Fragmin, a Microfilament Regulatory Protein from *Physarum polycephalum,* Is Phosphorylated by Casein Kinase II-Type Enzymes[†]

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ABSTRACT: Fragmin is a 42 kDa regulatory protein involved in actin microfilament organization in *Physarum polycephalum*. We show that fragmin is a target of casein kinase II (CK II) enzymes isolated from evolutionarily divergent species. In *Physarum* microplasmodia, two such kinases were identified. A serine residue located in the sequence Gly-Gly-Ser-Asp-Leu-Glu constitutes the phosphorylation site and was identified by phosphopeptide sequencing, mass spectrometry analysis, and inhibition studies with a synthetic peptide corresponding to this site. Interestingly, the actin−fragmin dimer (A−F) as well as the actin$_2$−fragmin trimer (A_2−F) are equally efficient targets, and phosphorylation had no effect on the actin-binding properties of fragmin. Actin−fragmin isolated from microplasmodia revealed a minor acidic fragmin isoform, suggesting that fragmin is phosphorylated *in vivo*. The actin−fragmin complex is also phosphorylated on the actin subunit by an endogenous actin−fragmin kinase [Gettemans, J., De Ville, Y., Vandekerckhove, J., & Waelkens, E. (1992) *EMBO J. 11*, 3185−3191]. We show that the two phosphorylation events act independently of each other.

The slime mold *Physarum polycephalum* displays a dynamic microfilament system [for a recent review, see Stockem and Brix (1994)] and is therefore a suitable organism for study of the nature and regulation of actin-based motility both at the biochemical and at the molecular level. An important regulatory component of this system is fragmin, an actin-binding protein that is structurally very similar to the amino terminal half of gelsolin (Kwiatkowski et al., 1986; Ampe & Vandekerckhove, 1987). Fragmin consists of three repeated segments, each approximately 120 residues long, at least two of which bind actin.

Many of the properties of fragmin resemble those of gelsolin, considered as an important regulator of the subcortical actin network in macrophages (Yin & Stossel, 1979) and other cells (Yin et al., 1981; Stossel, 1989). Like gelsolin, fragmin severs F-actin filaments and is able to nucleate actin filament formation in a Ca^{2+}-dependent manner *in vitro* (Gettemans et al., 1995). Under these conditions, fragmin binds two actin molecules. Addition of the Ca^{2+} chelator EGTA to the actin$_2$−fragmin trimer releases one actin subunit at the reversible Ca^{2+}-dependent site. The resulting actin−fragmin heterodimer (A−F)[1] efficiently caps F-actin filaments at the barbed ends and nucleates actin polymerization weakly but has lost its severing activity. This EGTA-resistant A−F complex is also a specific target for an 80 kDa *Physarum* actin−fragmin kinase (AFK) that phosphorylates the actin subunit at positions Thr-203 and Thr-202 (Furuhashi et al., 1992; Gettemans et al., 1992, 1993). This phosphorylation site is located in one of the actin−actin contact sites in the F-actin models (Holmes et al., 1990; Schutt et al., 1993) and provides an explanation for the inhibition of actin filament elongation at the pointed end of the protomer.

Like gelsolin, fragmin also binds phosphatidylinositol 4,5-bisphosphate (PIP_2) micelles, and once the phospholipid is bound, actin is no longer able to associate with this complex and vice versa (Gettemans et al., 1995).

We have investigated the extent of actin phosphorylation in *Physarum* cells by two-dimensional (2D) gel electrophoretic analysis of purified A−F complex. In addition to actin phosphorylation, a more acidic isoform of fragmin was noticed. We demonstrate that this isoform arises through phosphorylation of fragmin by casein kinase II-type enzymes. This modification could be similar in nature and in function to the phosphorylation previously mentioned for CapG, a homologous protein involved in macrophage motility (Onada & Yin, 1993).

We determined the phosphorylation site in fragmin and studied the effect of phosphorylation on interaction with actin and PIP_2 binding.

EXPERIMENTAL PROCEDURES

Materials. P-81 phosphocellulose paper and DEAE-cellulose (DE-52) were purchased from Whatman (Maidstone, U.K.). [γ-^{32}P]ATP (3000 Ci/mmol) and nitrocellulose

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[1] Abbreviations: A−F, actin−fragmin complex; AFK, actin−fragmin kinase; CK I, casein kinase I; CK II, casein kinase II; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; MOPS, morpholinopropanesulfonic acid; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; TFA, trifluoroacetic acid.

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were obtained from Amersham International (Buckinghamshire, U.K.). Hydroxyapatite (Bio-Gel HT) was from Bio-Rad (Richmond, VA), and trypsin and endoproteinase Asp-N (sequencing grade) from Boehringer Corp. (Mannheim, FRG). The ampholines were from Pharmacia/LKB (Uppsala, Sweden). Cellulose thin-layer plates were obtained from Merck (Darmstadt, Germany). Coomassie brilliant blue was from Serva (Heidelberg, Germany). Dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), heparin, polylysine, phosphatidylinositol 4,5-bisphosphate (PIP_2), β-casein (dephosphorylated, from bovine milk), and horse heart myoglobin were from Sigma (St. Louis, MO). PIP_2 was dissolved in water at a concentration of 2 mg/mL and sonicated for 10 min.

Proteins. The actin−fragmin complex, fragmin, and the actin−fragmin kinase (AFK) were purified from microplasmodia of *P. polycephalum* as described previously (Gettemans et al., 1992, 1993, 1995). Actin was prepared from rabbit muscle according to the procedure of Spudich and Watt (1971) and kept on ice in G-buffer (2 mM Tris-HCl, pH 7.6, 0.2 mM ATP, 0.5 mM β-mercaptoethanol, 0.2 mM $CaCl_2$, and 0.02% NaN_3). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Phosphorylation Assays. Routine phosphorylation of fragmin was measured by addition of 20 μL of a column fraction to either fragmin or the actin−fragmin complex (both 1.1 μM) in TDA buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT, and 0.02% NaN_3) supplemented with 10 mM $MgCl_2$ and 0.2 mM [γ-^{32}P]ATP (6 μCi/μmol) at 30 °C for the indicated times (final volume of 25 μL). The reaction was terminated by addition of one-fifth of a volume of concentrated sample buffer (Laemmli, 1970). The proteins were analyzed by 1D mini sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (see below) and autoradiography. Scanning of the autoradiographs was performed with a 2202 Ultroscanner (Pharmacia/LKB).

The A−F complex was phosphorylated in the actin subunit with purified AFK (Gettemans et al., 1993) in TDA buffer supplemented with 10 mM $MgCl_2$ and 0.2 mM [γ-^{32}P]ATP.

Synthetic peptides were phosphorylated in the same conditions as above. Twenty microliters of the enzyme fraction was mixed with the synthetic peptide solution (final volume of 25 μL containing 0.9 mM peptide). At appropriate times, the reaction was terminated by spotting 20 μL onto P-81 phosphocellulose paper disks. These were submerged in 75 mM H_3PO_4 and washed twice for 10 min (Marshak & Carroll, 1991). The radioactivity was measured in 1 mL scintillation liquid (Opti Phase HiSafe 3, LKB, Uppsala, Sweden) using a Wallac 1409 liquid scintillation counter (Pharmacia/LKB).

