Identification of a Cyclase-associated Protein (CAP) Homologue in *Dictyostelium discoideum* and Characterization of Its Interaction with Actin

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In a search for novel actin binding proteins in *Dictyostelium discoideum* we have isolated a cDNA clone coding for a protein of approximately 50 kDa that is highly homologous to the class of adenylyl cyclase-associated proteins (CAP). In *Saccharomyces cerevisiae* the amino-terminal part of CAP is involved in the regulation of the adenylyl cyclase whereas the loss of the carboxyl-terminal domain results in morphological and nutritional defects. To study the interaction of *Dictyostelium* CAP with actin, the complete protein and its amino-terminal and carboxyl-terminal domains were expressed in *Escherichia coli* and used in actin binding assays. CAP sequestered actin in a Ca^{2+} independent way. This activity was localized to the carboxyl-terminal domain. CAP and its carboxyl-terminal domain led to a fluorescence enhancement of pyrene-labeled G-actin up to 50% indicating a direct interaction, whereas the amino-terminal domain did not enhance. In polymerization as well as in viscometric assays the ability of the carboxyl-terminal domain to sequester actin and to prevent F-actin formation was approximately two times higher than that of intact CAP. The sequestering activity of full length CAP could be inhibited by phosphatidylinositol 4,5-bisphosphate (PIP_2), whereas the activity of the carboxyl-terminal domain alone was not influenced, suggesting that the amino-terminal half of the protein is required for the PIP_2 modulation of the CAP function. In profilin-minus cells the CAP concentration is increased by approximately 73%, indicating that CAP may compensate some profilin functions in vivo. In migrating *D. discoideum* cells CAP was enriched at anterior and posterior plasma membrane regions. Only a weak staining of the cytoplasm was observed. In chemotactically stimulated cells the protein was very prominent in leading fronts. The data suggest an involvement of *D. discoideum* CAP in microfilament reorganization near the plasma membrane in a PIP_2 -regulated manner.

INTRODUCTION

Actin binding proteins regulate and modulate the actin-based cytoskeleton of a cell. They are characterized by their ability and by the mode of interactions with G- or F-actin (Kreis and Vale, 1993; Stossel, 1993). The large number of F-actin binding proteins identified in various cells contrasts with

very few G-actin binding proteins. In *Dictyostelium discoideum* the profilins have been identified as members of this class (Haugwitz *et al.*, 1991, 1994). β -Thymosin-like proteins, which in mammalian cells are primarily responsible for stabilization of the G-actin pool, have not been encountered in *D. discoideum* (Safer and Nachmias, 1994; Haugwitz *et al.*, 1994). A novel G-actin-sequestering protein, ASP-56, recently isolated from porcine turned out to be a homologue of CAP from *Saccharomyces cerevisiae* (Gieselmann and Mann, 1992).

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CAP, a cyclase associated protein, was first described as the 70-kDa protein subunit of the yeast adenylyl cyclase complex (Field *et al.*, 1988). Knock-out mutants of *S. cerevisiae* had defects in signaling and altered cell shape indicating at least two functions of CAP (Fedor-Chaiken *et al.*, 1990; Field *et al.*, 1990). In a domain analysis the signaling defects were attributed to the amino-terminal one-third of CAP, which contains the putative adenylyl cyclase binding site (Gerst *et al.*, 1991). The morphological changes associated with an altered actin cytoskeleton and altered bud site selection could be reversed by expressing the carboxyl-terminal one-third of CAP in CAP-deficient cells. CAP has also been identified biochemically in *Schizosaccharomyces pombe* (Kawamukai *et al.*, 1992). CAP of *S. pombe* could only suppress the morphological and nutritional defects caused by the loss of the carboxyl-terminal domain of CAP in *S. cerevisiae*, but did not rescue the defects associated with deletion of the amino-terminal domain.

Furthermore CAP homologues in mammals have been identified via polymerase chain reaction (PCR) with degenerated primers aligned to the *S. cerevisiae* CAP (Matviw *et al.*, 1992; Vojtek and Cooper, 1993). All known homologues of CAP in human, mouse, and rat (MCH1) are about 37% identical to *S. cerevisiae* CAP and, like the yeast proteins, consist of two domains (Matviw *et al.*, 1992; Vojtek and Cooper, 1993; Zelicof *et al.*, 1993). Mammalian CAP proteins suppress the morphological defects in *S. cerevisiae* caused by the loss of the carboxyl-terminal domain of the endogenous CAP, but not the defects associated with the loss of the amino-terminal domain. These data suggest that the function of the carboxyl-domain of CAP is highly conserved during evolution. Recently a human CAP isoform has been isolated that is 65% identical to human CAP I (Yu *et al.*, 1994). A CAP homologue from hydra might be a mediator of nerve-cell determination and differentiation (Fenger *et al.*, 1994).

