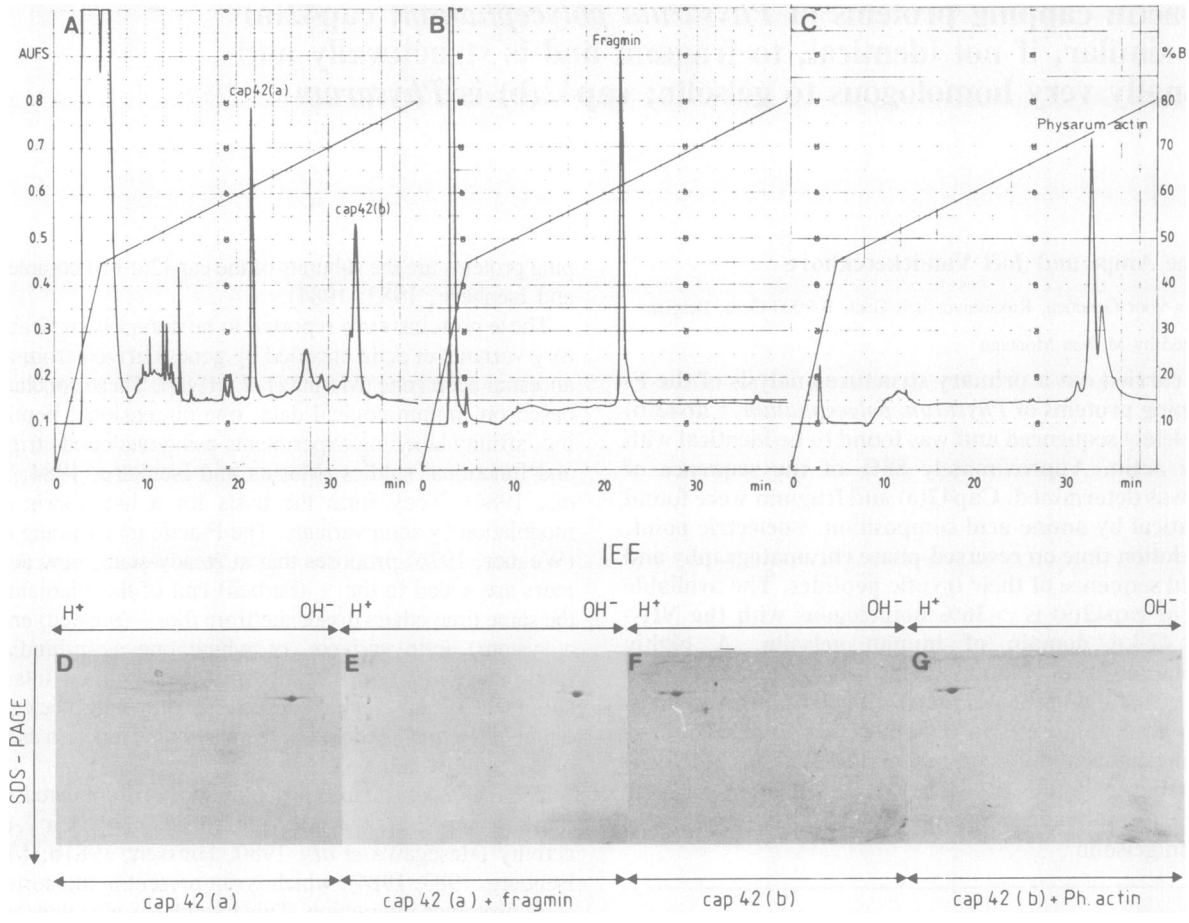

Fig. 1. *Physarum* F-actin capping proteins: characterization by reversed-phase chromatography and two-dimensional PAGE. Reversed-phase HPLC of the cap42(a+b) complex (**A**), fragmin (**B**), and *Physarum* actin (**C**). Chromatographic conditions are as described under Materials and methods. The slope of the gradient is given by a solid line and the percentage of eluent (solvent B: 0.1% TFA, 70% acetonitrile) is indicated at the right side of the traces. **Panels D–G** show a two-dimensional PAGE of HPLC-purified proteins: cap42(a) (**D**), a mixture of cap42(a) and fragmin (**E**), cap42(b) (**F**) and a mixture of cap42(b) and *Physarum* actin (**G**). Two μg of protein is loaded on the gels. Separation is as described in Materials and methods: isofocusing (IEF) is in the horizontal direction; H^+, the acidic side; OH^-, the basic side. SDS–PAGE is carried out in vertical direction. Proteins are detected with Coomassie Brilliant Blue.

tin comigrates with cap42(b) at a more acidic position (Figure 1F and G). (iii) The amino acid compositions of HPLC-purified cap42(a) and cap42(b) are very different from each other, but the former is very similar to that of HPLC-purified fragmin, while the latter is nearly identical to the composition of *Physarum* actin calculated from its known sequence (Vandekerckhove and Weber, 1978b) (Table I). By these three criteria, cap42(a) appears identical to fragmin, while cap42(b) cannot be distinguished from *Physarum* actin. On the other hand, cap42(a) and cap42(b) are very different proteins.

Determination of the primary structure of cap42(a)

Automated gas-phase amino acid sequence determination on cap42(a) failed to reveal phenylthiohydantoin (PTH) amino acid residues, indicating that the protein is NH_2-terminally blocked.