Partial Purification of the Physarum CK IIs. Microplasmodia of *P. polycephalum* were cultured as described by Daniel and Baldwin (1964). Cultures of 600 mL were grown in the dark at 24 °C by shaking at a frequency of 100 rev/min. Cells were collected by centrifugation at 4 °C. The pellets were washed twice with two volumes of Sörensen buffer (10 mM KH_2PO_4 and 10 mM Na_2HPO_4, pH 6.0) and once with one volume of 30 mM Tris-HCl (pH 8.0). Cells were homogenized for 2 × 4 min in 250 mM sucrose, 40 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, and 0.02% NaN_3 (3 mL/g of packed cells) using a Waring blender set at the highest speed. Cell debris was removed by centrifugation for 50 min at 16000*g*, and the supernatant (750 mL) was mixed batchwise with 180 mL of DEAE-cellulose (DE-52), equilibrated in TEDA buffer (20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM DTT, and 0.02% NaN_3). The slurry was poured into a column (5 × 11.5 cm), washed extensively with TEDA buffer supplemented with 1 mM PMSF and 1 mM benzamidine. The adsorbed proteins were eluted with a 900 mL linear gradient from 0 to 400 mM NaCl, and the absorbance of the eluate was measured at 280 nm. The flow rate was 50 mL/h. Fractions of 12 mL were collected and assayed for kinase activity. Two peaks of activity were detected, pooled separately, and referred to as kinase 1 and kinase 2. Each pool was dialyzed against 25 mM potassium phosphate (pH 7.1), 0.5 mM DTT, 1 mM EGTA, 1 mM PMSF, and 100 mM NaCl and loaded onto hydroxyapatite columns that were equilibrated with the same buffer (a 1.6 × 10 cm column for kinase 1 and a 1.6 × 5 cm column for kinase 2). Columns were washed with five column volumes of equilibration buffer, and proteins were eluted with a linear gradient of 25 to 450 mM potassium phosphate (pH 7.1) (80 mL for peak 1 and 40 mL for peak 2). The flow rate was kept at 10 mL/h, and the eluate was measured by absorbance at 280 nm. Fractions of 2.3 mL (peak 1) and 1 mL (peak 2) were collected and analyzed. The hydroxyapatite pools were dialyzed against TDA buffer, and each was applied onto a mono Q HR 5/5 column through a 10 mL superloop connected to a FPLC system (Pharmacia, LKB, Sweden), equilibrated with the same buffer. The column was developed with a linear gradient of 0 to 250 mM NaCl (total volume of 24 mL) (peak 1) or 100 to 570 mM NaCl (total volume of 22.5 mL) (peak 2) in TDA buffer supplemented with 1 mM PMSF and 1 mM benzamidine. The flow rate was maintained at 0.7 and 0.75 mL/min, respectively; fractions of 0.75 mL were collected and immediately stored at −80 °C.

Preparation of the Yeast CK II Enzyme. Yeast cells were grown in YPD (1% yeast extract, 2% Bacto-Peptone, and 2% D-glucose) by shaking at a frequency of 100 rpm at 28 °C. Cells were collected by low-speed centrifugation, washed with TEDA buffer, and sonicated on ice in 250 mM sucrose, 20 mM Tris-HCl (pH 7.5), 0.2 mM DTT, 0.5 mM EGTA, 0.5 mM EDTA, 1 mM PMSF, and 1 mM benzamidine for two periods of 6 min using a Vibra-Cell 500 W sonicator (Sonics and Materials, Danbury, CT) with the output control set at 40% duty cycle. The purification procedure was the same as described above. At this point, however, we used the synthetic peptide RRREEESEEE as a CK II-specific model substrate (Marshak & Carroll, 1991).

Phosphoamino Acid Analysis. Phosphorylated fragmin was dissociated and purified from actin by reversed-phase HPLC. A C_{18} column (4.6 × 250 mm, 5 μm particle size, 300 Å pore size; Vydac, Separations Group, Hesperia, CA) was equilibrated in 0.1% trifluoroacetic acid (TFA), and the proteins were eluted using a linear gradient of 0 to 70% acetonitrile in 0.1% TFA at a flow rate of 1 mL/min. The gradient was started 5 min after sample loading and finished after 70 min. Phosphorylated fragmin, eluting after 51 min, was Speed Vac (Savant Instruments, Farmingdale, NY) dried and hydrolyzed with 6 N HCl for 2 h at 110 °C. The hydrolysate was lyophilized and resuspended in electrophoresis buffer, formic acid (99%)/glacial acetic acid/water (22:78:900, pH 1.9), and applied onto a cellulose plate together with phosphoamino acid standards (5 μg each) and electrophoresed for 4 h at 210 V. The second dimension,

ascending chromatography, was run in 2-propanol/HCl/water (140:30:30). Radioactive phosphoamino acids were detected by autoradiography; the phosphoamino acid standards were visualized by ninhydrin staining.

Identification of the Phosphorylation Site through Mass Spectrometry and Phosphopeptide Amino Acid Sequence Analysis. Actin−fragmin (1.1 μM) was phosphorylated as described above. Fragmin, purified by reversed-phase HPLC, was evaporated to dryness, resuspended in 150 μL of 100 mM Tris-HCl (pH 8.2), and digested with trypsin (1 μg) for 4 h at 37 °C. The tryptic fragments were separated on a narrowbore C_4 reversed-phase column (21 × 200 mm) connected to a 140A HPLC instrument from Applied Biosystems Inc. (Foster City, CA). The column was equilibrated in 0.1% TFA at a flow rate of 80 μL/min. Peptides were eluted with a linear gradient of acetonitrile (0 to 70% in 0.1% TFA over 80 min). The phosphopeptides were identified by Cerenkov radiation. They were sequenced and subjected to mass spectrometry. Alternatively, the tryptic fragments were subdigested with endoproteinase Asp-N for 24 h at 37 °C, and the fragments were separated as above. Fragmin, phosphorylated fragmin, and the ^{32}P-labeled tryptic peptides were subjected to electrospray ionization mass spectrometry analysis (ESI-MS). The instrument [a single quadrupole mass analyzer VG PLATFORM (VG Biotech Fisons Instruments, Manchester, U.K.) and run according to the manufacturer's instructions) was calibrated with horse heart myoglobin (16 951.48 Da). Approximately 10 μL of the sample (containing between 1 and 5 pmol/μL protein or peptide) was introduced into the ion source with a HPLC pump (Pharmacia LKB-HPLC pump 2248) at a flow rate of 5 μL/min. Scans of the protein or peptide were made for 5 s between *m/z* 500 and 1500 and for 10 s between *m/z* 500 and 2000, respectively.

Amino acid sequence analysis was performed on a 477A model pulsed liquid-phase sequenator equipped with a 120A phenylthiohydantoin amino acid analyzer (Applied Biosystems Inc., CA). Differential sequence and mass spectrometry analysis (phosphorylated versus unphosphorylated forms) was used.

