The cyclase associated protein CAP as regulator of cell polarity and cAMP signaling in *Dictyostelium*

Angelika A. Noegel¹, Rosemarie Blau-Wasser¹, Hameeda Sultana¹, Rolf Müller¹, Lars Israel²,
Michael Schleicher²*, Hitesh Patel³, Cornelis J. Weijer³

¹)Center for Biochemistry, Medical Faculty, University of Cologne, 50931 Köln, Germany,
²)Institute of Cell Biology, Ludwig-Maximilians-Universität, 80336 München, Germany,
³)School of Life Sciences, Division of Cell and Developmental Biology, University of Dundee, Wellcome Trust Biocentre, Dow Street, Dundee, DD1 5EH, UK

Running title: CAP in *Dictyostelium*

*Corresponding author: Michael Schleicher
Institut für Zellbiologie
Ludwig-Maximilians-Universität
Schillerstr. 42
80336 München, Germany

Telephone: +49 89 5996 876
Fax: +49 89 5996 876
email: schleicher@lrz.uni-muenchen.de

**Key words:** adenylyl cyclase/cAMP relay/F-actin/cell migration/phototaxis/cGMP
ABSTRACT

CAP (cyclase associated protein) is an evolutionarily conserved regulator of the G-actin/F-actin ratio and, in yeast, is involved in regulating the adenylyl cyclase activity. We show that cell polarization, F-actin organization and phototaxis are altered in a Dictyostelium CAP knockout mutant. Furthermore, in complementation assays we determined the roles of the individual domains in signaling and regulation of the actin cytoskeleton. We studied in detail the adenylyl cyclase activity and found that the mutant cells have normal levels of the aggregation phase specific adenylyl cyclase (ACA) and that receptor mediated activation is intact. However, cAMP relay which is responsible for the generation of propagating cAMP waves that control the chemotactic aggregation of starving Dictyostelium cells was altered, and the cAMP induced cGMP production was significantly reduced. The data suggest an interaction of CAP with adenylyl cyclase in Dictyostelium and an influence on signaling pathways directly as well as through its function as a regulatory component of the cytoskeleton.

INTRODUCTION

CAP/ASP56, a regulator of the F-actin/G-actin ratio has been identified in many species (Hubberstey and Mottillo, 2002). In yeast, CAP/Srv2 was identified as an adenylyl cyclase associated protein by a biochemical and a genetic approach (Fedor-Chaiken et al., 1990; Field et al., 1990). Mutations in CAP affected the regulation of the adenylyl cyclase and the cytoskeleton. These characteristics led to the suggestion that CAP is a bifunctional protein with roles in signaling and regulation of the cytoskeleton which have been attributed to individual domains of the protein (Goldschmidt-Clermont and Janmey, 1991). The amino terminal region contains the adenylyl cyclase binding site (Nishida et al., 1998). The overall structure of this domain consists of an α-helix bundle composed of six antiparallel helices.
(Ksiazek et al., 2003). It is followed by a proline rich region which interacts with proteins containing an SH3 domain, whereas the C-terminus is responsible for binding to monomeric actin (Freeman et al., 1995; Gottwald et al., 1996; Wesp et al., 1997). The Drosophila homologue was identified in two independent screens. Benlali et al. (2000) identified CAP in a screen for mutations that disrupted eye development by increasing the F-actin levels and inducing premature photoreceptor differentiation. Baum et al. (2000) discovered CAP when searching for mutations that perturbed actin organization. CAP preferentially accumulated in the oocyte where it inhibited actin polymerization resulting in a loss of asymmetric distribution of mRNA determinants within the oocyte.

Cell polarization is defined as an asymmetry of cell shape and cellular functions which is stable for some time and requires localized signaling, directed cytoskeletal rearrangements and distinct recruitments of proteins and supramolecular complexes (for reviews see Bretscher, 2003; Nelson, 2003). During growth Dictyostelium cells do not display a fixed polarity. They constantly change their shape and form new ends in response to external signals, which target them toward a food source. However, after the onset of starvation periodic signals of the chemoattractant cAMP lead to polarization of the cells and initiate the development into a multicellular organism. In response to cAMP proteins such as the cytosolic regulator of adenylyl cyclase (CRAC), a PH-domain containing protein, and protein kinase B (PKB) associate temporarily with newly formed polarized regions of the cell and help to initiate extension of pseudopods (Parent et al., 1998; Meili et al., 1999; Corner and Parent, 2002). This process requires distinct signaling molecules, the chemotactic machinery as well as components of the cytoskeleton such as CAP which in Dictyostelium is involved in actin cytoskeleton rearrangements and relocates quickly to newly extending pseudopods upon a cAMP stimulus (Gottwald et al., 1996).