Cap42(a) was cleaved chemically either with BNPS-skatole or with CNBr. The resulting fragments were purified by reversed-phase HPLC (results not shown) and characterized by their full-length (W1, W5) or partial NH_2-terminal sequence (W2, W1–W3, W3, W4, M1) (Figure 2). In a second approach, cap42(a) was degraded with either trypsin, chymotrypsin or the *Staphylococcus aureus* V8 protease. The resulting peptides were separated on Whatman 3MM paper and characterized by their amino acid composition (data not shown) and full-length sequence. Overlaps of these peptides allow us to deduce the sequence of two contiguous blocks covering 164 and 195 residues, respectively (Figure 2). The COOH-terminal sequence of cap42(a) was deduced by time course amino acid analysis following carboxypeptidase Y action (results not shown). The Glu-Asp-Phe-Phe-Asp-COOH sequence identifies the region containing 195 residues, as the COOH-terminal part of cap42(a). Figure 2 documents the overlaps and summarizes the available sequences of cap42(a).

Apart from the missing blocked NH_2-terminal region we have also no information on an internal region separating the two large sequence stretches. None of the three types of proteases used yielded overlapping peptides in the region and the tryptophan cleavage product (W3) could not be sequenced over a sufficient length to link both long segments. By homology with the gelsolin sequence (see below), we expect this region to contain 10–15 amino acids (see Discussion).

Further, we noticed that methionine was absent from the available sequences, although three of these residues were found in the intact cap42(a) (Table I). The CNBr fragment M1 has a mol. wt of 42 kd (data not shown) suggesting a clustering of the three methionine residues at the NH_2-terminus of the protein, and the absence of this amino acid in the internal region. Based on the specificity of CNBr, we position the most COOH-terminal meth-

Table I. Amino acid composition of the *Physarum* capping proteins

	Cap42(a)	Fragmin	Cap42(b)	Actin[a]
Cys[b]	–	–	3.6	4
Asx	37.3	37.0	30.5	30
Thr	18.4	18.4	24.7	25
Ser	21.3	21.2	25.4	25
Glx	47.1	46.8	40.8	41
Pro	16.3	15.8	19.0	19
Gly	40.5	40.2	30.4	30
Ala	35.8	35.9	29.3	29
Val	36.3	36.3	21.8	22
Met[b]	2.8	2.7	15.6	16
Leu	37.8	36.8	27.7	28
Ile	16.0	17.2	26.5	27
Tyr	14.0	14.0	14.8	15
Phe	20.5	20.8	13.2	13
Lys	32.8	32.7	20.5	20
His	13.0	13.0	7.8	8
Arg	15.1	14.9	18.3	18
Trp	n.d.	n.d.	n.d.	4
His[m]	–	–	+	1
Total	404	404	370	375
Mol. wt	42342	42342	40786	41667

[a]Taken from the sequence given by Vandekerckhove and Weber (1978b).
[b]Calculated as cysteic acid and methioninesulphon.
His[m] denotes the methylhistidine residue present in *Physarum* actin.
The totals of fragmin, cap42(a) and cap42(b) are without the tryptophan content, the latter was not determined (n.d.).

ionine of cap42(a) in front of the sequence of peptide M1 (Figure 2). The two remaining methionines could not be unambiguously located due to very low cleavage yields at the other potential sites. Since it is also difficult to prepare sufficient amounts of the protein (in total 5 mg were used for both structural and functional studies), no further attempts were made to obtain a complete sequence. The available cap42(a) sequence is, however, sufficient to predict some functional properties by its structural homology with other actin-binding proteins (see Discussion).

The partial amino acid sequence of fragmin

Like cap42(a), fragmin was NH_2-terminally blocked. The two dimensional trypsin peptide map of fragmin was identical to that of cap42(a) (data not shown). Moreover, no difference was found between the composition (data not shown) and the sequence (Figure 2) of the fragmin peptides and that of the corresponding cap42(a) peptides. Approximately 82% of the total fragmin sequence is covered by these studies (tryptic peptides in Figure 2). The 25 extra residues in cap42(a), not available from the sequence information of tryptic peptides (Figure 2) were assigned from overlapping chymotryptic peptides and chemical cleavage fragments. Since both proteins could not be further distinguished by composition (Table I) or by chromatographic and electrophoretic procedures (Figure 1), we conclude that they are very likely identical molecules (see also Discussion).

Structural analysis of cap42(b)

Amino acid analysis (Table I), HPLC and two-dimensional gel electrophoresis (Figure 1) suggest strong homology between cap42(b) and *Physarum* actin. Therefore, we sequenced cap42(b) using the strategy described previously for the analysis of *Physarum* actin (Vandekerckhove and Weber, 1978b). No difference was found with actin. In addition, a search for possible differences in the nature and degree of post-translational modification was negative: as in *Physarum* actin, histidine at position 73 was methylated and, as judged from the paper electrophoretic mobility of the NH_2-terminal tetrapeptide, the NH_2-terminal residue was equally acetylated. Thus cap42(b) and *Physarum* actin are indistinguishable proteins.

At this stage, our results contradicted those of Maruta *et al.* (1984), who identified cap42(b) as a nonpolymerizable and capping variant of actin. To resolve this discrepancy, we carried out the same protein-chemical analysis on a sample of cap42(b) kindly provided by Dr Maruta (München, FRG). Reversed-phase chromatography showed a peak coeluting with *Physarum* actin and a more hydrophilic component (Figure 3A). SDS–PAGE identified the latter as a 22-kd fragment (Figure 3B, lane 1) present in nearly equimolar amounts to cap42(b) (Figure 3B, lane 2), and the intact cap42(b) peak as a 42-kd polypeptide (Figure 3B, lane 3).

Cap42(b) was again identified as *Physarum* actin by all the criteria described above. The 22-kd fragment was analysed by tryptic finger-printing. Amino acid composition and sequence analysis identified all the peptides as derived from the NH_2-terminal half of cap42(a). As in cap42(a), the 22-kd chain contains a blocked NH_2-terminus and peptide ILDG...DKYR was identified as the most COOH-terminally located peptide (see Figure 2). From these results, we conclude that the 22-kd polypeptide spans the NH_2-terminal half of cap42(a). Note that the NH_2-terminal sequence block of cap42(a) terminates at the same region as the 22-kd polypeptide present in the cap42(b) preparation of Maruta and Isenberg (1984).