Peptide Synthesis. The CK II model peptide RRREEESEEE (Marshak & Carroll, 1991) and the fragmin peptides RRRKKVKLEASQHEDAWKG and RRRDAAAGGSDLEAD were synthesized on an Applied Biosystems (Foster City, CA) automated 431A peptide synthesizer. The synthesis was performed using the fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry procedure following the manufacturer's instructions. The peptides were cleaved from the resin with TFA and desalted in water using a Sephadex G-25 gel filtration column (30 × 2.6 cm). The peptides were further purified by preparative C_4 reversed-phase HPLC, lyophilized, and stored at −20 °C until further use.

Gel Electrophoresis. SDS−polyacrylamide gel electrophoresis was carried out according to Matsudaira and Burgess (1978). The 10% gels were stained with 0.25% Coomassie brilliant blue in 10% acetic acid and 45% ethanol and destained in 5% ethanol and 7.5% acetic acid.

Isoelectric focusing was performed as described by Garrels (1979) using 1.6% ampholines (pH 5−7) and 0.4% ampholines (pH 3.5−10). The sample was prepared as follows. Fragmin was isolated from the actin−fragmin complex (0.6 μM) using a C_{18} reversed-phase HPLC column and lyophilized (see above). Phosphorylated actin−fragmin (0.6 μM) was first passed through a Sephadex G-25 column to remove free nucleotide, and fractions of 100 μL were collected manually. Phosphorylated fragmin was obtained by reversed-phase HPLC as above. Proteins were resuspended in 50 μL of lysis buffer (9.5 M urea, 2% Nonidet P 40, 100 mM DTT, and the ampholine mixture). The second dimension was SDS−polyacrylamide gel electrophoresis.

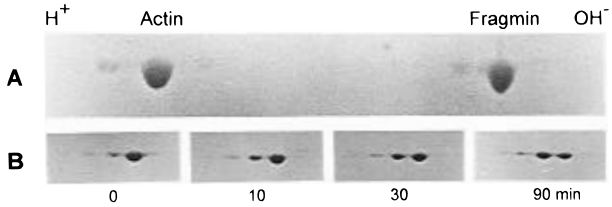

FIGURE 1: *In vivo* and *in vitro* phosphorylation of fragmin. (A) 2D gel electrophoresis of purified actin−fragmin complex showing *in vivo*-phosphorylated minor isoforms of actin and fragmin. (B) Time course of fragmin phosphorylation *in vitro* by *Physarum* kinase 2, analyzed through 2D gel electrophoresis. Isofocusing was in the horizontal direction and SDS−PAGE in the vertical direction. Gels were stained with Coomassie brilliant blue.

Immunoblotting of the Physarum Kinase 1 and Kinase 2. Kinase preparations were subjected to electrophoresis on 12.5% polyacrylamide gels, transferred to PVDF, and probed with antibodies. A reference of CK II and CK I, both prepared from pig spleen, was included. Western blotting was performed as described by Towbin et al. (1979). Chicken polyclonal CK II antibodies were raised against purified CK II holoenzyme and react with α/α' and β subunits. Anti-peptide antibodies specific for the α, α', and β subunits were prepared separately in rabbits. These CK II antibodies were kindly provided by Dr. P. Agostinis (Afdeling Biochemie, K. U. Leuven, Belgium).

Miscellaneous. Gel filtration was performed on a Superose 12 HR 10/30 column (Pharmacia, Uppsala, Sweden) equilibrated in 20 mM MOPS (pH 7.4), 50 mM KCl (or 750 mM KCl), and 0.02% NaN_3. The flow rate was maintained at 0.4 mL/min.

RESULTS

(1) Phosphorylation of Fragmin. 2D gel electrophoresis of the purified actin−fragmin complex reveals more than one isoform of both actin and fragmin (Figure 1A). A similar observation with respect to fragmin was made by Hinssen (1981). Data on actin phosphorylation as well as on the purification and characterization of the corresponding kinase have been reported previously (Gettemans et al., 1992, 1993). When fragmin was phosphorylated with partially purified kinase from *P. polycephalum* and subsequently analyzed by 2D gel electrophoresis, we noticed a marked relative increase of the minor (acidic) fragmin isoform (Figure 1B), suggesting this minor spot represents *in vivo*-phosphorylated fragmin. Western blotting with affinity-purified actin−fragmin polyclonal antibodies showed that this protein indeed represents a fragmin isoform (not shown). A more thorough screening procedure demonstrated that a similar kinase activity was also present in DEAE column fractions of *Dictyostelium discoideum*, bovine spleen, and *Saccharomyces cerevisiae* cytosolic extracts.

We partially purified this enzyme from *Physarum* by sequential chromatography on DEAE-cellulose, hydroxyapatite, and a mono Q column using fragmin as substrate for screening of the column fractions.

Interestingly, DEAE-cellulose column chromatography of crude cytosolic extracts revealed two peaks of fragmin kinase

