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Physarum actin is phosphorylated as the actin-fragmin complex at residues Thr203 and Thr202 by a specific 80 kDa kinase

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The *Physarum* EGTA-resistant actin-fragmin complex, previously named cap 42(a+b), is phosphorylated in the actin subunit by an endogenous kinase [Maruta and Isenberg (1983) *J. Biol. Chem.*, 258, 10151-10158]. This kinase has been purified and characterized. It is an 80 kDa monomeric enzyme, unaffected by known kinase regulators. Staurosporine acts as a potent inhibitor. The actin-fragmin complex is the preferred substrate. The phosphorylation is inhibited by micromolar Ca^{2+} concentrations, but only in the presence of additional actin. Polymerized actin (vertebrate muscle and non-muscle isoforms) and actin complexes with various actin-binding proteins are poorly phosphorylated. The heterotrimer consisting of two actins and one fragmin, which is formed from cap 42(a+b) and actin in the presence of micromolar concentrations of Ca^{2+}, is also a poor substrate. From the other substrates tested, only histones were significantly phosphorylated, in particular histone H1. In the same manner, casein kinase I could also phosphorylate the actin-fragmin complex. The major phosphorylation site in actin is Thr203. A second minor site is Thr202. These residues constitute one of the contact sites for DNase I [Kabsch *et al.* (1990) *Nature*, 347, 37-44] and are also part of one of the predicted actin-actin contact sites in the F-actin model [Holmes *et al.* (1990) *Nature*, 347, 44-49].

Key words: cap 42(a+b)/microfilaments/phosphorylation/ *Physarum* actin

Introduction

Cap 42(a+b) is an EGTA-resistant protein complex isolated from microplasmodia of *Physarum polycephalum* and composed of cap 42(a) and cap 42(b) in a 1:1 molar ratio (Maruta and Isenberg, 1983; Maruta *et al.*, 1983). At a complex:actin ratio of 1:1000 (or lower), this complex induces actin polymerization by promoting the nucleation step. It also caps the actin filaments at the barbed or fast growing end (Maruta and Isenberg, 1983). It has also been shown that cap 42(a+b) can be phosphorylated at a threonine residue by a *Physarum* kinase by which F-actin capping activity becomes Ca^{2+}-dependent (Maruta *et al.*, 1983). These properties suggest that the complex may play an important role in organizing the microfilament system of *Physarum* plasmodia.

In order to understand better the function of this complex, we have previously characterized its components and shown that cap 42(a) is fragmin, whereas cap 42(b) is *Physarum* actin (Ampe and Vandekerckhove, 1987). Fragmin was isolated by Hasegawa *et al.* (1980) and Hinssen (1981) as a Ca^{2+}-dependent actin-binding protein. Its amino acid sequence is very similar to that of the NH_2-terminal half of gelsolin (Kwiatkowski *et al.*, 1986) and villin (Arpin *et al.*, 1988; Bazari *et al.*, 1988). It is also very similar along its entire length with *Dictyostelium* severin (Andre *et al.*, 1988) and with the recently described g Cap 39 (Yu *et al.*, 1990; Johnston *et al.*, 1990), MCP (Young *et al.*, 1990) and Mbh1 (Prendergast and Ziff, 1991). These proteins interact with actin only in the presence of Ca^{2+} concentrations exceeding 10^{-6} M. All of them bind to the fast growing (+) end of actin filaments and most of them exhibit F-actin severing activities.

The properties of fragmin are very similar to those of gelsolin. At high Ca^{2+} concentrations, fragmin binds two actin molecules (Ampe and Vandekerckhove, 1987). At lower Ca^{2+} concentrations, one of the two actin molecules dissociates, leaving the EGTA-resistant fragmin-actin complex. For gelsolin, villin and g Cap 39 it has been shown that the EGTA-resistant complex can be dissociated by phosphatidylinositol bisphosphate (Janmey and Stossel, 1987; Janmey and Matsudaira, 1988; Yu *et al.*, 1990). This property, which links the control of actin organization to the phosphatidylinositol bisphosphate cycle, has not yet been demonstrated for the actin-fragmin complex. However, it has been suggested that phosphorylation of this complex might be another mechanism to regulate actin organization (Furuhashi and Hatano, 1990). Furthermore, Shibayama *et al.* (1985) have demonstrated that the phosphorylation of the actin-fragmin complex depends on the growth phase of the slime mold, suggesting a link between the actin organization and the cell cycle.

In the present study we have further investigated this process. The *Physarum* actin-fragmin kinase was purified. Its enzymatic properties were studied using a series of well known kinase modulators. The substrate specificity was determined and a series of other kinases were screened for their potential actin-fragmin kinase activity. Furthermore, we have located the phosphorylation sites in the actin molecule.

The results reported here suggest that an actin phosphorylation-dephosphorylation mechanism might be an alternative way to regulate the organization of the microfilament system in *P.polycephalum*.

Results

Characterization of the actin-fragmin kinase

Purification. The actin-fragmin kinase (AFK) was purified from extracts of *P.polycephalum* microplasmodia in eight chromatographic steps using columns of DEAE-cellulose, hydroxylapatite, phosphocellulose P11, Heparin-Sepharose, mono Q, mono P, Superose 12 and mono Q. Details of the

purification procedure will be published elsewhere (Gettemans,J., De Ville,Y., Vandekerckhove,J. and Waelkens,E., in preparation). The AFK corresponded to a 80 kDa band in SDS−PAGE (Figure 1).