CAP deficiency could also be overcome by overexpression of profilin in *S. cerevisiae* (Vojtek *et al.*, 1991). Profilin binds to and sequesters G-actin, promotes the ADP/ATP-exchange on actin, and is a phosphatidylinositol 4,5-bisphosphate (PIP₂) binding protein (Theriot and Mitchison, 1993; Carlier and Pantaloni, 1994). Rescue of the cytoskeletal defects was furthermore achieved with profilin from *Acanthamoeba*. Interestingly only the isoform II that had a higher affinity for PIP₂ was active in this assay. This led to the speculation that the interaction of profilin II with polyphosphoinositides results in the rescue (Goldschmidt-Clermont and Janmey, 1991). Overexpression of a protein specified by the SNC1 gene locus of *S. cerevisiae* compensates the loss of the carboxyl-domain function of CAP under RAS2-activated conditions (Gerst *et al.*, 1992). The SNC1 locus encodes a homologue of the

vertebrate synaptic vesicle-associated membrane proteins or synaptobrevins. These highly conserved proteins are thought to play a role in vesicle fusion and neurotransmitter release.

In a search for novel actin binding proteins we identified a *Dictyostelium* cDNA that codes for a 50-kDa protein with about 40% identity to CAP from *S. cerevisiae*. We have expressed this protein and its amino-(N-) and carboxyl-terminal domains (C-terminal domain) in *E. coli* and analyzed their interaction with actin. *Dictyostelium* CAP sequesters G-actin. For this activity the C-terminal domain is sufficient labeling with specific antibodies localized CAP at polar regions of migrating cells.

MATERIALS AND METHODS

Isolation and Analysis of CAP cDNA

A cDNA-clone encoding *D. discoideum* CAP was isolated by differential screening of a λ gt11 cDNA library (Lacombe *et al.*, 1986) with polyclonal antibodies against severin. The cDNA was amplified by PCR using primers of the flanking regions of the λ gt11 phage, cloned into a pUC19 vector (Yanisch-Perron *et al.*, 1985), and sequenced by the chain termination method (Sanger *et al.*, 1977) using uni and reverse primers as well as sequence-specific oligonucleotide primers (19 to 21 mers). The sequence was analyzed using the University of Wisconsin GCG software, version 8.0/1994 (Devereux *et al.*, 1984). Sequences of different species were aligned with the program "Clustal" and compared with the program "bestfit."

Cloning, Expression, and Purification of CAP and Selected Domains in *E. coli*

The CAP cDNA containing the endogenous start and stop codons was cloned into the *Pst*I and *Bam*HI restriction sites of the pT7-7 expression vector (Tabor, 1990). *E. coli* BL21 harboring this plasmid were grown at 30°C to an A₆₀₀ of 0.9. For expression of the protein, IPTG was added (0.5 mM final concentration) and the cells were further incubated for 6 h at 30°C. They were harvested by a 10,000 × g spin, washed once in MEDA-buffer (10 mM morpholinopropane sulfonic acid, pH 7.0, 1 mM EGTA, 0.2 mM dithiothreitol [DTT], 0.02% Na₂S₂O₃), and resuspended in this buffer. After adding 1 mM benzamidine and 0.5 mM phenylmethylsulfonyl fluoride, the cells were lysed in a Parr-bomb (Parr Instrument Company, Moline, IL; 10 min, >14 MPa) and subsequently ultrasonified 5 times for 10 s. The lysate was centrifuged for 30 min at 40,000 × g. The supernatant containing the recombinant protein was loaded onto a DEAE-cellulose column (DE-52; Whatman, Maidstone, UK) equilibrated in the same buffer. Proteins were eluted by a linear gradient of 0 to 300 mM NaCl. CAP eluted at about 100 mM salt. The fractions containing CAP protein were dialyzed against MEDA-buffer, concentrated in a Centriprep-30 concentrator (Amicon, Beverly, MA), and then purified by a Sephacryl S300 gel filtration column (110 × 1.7 cm) (Pharmacia, Freiburg, Germany). The protein was concentrated in Centriprep to ~1.5 mg/ml. Aliquots could be stored at -70°C for several months without losing activity. The N-terminal domain of CAP encompassing amino acid residues (aa) 1 to 215 was amplified by PCR using the CAP cDNA as template. Primers with "add on" sequences containing restriction sites allowed the cloning of the fragment into the pT7-7 vector without additional amino acids. Expression and lysis of the bacteria was done as described for CAP. After centrifugation of the lysate the N-terminal domain remained in the pellet. The pellet was first extracted with 1 M urea and then with 4 M urea in TEDA-buffer (10 mM Tris/HCl, pH 8.7, 1 mM EGTA, 1 mM DTT, 0.02% Na₂S₂O₃). The N-terminal domain solubi-

lized by 4 M urea was applied to a cation exchange column (QA52; Whatman) and eluted with a linear NaCl gradient (0–300 mM NaCl) between 50 and 100 mM NaCl. The fractions containing the N-terminal domain were dialyzed against G-buffer (2 mM Tris/HCl, pH 8.0, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM CaCl₂) and concentrated on Centricon-10 (Amicon). The C-terminal domain of CAP encompassing the last 210 aa of CAP was amplified by PCR and cloned into the pT7-7 expression vector using the ATG of the *Nde*I-site as start codon. The C-terminal domain was purified similar to CAP, except that MEDA-buffer was replaced by MES-buffer, pH 6.7 (10 mM 2-morpholinoethanesulfonic acid, 1 mM EGTA, 0.2 mM DTT, 0.02% NaN₃). The C-terminal domain was dialyzed against G-buffer and concentrated with Centrprep-10.