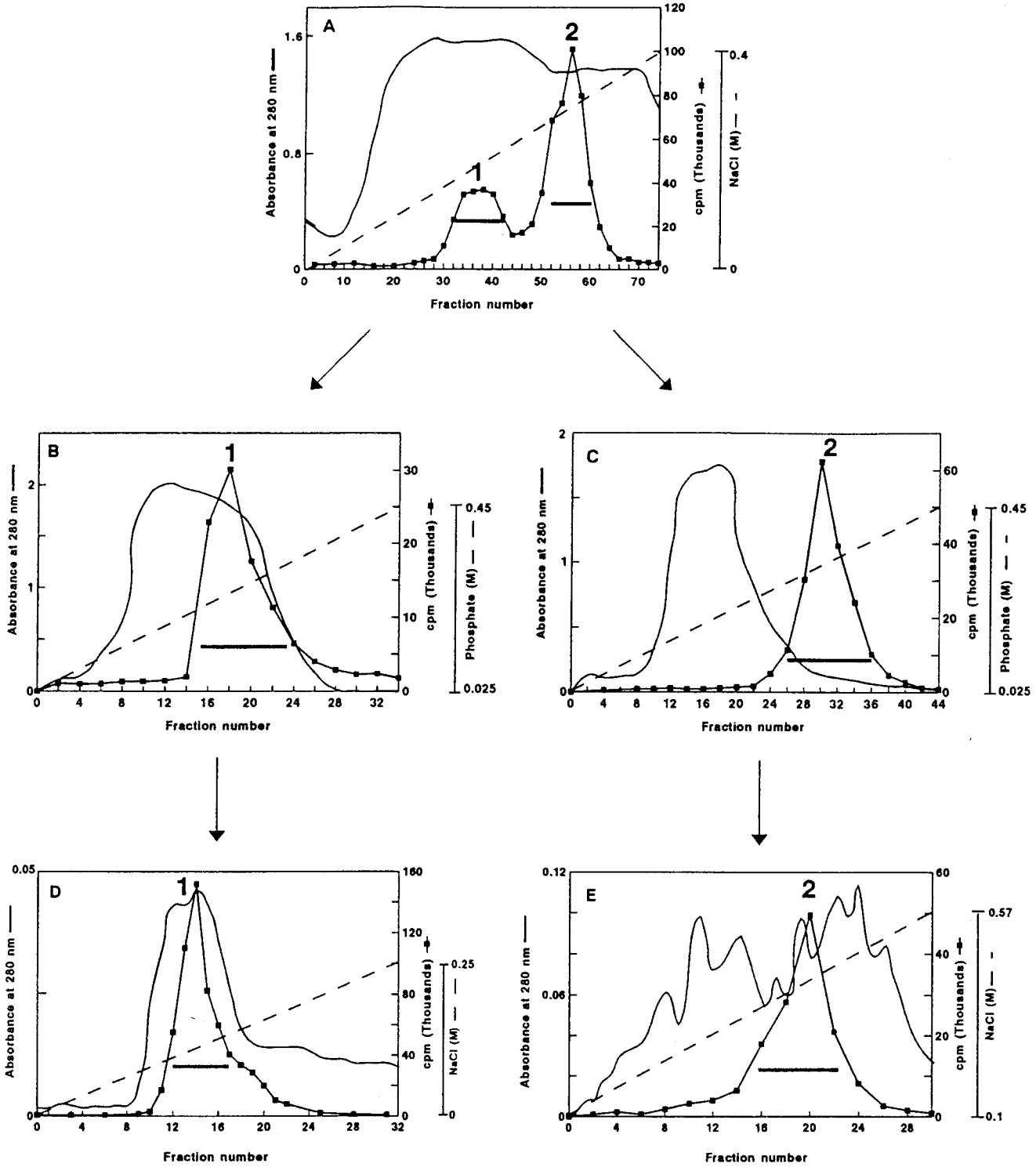

FIGURE 2: Separation and partial purification of *Physarum* fragmin kinases. The clarified *Physarum* extract was chromatographed on DEAE-cellulose (A). Peak 1 (fraction numbers 32−42) and peak 2 (fraction numbers 52−60) were collected separately and loaded onto a hydroxyapatite column (B and C). Active fractions (indicated by the horizontal bar) were pooled and applied onto a mono Q column (D and E). (■) Fragmin kinase activity (counts per minute incorporated in fragmin). Salt gradients are indicated (− − −). Protein elution was measured by absorbance at 280 nm (—).

activity, one eluting at 175−225 mM NaCl (peak 1) and a second peak eluting at 275−325 mM NaCl (peak 2) (Figure 2A). After hydroxyapatite chromatography, the kinase 1 activity eluted at 225−325 mM phosphate (Figure 2B), whereas kinase 2 activity was detected at 275−375 mM phosphate (Figure 2C). In mono Q column chromatography, the kinase 1 eluted at 80−120 mM NaCl (Figure 2D) and kinase 2 at 400−460 mM NaCl (Figure 2E). At this stage, preparations were obtained that proved rather unstable, and the kinases were therefore stored immediately at −80 °C. We refer to these preparations as fragmin kinase 1 and fragmin kinase 2, respectively.

Figure 3 shows a time course analysis of fragmin phosphorylation with the enriched *Physarum* fragmin kinases. In the experimental conditions used, we measured a time-dependent phosphorylation up to 90 min by kinase 1 (Figure 3A) and kinase 2 (Figure 3B). Phosphorylation of fragmin in the actin−fragmin complex was as efficient as phosphorylation of fragmin alone (not shown). Because the actin−fragmin heterodimer is more easily purified than free

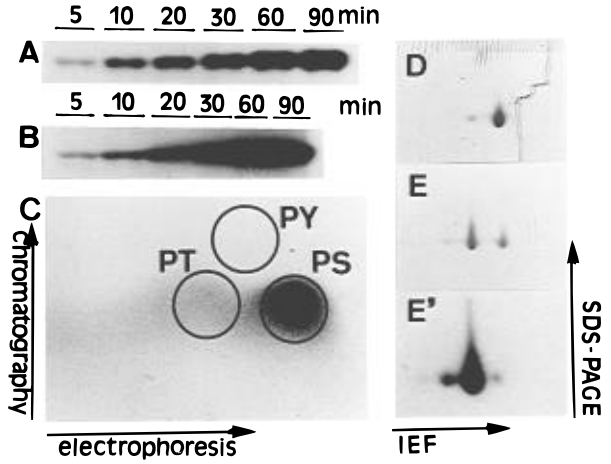

FIGURE 3: Phosphorylation rates; 1D and 2D gel electrophoresis of phosphorylated fragmin and phosphoamino acid analysis. Time course analysis of fragmin phosphorylation by *Physarum* kinase 1 (A) and kinase 2 (B). Fragmin was phosphorylated for various time intervals (indicated on top) and analyzed by SDS−PAGE and autoradiography. (C) Phosphoamino acid analysis of ^{32}P-labeled fragmin by kinase 2. The phosphoamino acids were resolved by electrophoresis at pH 1.9 in the first dimension and by ascending chromatography in the second dimension. The positions of the phosphoamino acid standards, detected by ninhydrin staining, are shown (PS, phosphoserine; PT phosphothreonine; and PY, phosphotyrosine). 2D gel electrophoresis of unphosphorylated (D) and phosphorylated fragmin (E), detected by staining with Coomassie brilliant blue. Phosphofragmin was detected by autoradiography (E′). Isoelectric focusing (IEF) was in the horizontal direction and SDS−PAGE in the vertical direction.

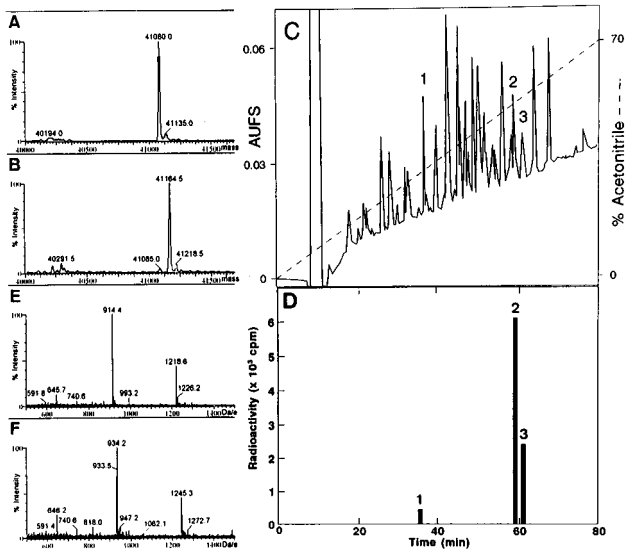

FIGURE 4: ESI-MS analysis of phosphorylated fragmin and separation of ^{32}P-labeled tryptic peptides by reversed-phase HPLC. Mass-transformed spectrum of unphosphorylated fragmin (A) and phosphorylated fragmin (B). In both cases, the molecular mass is indicated on top of the major peak. (C) Reversed-phase HPLC chromatogram of the tryptic peptides of phosphorylated fragmin. The absorbance at 214 nm is shown with a full line. The percentage of acetonitrile is indicated by the broken line. Radioactive peptides are numbered 1−3 (AUFS, absorption unit full scale). (D) Radioactivity (Cerenkov radiation) associated with each of the three phosphopeptides. (E) ESI-MS analysis of unphosphorylated peptide 2 (see C). The *m/z* (mass to charge ratio) of 914.4 corresponds to the quadruply charged non-phospho form of tryptic peptide 2. A value of 1218.6 corresponds to the triply protonated peptide. The molecular weight of this peptide is thus 3653.1 (±0.36) (F) The phosphopeptide 2 produced signals at *m/z* 934.2 and 1245.3 corresponding to the quadruply and triply protonated phosphopeptide. The derived molecular weight is 3732.6 (±0.15) (Da/e, daltons/electron).

fragmin, we used this complex in most of the following experiments.