CAMP signaling is essential for the chemotactic aggregation of individual Dictyostelium cells into multicellular aggregates and for progression through late development (Firtel and Meili,
2000). The aggregation centers produce cAMP pulses, which are detected, amplified and relayed to the surrounding cells. The cAMP is sensed by a cAMP receptor on the cell surface, which couples to a heterotrimeric G-protein. The Gβγ complex is set free and, together with CRAC, it activates the adenylyl cyclase (ACA) and leads to synthesis of cAMP (cAMP relay) (Firtel and Chung, 2000). CRAC transiently associates with the plasma membrane at the stimulated edge (Insall et al., 1994; Parent et al., 1998). In addition to CRAC other factors exist that affect activation of ACA like pianissimo, ERK and aimless (Verkerke-Van Wijk and Schaap, 1997). cAMP also initiates a network of signaling pathways such as cGMP signaling which is responsible for changes in the cytoskeleton (Liu and Newell, 1988).

CAP bsr, a Dictyostelium mutant in which the CAP gene has been inactivated by homologous recombination in such a way that the expression of the full length protein was reduced to less than 5% of the protein concentration in wild type AX2, revealed changes during growth and development. Growing cells were heterogeneous with regard to cell size and were often multinucleated. The mutant had an endocytosis and a chemotaxis defect. When chemotactic motility was assayed by applying a cAMP gradient, the cells did not properly orientate in the direction of the chemotactic agent. Development was significantly delayed and developmentally regulated genes such as csA (contact site A) and cAR I (cAMP receptor I), were expressed significantly later than in wild type. However, the mutant was able to complete the developmental cycle and to form fruiting bodies containing viable spores (Noegel et al., 1999).

Here we studied the responses of mutant cells to exogenous cAMP stimuli and tested specifically the cAMP relay and events that are associated with cAMP initiated changes in cell polarity, F-actin accumulation, cGMP production, and directed migration during the slug stage. The data suggest that loss of the cyclase associated protein caused a drastically lowered
sensitivity to external signals resulting in reduced cell polarity and altered cAMP waves during aggregation.

**MATERIALS AND METHODS**

*Strains and developmental conditions*

*D. discoideum* strain AX2, the CAP deficient mutant CAP bsr, the adenylyl cyclase deficient mutant aca (Pitt et al., 1992) and transformants were cultured at 21°C as described before (Noegel et al., 1999). For developmental studies exponentially growing cells were harvested from liquid medium, washed twice in Soerensen phosphate buffer (17 mM Na+/K+-phosphate buffer, pH 6.0) and shaking was continued for the indicated times in Soerensen buffer at a density of 1 x 10^7 cells/ml. EDTA-sensitivity of cell-cell contacts was determined as described (Faix et al., 1990). For rescue experiments CAP bsr cells were transformed with vectors allowing the expression of GFP fusion proteins (Noegel et al., 1999). Full length CAP cDNA was cloned into pDEXRH vector leading to expression of CAP carrying GFP at its amino terminus (Westphal et al., 1997). N- and C-terminal deletion constructs of CAP, N-CAP (aa 1-215), N-Pro-CAP (aa 1-254), C-CAP (aa 254-464) and Pro-C-CAP (aa 216-464), were generated by PCR and cloned into pDdA15GFP (Gerisch et al., 1995). The GFP tag was at the C-termini of the proteins. Control of transcription was under the actin15 gene promoter and actin8 gene terminator. Plasmids were introduced using the CaCl2 technique (Nellen et al., 1984), selection was with G418. Transformants were cloned and analyzed by fluorescence microscopy. Protein levels were determined by western blotting employing GFP-specific monoclonal antibodies and antibodies that recognized specifically the N- or the C-terminal domain of CAP (Gottwald et al., 1996).

*Mutant analysis*
For determination of the cell size, cells were harvested, washed and then resuspended at a density of 1 x 10^7 cells/ml in Soerensen phosphate buffer and shaking was continued for another hour at 21°C and 160 rpm in the presence of 10 mM EDTA. Cell polarity was determined for aggregation competent cells. Cells at the appropriate time points were allowed to settle on coverslips and fixed with cold methanol (-20°C). Cells were stained with monoclonal antibodies. Detection was with Cy3-labeled anti-mouse IgG.