Antibody Production

For monoclonal antibody (mAb) production BALB/c mice were immunized with purified recombinant CAP or N-terminal domain using Alugel S (Serva, Heidelberg, Germany). Using PAIB3AG8I myeloma lines, fusion was performed 2 days after the last boost essentially as described previously (Schleicher *et al.*, 1984). Hybridomas were screened for their ability to recognize CAP in *D. discoideum* extracts on Western blots. The binding regions of the antibodies were localized more precisely using recombinant CAP domains. The antibodies designated as 223–391-1, 223–445-1, and 230–18-8 were used in these studies. Polyclonal antisera were raised in rabbits by subcutaneous injection of purified recombinant CAP according to the method of André *et al.* (1989).

Actin Purification and Labeling

Actin was prepared from rabbit skeletal muscle as described (Spudich and Watt, 1971) and subjected to a S300 gel filtration. It was labeled with *N*-(1-pyrenyl)iodoacetamide (Kouyama and Mihashi, 1981) following the modifications of Cooper *et al.* (1983). Aliquots were frozen in liquid nitrogen and stored at –70°C. Actin or pyrene-labeled actin were centrifuged before use for 10 min at 15,000 × *g* to remove precipitates.

Low-Shear Viscometry

Viscometry was performed in a falling ball viscometer at 25°C (MacLean-Fletcher and Pollard, 1980). Polymerization (150 µl volume) was started by addition of 5 µM G-actin to buffered MgCl₂ (buffer A, final concentration: 2 mM MgCl₂, 10 mM imidazole, pH 7.4, 1 mM ATP, and 0.2 mM CaCl₂ or 1 mM EGTA). In parallel, experiments were done under high salt conditions using buffer A plus 100 mM KCl. The viscosity of an actin solution polymerized for 30 min was set to 100%.

Fluorescence Spectroscopy

Interaction of CAP and its domains with pyrene-labeled G-actin was determined in a luminescence spectrometer (model LS50B, Perkin-Elmer, Beaconsfield, Buckinghamshire, UK) as a fluorescence enhancement under nonpolymerizing conditions (G-buffer with 0.2 mM CaCl₂ or 1 mM EGTA) at 25°C (Lee *et al.*, 1988; Eichinger and Schleicher, 1992). Emission was measured at an excitation wavelength of 345 nm; excitation spectra were obtained at an emission wavelength of 385 nm. The kinetic measurements were performed at an excitation wavelength of 345 nm and an emission wavelength of 385 nm always using the same slit widths. Five micromolar G-actin containing 10% pyrene-labeled actin was induced to polymerize by adding buffer A in the presence or absence of CAP or its C-terminal domain. For quantitative calculations CAP or its C-terminal domain were taken as monomers. Aliquots of 400 µl were measured at 25°C, and data were plotted as fluorescence increase in arbitrary units.

Immunofluorescence Microscopy and Image Analysis

D. discoideum cells of various developmental stages were allowed to adhere onto glass coverslips for 30 min, fixed with methanol for 10 min at –20°C or with picric acid and paraformaldehyde for 30 min at room temperature (Jungbluth *et al.*, 1994), and processed as described by Weiner *et al.* (1993). Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) diluted 1:200, Cy3-labeled goat anti-mouse IgG, or Cy3-labeled goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Avondale, PA) diluted 1:2,000, were used as second antibodies. Profilin I was detected with mAb 153–246-10 (Haugwitz *et al.*, 1991). Actin was labeled with the mAb described by Simpson *et al.* (1984).

The cells embedded in gelvatol (Langanger *et al.*, 1983) were examined with a Zeiss Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany). Confocal laser microscopy was performed using a Zeiss LSM-410 microscope (Zeiss, Oberkochen, Germany). For excitation, a green HeNe laser (Zeiss) was used for Cy3 at a wavelength of 543 nm and an Argon-Ion laser at a wavelength of 488 nm for FITC. Emitted light was separated for Cy3 by a low pass filter (LP 570, Zeiss) and for FITC by a band pass filter (BP 510–525, Zeiss). The number of optical sections taken at distances of 100 or 300 nm varied between 20 and 30 per cell. For data analysis AVS 5.01 system (Advanced Visual Systems, Waltham, MA) was run on a DEC alpha (Digital Equipment Corporation, NH).

Chemotactic Stimulation

Chemotaxis experiments were performed essentially as described by Gerisch and Keller (1981). The cells, developed for 6 h, were transferred onto glass coverslips with an engraved grid (CELLocate; Eppendorf, Hamburg, Germany) to relocate cells after fixation (Kreitmeyer *et al.*, 1995). Photographs were taken on an Axiovert microscope (Carl Zeiss). Micropipettes filled with 10^{–4} M cAMP were used to stimulate the cells. The migrating cells were fixed with methanol (–20°C), and labeled with mAb 223–391-1.