Molecular mass. The molecular mass of the native AFK was determined by sucrose density gradient centrifugation and by Superose 12 gelfiltration. In the first experiment, we measured a molecular mass of 70 kDa. By gel filtration, we found values between 90 and 110 kDa. These results are in close agreement with the 80 kDa band seen by SDS−PAGE and indicate that the native AFK is a monomeric 80 kDa protein.

Substrate specificity. The substrate specificity of the AFK was determined using the proteins listed in Table I. The *Physarum* actin−fragmin complex appeared to be the most effective substrate (Figure 2A). Free *Physarum* actin and rabbit skeletal muscle actin were only poorly phosphorylated, whereas thrombocyte actin was not phosphorylated. The rate and extent of actin phosphorylation was dramatically increased when the *Physarum* actin−fragmin complex was used. Interestingly, the $(actin)_2$−fragmin complex, which is formed in the presence of micromolar concentrations of Ca^{2+} and additional skeletal muscle actin (see below) and a synthetic peptide corresponding to the actin phosphorylation site, are not phosphorylated. From the other substrates, histone H1 (Figure 2A) appeared to be the substrate of preference.

Modulation of the actin−fragmin kinase activity. The AFK activity was also measured in the presence of various enzyme effectors (see Table II). Among these, only staurosporine exhibited a strong inhibitory activity, whereas the staurosporine analogues were nearly uneffective. Ca^{2+} and EGTA could modulate the AFK activity, but these effects are substrate- rather than enzyme-directed (see further).

Since the S6 kinase activity is regulated by the MAP2

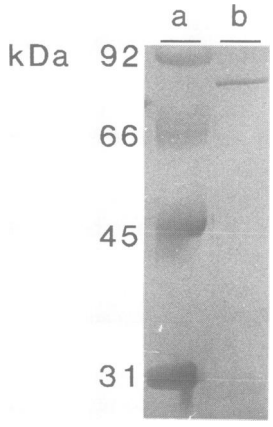

Fig. 1. The purity and molecular weight of AFK. The purity of the isolated AFK was investigated by 10% SDS−PAGE and subsequent silver staining (lane b). The molecular weight markers are indicated (lane a).

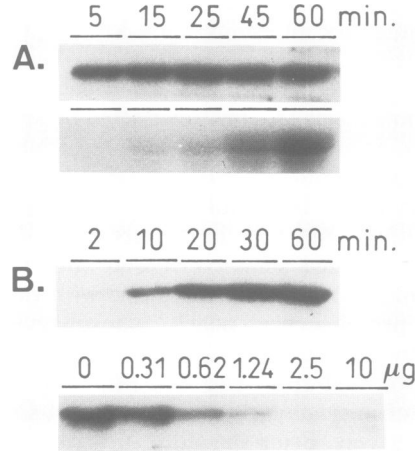

Fig. 2. Phosphorylation assays. **A.** Phosphorylation of the actin−fragmin complex (upper line) and histone H1 (lower line) by the AFK. The substrate (2 mg/ml) was phosphorylated by AFK for various time intervals (min). The phosphorylation was analysed by SDS−PAGE and autoradiography. **B.** Phosphorylation of the actin−fragmin complex by casein kinase I (upper line) and the inhibition of this phosphorylation by DNase I (lower line). The actin−fragmin complex (2 mg/ml) was phosphorylated by casein kinase I for up to 60 min. The DNase I-mediated inhibition of this phosphorylation was studied with various concentrations of DNase I (2 mg/ml stock solution), using a phosphorylation time (30 min) corresponding to a half-maximal phosphorylation of the actin−fragmin complex.

Table I. Substrate specificity of AFK

Protein	Extent of phosphorylation by AFK
Actin−fragmin	+++
Physarum actin	±
Rabbit skeletal muscle actin	±
Thrombocyte actins	−
Actin−thymosin	±
Actin−DBP	±
Actin−(N15 of gelsolin)	±
Actin−gelsolin	±
Casein	−
Myosin light chain	−
Histone f2b	+
Histone 2A	+
Histone H1	++
S6−peptide[a]	−
Actin−peptide[b]	−

[a]Pelech *et al.* (1987)
[b]Synthetic hexadecapeptide corresponding to the actin phosphorylation site, see Materials and methods.

Table II. Modulation of AFK by enzyme effectors

Effector	Concentration range	AFK activity
Phosphatidylserine−diolein	20−320 µg/ml	no effect
PKA inhibitory peptide	0−5 µM	no effect
Staurosporine	10−400 nM	inhibition[a]
CGP 42 700	0.1−10 µM	no effect
CGP 41 251	20−1000 nM	no effect
CGP 44 800	20−1000 nM	no effect
Trifluoroperazine	1−150 µM	no effect
Calmidazolium	450 µM	no effect
EGTA	0.5−8 mM	no effect or stimulation[b]
Heparin	5−80 µg/ml	no effect
Polylysine	50−800 µg/ml	no effect

[a]I_{50} = ± 150 nM.
[b]In the presence of free actin and Ca^{2+} (see text).

kinase (Ahn and Krebs, 1990), the phosphorylation of the S6 peptide was also investigated following a preincubation of the AFK with MAP2 kinase. However, no S6 kinase activity could be generated.