We verified the identity of our cap42(a+b) preparation by phosphorylating the complex according to published procedures (Maruta *et al.*, 1983). As judged from the autoradiogram and Coomassie-stained pattern of a two-dimensional gel analysis, cap42(b) showed 60% phosphorylation (results not shown). This indicates that our cap42(a+b) complex behaved similarly to that of previous isolates and that *Physarum* actin [former cap42(b)] is the substrate for the endogenous kinase.

The binding of cap42(a) to actin is similar to that of gelsolin

The structural homology observed between cap42(a) and human gelsolin (see Discussion) and between cap42(b) and *Physarum* actin, prompted us to reinvestigate the binding of actin to cap42(a) in the light of recent studies on actin–gelsolin interactions (Bryan and Kurth, 1984; Coué and Korn, 1985; Janmey *et al.*, 1986). In particular, we were interested to know if, like gelsolin, cap42(a) also contained two actin-binding sites: one Ca^{2+}-dependent and a second Ca^{2+}-independent.

Our cap42(a+b) preparation (see Materials and methods) was slightly contaminated with a 90-kd protein, assumed to be the *Physarum* kinase (Maruta *et al.*, 1983). It was passed through an actin–Sepharose column equilibrated in a low-salt buffer containing 2 mM EGTA. Both, cap42(a+b) and the contaminating 90-kd protein were not adsorbed and were recovered in the void volume (Figure 4A, lane 1). When the column was re-equilibrated with the low-salt buffer, now containing 2 mM Ca^{2+}, the complex was retained and only the 90-kd protein appeared in the flow-through (Figure 4A, lane 2). Subsequently, cap42(a+b) could be eluted with the EGTA-containing buffer (Figure 4A, lane 3). Under our experimental conditions (4°C and absence of Mg^{2+}), no detectable phosphorylation was observed due to the contaminating kinase(s). This could be concluded from controls using 2D-PAGE. The use of low-salt buffer and the presence of actin [both actin immobilized on Sepharose and actin present in the cap42(a+b) complex as the former cap42(b)] at subcritical monomer concentrations (< 1 mg/ml) makes binding of

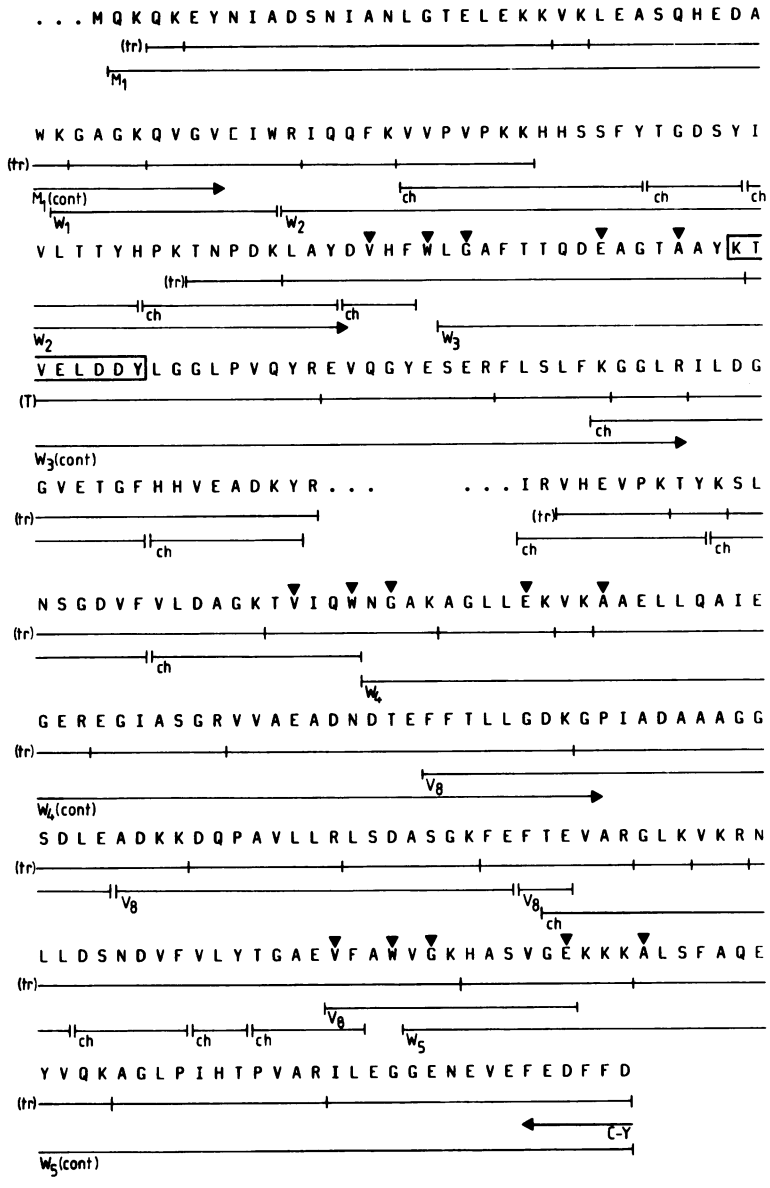

Fig. 2. Summarized results of the amino acid sequence determination of cap42(a) and fragmin. The nearly complete amino acid sequence of cap42(a) is derived from overlapping tryptic, chymotryptic, V8-protease, tryptophan and methionine cleavage products. All the tryptic peptides (tr) are given and only those chymotryptic (ch) and V8-protease (V8) peptides necessary to document overlaps. Horizontal arrows (→) underline the identified residues of the BNPS-Skatole fragments (W) or the CNBr fragment (M1). The peptides are ordered in two contiguous stretches interrupted by a region of probably 12 amino acids. The second segment is the COOH-terminal region of cap42(a). The first segment starts with the third methionine of cap42(a) and is preceded by ~30 residues containing the two remaining methionines. Residues are not numbered here as the sequence is not complete, however, they will be numbered in Figure 5 by homology with the human gelsolin sequence. The sequence in which fragmin is compared with cap42(a) is covered by the tryptic peptides. Residues constituting the three-times repeated motif are indicated with black arrows. Residues forming the potential pp60src kinase substrate are shown in a box.