Miscellaneous Methods

D. discoideum AX2 and profilin I/II-minus mutants (Haugwitz *et al.*, 1994) were cultivated in nutrient medium as described by Watts and Ashworth (1970) in suspension culture at 21°C. To induce development, cells were washed twice in 17 mM Soerensen phosphate buffer, pH 6.0 (Malchow *et al.*, 1972), resuspended at a density of 10⁷ cells/ml in the buffer, and starved for 6 h. Development on filters was as described by Noegel *et al.* (1985). DNA and RNA were analyzed according to the method of Sambrook *et al.* (1989). SDS-PAGE was performed according to the method of Laemmli (1970) and immunoblotting was performed using the method of Towbin *et al.* (1979). Actin was determined at A₂₉₀ (Wegner, 1976); other proteins were determined by the method of Bradford (1976) using bovine serum albumin as a standard. PIP₂ (Sigma Chemical Company, St. Louis, MO) was dissolved in G-buffer according to the method of Haus *et al.* (1991).

RESULTS

Isolation of the *D. discoideum* CAP Gene and Sequence Analysis

In a search for novel actin binding proteins we have used a polyclonal antibody generated against severin to screen a λgt11 expression library. This antibody strongly cross-reacted with a protein of an apparent MW of 50,000. Several cDNA clones were isolated that reacted with the polyclonal antibody but not with several mAbs directed against severin. The largest clone harbored an *Eco*RI fragment of 1600 bp. This

fragment contained one open reading frame starting in position 66 of the sequence with an ATG that codes for 464 amino acids. The deduced protein sequence was closely related to members of the CAP family. The *D. discoideum* CAP has 39% identity and 61% similarity to the *S. cerevisiae* protein (Figure 1) (Field *et al.*, 1988, 1990). Other CAP homologues known from *S. pombe* (Kawamukai *et al.*, 1992), mammals (Matviw *et al.*, 1992; Vojtek and Cooper, 1993; Zelicof *et al.*, 1993), and *Chlorohydra viridissima* (Fenger *et al.*, 1994) are related to each other to a similar extent. All known CAP proteins carry an extended polyproline stretch dividing the protein into two domains. In the *D. discoideum* protein this motif is not so prominent; it is replaced by an amino acid stretch between residues 215 and 255 consisting of 20% proline residues (Figure 2).

Full length CAP as well as the N-terminal and the C-terminal domains as defined by Gerst *et al.* (1992)

were expressed in *E. coli* (Figures 2 and 3). The proline-rich part between residues 215 to 255 was not included. Full length CAP and the N-terminal domain were used to immunize mice for the production of mAb (Figure 3).

Actin Binding Properties of *D. discoideum* CAP and Its Domains

Interaction of *D. discoideum* CAP and its domains with G-actin was investigated by analyzing the fluorescence enhancement of 300 nM pyrene-labeled G-actin (Lee and Pollard, 1988). CAP led to an emission enhancement up to 50% with respect to the G-actin spectra, indicating an interaction of CAP and G-actin (Figure 4A). The C-terminal domain of CAP enhanced the fluorescence in the presence of labeled G-actin by 35%. The N-terminal domain

Dd	1	MSEAT-----IVE	LLKRLDQAT	TRLEAV-----	EKS	IASGVASSSSSSSS
Hs	1	MAD-----	MON	VERLERAVGRLEAV-----	SH--	TSDMHRGYADSP
Sc	1	MPD	SKYTMQGYNLV	KLLKRLEE	ATARLEDV	TIYQEGYIQNKLEASKNNKP
Dd	40	PSSGAAGPSSA-----			SVKE	FONLVDQHITPFVALSKKLA
Hs	36	SKAGAA-----	P-----		YVQAF	DSLLAGPVAEYLKISKETIG
Sc	51	SDSGADANTTNEPSA	ENAPEVEQDPKCITAFQSY	IGENIDPLVELSGKID		
Dd	75	PEVGNQVEQLVKA	IDA	EKALINTASQSKKP--	SOETLLE	LILIKPLNNFAAE
Hs	67	GDVQKHAEMVHT	GLKLERALLVTASQ	CCQP--	AENKLS	DLIAPIS
Sc	101	TVVLDALQLLKG	GFQSQ	LTFLRAAVRSR	KEDYSSQ	TFADSLRPINENI
Dd	123	VGKIRDSNRS	SKFFNLSAISES	IGFLSWVVEPTPGPHVAEMRGS	AEFY	
Hs	115	VITFREKNR	GSKLENHLSAVSESIQAL	GWAMAPKPGPYVKEMND	AMFY	
Sc	151	LGQLKESNR	QSKYFAYLSALSEGAP	LFWSVAVD--	TPVSMV	TDFKDAQQFW
Dd	173	TNRILKEFKG	VNQDQVDWVSNV	NFLKDL	EKYIKQYHTTGLTWN	PKG---
Hs	165	TNRVLKEYK	DVDKKHVDWVKAYLSI	WTELQAYIKE	FHTTGLAWSK	TG---
Sc	200	TNRILKEYR	ESDPNAV	EWVKKFLASFDNL	KAYIKE	FHTTGVSWKKDGMDF
Dd	220	----GD	AK-----	SATPAPASS--	APAA	PVAPAVS--STPVESKKGP---
Hs	212	----PV	AKELSGLP	SGPSAGSG--	PPPPPP	PGPPPPPVSTSSGSDESA---
Sc	250	ADAMAQ	STKNTGATSS	SPSPASATAAP	APPPPP	PPAPPASVFEISNDT
Dd	254	-----	GLGAVEGELSK	GDGVTSG	LKKVTNDMKS	KN--FTDKSSVVKAA
Hs	253	-----	SRSALFAQINQ	GESITHALKE	VSDDMKTHKNP	ALKAQSGPVRSG
Sc	300	SDANKG	GIGAVFAELNQ	GENITKGLKKV	DKSQQTHKNPE	LROSSTVSSTG
Dd	295	-----	DTKVAKVDA	PSR----	PAVFALQGNKWS	IEYQVNNKEIVIAEPD
Hs	297	PKPFSAP	KPQTSPSP	KRATKKEPAVLE	LEGKWRVENQ	ENVSNLVIEDTE
Sc	350	SKSGPP	PRPKK---	PSTLKT	KRPKRKELV	GNKWFIENYENETESLVIDAN
Dd	335	SRQTVY	IFQCVNSLVQIKG	VNAITLDGCKKTS	IVFENAISS	CEVVCNCG
Hs	347	LKQVAY	IYKCVNTTLQIKG	KINSITVDNCKKLGLV	FDVVGIVET	INSKD
Sc	397	KDESIF	IGKCSQVLVQIKG	VNAISLS	SETESC	SVVLDSSISGMDVIRSNK
Dd	385	VEIQVT	GRVPSIAIDKTS	GCQIYLSKDSLE	TEIVSSKSSEM	NVLIPGATE
Hs	397	VKVQVM	GKVPITISIN	KTDGCHAYLSKNSL	DCEIVSAKSSEM	NVLIP--TE
Sc	447	FGIQV	NHSLPQISIDKSD	GGNIYLSKESL	NTEIYTS	CSTAINVNLP-IGE
Dd	435	NDDL	VELAIP	EQYKTSVKGN	KLHTEST	SHI-
Hs	445	GGDF	NEFPVPEQ	FKTLWNGOKL	VTTVTET	AG
Sc	496	DDDY	VEFPIPEQ	MKHSFADG	KFKSAV	FEHAG