The effect of Ca^{2+} on the actin–fragmin phosphorylation. Maruta and coworkers (Maruta *et al.*, 1983) reported that in the crude extract, Ca^{2+} could reversibly inhibit the phosphorylation of the actin–fragmin complex. Inhibition was observed at micromolar concentrations of Ca^{2+} and was reversed by EGTA. We have studied this effect using highly purified preparations of the actin–fragmin complex and the AFK, and observed a Ca^{2+}-mediated inhibition only at millimolar concentrations (I_{50} = 3 mM) of Ca^{2+} (Figure 3A). This 'high zone' inhibition of the actin–fragmin complex phosphorylation by Ca^{2+} was found to be unspecific and could also be demonstrated with casein kinase I. The I_{50} of the Ca^{2+}-mediated inhibition was reduced to micromolar concentrations (I_{50} = 25 μM) when actin (250 μg/ml) was added to the assay mixture, indicating that the 'low zone' inhibition was mediated by actin (Figure 3B). Thus, either excess actin binds and inhibits the kinase, or the added actin binds to the actin–fragmin complex (in the presence of micromolar concentrations of Ca^{2+}) and renders a substrate insensitive to the kinase. Since the phosphorylation of histone H1 is not inhibited by added actin (results not shown), the inhibition is most likely due to the formation of an $(actin)_2$–fragmin complex. This formation takes place only at Ca^{2+} concentrations $>10^{-6}$ M (Ampe and Vandekerckhove, 1987).

Comparison with other kinases. The phosphorylation of the actin–fragmin complex in the presence of 2 mM EGTA was also investigated using a series of other kinases. In particular, interest has been paid to the histone H1 kinase activity of the AFK. Such activity has been found to be the privilege of only a limited number of protein kinases including protein kinase A (Langan, 1978), protein kinase C (Takai *et al.*, 1977), and the cdc2 kinase (Brizuela *et al.*, 1989). Under our assay conditions, these kinases did not phosphorylate the actin–fragmin complex. The lack of phosphorylation by protein kinase A and protein kinase C is in agreement with the results obtained with the PKA inhibitory peptide and with Ca^{2+} and phosphatidylserine–diolein, respectively. However, it was questioned whether the *Physarum* AFK could be an aberrant or evolutionarily distinct variant of the *Xenopus* cdc2 kinase. Arguments for this assumption were based on reports of a cell cycle regulated phosphorylation of the actin–fragmin complex (Shibayama *et al.*, 1985) and a cell cycle dependent histone H1 kinase activity in *Physarum* (Ducommun *et al.*, 1990). Therefore the phosphorylation of the actin–fragmin complex by the *Xenopus* cdc2 kinase was also investigated under conditions favourable for optimal enzymatic activity (see Materials and methods). However, the cdc2 kinase only showed a weak phosphorylation of the complex compared with its strong histone H1 activity used as a control. Because $p13^{suc1}$–Sepharose can be used as an affinity matrix for the purification of $p34^{cdc2}$ homologues and associated proteins in every organism studied so far (Arion *et al.*, 1988; Draetta *et al.*, 1989; Booher *et al.*, 1989; Dunphy *et al.*, 1988; Pondaven *et al.*, 1990), the binding of the AFK to $p13^{suc1}$–Sepharose was also investigated. However, the results did not allow us to classify AFK as a member of the cdc2 kinase family.

Several other kinases, including casein kinase I and II, GSK-3 (glycogen synthase kinase 3) and phosphorylase kinase, were also tested with respect to their potential AFK activity, again using the same phosphorylation conditions as used for the *Physarum* AFK. From these, only casein kinase I displayed AFK-like activity (Figure 2B). However, the molecular mass difference between AFK (80 kDa) and casein kinase I (44 kDa) and the inability of AFK to phosphorylate casein (see above) oppose the classification of the AFK as a casein kinase I.

Our studies allow us to conclude that AFK is an 'independent' protein kinase, unaffected by various known kinase modulators. The effect of Ca^{2+} in the physiological concentration range, occurring only in the presence of excess of free actin, is substrate- rather than enzyme-directed.