cap42(a+b) to the actin−Sepharose through actin−actin protomer interaction very unlikely. Instead, the results argue for the existence of (an) additional Ca^{2+}-dependent, EGTA-reversible, actin-binding site(s) in cap42(a). This also predicts that a ternary complex, consisting of cap42(a) and two actin molecules [the former cap42(b) and an additional actin] should exist. Indeed, such a 130-kd complex can be isolated by gel permeation HPLC (Figure 4B). Analysis of the 130-kd and 90-kd complex by SDS−PAGE showed that relative to cap42(a), the 130-kd peak contained twice as much actin as the 90-kd peak (Figure 4B, lanes 4 and 6), while the ^{32}P-label remained unchanged (Figure 4b, lanes 5 and 7). Thus, the 130-kd complex is a trimer consisting of cap42(a), phosphorylated cap42(b) or actin, and a second actin, while the 90-kd complex represents the originally phosphorylated cap42(a+b) complex. These results also show that phosphorylation does not induce complex dissociation and that the phosphorylated actin of the complex [former cap42(b)] is not exchangeable with the exogenous actin.

In conclusion, the Ca^{2+}-dependent adsorption of cap42(a+b) on immobilized actin, and the formation of a stable 130-kd complex containing two actins per cap42(a), are most easily explained by assuming that cap42(a) (and very likely fragmin) displays two actin-binding sites, one of which is Ca^{2+}-dependent.

Discussion

The amino acid sequence of cap42(a)

The available sequence of cap42(a) covers two long stretches of 164 and 195 amino acids respectively. Comparison with the

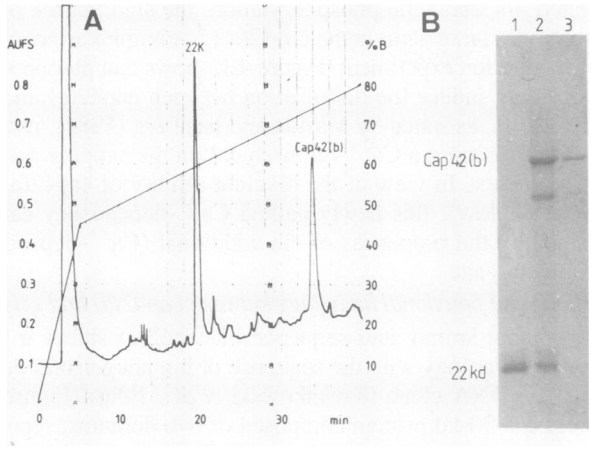

Fig. 3. Isolation of the NH_2-terminal half of cap42(a). Purification and identification of a 22-kd fragment of cap42(a) by reversed-phase HPLC (**A**) and one-dimensional SDS−PAGE (**B**). Reversed-phase HPLC conditions are as in Figure 1. The 22-kd cap42(a) fragment was found in a sample of cap42(b) prepared according to Maruta and Isenberg (1984). The gel separation patterns are from the HPLC-purified 22-kd fragment (B, **lane 1**), the contaminated cap42(b) sample prior to purification (B, **lane 2**) and the HPLC-purified cap42(b) or actin (B, **lane 3**).

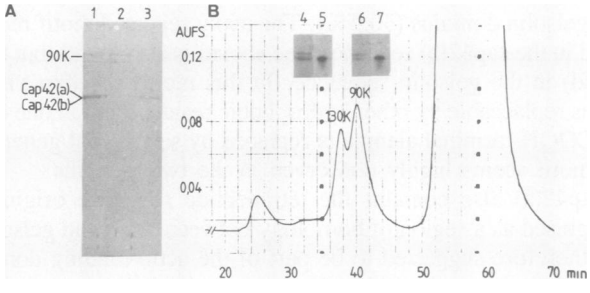

Fig. 4. Ability of cap42(a) to bind two actins. (**A**) The cap42(a+b) complex was passed over an actin−Sepharose column (see Materials and methods) and eluates analysed by one-dimensional PAGE. **Lane 1**, flow-through in the presence of EGTA; **lane 2**, flow-through in the presence of Ca^{2+}; **lane 3**, EGTA eluate of the Ca^{2+}-dependent complex. Here, cap42(a) and cap42(b) are marginally separated. One of the ±90-kd bands represents the *Physarum* kinase present as contaminant in the cap42(a+b) preparations (see text). They serve here as internal reference proteins. (**B**) Gel permeation HPLC of phosphorylated cap42(a+b) supplemented with actin in the presence of Ca^{2+}. The eluate is monitored by absorbancy at 214 nm and the flow-rate is 0.5 ml/min. Peak fractions at 130 kd and 90 kd are analysed by one-dimentsional SDS−PAGE. Insets show the Coomassie-stained pattern and autoradiograms, respectively, of the 130-kd (**lanes 4** and **5**) and 90-kd complex (**lanes 6** and **7**).

amino acid composition (Table I) shows that these 359 residues account for ~88% of the total sequence. Indeed, cap42(a) is expected to contain ~409 amino acids and is therefore longer than actin by ~44 residues. Notwithstanding this relatively large size difference, cap42(a) migrates only slightly more slowly than *Physarum* actin in SDS−PAGE.