Figure 1. Multiple alignment using the program "Clustal" (GCG package) of the CAP amino acid sequences from *Dictyostelium discoideum* (Dd), *Homo sapiens* (Hs) (Matviw *et al.*, 1992), and *Saccharomyces cerevisiae* (Sc) (Field *et al.*, 1990). Identical amino acids are boxed. *Dictyostelium* CAP exhibits approximately 40% identity and 60% similarity to CAP from human and yeast. The accession number for this sequence is U43027.

Figure 2. Schematic presentation of the *Dictyostelium* CAP domain structure following the domain classification of *S. cerevisiae* by Gerst *et al.* (1992). The amino-terminal domain encompassing residues 1–215 is separated by the proline-rich region (shown above the drawing) from the carboxyl-domain encompassing residues 255 to the end. Amino-terminal, carboxyl-terminal domain and the complete CAP were expressed in *E. coli* using the pT7–7 expression vector. The monoclonal antibodies (mAb) specific for the different domains are indicated below.

added at a 1:1 molar ratio to G-actin did not increase the fluorescence (Figure 4A). At increasing concentrations of CAP or C-terminal domain, the fluorescence enhancement saturated (Figure 4B). With complete CAP, maximal fluorescence enhancement was achieved at a molar ratio of about 1:1 (CAP to G-actin), whereas the C-terminal domain did not show such a distinct level of saturation. The measured fluorescence increase in Ca^{2+} and in EGTA-buffer were similar, indicating that neither the interaction of CAP nor of the C-terminal domain with G-actin is Ca^{2+} sensitive. Bovine serum albumin as a control did not enhance the fluorescence.

The effect of CAP proteins on actin polymerization was studied in assays using 10% pyrene-labeled actin in a Mg^{2+} -buffer containing 1 mM ATP (Figure 5, A

and B). During the polymerization of G- to F-actin the fluorescence emission of pyrene increases up to 20-fold thus allowing for monitoring of the actin elongation in a very sensitive way (Kouyama and Mihashi, 1981). CAP reduced the rate of actin elongation in a dose-dependent manner (Figure 5A). At a 1:1 ratio of CAP to actin monomers the polymerization was completely inhibited during the time assayed. The C-terminal domain inhibited the actin polymerization even at a ratio of 1:2 (Figure 5B). CAP and C-terminal domain prevented actin polymerization also in the presence of 1 mM EGTA, ruling out a Ca^{2+} dependence. The inhibition of actin polymerization by CAP was only marginally affected by the addition of high salt (100 mM KCl).

To investigate the interaction of CAP and C-terminal domain with polymerized actin, the protein was added to F-actin previously polymerized for 60 min and carefully mixed. A net depolymerization could be observed leading to a 50% decrease in fluorescence after 60 min (Figure 5C). The rate of depolymerization measured with CAP was not as high as the one observed with the C-terminal domain.

The effect of CAP and its C-terminal domain on actin polymerization was also observed in low-shear viscometry (MacLean-Fletcher and Pollard, 1980). When actin was polymerized in the presence of CAP the viscosity measured was reduced in a dose-dependent manner. Using molar ratios of one CAP molecule to two actin monomers the relative viscosity was decreased to 10% of the control viscosity even in the presence of 100 mM KCl. The formation of actin gels is thus efficiently inhibited by CAP, suggesting a sequestering activity. As observed in the fluorescence assays, viscometry revealed an increased activity of the C-terminal domain and the activities of CAP and its C-terminal domain did not depend on Ca^{2+} .