^{32}P-phosphorylated fragmin was subjected to phosphoamino acid analysis. Comparison of the autoradiogram and the ninhydrin-stained pattern of the standard phosphoamino acids revealed that serine was the major phosphorylated amino acid (Figure 3C). An identical result was obtained for both kinases. When fragmin, phosphorylated with kinase 2, was analyzed by 2D gel electrophoresis, we noticed a considerable increase in the intensity of a more acidic spot, carrying most of the radioactivity (Figure 3D−E′). A minor, more acidic variant, containing approximately 5% of the total radioactivity, was also observed (Figure 3E,E′). This spot may represent a doubly phosphorylated form of fragmin. Similar data were also obtained with kinase 1 (not shown).

Metabolic ^{32}P labeling of *Physarum* microplasmodia (V. De Corte et al., in preparation) and immunoprecipitation of the actin−fragmin complex with polyclonal anti-fragmin antibodies demonstrated that fragmin was weakly phosphorylated. The ^{32}P label was also located in the peptide that was identified as the target site *in vitro* (data not shown).

(2) Identification of the Phosphorylation Site in the Primary Structure of Fragmin. Electrospray ionization mass spectrometry of fragmin yielded *m/z* values corresponding to a molecular weight of 41 080 (Figure 4A). *In vitro*-phosphorylated fragmin yielded a value of 41 164.5 (Figure 4B). This increase is in agreement with the addition of a single phosphoryl group. Because the technique used here is semiquantitative, it was not possible to detect additional minor phosphorylation sites. For more quantitative data, we refer to the 2D gel electrophoretic analysis (Figure 3D−E′). Both kinases gave similar results, but only the analysis of fragmin phosphorylation by kinase 2 is shown here.

Fragmin that was terminally phosphorylated with kinase 2 was digested with trypsin, and the peptides were separated by reversed-phase HPLC (Figure 4C). Radioactivity was identified by Cerenkov radiation and coincided with three peptide peaks (Figure 4D). Aliquots of each of these peptides were subjected to automated Edman degradation and to ESI-MS analysis.

The first peptide (1) contained a very low amount of label and showed the sequence LEASQHEDAWK, corresponding to a region in the amino terminal part of fragmin. Interestingly, the PTH-serine residue was recovered in normal yields, suggesting the absence of phosphorylation. This result was confirmed by peptide mass analysis, indicating a value of 1313.3 Da, in accordance with the unphosphorylated form of the peptide (not shown). These results could be interpreted in different ways. Peptide 1 is only marginally phosphorylated (below the detection limit level of mass spectrometry or sequence analysis) and we only detect the ^{32}P-phosphorylated peptide, coeluting with its unphosphorylated form. Alternatively, peptide 1 could have been contaminated by a minor ^{32}P-phosphorylated peptide of unknown nature.

The second phosphopeptide (2 in Figure 4C) contains 36 amino acids, VVAEADNDTEFFTLLGDKGPIADAAAGGXDLEADKK. Remarkably, the lysyl−glycine peptide bond was not cleaved by trypsin. Sequence analysis revealed a gap at cycle 29 (X), which should normally yield a serine residue in the fragmin sequence. It most likely represents the phosphorylated residue as it is known that phosphoserine is decomposed in the course of Edman degradation (Martensen, 1984). The molecular weight of the peptide was

determined by ESI-MS (Figure 4E) and corresponded with that calculated for the phosphopeptide derivative (3732.6). When the elution profiles of the tryptic peptides from unphosphorylated (not shown) and phosphorylated fragmin were compared, peptide 2 eluted at the same position. However, its molecular weight (3653.1) was in accordance with that of the unphosphorylated form (Figure 4F). Amino acid sequence analysis of the unphosphorylated peptide showed a serine residue at position 29. Since this peptide contains no serine residues at other positions, we conclude that this serine represents the phosphorylated site. To confirm this result, we subdigested peptide 2 with endoproteinase Asp-N and isolated the phosphopeptide by reversed-phase HPLC (not shown). The radioactive peptide displayed the sequence DAAAGGXDLEA, with X again representing the decomposed phosphoserine. Its unphosphorylated counterpart showed normal PTH-serine yields. Mass spectrometry analysis on the phosphorylated and unphosphorylated peptides confirmed this conclusion (not shown).

The third radioactive peptide showed the same amino terminal sequence as peptide 2 but displayed a molecular weight of 4626.0. Although we were not able to determine its complete sequence, the experimentally determined molecular weight suggested that peptide 3 consisted of peptide 2 with a carboxy terminal extension, up to the next tryptic cleavage site. No additional serine is present in the carboxy terminal tail. Thus, peptides 2 and 3 carry the same phosphorylated serine residue. Identical results were observed for fragmin that was phosphorylated with fragmin kinase 1 (results not shown).

In conclusion, fragmin is similarly phosphorylated by the two *Physarum* kinases; the major site contains up to 95% of the phosphate and is located in the DAAAGGSDLEA sequence.

(3) The Fragmin Kinases 1 and 2 Belong to the CK II Family. The identified phosphorylation site in fragmin corresponds with the consensus CK II phosphorylation motif, Ser/ThrXXA (with A being an acidic residue) (Krebs et al., 1988; Meggio et al., 1994). To further confirm that fragmin is a target for CK II-type kinases, we studied the effects of specific CK II modulators.

At a concentration of 0.8 μg/mL, heparin exerted complete inhibition of fragmin phosphorylation. This effect was noticed for kinase 1 as well as for kinase 2 (Figure 5A). The effects of polylysine on kinase 2 are in line with the known stimulatory activity of other CK II kinases (Hathaway & Traugh, 1984). A stimulatory effect reaching its maximal value at 60 μg/mL was measured (Figure 5B). Kinase 1 however was inhibited by polylysine, even at a concentration of 20 μg/mL (Figure 5B). Interestingly, both kinases were stimulated by polylysine when β-casein was used as the substrate (Figure 5C).