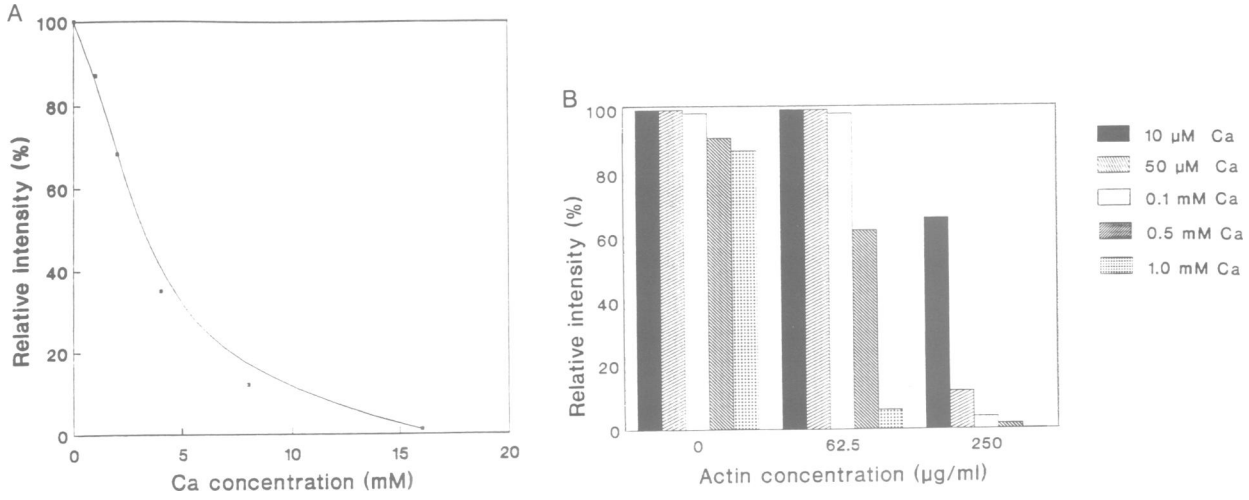

Fig. 3. Inhibition of the AFK activity by Ca^{2+} and actin. The phosphorylation of the actin–fragmin complex (2 mg/ml) was studied in the absence and presence of Ca^{2+} and actin. **A.** The inhibition of the actin–fragmin complex phosphorylation by Ca^{2+} (0–16 mM) in the absence of free actin. The I_{50} is 3 mM. **B.** The effect of additional free actin on the Ca^{2+}-mediated inhibition. Proportional to the concentration of free actin, the I_{50} is shifted to a lower concentration. At a free actin concentration of 250 μg/ml, the I_{50} for the Ca^{2+}-mediated inhibition could be calculated as 25 μM.

Localization of the phosphorylation site(s) in Physarum actin

The extent of phosphorylation of the actin–fragmin complex was measured by two-dimensional gel electrophoresis after a 2 h phosphorylation at 37°C. Coomassie Blue staining and autoradiography revealed that 50% of the actin was in the monophosphorylated form and that 5–10% was in the diphosphorylated form (results not shown). One milligram of this material was digested by trypsin. The peptides were separated on paper electrophoresis at pH 6.5 and the ^{32}P-labelled peptides were detected by autoradiography. Two bands (P1 and P2) could be visualized. These bands were eluted from the paper and subjected to a second paper electrophoresis at pH 3.5 (Figure 4). Each peptide was eluted again and subjected to a C18 reversed phase HPLC. Sequence analysis of the major [^{32}P]peptide (P1) yielded the sequence GYSFTTXAER, corresponding to the region 197–206 of *Physarum* actin (Vandekerckhove and Weber, 1978). The missing residue at position 203 was probably the phosphorylated amino acid, as phosphorylated amino acids cannot be detected by the standard gas phase sequencing procedure. In order to confirm this, the P1 peptide was dephosphorylated by *Escherichia coli* alkaline phosphatase, repurified and again sequenced, revealing a threonine residue