Figure 3. Expression of CAP domains in *E. coli*, specificity of the mAbs, and presence of CAP in growing and developing *D. discoideum* cells. Purified recombinant proteins were separated on SDS-PAGE and stained with Coomassie blue (lane 1, CAP; lane 2, N-terminal domain; lane 3, C-terminal domain; 12% acrylamide). Total cell homogenates of growing (lane 4) and developing (lane 5) *Dictyostelium* cells (2×10^5) were subjected to SDS-PAGE and the resulting Western blot was probed with mAb 223–445-1. Western blot of total homogenates of *E. coli* cells expressing complete CAP is shown in lane 6, the N-terminal domain in lane 7, and the C-terminal domain in lane 8. For CAP and the C-terminal domain, mAb 223–445-1 was used; for the N-terminal domain, mAb 230–18-8 was used. The sizes in kDa of the molecular mass markers are shown on the left.

The Interaction of CAP with Actin Is PIP_2 Regulated

Different concentrations of PIP_2 were tested in actin polymerization assays. CAP and PIP_2 were mixed, incubated for 1 min, and the polymerization was started by adding G-actin. At concentrations of 40 μM

Figure 4. (A) Fluorescence enhancement of pyrene-labeled G-actin (300 nM) upon binding of recombinant CAP and its domains. The proteins were used in a 1:1 molar ratio to actin. The excitation scans (320–360 nm) were recorded at an emission wavelength of 385 nm, and the emission scans (370–420 nm) were recorded with excitation of 345 nm and corrected for fluorescence of buffer and CAP-protein alone. (B) Increase of the pyrene-labeled G-actin fluorescence upon the interaction with increasing amounts of CAP or C-terminal domain. G-actin (300 nM) was recorded at an excitation wavelength of 345 and an emission of 385 nm.

or less, PIP₂ did not interfere with the actin-sequestering activity of CAP (Figure 6A). Higher concentrations of PIP₂ led to a decrease of its sequestering activity as indicated by increased fluorescence due to actin polymerization. At a molar ratio of CAP to PIP₂ of 1:40, CAP no longer interacted with actin and polymerization was indistinguishable from control curves measured with actin and PIP₂ alone. The sequestering activity of the C-terminal domain was not inhibited even at high concentrations of PIP₂ (Figure 6B). This result indicates that for PIP₂ binding the N-terminal

half or the proline-rich region of the molecule is required.

Overexpression of CAP in Profilin I/II-Minus Cells

To test whether CAP expression is altered in profilin-minus cells, we determined the amount of CAP mRNA and CAP protein in this mutant. A quantitative evaluation of Northern and Western blots showed that the mRNA level is increased by 60% and the protein concentration by approximately 73% (Figure

Figure 5. Polymerization of G-actin (5 μ M) as measured by a fluorescence increase in the presence of different concentrations of CAP (A) and C-terminal domain (B). (C) Depolymerization of polymerized pyrene-labeled F-actin induced by addition (arrow) of the C-terminal domain in a 1:1 molar ratio to actin. Addition of buffer alone leads to a dilution and disturbance of the sample.

Figure 6. PIP₂ sensitivity of CAP. (A) PIP₂ inhibits the sequestering activity of CAP. CAP (2.5 μ M) was preincubated with increasing PIP₂ concentrations for 1 min and then added to the polymerization assay (5 μ M actin). (B) PIP₂, at concentrations of 100 μ M that inhibited the activity of full length CAP almost completely, did not have any effect on the activity of the C-terminal domain.

7). These data suggest that the lack of G-actin sequestering profilins triggers an overexpression of CAP.

CAP Expression during the *D. discoideum* Life Cycle

Starvation of *D. discoideum* initiates a developmental cycle that culminates in the formation of fruiting bodies. The morphological changes are paralleled by changes in the expression pattern of various gene products. To examine whether the mRNA of CAP is continuously present we analyzed RNA isolated from

distinct developmental stages. CAP cDNA recognized a single mRNA species of about 1.6 kb that was present in nearly unaltered amounts during all stages of development (Figure 8). At the protein level equal amounts of CAP were found in growing and developing cells with mAbs. Using known amounts of recombinant CAP as a reference, we estimated the cellular concentration of CAP to be 5 μ M.

Subcellular Localization of CAP

D. discoideum cells that are allowed to settle onto coverslips adhere and begin to migrate by extending pseudopodia. In such cells CAP is specifically enriched in peripheral regions (Figure 9, C, F, and G). Usually no continuous labeling of the plasma membrane was observed. The cytoplasm showed a weak overall staining of CAP. Thin filopodia of migrating amoebae always contained CAP. Confocal microscopy showed that the regions where the cells attach to the substratum were not enriched in CAP. Cells fixed with picric acid/paraformaldehyde showed a similar distribution. Aggregation-competent *D. discoideum* cells that are starved for 6 h exhibit an elongated cell shape. In these cells CAP staining was very prominent at the anterior and posterior regions of the plasma membrane (Figure 9C). In contrast, profilin localization

Figure 7. Expression of CAP in profilin-minus mutants. AX2 wild-type and profilin-minus cells (pI/II-) were assayed in Northern (left) and Western blot (right) analysis for overexpression of CAP. The relative concentrations of specific mRNA and protein were determined by densitometry. The protein determinations represent the average value of 20 measurements.