The CK II enzyme from yeast could also efficiently phosphorylate fragmin, and in addition, it proved more stable as compared to the *Physarum* kinases. The phosphorylation sites in fragmin were identical to those described for the *Physarum* kinases (results not shown). The yeast CK II activity was inhibited by heparin (0.8 μg/mL) (Figure 5A) and stimulated at least 5-fold by polylysine (60 μg/mL) (Figure 5B). Therefore, it resembles *Physarum* kinase 2 more than it does *Physarum* kinase 1.

In order to further characterize the two *Physarum* kinases in more detail, we determined their molecular sizes and immunological relationship with mammalian CK II enzymes.

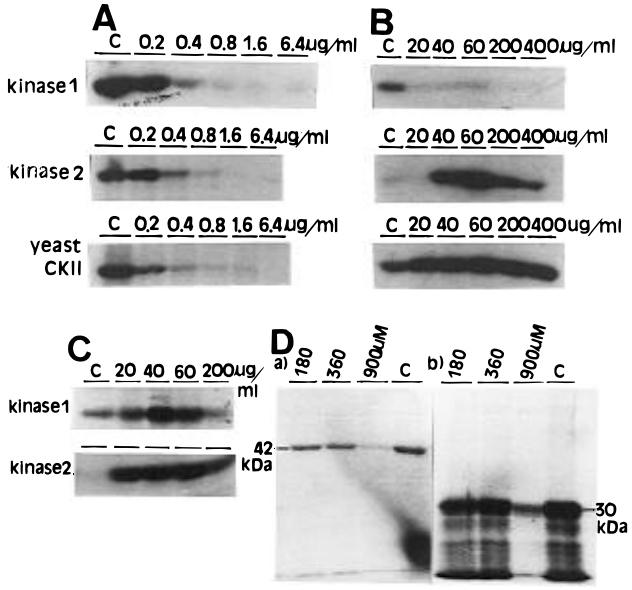

FIGURE 5: Modulation of fragmin phosphorylation by heparin, polylysine, and PIP_2. Comparison of fragmin kinases 1 and 2 with yeast casein kinase II. (A) Inhibition of fragmin phosphorylation by heparin. A−F (1.8 μM) was phosphorylated for 60 min in the presence of the indicated amounts of heparin and analyzed by SDS−PAGE and autoradiography [c, control (no heparin)]. (B) Modulation of fragmin phosphorylation by polylysine. A−F (1.8 μM) was phosphorylated for 10 min in the presence of increasing concentrations of polylysine and analyzed as in A [c, control (no polylysine)]. Note the inhibiton of kinase 1. (C) Polylysine stimulation of β-casein phosphorylated by fragmin kinases 1 and 2. β-Casein (10 μM) was phosphorylated for 10 min in the presence of the indicated amounts of polylysine. (D) Effect of PIP_2 on the phosphorylation of fragmin. (a) A−F (5.5 μM) was preincubated with 180, 360, or 900 μM PIP_2 for 30 min at 25 °C and then phosphorylated. (b) β-Casein (18 μM) was preincubated with the same amounts of PIP_2 and then phosphorylated. In the control experiments, no PIP_2 was added.

Physarum kinase 1 eluted as a 50−55 kDa molecule during gel filtration. Western blot analysis using chicken polyclonal CK II antibodies (reacting with the α, α′, and β subunits) revealed that kinase 1 consisted of a single subunit migrating at the position of α′ following SDS−PAGE (Figure 6A, lane c). This was confirmed using rabbit peptide antibodies specific for the CK II β subunit, which did not react with *Physarum* kinase 1 (Figure 6A, lanes b and d).

Physarum kinase 2 eluted as a 300−350 kDa multimeric complex at physiological salt concentrations but showed a more reduced size (150−160 kDa) when passed through a gel filtration column equilibrated with 0.75 M KCl. The higher apparent molecular size was probably due to self-aggregation under lower ionic strength conditions. Interestingly, Western blot analysis using a mixture of rabbit peptide antibodies specific for the α, α′, and β CK II subunits demonstrated that *Physarum* kinase 2 is an oligomeric enzyme similar to other CK IIs (see Figure 6B, lane g).

A synthetic peptide corresponding to the phosphorylation site in fragmin (RRRDAAAGGSDLEAD) and carrying an amino terminal polyarginine tag (to facilitate binding to phosphocellulose disks during the biochemical assay) was efficiently phosphorylated by both *Physarum* kinases (Figure 7A). In addition, this peptide also inhibited the phosphorylation of fragmin in a concentration-dependent manner. Full inhibition was measured at a 30-fold molar excess over fragmin (Figure 7B). The CK II model peptide (RRREEESEEE) was also efficiently phosphorylated, but when a

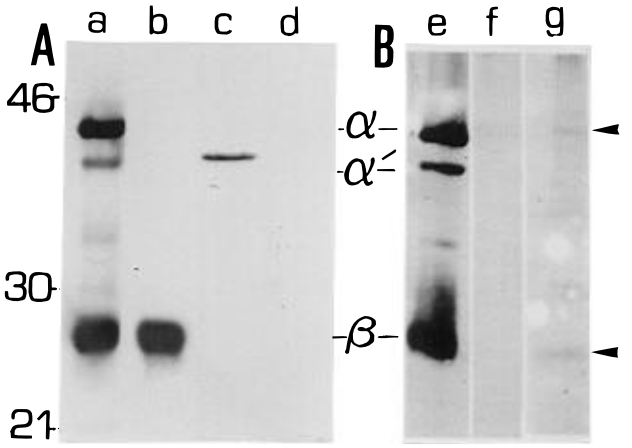

FIGURE 6: Immunocharacterization of *Physarum* kinase 1 and 2 using CK II specific antibodies. (A) *Physarum* kinase 1. Kinase preparations included a reference CK II from pig spleen (lanes a and b) and the partially purified *Physarum* kinase 1 (lanes c and d). Chicken polyclonal CK II antibodies were used in lanes a and c, whereas rabbit anti-peptide antibodies specific for the β subunit were used in lanes b and d. Both antibodies were diluted $^1/_{1000}$. The position and the molecular masses (in kilodaltons) of the marker proteins are shown at the left. The migration positions of the α, α′, and β subunits of pig spleen CK II are shown at the right. (B) *Physarum* kinase 2. A mixture of rabbit peptide antibodies specific for the α, α′, and β subunits (each diluted $^1/_{1000}$) were used in lane e (CK II from pig spleen, 0.05 μg loaded), lane f (CK I from pig spleen, 0.055 μg loaded), and lane g (partially purified *Physarum* kinase 2). Note that there is no cross-reactivity of the α, α′, and β subunit CK II specific antibodies with CK I. The α and β subunits in the *Physarum* kinase 2 preparation are indicated with an arrow.