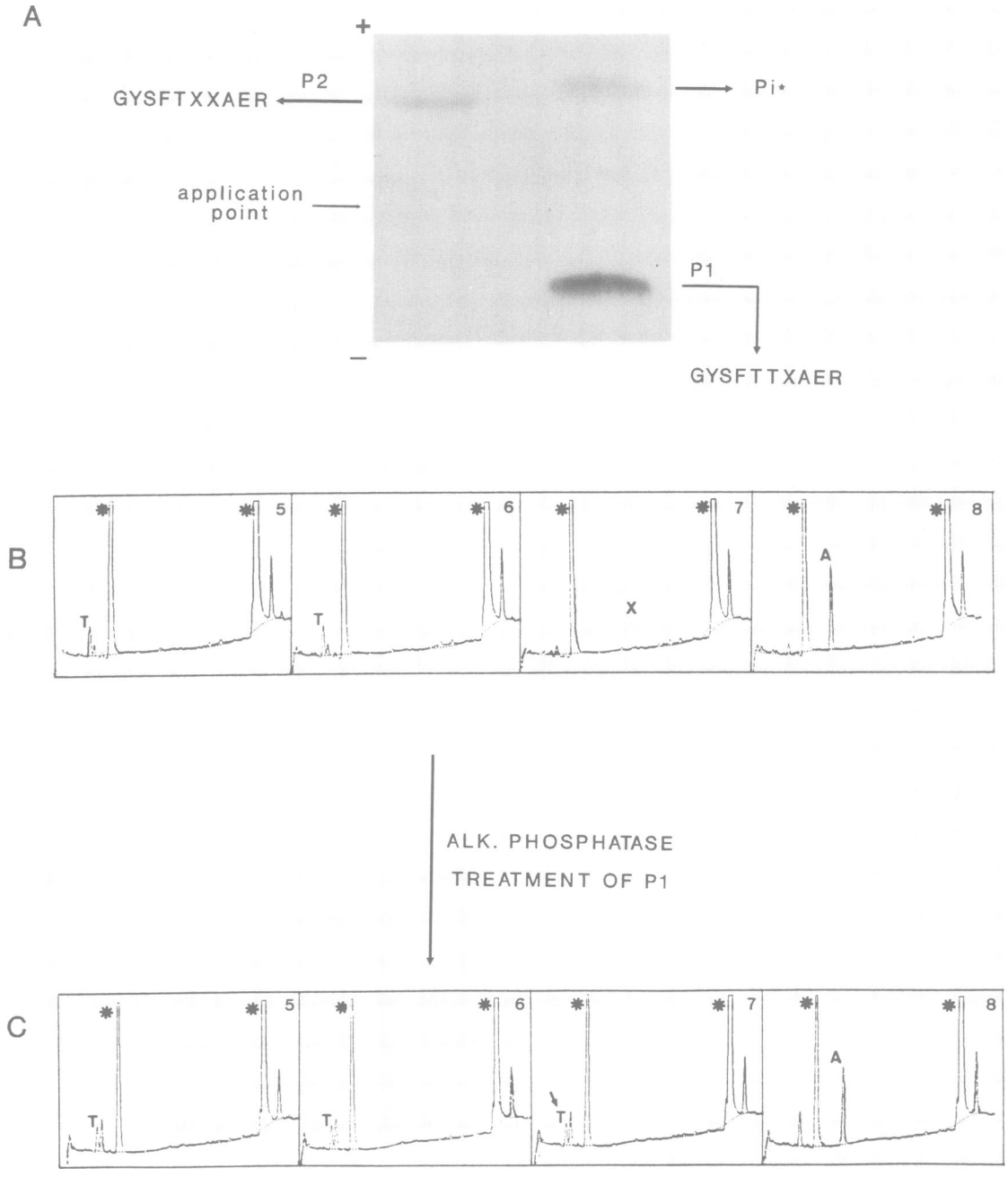

Fig. 4. Electrophoretic separation and amino acid sequencing of ^{32}-P labelled peptides. **A.** The phosphopeptides were separated by paper electrophoresis at pH 3.5 and detected by autoradiography. The sequences of P1 the single phosphorylated and P2 the doubly phosphorylated peptide are indicated. P_i is inorganic phosphate. **B.** Traces of the PTH–amino acid identification of cycles 5–8 of peptide P1. **C.** Traces of the same cycles of the dephosphorylated P1. Asterisks indicate the byproducts dimethylphenylthiourea and diphenylthiourea. The chromatograms of panel C show glycine as contaminant.

at position 203 (Figure 4). The second phosphopeptide (P2) was analysed in a similar manner. This peptide corresponded to the same region in actin but was phosphorylated at both Thr203 and Thr202. Since no evidence was found for a single phosphorylation at position 202, Thr203 is the first and major phosphorylation site for the AFK, whereas an additional phosphorylation can occur at position 202. These results are in agreement with an earlier report by Maruta and coworkers (Maruta and Isenberg, 1983), who identified the phosphorylated residue as a threonine.

Residue Thr203 is located in the minor contact site of actin and pancreatic DNase I (Kabsch *et al.*, 1990). This explains why DNase I is able to block the phosphorylation of the actin−fragmin complex (Maruta *et al.*, 1984) by masking the actin phosphorylation site. Inhibition of the phosphorylation of the actin−fragmin complex by DNase I may be used as a specific criterion to distinguish AFK-specific actin phosphorylation from other types of actin phosphorylation. In this respect it is worth noting that the reported actin−fragmin phosphorylation by casein kinase I is inhibited by DNase I in a similar and dose-dependent manner, suggesting that the phosphorylation is also at the DNase I binding loop (Figure 2B).

Discussion

We have characterized a monomeric 80 kDa *Physarum* actin−fragmin kinase, which phosphorylates actin in a presumed F-actin contact site and which might be involved in the microfilament organization of this organism. The inhibition of the actin−fragmin complex phosphorylation by Ca^{2+} comprises two aspects. With highly purified preparations of the actin−fragmin complex and AFK, inhibition is only observed at millimolar concentrations (I_{50} = 3 mM) of Ca^{2+}. This 'high zone' inhibition is also observed during histone H1 phosphorylation and with casein kinase I, suggesting that it is probably irrelevant physiologically. In the presence of excess of actin in the phosphorylation mixture, either added to the assay mixture or by analysis of AFK activity in crude extracts, phosphorylation is antagonized by micromolar concentrations of Ca^{2+}. This 'low zone' inhibition could provide a possible link between actin organization and intracellular concentrations of free Ca^{2+}. It has been suggested that the Ca^{2+} sensitivity of the actin−fragmin complex phosphorylation could be a property of the AFK itself (Maruta *et al.*, 1983), or that it is mediated through an unknown factor or a Ca^{2+}-sensitive phosphatase (Furuhashi and Hatano, 1990). Our results show that the low zone Ca^{2+} sensitivity is due to the presence of excess of actin. Actin is not an inhibitor of the AFK since it does not affect the phosphosphorylation of histone H1 by AFK. Consequently, the Ca^{2+} effect is most probably due to a modulation of the substrate by actin, probably through the formation of an $(actin)_2$−fragmin trimer at micromolar Ca^{2+} concentrations (Ampe and Vandekerckhove, 1987), thereby generating an AFK inhibitor or a sterically hindered substrate.