Figure 8. Northern blot analysis of total RNA (10 μ g per lane) of *D. discoideum* strain AX2 isolated at different developmental stages indicated in hours. At time 0 the cells were harvested, washed, and deposited on nitrocellulose filters (t12, aggregation; t18, culmination). The blot was probed with the full length CAP cDNA. The mRNA of CAP is present at all developmental stages.

Figure 9. Immunolocalization of CAP to polar regions of *Dictyostelium* cells and colocalization of actin and CAP. *Dictyostelium* cells starved for 6 h (A–C) or growing cells (D–H) were fixed with methanol. Indirect immunofluorescence was performed with mAb against *D. discoideum* actin (Simpson *et al.*, 1984) and an FITC-labeled goat anti-mouse IgG (B and E). CAP was detected by a polyclonal sera and a Cy3-labeled goat anti-rabbit IgG (C, F, and G). (A and D) Corresponding phase-contrast images. Double-immunofluorescence of CAP (G) and profilin I (H) revealed a different localization of both G-actin-sequestering proteins. Profilin was detected with specific mAb 153-246-10 (Haugwitz *et al.*, 1991) and an FITC-labeled goat anti-mouse IgG. Pictures in panels G and H were taken by confocal microscopy. Bars, 10 μ m.

differed profoundly from CAP (Figure 9H). Profilins were distributed all over the cell in a nearly homogeneous way and did not show any accumulation near the plasma membrane whereas CAP was mostly found in these regions. Double immunolocalization of actin and CAP revealed that CAP colocalized with actin especially in highly motile areas at the leading edge and in cortical regions of the cell (Figure 9, B and C, E and F).

The polarity and the direction of movement of aggregation-competent *Dictyostelium* cells can be manipulated by local stimulation with cAMP through micropipettes (Gerisch *et al.*, 1975). Seconds after stimulation the cells reorient toward the tip of the micropipette. To follow CAP distribution during che-

motactic movement, cells were recorded at intervals before and during stimulation and then immediately fixed to observe whether CAP is redistributed. In Figure 10 a typical experiment is shown. In the chemotactically responding cells we find that CAP is predominantly localized in the newly formed pseudopod.

DISCUSSION

The link between signal transduction and subsequent alterations in the cytoskeleton is a central theme in cytoskeleton research. A protein that may provide this link is the cyclase-associated protein CAP, first found in *S. cerevisiae* (Field *et al.*, 1988). We have identified the CAP-homologue from *D. discoideum* and investi-

gated its properties. In vitro *D. discoideum* CAP acts as a G-actin-sequestering protein in a PIP_2 -sensitive manner. In the cells it could be localized to regions near the plasma membrane where actin also accumulates.

A comparison of the amino acid sequence of *Dictyostelium* CAP to the ones of the other members of this protein family revealed identities of about 40% and similarities of 60%. The N- and the C-terminal domains of all CAP homologues are separated by one (*S. cerevisiae*, *S. pombe*) or two polyproline stretches (*Chlorohydra viridissima*, mammalian CAPs), which have been discussed as SH3 protein binding motif (Freeman *et al.*, 1995). In *Dictyostelium* CAP the polyproline stretches are replaced by a proline-rich sequence that might still have the potential to bind to SH3 domains, because the sequence APASSAAPV (position 228–238) resembles the SH3 consensus binding sequence XPXXPPYXPX (Y indicates a hydrophobic residue; prolines 2, 7, and 10 are essential) as analyzed for the SH3 domain of Abl (Ren *et al.*, 1993; Williamson, 1994). A second feature of the CAP sequence is a conserved amino acid repeat in the N-terminal domain of all CAP proteins (Vojtek and Cooper, 1993). This repeat is localized in *S. cerevisiae* at positions 19–21 and 26–28 and has the potential to form an amphipathic helix, which has been implicated in protein-protein interactions (Cohen and Parry, 1986). This region, which in *Dictyostelium* CAP is altered to RLD/RLE (positions 12–14 and 19–21), has been suggested to function as adenylyl cyclase binding motif (Fenger *et al.*, 1994).

The G-actin sequestering activity of *Dictyostelium* CAP could be attributed to the C-terminal domain, because when comparing the N- and C-terminal domains separately, only the C-terminal domain was able to enhance the fluorescence of pyrene-labeled G-actin. The C-terminal domain also inhibited actin polymerization, and reduced the viscosity of an actin solution with even higher efficiency than the whole protein. This indicates a modulation of the sequestering characteristics of CAP by the N-terminal domain. An actin monomer binding activity was also recently reported for yeast CAP (Freemann *et al.*, 1995).