Examination of the available cap42(a) sequence reveals three weakly homologous repeated regions. They are easily recognized by comparing the sequences surrounding the third, fourth and fifth tryptophan residue (Figure 2, arrows). Here a consensus motif of the type $Vx_2WxGx_6Ex_3A$ can be recognized. Such motifs, in which strictly conserved amino acids are separated by a constant number of variable amino acids (indicated by x) are generally assigned to folding patterns associated with specific functions (see for instance, Kamps *et al.*, 1984). A search for homology did not reveal proteins with similar determinants. Thus the role of this sequence determinant remains unknown.

Cap42(a) and fragmin are very similar, if not identical, proteins

Although only 82% of the total expected fragmin sequence is covered by the tryptic peptides, all available protein-chemical arguments point to identity of fragmin and cap42(a). These results are in accordance with the one-dimensional SDS−PAGE peptide analysis (Hinssen, 1981a), but disagree in two other aspects: the lack of severing activity of cap42(a) (see below) and the exclusive phosphorylation of fragmin by the tyrosine-specific pp60 kinase (Maruta *et al.*, 1984), of which the target tyrosine is mostly (but not always) located at the COOH-terminal side of a cluster of acidic residues (Glenney and Tack, 1985; Patschinsky *et al.* 1982; De *et al.*, 1986). Such a sequence is found in both cap42(a) and fragmin (Figure 2, boxed residues), and therefore, it is difficult to understand why both proteins are not equally phosphorylated by the $pp60^{src}$ kinase. Thus, more rigorous comparison seems to be warranted.

Cap42(b) and Physarum actin are products of the same structural gene

The complete primary structure of cap42(b) shows no difference with *Physarum* actin (Vandekerckhove and Weber, 1978b). However, there is still a remote possibility that both proteins differ in a yet unidentified post-translational modification which might have remained undetected. If such a modification were present, then it should involve a small, neutral group, since it does not change the mol. wt or the isoelectric point of cap42(b). However, we consider this possibility as very unlikely, especially since the cap42(b) preparation from Maruta and Isenberg (1984) was shown in this study to be 'contaminated' by a 22-kd fragment comprising the NH_2-terminal half of cap42(a). This fragment probably arose by proteolytic cleavage of the cap42(a) molecule, present in the cap42(a+b) complex used as source for cap42(b) preparations (Maruta and Isenberg, 1984). The use of formamide to elute the complex from the DNase I Sepharose column and to keep the complex dissociated in the hydroxyapatite chromatography may have rendered cap42(a) more sensitive to proteolytic attack. Note that we have also been unable to obtain overlapping enzymatic fragments from that same region during analysis of the structure of cap42(a) (see above), again suggesting an unusual sensitivity to trace amounts of unspecific proteases, contaminating those used in this sequence work. The NH_2-terminal part of cap42(a) either remained bound to cap42(b) even in 8.8 M formamide or was incompletely separated from the latter by the step gradient used in the purification procedure of Maruta and Isenberg (1984). The presence of this fragment may be the reason for the previously measured F-actin capping activity of cap42(b) (actin) and may also have disturbed the radioimmune assays, explaining the observed immunological cross reactivity between actin, cap42(b), cap42(a) and fragmin (Maruta *et al.*, 1984).

Purified *Physarum* actin is not phosphorylated by the endogenous kinase (Maruta *et al.*, 1983), but becomes a substrate when present in the cap42(a+b) complex. Thus, interaction between cap42(a) and cap42(b) (actin) as the EGTA-insensitive complex seems to be a prerequisite for efficient actin phosphorylation. Similarly, one can assume that binding of the 22-kd cap42(a) NH_2-terminus to cap42(b) or actin may explain the reported phosphorylation of cap42(b) (Maruta and Isenberg, 1984).

Phosphorylation of actin has been noticed in various instances. Generally, different types of membrane-bound kinases were found to be responsible for this modification (Grazi and Magri, 1979;