Inhibition of phosphorylation by Ca^{2+} was also noticed with *Physarum* myosin. As for the actin−fragmin complex phosphorylation, it was suggested that this inhibition could involve a substrate modulation rather than a kinase inactivation (Kohama, 1990). A possible functional link between the two systems through the same kinase, might be an interesting aspect for further investigation.

Monomeric actin (different isoforms were investigated), actin in complex with different actin-binding proteins and a synthetic peptide corresponding to the phosphorylation site in actin, are weakly or not phosphorylated. This leaves the actin−fragmin complex as the preferred substrate.

The substrate specificity and the studies with various known kinase modulators and inhibitors suggest that AFK is an independent kinase. Although it displays histone H1 kinase activity, we were not able to demonstrate further similarity with the cdc2 kinase family. Under identical phosphorylation conditions as those used in our standard assays, only casein kinase I was able to phosphorylate the actin−fragmin complex. There was no further evidence for a similarity with this class of kinases since AFK was unable to phosphorylate casein.

Although AFK seems to be an unique kinase, difficult to classify in any of the known kinase families, *Physarum* AFK could still represent an evolutionarily distinct variant with properties different from the kinase present in higher organisms. In this respect it should be noted that a *Dictyostelium* kinase, lacking regulation by Ca^{2+} and calmodulin, could only be classified as a myosin light chain kinase following amino acid sequence analysis and homology studies (Tan and Spudich, 1990).

Our observations are distinct from the actin phosphorylation by the cAMP-dependent protein kinase from rat liver plasma membranes (Grazi and Magri, 1979) and also from the actin phosphorylation by protein kinase A and protein kinase C in rabbit brain and muscle cells (Ohta *et al.*, 1987). It is not yet clear whether the AFK resembles the amoeba G-actin kinase reported earlier by Sonobe *et al.* (1986). The latter is a Ca^{2+}-dependent, but calmodulin-independent kinase that phosphorylates actin as the profilactin complex, while F-actin is a poor substrate. So far, the nature and the localization of the phosphorylated residue of this substrate are not known. In addition, little is known about the properties of the amoeba kinase involved.

Phosphorylation of the *Physarum* actin−fragmin complex was first reported by Maruta and Isenberg (1983), although at that time, the identity of cap 42a as fragmin and cap 42b as actin was not established. The cap 42(a+b) kinase mentioned in their studies is probably identical to the AFK reported here. The cap 42(a+b) kinase is similar to AFK in using the EGTA-resistant actin−fragmin as the major substrate, whereas free actin or fragmin are not phosphorylated (Maruta *et al.*, 1983). The Ca^{2+} dependency in the presence of excess of actin is identical for both enzymes. However, while a 110 kDa value (similar to that of the AFK) is reported for the cap 42(a+b) kinase at an early stage of the purification, a much lower value (35 kDa) is obtained after a DNase I affinity chromatography (Maruta and Isenberg, 1984). Since we have been unable to confirm this result, the lower molecular product may represent a degradation product.

AFK is probably also identical to factors called actin kinase by Furuhashi and Hatano (1990). First, the AFK and the actin kinase display the same molecular weight. Secondly, in the presence of excess of actin, both phosphorylations are similarly modulated in the same concentrations range of Ca^{2+} (the specific actin effect was not studied previously). Thirdly, previous studies (Ampe and Vandekerckhove, 1987) have shown that the actin−fragmin complex is structurally very similar, if not identical with cap 42(a+b). Furthermore, we noticed only one EGTA-resistant actin containing an

85 kDa complex that could be phosphorylated in conditions favourable for both AFK and actin kinase. Therefore, the AKF and the actin kinase phosphorylate the same substrate. Fourthly, the corresponding kinase elutes as a single component in all purification steps performed, suggesting the absence of a second kinase with similar properties.

The only remaining discrepancy resides in the effect of Ca^{2+} on the F-actin capping activity of the phosphorylated complex. Although Furuhashi and Hatano (1990) reported no Ca^{2+} effect, in agreement with the results of Maruta and Isenberg (1983) we find that this activity is regulated by Ca^{2+}. The effect of excess actin on the phosphorylation, which was not noticed previously, could be one of the reasons of this discrepancy.

The AFK could also be similar to a kinase that is thought to be inhibited following staurosporine treatment of human neutrophils, resulting in a concentration-dependent reorganization of the microfilament system (Niggli and Keller, 1991). Indeed, AFK is also highly sensitive to staurosporine and much less sensitive to the staurosporine analogues: CGP 41 251 and CGP 42 700. This suggests that the regulation of actin organization through actin phosphorylation might not be restricted to lower organisms.

The major phosphorylation site is located at Thr203 and a minor site at Thr 202. These residues are part of the contact site for DNase I (Kabsch *et al.*, 1990), explaining the inhibitory effect of DNase I on the phosphorylation of the actin–fragmin complex (Maruta and Isenberg, 1984) and the failure of DNase I to bind to the phosphorylated actin–fragmin complex. The phosphorylated residues are also part of the actin–actin contact sites predicted by the F-actin model of Holmes *et al.* (1990). Therefore phosphorylation of the actin–fragmin complex could be considered as a mechanism to block addition of actin monomers to the actin–fragmin complex, resulting in a Ca^{2+}-dependent and cell cycle regulated actin nucleation.