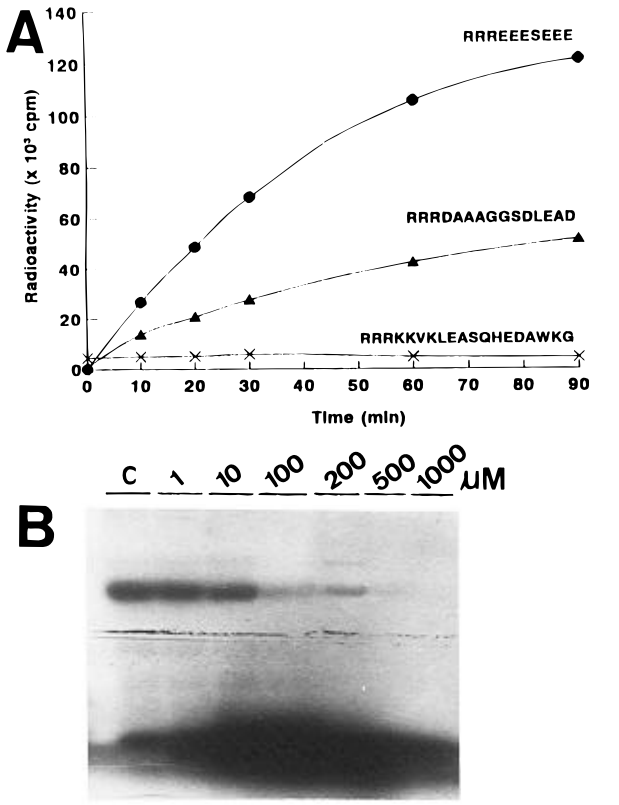

FIGURE 7: Phosphorylation of synthetic peptides by *Physarum* CK II. (A) Comparison of the phosphorylation rates of the CK II ESE model peptide substrate and two synthetic fragmin peptides corresponding to the presumed phosphorylation sites. Each peptide (0.9 mM) was phosphorylated for various time intervals (minutes). (B) Competition between the synthetic peptide RRRDAAAGGS-DLEAD and fragmin for CK II. Fragmin (3.4 μM) was preincubated with increasing amounts (ranging from 1 μM to 1 mM) of the synthetic peptide corresponding to the major phosphorylation site. CK II was added after 10 min. Phosphorylation was analyzed by SDS−PAGE and autoradiography [c, control (without added peptide)]. Notice the strong phosphorylation of the peptide (lower edge of the gel).

similar study was carried out with the synthetic peptide 1 (RRRLEASQHEDAWK, see above), no phosphorylation was measured (Figure 7A). This suggests that the presumptive minor phosphorylation site is not located in this sequence.

(4) Effects of the Phosphorylation on Fragmin Function. Like gelsolin, fragmin binds two actin monomers in the presence of micromolar amounts of Ca^{2+}. In EGTA, one actine molecule is released from the trimeric complex. In addition, fragmin and A−F also bind PIP_2 (Gettemans et al., 1995). We have investigated the effect of phosphorylation of fragmin on actin and PIP_2 binding.

Free fragmin as well as the actin−fragmin complex and the $actin_2$−fragmin trimer (formed by addition of a 2-fold molar excess of muscle actin to actin−fragmin in the presence of 0.2 mM Ca^{2+}) were phosphorylated with the same kinetics (not shown). This indicates that the phosphorylation site is not part of one of the actin-binding contacts with fragmin. In the same line, we did not detect dissociation of the A−F complex in the course of phosphorylation. This was measured by Superose-12 gel filtration of the unphosphorylated and phosphorylated A−F complex. No differences were noticed in the elution volume (Figure 8A,C).

We have performed F-actin severing and nucleation assays with phosphorylated fragmin (or phosphorylated actin−fragmin) and found that phosphorylation of fragmin or the fragmin subunit in the complex by CK II did not affect these properties (data not shown).

Interestingly, when the actin subunit of the A−F complex was first phosphorylated by AFK, subsequent phosphorylation of the fragmin subunit by CK II proceeded at the same rate as when actin was not previously phosphorylated (Figure 9A). The same result was obtained when fragmin was first phosphorylated (Figure 9B). This indicates that the phosphorylations of the two subunits of the complex proceed independently from each other.

The effect of PIP_2−fragmin binding on the CK II-catalyzed phosphorylation was studied by preincubation of increasing concentrations of PIP_2 with a constant amount of the A−F complex, followed by measurement of the degree of phosphorylation by CK II after 30 min. Only at high concentrations of PIP_2 (900 μM) did we notice an inhibitory effect (Figure 5D, lane a). This inhibition is probably not specific since it was also observed when β-casein was used as substrate (Figure 5D, lane b). In a second experiment, we measured binding of PIP_2 to fragmin after phosphorylation. Phosphorylated $A-F^P$ was first passed over a gel filtration column to remove free Mg^{2+} that could disturb micelle formation (Janmey et al., 1987). Then $A-F^P$ was incubated with a 65-fold molar excess of PIP_2 and passed over the same column. The phosphorylated complex bound to PIP_2 as efficiently as unphosphorylated complex (Figure 8B,D). These experiments demonstrate that phosphorylation of fragmin by CK II has no major effect on the interaction with PIP_2, nor did interaction with PIP_2 specifically influence the rate of phosphorylation.

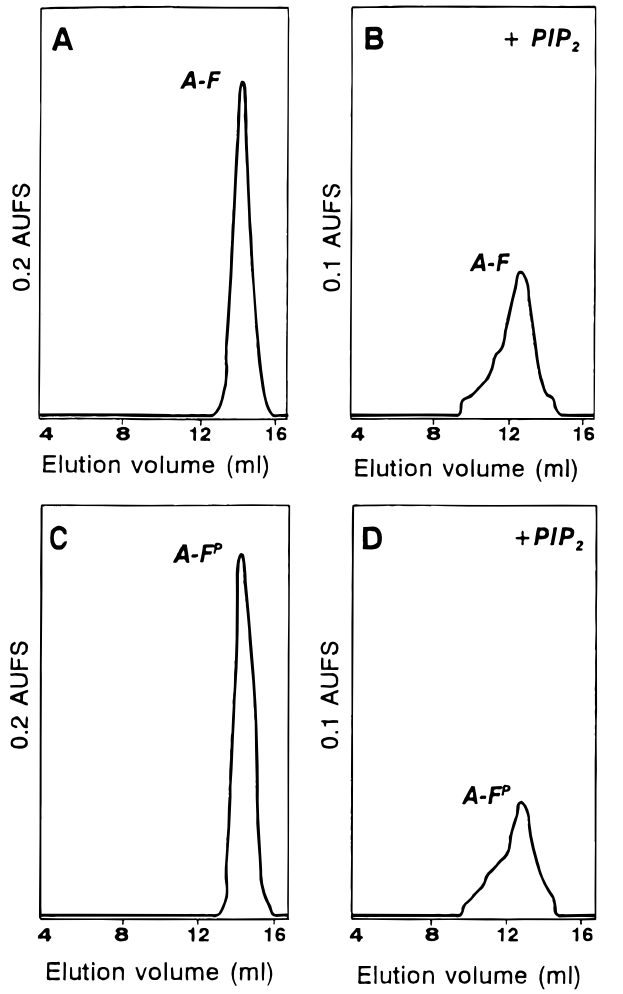

FIGURE 8: CK II phosphorylation of fragmin does not dissociate the A−F complex, and A−F^P is still able to bind PIP_2. (A) Superose 12 size exclusion chromatography of A−F (8.5 μM) in the absence of PIP_2 (control). (B) The peak fraction of panel A was incubated with a 65-fold molar excess of PIP_2 for 5 h at 25 °C and again chromatographed through the column. Note the shift in elution volume to a higher apparant M_r. (C) Superose 12 chromatography of A−F^P and (D) A−F^P (peak fraction) incubated with the same molar excess of PIP_2 as in B.