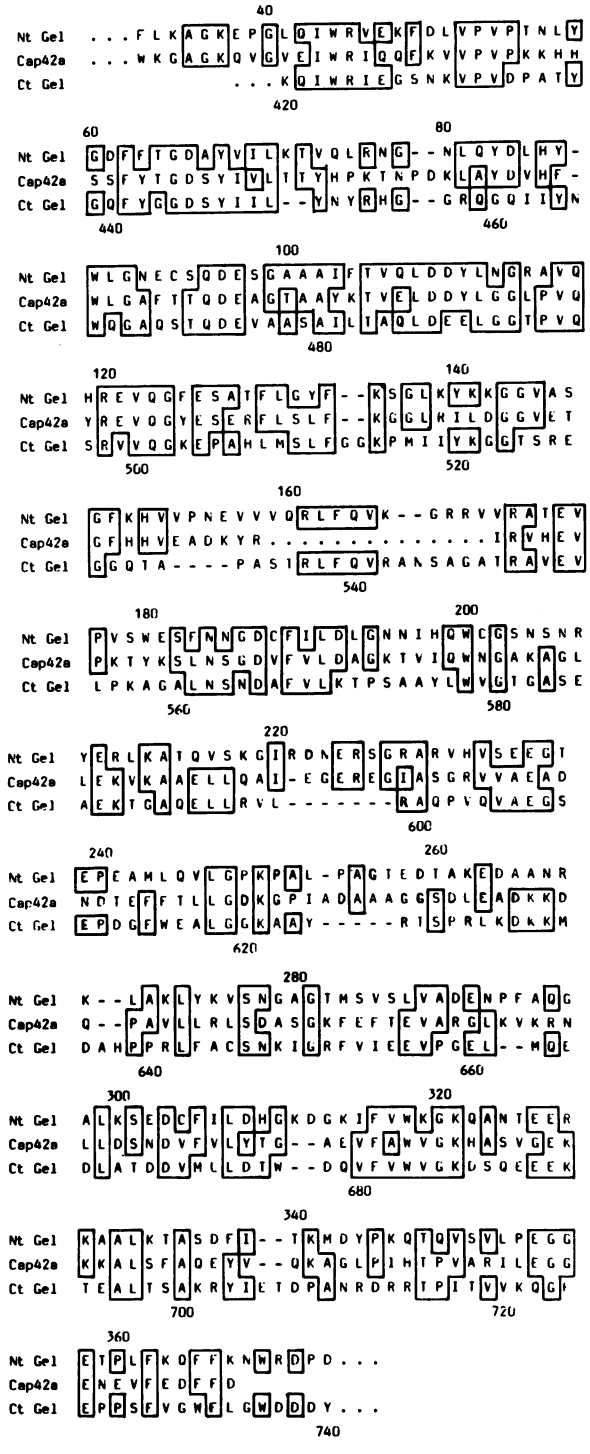

Fig. 5. Alignment of cap42(a) with the homologous domains of human gelsolin. Cap42(a) (**middle lane**) is aligned with the HN_2-terminal (**upper lane**) and COOH-terminal (**lower lane**) domain of human plasma gelsolin. Homology starts from number 35 in plasma gelsolin and 38 residues COOH-terminal of the start of the available sequence of cap42(a). Residues are numbered according to the plasma gelsolin sequence. Maximal homology was obtained by introducing gaps or inserts in the proteins. Identical residues are boxed. The gelsolin sequence was taken from Kwiatkowski *et al.* (1986).

Hofstein *et al.*, 1980; Steinberg, 1980; Machicao *et al.*, 1983). So far, only in one case (*Amoeba* actin) has it been shown that formation of a heterodimer between actin and a second protein (here *Amoeba* profilin) induces the phosphorylation of actin by an endogenous kinase (Sonobe *et al.*, 1986). As for the profilin-mediated *Amoeba* actin phosphorylation, the significance of the phosphorylation of actin in the cap42(a+b) complex is not clear. Our gel filtration experiment (Figure 4B) shows that phosphorylation does not induce the dissociation between cap42(a) and actin. However, as stated by Maruta and Isenberg (1984), it could be a way to impose a Ca^{2+}-requirement on the capping activity of the complex. In view of the bivalent affinity of cap42(a) for actin (see below), this newly gained Ca^{2+}-dependency can be explained by the properties of the additional (Ca^{2+}-dependent) actin binding-site.

Structural and functional homology between cap42(a) and gelsolin

The available amino acid sequence of cap42(a) shows a high degree of homology with the sequence of human gelsolin derived from a cDNA clone (Kwiatkowski *et al.*, 1986) (Figure 5). Gelsolin is a 90-kd protein composed of two domains, repeated in tandem. The available NH_2-terminal sequence of cap42(a) shows no similarity with the extreme NH_2-terminal region of the cytoplasmic or secreted form of gelsolin (Yin *et al.*, 1984; Kwiatkowski *et al.*, 1986). The homology starts close to the first tryptophan residue of cap42(a) and extends through the COOH-terminus. It amounts to 36% when compared with the NH_2-terminal domain sequence of gelsolin. This value is higher than the percentage homology between cap42(a) and the COOH-terminal gelsolin domain (30.5%) and also than that found between the two gelsolin domains (30.5%). The triply repeated motif recognized in the cap42(a) sequence (see above) is also found (but then 6-fold) in the gelsolin sequence. In this motif, the first valine seems replaceable by other hydrophobic residues and in one case the COOH-terminal alanine is replaced by serine, but generally the motif seems highly conserved in the two proteins.

Cap42(a) also contains the tetrapeptide sequence originally recognised as a region of homology between actin and gelsolin, and therefore suggested to be part of the actin-binding domain (Kwiatkowski *et al.*, 1986). Here, the tetrapeptide sequence is DEAG (residues 96–99), similar to that of vertebrate muscle actins (Collins and Elzinga, 1975), while the previously recognized tetrapeptide of gelsolin was DESG, similar to that of vertebrate non-muscle or invertebrate actins (Elzinga and Lu, 1976; Vandekerckhove and Weber, 1978a, 1984).

Apart from a significant structural homology between cap42(a) and gelsolin, we have also demonstrated functional similarity between the two proteins. Like gelsolin (Brian and Kurth, 1984; Coué and Korn, 1985), cap42(a) can bind two actin molecules, one of these interactions being Ca^{2+}-mediated and reversible (this study), the other actin [formerly cap42(b)] forming a heterodimer [cap42(a+b)] which does not dissociate in the presence of EGTA (Maruta *et al.*, 1983). The binding of free cap42(a) (Maruta and Isenberg, 1983) and fragmin (Hasegawa *et al.*, 1980; Hinssen, 1981b) to actin is Ca^{2+}-dependent. These results are consistent with the model for cooperative actin-binding proposed for gelsolin (Janmey *et al.*, 1986): first, actin binds to a Ca^{2+}-dependent site of cap42(a) thereby 'opening' a second (Ca^{2+}-independent) actin-binding site yielding a heterotrimer. Consecutive removal of Ca^{2+} only dissociates the Ca^{2+}-dependent actin, leaving the dimer cap42(a+b). A similar mechanism has recently been suggested for the interaction between actin and the 45-kd actin-modulating protein of sea urchin eggs (Ohnuma and Mabuchi, 1986).