Materials and methods

Materials

Physarum cell culture. P.polycephalum cultures were kindly provided by Prof. Dr Stockem, University of Bonn, Germany. The slime mold was grown as described by Daniel and Baldwin (1964). Cultures of either 100 ml or 7000 ml (preparation culture) were agitated by shaking at a frequency of 100 rev/min at 24°C. Typically, 100 g of microplasmodia were obtained from a 7000 ml culture.

Proteins. Physarum actin and rabbit skeletal muscle actin were prepared according to established procedures (Spudich and Watt, 1971; Gordon *et al.*, 1976). Thrombocyte actin was purified essentially as in Gordon *et al.* (1976), following the preparation of a human platelet extract (Kurth *et al.*, 1983). Actin complexes with vitamin D-binding protein (Van Baelen *et al.*, 1980; Vandekerckhove and Sandoval, 1983), with the N-terminal half of gelsolin (Bryan and Kurth, 1984) and with thymosin β4 (Safer *et al.*, 1990), were prepared by incubating equimolar concentrations of actin and the actin-binding protein, followed by a separation of the complex on mono Q FPLC. Histone H1 and DNase I were from Boehringer Mannheim. Histone 2A, histone f2b, mixed casein and the *E.coli* alkaline phosphatase were from Sigma. Myosin light chains and the kinases used for the comparative study (protein kinase A, protein kinase C, casein kinase I and II, phosphorylase kinase, *Xenopus Laevis* cdc2 kinase and glycogen synthase kinase III), were kindly provided by Prof. Dr J.Goris, University of Leuven, Belgium.

Chemicals. Trifluoroperazine and calmidazolium were obtained from Janssen Chimica (Belgium). Dithiotreitol, phenylmethanesulfonylfluoride, heparin, polylysine, phosphatidylserine, diolein and the PKA inhibitory peptide (with a sequence corresponding to the inhibitory site of cAMP-dependent protein kinase inhibitor) (Cheng *et al.*, 1986), were purchased from Sigma. DEAE-cellulose (DE52) was obtained from Whatman Co., Ltd. Mono Q, Superose 12 and CNBr-activated Sepharose 4B were from Pharmacia. [^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham International.

General protein and chemical procedures

Proteins (1–3 μg) were analysed by 10–17% (mini slab) SDS–PAGE according to Laemmli (1970). Molecular weight standards for gel electrophoresis were from Bio-Rad. Protein bands were visualized by Coomassie brilliant blue or silver staining. The position of the radioactive proteins was detected by autoradiography on Kodak X-AR film at room temperature in the presence of an intensifying screen.

Paper electrophoresis was carried out on Whatman 3MM paper in toluene or varsol containing electrophoresis tanks (Vandekerckhove and Van Montagu, 1974). [^{32}P]peptides were detected by autoradiography (see above). HPLC separations were carried out on a Waters–Millipore apparatus equipped with a C18 reversed phase column (Vydac Separations Group, USA). The column was equilibrated in 0.1% TFA and the adsorbed peptides were eluted with 70 ml of a 0–70% linear gradient of acetonitrile in 0.1% TFA. The flow rate was 1 ml/min. Peptides were detected by radioactivity.

A peptide with the sequence ERGYSFTTTAEREIVR, corresponding to residues 195–210 in *Physarum* actin and containing the threonine residues target for phosphorylation by AFK (see Results), was synthesized with an Applied Biosystems 431A automated peptide synthesizer. The synthesis was done following the F-moc chemistry procedure. Peptide that had been cleaved from the resin was desalted in water over a G-25 column (30 × 2.6 cm). The peptide was further purified by preparative HPLC on a 1 × 25 cm C4 reversed phase column (Vydac, Separations Group, USA) desalted as above and stored in lyophilized form at −20° C. The molecular weight of the AFK was determined by Superose 12 gelfiltration and by sucrose density gradient centrifugation. Superose 12 gelfiltration was performed in 10 mM Tris–HCl pH 7.5, 1 mM EGTA, 1 mM DTT and 0.02% NaN_3 (TEDA buffer) supplied with 150 mM NaCl. Up to 200 μl of the purified AFK was applied on the column. The flow rate was set to 0.25 ml/min and the absorbance was measured at 280 nm. Fractions of 0.5 ml were collected and assayed for AFK activity (see below). For the sucrose density gradient centrifugation, a 100 μl sample was applied on to 4.5 ml of 5–20% sucrose density gradients and centrifuged at 4° C for 15 h at 40 000 r.p.m. in a Beckman SW 50.1 rotor. [^{14}C]bovine serum albumin was used as an internal marker. Twenty fractions were collected from each gradient and analyzed for AFK activity and for ^{14}C labelling.

Amino acid sequence analysis was carried out with a 477 model liquid phase sequenator equipped with a 120A phenylthiohydantoin amino acid analyser (Applied Biosystems Inc., USA).

Purification of the actin–fragmin complex

Highly purified actin–fragmin complex was obained by a modification of the procedure of Maruta and Isenberg (1983).

Preparation of the crude extract. The microplasmodia were collected by low speed centrifugation, washed twice with 2 vol of Sorensen buffer (10 mM KH_2PO_4 and 10 mM Na_2HPO_4, pH 6.0), followed by a wash with 1 vol of 30 mM Tris–HCl pH 8.0. Plasmodia were then homogenized using a Waring blender in 2 vol of extraction buffer (10 mM Tris–HCl pH 7.5, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF and 0.5 M sucrose). The homogenate was centrifuged for 60 min at 12 000 *g*. This and all consecutive steps were carried out at 4° C.