We have found that PIP_2 can regulate the binding of *Dictyostelium* CAP to G-actin and that a putative PIP_2

Figure 10 (cont). Enrichment of CAP at the leading edges of chemotactically stimulated *Dictyostelium* cells. Aggregation-competent cells migrate toward the tip of the micropipette filled with the chemoattractant cAMP (10^{-4} M). Photographs were taken 2 (A), 60 (B), and 120 s (C) after insertion of the pipette. Thereafter the cells were immediately methanol fixed and labeled with mAb 223-391-1 and Cy3 labeled goat anti-mouse IgG (E). The corresponding phase-contrast is shown in panel D. The cells, which migrated toward the cAMP source, show an accumulation of CAP in the regions of the leading edges. The cell on the right side of the photograph did not react to the cAMP stimulus. Although the cell is rather faintly labeled, it can be seen that CAP is not restricted to the leading edge.

Figure 10.

binding site could be localized to the N-terminal half of the protein. Other actin binding proteins from *Dictyostelium* like the F-actin capping proteins protovillin (Hofmann *et al.*, 1992), cap32/34 (Haus *et al.*, 1991), and the actin filament-fragmenting protein severin (Yin *et al.*, 1990; Eichinger and Schleicher, 1992) were similarly characterized as PIP₂-sensitive actin binding proteins. It has been suggested that PIP₂ acts in an inhibitory way, and increased PIP₂ concentrations in the cell could transiently stimulate actin polymerization predominantly near the plasma membrane by preventing the action of proteins like cap 32/34 and severin. In parallel the profilins, inactivated by increased PIP₂ concentrations, will release monomeric actin that is then available for polymerization (Stossel, 1989; Ridley, 1994). The regulation of CAP by PIP₂ would fit well into this cooperative mechanism of regulating the dynamics of the actin cytoskeleton.

CAP is enriched at peripheral regions of *Dictyostelium* cells; a continuous plasma membrane stain is, however, not observed. Colocalization studies revealed that in regions where actin was accumulated, CAP was enriched too. However, in experiments in which developing cells were lysed with Triton X-100 after stimulation with cAMP and fractionated into Triton-insoluble and Triton-soluble material, we observed that CAP was nearly exclusively present in the supernatant and not in the Triton-insoluble pellet. These findings can be reconciled by assuming that the distribution of CAP in membrane near regions reflects the pattern of adenylyl cyclase distribution. However, up to now there are no immunolocalization data available on *D. discoideum* adenylyl cyclase. In aggregation-competent cells, CAP was enriched at polar regions and the cytoplasmic stain was reduced. The mRNA corresponding to the *Dictyostelium* adenylyl cyclase increases during aggregation, which could result in higher concentration of adenylyl cyclase at the plasma membrane (Pitt *et al.*, 1992). The increased accumulations of CAP near the membrane could then be due to increased levels of adenylyl cyclase in these cells. Localization data from mouse fibroblasts using an epitope-tagged CAP showed a cytoplasmic distribution. In migrating cells the fusion protein could be seen at the leading edge but never at posterior regions (Vojtek and Cooper, 1993). We observed an obvious enrichment of CAP at leading edges in chemotactic-stimulated aggregation-competent *Dictyostelium* cells, which migrate toward micropipettes filled with cAMP.

In contrast to the *Dictyostelium* profilins that in growing cells are not significantly concentrated at the membrane and rather show a cytoplasmic distribution, the distribution of CAP appears to be spatially restricted, suggesting that both proteins are involved in different aspects of cytoskeletal rearrangements. This is also indicated by the abundance of profilin and

CAP. The overall concentrations of profilins in wild-type *Dictyostelium* cells (AX2) were found to be about 19 μ M for isoform I and 28 μ M for isoform II (Haugwitz *et al.*, 1994). We estimated a cellular concentration of about 5 μ M for CAP. *Dictyostelium* mutants lacking both profilin isoforms still contain about 55 μ M unpolymerized actin (Haugwitz *et al.*, 1994) and therefore the abundance of CAP cannot account for the remaining G-actin. However, locally CAP could reach concentrations near the plasma membrane high enough to act as a major sequestering protein in microcompartments around adenylyl cyclase complexes.

A further indication that the actin-binding function of CAP might play a role in vivo was its overexpression in profilin-minus cells. A complete lack of profilin results in significantly aberrant cytokinesis, growth, and development (Haugwitz *et al.*, 1994). These defects most likely are caused by the reduced ability of the mutant cell to sequester actin. It is tempting to speculate that the profilin-minus mutant tries to overcome the lack of G-actin sequestering profilin by overexpression of other G-actin binding proteins like CAP. Although the increase of CAP RNA and protein is significant, the total amount cannot substitute the lack of the abundant profilin isoforms.

In *D. discoideum* amoebae, cAMP works as an important signaling molecule involved in coordinated cell movement and cell-type differentiation. Extracellular cAMP binds to the cAR receptor, and signaling by the $\beta\gamma$ -subunit of the G protein is necessary for the activation of adenylyl cyclase (Devreotes, 1994). Moreover, a mitogen-activating kinase kinase (ERK2) and a cytosolic regulator of adenylyl cyclase are involved in the regulation of adenylyl cyclase (Lilly and Devreotes, 1994; Segall *et al.*, 1995). With the help of various mutants recently described and genetic alteration of CAP in *Dictyostelium*, it might be possible to identify the role of CAP in these pathways.

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