Janmey *et al.* (1985) and Ohnuma and Mabuchi (1986) have reported that only the free actin-modulating proteins are able to fragment F-actin filaments, while the Ca^{2+}-independent heterodimers can only cap the filaments. Fragmin clearly follows these rules (Hinssen, 1981b), but cap42(a) does not apparently display

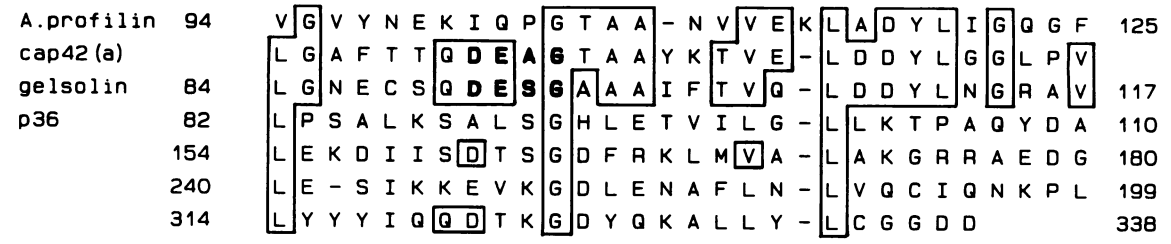

Fig. 6. Alignment of the COOH-terminal sequence of *Acanthamoeba* profilin and regions of cap42(a) and other actin-binding proteins. The sequence of *A.* profilin represents the 29 COOH-terminal residues of this protein and is taken from Ampe *et al.* (1985). The gelsolin sequence is from Kwiatkowsky *et al.* (1986) and that of the four repeats of p36 is from Saris *et al.* (1986). Amino acids that are identical with those of cap42(a) are boxed. A single gap is inserted to achieve optimal alignment. The tetrapeptide defining homology of cap42(a) and gelsolin with actin is shown in thick lettering. Homology between p36 and gelsolin in that region was previously recognized by Burgoyne (1987).

F-actin severing activity in its free form. Further studies are needed to determine if this reflects a real difference between fragmin and cap42(a). Possibly an F-actin severing activity of cap42(a) has been overlooked. Different Ca^{2+} concentrations seem to be required for both capping and severing activities of villin (Mooseker *et al.*, 1980; Walsh *et al.*, 1984) and this might be true of cap42(a).

Although no structural information is available for the 45-kd actin-modulating protein of sea urchin eggs, the functional similarities between the former protein, gelsolin and cap42(a), and the structural homology between the latter two, suggest the existence of a family of proteins conserved throughout evolution which modulate F-actin by a general mechanism. In the more primitive eukaryotes, this activity is displayed by proteins of the size of cap42(a) (fragmin, severin and the 45-kd protein of sea urchin eggs). In higher eukaryotes (vertebrates) F-actin seems to be also modulated by double-sized molecules which probably arose during evolution by a gene-duplicating event giving rise to the gelsolin-like proteins.

The observation that proteins of half the size of gelsolin are able to bind an equal amount of actin molecules, may help in understanding the conflicting data in the literature on the localization of the actin-binding sites in gelsolin. Bryan and Hwo (1986) proposed a model where both sites are located in the NH_2-terminal half of gelsolin, while Chaponnier *et al.* (1986) found that both halves of gelsolin each bind one actin. The results of Ohnuma and Mabuchi (1986) and those presented here favour the idea of two actin-binding sites situated in the NH_2-terminal half. However, in view of the repeated sequences of gelsolin [as in cap42(a)], one has to consider the alternative possibility of multiple potential actin-binding sites of which maximally two can be occupied at the same time due to steric hindrance. It is also possible that capping activity might be controlled by actin-binding sites different from those responsible for the F-actin severing activity. In this situation, proteolytic cleavage of gelsolin using enzymes with different specificities may have 'opened' or 'destroyed' different actin-binding sites.

Common sequences in actin-binding proteins?

Comparison of the available cap42(a) sequence with that of other actin-binding proteins reveal a region which is highly homologous to the COOH-terminal part of *Acanthamoeba* profilin-I and -II (Ampe *et al.*, 1985; 1987) (Figure 6). With one deletion, compensated by one addition four residues later, the homologous stretch extends over 16 residues in both proteins. In cap42(a) and gelsolin it is located COOH-terminally to the DEA/SG tetrapeptide but the latter sequence is absent in *Acanthamoeba* profilin. Since electron microscopic data (Pollard and Cooper, 1984) have suggested *Acanthamoeba* profilin may act as a barbed-end capping protein, it is tempting to speculate that this common region is (part of) a F-actin-capping domain.

Thus, *Acanthamoeba* profilin may contain two actin-binding sites: the sequence common with cap42(a) or fragmin and gelsolin, located at its extreme COOH-terminus and the region containing the NH_2-terminal residues which by homology with calf spleen profilin (Nyström *et al.*, 1979) were suggested to participate in the interaction of profilin with actin (Ampe *et al.*, 1985).

The common domain in cap42(a) and *Acanthamoeba* profilin is partially overlapping a consensus sequence previously recognized by Burgoyne (1987) and shared by gelsolin (Kwiatkowski *et al.*, 1986) and the internal repeats of p36 (Saris *et al.*, 1986) and p35 (Huang *et al.*, 1986) (Figure 6). Further functional similarities between these actin-binding proteins have to be verified by future experiments.

In conclusion, fragmin and cap42(a) are probably identical, while cap42(b) is indistinguishable from actin. This reduces the number of *Physarum* F-actin capping proteins from three to one. The previously reported functional and structural similarities (Maruta *et al.*, 1984) can be explained by cross-contamination of samples prepared in weakly denaturing conditions. No significant sequence homology was found between actin and the *Physarum* capping proteins, in disagreement with the concept of actin variants functioning as capping proteins. This is also the case for all the other F-actin capping proteins for which the sequence is known (Kwiatkowski *et al.*, 1986). Thus, these proteins do not function by means of an actin domain. In contrast, significant sequence homology is found between fragmin or cap42(a) and human gelsolin. These findings are in line with the identification in cap42(a) of a Ca^{2+}-dependent and a Ca^{2+}-independent acting-binding site. This suggests the existence of a general mechanism of Ca^{2+}-dependent F-actin modulation by a group of very similar proteins which probably arose during evolution from a smaller ancestor molecule. The identification of a sequence homologous with the COOH-terminus of *Acanthamoeba* profilin forms the basis for a further search for additional homology within the group of actin-binding proteins.