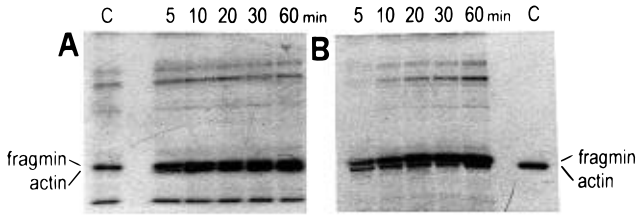

FIGURE 9: Fragmin phosphorylation by CK II is not affected by previous modification of the actin moiety or vice versa. (A) The actin−fragmin complex was phosphorylated by AFK in the actin subunit (lower band). At the indicated intervals, aliquots were withdrawn and each was further phosphorylated during 20 min with kinase 2. Note that the degree of subsequent fragmin phosphorylation was not affected by the degree of actin phosphorylation [c, control, 20 min phosphorylation of A−F with CK II only]. (B) Fragmin (upper band) was first phosphorylated with kinase 2 for the indicated time intervals. Aliquots were then treated for 5 min with AFK. The degree of actin phosphorylation was independent of that of fragmin phosphorylation [c, control, 5 min actin phosphorylation by AFK only].

DISCUSSION

We have demonstrated that extracts of *Physarum* microplasmodia contain two kinase activities, phosphorylating fragmin at identical sites. These activities can be separated from each other by ion exchange chromatography into a basic monomeric enzyme (kinase 1) and an oligomeric more acidic (kinase 2) variant. The major phosphorylation site (DAAAGGSDLEA) was characterized in detail by mass spectrometry and automated Edman degradation and corresponds to the consensus sequence specific for the CK II family (Ser/ThrXXA) (Krebs et al., 1988; Meggio et al., 1994).

Although the two kinases behave differently with respect to polylysine modulation (Figure 5B) and display a different isoelectric point, there are a number of arguments that support their classification as CK II enzymes. First, the two fragmin kinases are equally inhibited by similar concentrations of heparin, a well-known inhibitor of CK II enzymes (Hathaway et al., 1980). Second, CK II isolated from yeast also phosphorylates fragmin at the same site as the two *Physarum* kinases. Third, fragmin phosphorylation by both kinases is inhibited by the synthetic peptide derived from the phosphorylation site. Fourth, kinase 1 and 2 phosphorylate β-casein, a common substrate of CK II enzymes, with similar efficiencies (Hathaway & Traugh, 1982). Interestingly, with β-casein as substrate, kinase 1 as well as kinase 2 were inhibited by heparin and stimulated by polylysine, providing further support for their classification in the CK II family. Finally, we determined the M_r in gel filtration of both *Physarum* kinase 1 and 2 as 50−55 and 150−160 kDa, respectively. *Physarum* kinase 1 shares the properties of a CK II enzyme, although it displays an unusually low molecular mass. Western blot analysis with chicken CK II antibodies revealed the presence of one catalytic subunit. An identification of *Physarum* kinase 1 as CK I (both eluting from DEAE-cellulose at low salt concentrations) can be excluded because fragmin is not phosphorylated by rabbit spleen CK I (a sample kindly provided by Prof. Dr. J. Goris, University of Leuven, Belgium) (data not shown).

In general, CK II enzymes from most organisms are composed of ±130 kDa heterotetramers, consisting either of an $\alpha_2\beta_2$ or an $\alpha\alpha'\beta_2$ subunit configuration (Hathaway & Traugh, 1982; Edelman et al., 1987; Pinna, 1990). In addition to the heterogeneity of the α subunit, a heterogeneity of the β subunit was reported recently (Bidwai et al., 1994). The existence of monomeric CK II enzymes has been reported in a few instances only. It was either based on genetic evidence from experiments in *Caenorhabditis elegans* (Hu & Rubin, 1990) or based on biochemical data from studies in plant cells (Dobrowolska et al., 1992). *Physarum* kinase 1 may represent another example of the monomeric CK II family. In contrast, *Physarum* kinase 2 belongs to the family of more common oligomeric CK II-type enzymes.

By reconstructing the fragmin 3D structure, using the structural information of the gelsolin S-1 fragment (McLaughlin et al., 1993), we are able to locate the phosphorylation site (DAAAGGSDLEA) in a region that interconnects domains 2 and 3 of fragmin. This region is not expected to coincide with actin-binding site(s), and this is in accordance with the finding that CK II phosphorylation does not dissociate the preformed actin−fragmin complex or block complex formation. In addition, neither phosphorylation of the actin subunit by the actin−fragmin kinase nor phosphorylation of the fragmin subunit by CK II is influenced when the other subunit was previously phosphorylated. Thus, both phosphorylation mechanisms, although acting on the same actin−fragmin dimer, seem to act independently of each

other. In the same line, binding of PIP_2 to phosphorylated fragmin or phosphorylation of the PIP_2−fragmin complex was not altered when compared to control experiments. These findings contrast with those described for profilin, where phosphorylation by protein kinase C (PKC) *in vitro* is specifically stimulated by PIP_2 (Hansson et al., 1988). Thus, phosphorylation of fragmin by CK II does not seem to affect its most conspicuous regulatory functions on the organization of actin microfilaments *in vitro*. This observation raises the interesting hypothesis that this modification could be a step in acquiring new, as yet unidentified functions. For instance, fragmin was reported to be also a target for the src-kinase (Maruta et al., 1984), but so far, little is known about how both phosphorylations affect each other or how tyrosine phosphorylation of fragmin affects its biological properties.

An additional argument for a defined function may come from preliminary studies on the dephosphorylation of fragmin, which occurs only in the presence of a highly specific phosphatase (unpublished results). This observation also suggests that fragmin phosphorylation is not a silent event.

Onada and Yin (1993) demonstrated phosphorylation of CapG, a protein which on the basis of size and sequence can be considered as the fragmin analogue of macrophages (Ampe & Vandekerckhove, 1987; Johnston et al., 1990). Gelsolin was not found to be phosphorylated. Neither the kinase nor the phosphorylation sites were identified, but at least four putative CK II and three PKC sites were predicted. Alignment of both sequences reveals little homology in the region surrounding the phosphorylated serine residue in fragmin. We also found that gelsolin is not phosphorylated by the *Physarum* CK IIs (not shown). The authors showed that phosphorylated forms of CapG were preferentially associated with nuclear preparations. A similar calcium/calmodulin-dependent protein kinase II phosphorylation-induced nuclear translocation also applies to cofilin (Ohta et al., 1989). It will be of interest to further investigate whether phosphorylation of fragmin is also associated with a similar intracellular redistribution.

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