DEAE chromatography. The extract was loaded onto a 300 ml DEAE-cellulose column. The column was washed extensively with TEDA buffer (10 mM Tris–HCl pH 7.5, 1 mM EGTA, 1 mM DTT and 0.02% NaN_3). Subsequently, the absorbed proteins were eluted stepwise with 75 mM, 150 mM and 300 mM NaCl in TEDA buffer. As analysed by SDS–PAGE, the actin–fragmin complex and the actin–fragmin kinase eluted from the column at 150 mM NaCl. These fractions were pooled and concentrated by 80% ammonium sulphate precipitation. The pellet was dissolved in TDA buffer (TEDA buffer without EGTA) and subsequently dialysed against TDSA buffer (10 mM Tris–HCl pH 7.5, 1 mM DTT, 0.25 M sucrose and 0.02% NaN_3). The solution was adjusted to 1.5 M ammonium sulphate, stirred for 10 min and centrifuged for 15 min at 15 000 *g*. The supernatant was then made up to 2 M ammonium sulphate (60% saturation), allowed to precipitate for 15 min and centrifuged for another 15 min at 15 000 *g*. The resulting pellet was redissolved in a minimum volume of TEDA buffer and dialysed for 16 h against 100 vol of the same buffer.

Mono Q FPLC. The dialysed material was applied onto a mono Q HR 5/5 column equilibrated in TEDA buffer. The proteins were eluted from this column using a linear gradient or 0–300 mM NaCl in TEDA buffer. As analysed by SDS–PAGE, fractions eluting at 220–230 mM NaCl almost exclusively contained the actin–fragmin complex, whereas the actin–fragmin kinase eluted at 130–170 mM NaCl.

Superose 12 HR 10/30 gel filtration. Following the mono Q FPLC, the fractions containing the actin−fragmin complex were concentrated and loaded on a Superose 12 gelfiltration column that had been equilibrated in 150 mM NaCl in TEDA buffer. The elution position of the actin−fragmin complex was determined by SDS−PAGE. The peak fractions were pooled and concentrated to a final concentration of 2 mg/ml by dialysis against 60% glycerol in TEDA.

Purification of the actin−fragmin kinase

The purification of the actin−fragmin kinase was separated from that of actin−fragmin. In order to reach a homogeneous preparation, it was necessary to use eight chromatographic steps. Details on the purification will be published separately (Gettemans,J., De Ville,Y., Vandekerckhove,J. and Waelkens,E., in preparation).

p13^{suc1}−Sepharose chromatography

p13^{suc1}−Sepharose was prepared by the procedure described by Brizuela *et al.* (1987). For the p13^{suc1} affinity absorption assays, the kinase samples were first diluted 2-fold in H1 kinase buffer [50 mM Tris−HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 80 mM sodium α-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$ and 1 mM DTT to stabilize the potential MPF activity (Wu and Gerhart, 1980)]. The samples were then transferred to an Eppendorf tube containing p13^{suc1}−Sepharose (50 μl of packed beads per 100 μl of extract) equilibrated with H1 kinase buffer and rotated end-over-end for 120 min at 4°C. The beads were pelleted and washed three times in bead buffer (50 mM Tris−HCl pH 7.4, 1 M NaCl, 5 mM EDTA, 5 mM EGTA and 0.5% Tween-20) and then twice in H1 kinase buffer. The beads were then used immediately for kinase activity determination.

Phosphorylation assays

Protein samples were incubated at room temperature with a solution of 2 mg/ml of the substrate (actin−fragmin complex, histone f2b, histone 2A, histone H1, casein or myosin light chains). The phosphorylation mixture contained 2 mM EGTA, 10 mM $MgCl_2$ and 150 μM [^{32}P]ATP (specific activity: 1 μCi/nmol) in TDA buffer. Reactions were terminated by the addition of 4 × concentrated SDS gel sample buffer (Laemmli, 1970) and boiling for 3 min. The degree of phosphorylation was quantified by 12.5−15% mini slab gel electrophoresis followed by Coomassie brilliant blue staining and autoradiography. The electrophoresis was terminated before the front reached the lower buffer chamber. This gel strip was excised and removed before the gel was stained. The stained gel was further washed several times with water and dried before autoradiography.

The synthetic hexadecapeptide (500 μg) was phosphorylated by the purified AFK in a phosphorylation mixture (150 μl) containing 2 mM EGTA, 10 mM $MgCl_2$, 2 mM ATP (specific activity : 0.15 μCi/nmol) and 1 M NaCl in 20 mM Tris−HCl pH 7.4. The mixture was incubated 2 h at 37° C and the reaction was terminated by heating for 2 min at 100° C. The phosphorylated peptide was separated from the free ATP by gel filtration using a disposable PD10 column (Pharmacia) equilibrated in 0.5% NH_4HCO_3. The peptide containing fractions were concentrated in a Savant Speed Vac Concentrator and appropriate aliquots were applied on Whatman 3MM paper for electrophoresis and autoradiography.

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