Materials and methods

Materials and proteins

Cells of *Physarum polycephalum* were kindly provided by Dr G.Isenberg (München, FRG). Microplasmodia were grown according to Daniel and Baldwin (1964). Harvesting and extraction of the cells, and purification of cap42(a+b) was as described by Maruta *et al.* (1983), except that the final gel filtration step was omitted. These cap42(a+b) preparations were variably contaminated by small amounts of a 90-kd protein. Aliquots (500 μg) of the cap42(a+b) preparation, were loaded on a C4-reversed-phase column (0.5 × 25 cm), (Baker Chemical Co.) connected with a HPLC apparatus (Waters, Millipore Inc.). Solvent A was 0.1% TFA and solvent B was 70% acetonitrile in 0.1% TFA. The flow-rate was kept at 1 ml/min and the absorbance was monitored at 214 nm. Gradients were

formed as indicated in the figures. Proteins or peptides were collected by hand and stored in the elution buffer at −70°C until use. This procedure yielded HPLC-purified cap42(a) and cap42(b). *Physarum* actin was prepared using the method of Gordon *et al.* (1976). Fragmin was a kind gift of Dr H.Hinssen (Salzburg, Austria). Cap42(b), purified according to the procedure of Maruta and Isenberg (1983, 1984), was kindly supplied by Dr Maruta (München, FRG). These samples were further treated by reversed-phase HPLC as described above.

Generation and purification of peptides

Enzymatic digests were carried out with trypsin, chymotrypsin and *Staphylococcus aureus* V8 protease according to established procedures. For each enzymatic cleavage ~0.8 mg (± 20 nmol) was used. Peptides were separated on Whatman 3MM paper by a combination of electrophoretic and chromatographic systems (for details, see e.g. Ampe *et al.*, 1985), and detected by a dilute fluorescamine stain. Only tryptic peptides were analysed from fragmin (± 1 mg), the samples of cap42(a) and cap42(b) provided by Dr Maruta (München, FRG) and the 22-kd fragment present in this cap42(b) sample. HPLC-purified cap42(b) and *Physarum* actin were first performic-acid-oxidized (Hirs, 1967) and their amino acid sequence determined as described previously (Vandekerckhove and Weber, 1978b). Carboxypeptidase Y degradation was done on 0.5 mg cap42(a) using the conditions described by Hayashi *et al.* (1973).

Cap42(a) (0.4 nmol) was treated with 2-(2-nitrophenylsulphenyl)-3-methyl-3-bromoindolenine (BNPS)-Skatole (Fontana, 1972), and the peptide mixture was separated on the C4 reversed-phase column eluted as described for protein purification. CNBr-cleavage (Gross and Witkop, 1962) was performed on 2 nmol of cap42(a). Peptides were purified as described above.

Amino acid analysis and sequence determination

Amino acid analysis was performed either by classical ion exchange post-column derivatization using *O*-phthaldialdehyde (Benson and Hare, 1975) or by precolumn derivatization followed by HPLC (Bidlingmeyer *et al.*, 1984). The amino acid sequence was determined with a gas-phase sequencer (Applied Biosystems Inc., USA) operated essentially as described by Hewick *et al.* (1981). In a later phase of this work (the analysis of the chemical fragments), the PTH amino acid derivatives were identified with an on-line analyser (120A Applied Biosystems Inc., USA).

Actin−binding studies on cap42(a)

Actin-Sepharose was prepared as described previously (Vandekerckhove and Sandoval, 1982) and a column was made (2.5 × 0.4 cm) in EGTA-G buffer (10 mM Tris−HCl, 0.2 mM ATP, 1 mM DTT, pH 7.6) (Spudich and Watt, 1971) supplemented with 2 mM EGTA. A 100-μl solution of cap42(a+b) (40 μg) in EGTA-G buffer was loaded on the column and eluted with 4 ml buffer. Then, the same actin column was re-equilibrated with 5 ml of Ca^{2+}-G buffer (G buffer in which EGTA was replaced by 2 mM Ca^{2+}). The same amount of cap42(a+b), now dissolved in Ca^{2+}-G buffer was loaded onto the column and eluted consecutively with 4 ml Ca^{2+}-G buffer and 4 ml EGTA-G bufer. Each time, fractions of 500 μl were collected in Eppendorf tubes, precipitated with trichloroactic acid/deoxycholate (TCA/DOC) (Peterson, 1983), and analysed on mini SDS−PAGE gels run according to Laemmli (1970). All steps in this experiment were carried out at 4°C.

Cap42(a+b) was phosphorylated using the endogenous kinase fraction separated from cap42(a+b) during the hydroxyapatite chromatography (Maruta *et al.*, 1983) and incubated with an equimolar amount of *Physarum* actin in Ca^{2+}-G buffer. The sample was then equilibrated versus 0.2 M KH_2PO_4 (pH 7.0) buffer by passing over a G-10 Sephadex column (1.2 × 7 cm) and the protein fractions concentrated by Centricon tubes. The concentrated mixture was then loaded in a 200-μl volume of a HPLC gel permeation TSK-SW3000 column (LKB, Sweden) and eluted with 0.2 M KH_2PO_4 (pH 7.0) buffer at a flow-rate of 0.5 ml/min. Proteins were measured by adsorbance at 220 nm. Peak fractions were collected and precipitated with TCA/DOC for SDS−PAGE analyses.

Other techniques

Aliquots of ±2 μg of protein were separated on two-dimensional gel electrophoresis as described by Garrels (1979) using 15% polyacrylamide gels for the second dimension.

The computer programs used for the protein homology searches were from the Institut Pasteur (SASIP, Paris, France; Claverie, 1984) and from Los Alamos National Laboratory (Los Alamos sequence analysis package; Kanchisa, 1982), the databank was from Protein Identification Resources (National Biomedical Research Foundation, Georgetown University, Washington, DC).

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