**cAMP relay experiments**

For determination of the cAMP relay response the cells were shaken at a density of 2 x 10^7 cells/ml in development buffer (DB) as described in Patel et al. (2000). The cells were either not pulsed or pulsed at 6 minute intervals with cAMP to a final concentration of 50 nM. The assay of the cAMP relay response and the adenyl cyclase assay and quantification of adenyl cyclase amounts were done as described in Patel et al. (2000).

**Phototaxis analysis**

To analyze slug behaviour, 5 x 10^6 amoebae were inoculated onto a circular, 0.5 cm² origin at the center of a water agar plate. Slugs were allowed to form and migrate towards light (Fisher et al., 1983). After 48 hours slugs and slime trails were transferred to nitrocellulose filters (BA85, Schleicher and Schuell, Dassel, FRG) and stained with Amido Black.

**Determination of F-actin levels**

For actin detection in immunoblots and methanol fixed cells we used monoclonal antibody act1 (Simpson et al., 1984). F-actin was detected in picric acid/paraformaldehyde fixed cells with TRITC phalloidin (Sigma, Deisenhofen, Germany). For analysis of F-actin accumulation after stimulation with cAMP we used the method described by McRobbie and Newell (1984).
Alternatively determination was done with TRITC-phalloidin (Haugwitz et al., 1994). Both methods gave comparable results.

**cGMP determination**

Cells were starved for the appropriate time at $2 \times 10^7$ cells/ml or $4 \times 10^7$ cells/ml followed by stimulation with 0.1 $\mu$M cAMP. Responses were terminated by lysing the cells with 0.5% Triton X100 in 0.1 M HCl (final concentration each). Incubation was for 10 minutes at room temperature. Cells were viewed under a microscope to control lysis. The samples were centrifuged for 5 min at 10,000 x g and the cGMP content was determined using a commercially available kit (cyclic GMP (low pH) immunoassay, R&D, Wiesbaden, FRG). Samples were either used directly or stored at –20°C. Determinations were done in duplicate or triplicate and the assays were performed at least three times. To ensure that the appropriate developmental stages had been reached, samples were taken in parallel and assayed for the presence of the csA protein.

**Analysis of cell shape and cell migration**

Aggregation competent wild type and mutant cells were plated onto glass coverslips in small plastic dishes, and cell migration was recorded at intervals of 10 sec using a Zeiss Axiovert-200 inverted microscope and the Axiovision software. The time lapse movies were analyzed with the DIAS program (Solltech Inc., Oakdale, USA; Wessels et al., 1998). For shape analysis the outlines of the single cells were drawn manually. Several hundreds of cells have been recorded under different conditions including after development in shaking cultures or adhered to the plastic surface in submerged cultures. Chemotaxis experiments were performed with micropipettes and a micromanipulator system (Eppendorf, Hamburg, FRG) essentially as described (Gerisch and Keller, 1981).
**Yeast two hybrid interaction**

Full length *Dictyostelium* CAP was cloned into pAS2 (Harper et al., 1993), a 419 bp fragment corresponding to the C-terminus of the *Dictyostelium* adenylyl cyclase (position 4394 to 4813 of the published sequence, Pitt et al., 1992) was amplified by PCR using the following primers 5’ GTT GCA ATT TCA AGA GTA GT 3’ and 5’ TTC TTA ACT TGA AAG ATG GA 3’, and cloned into pACT2.

**Miscellaneous methods and monoclonal antibodies used**

Changes of myosin II in detergent-insoluble cytoskeletons were analyzed in aggregation competent wild type and mutant cells after treatment with caffeine, stimulation with cAMP and lysis as described (Chung and Firtel, 1999). SDS-PAGE, immunoblotting and immunofluorescence were done as described (Gottwald et al., 1996). mAb 223-445-1 had been generated against the C-terminal domain of CAP and 230-18-8 against the N-terminal domain (Gottwald et al., 1996). Contact site A antibody 33-294-17 (Berthold et al., 1984) was used to monitor the developmental stage, mAb act1 recognized actin (Simpson et al., 1984). Antibody K3-184-2 was raised against recombinant GFP; it recognizes wild type GFP and the red-shifted isoform S65T. Microscopic analysis was done as described (Noegel et al., 1999).

**RESULTS**

**CAP is required for cell polarization**

In *Dictyostelium*, CAP shows a temporary enrichment in extending pseudopods during chemotaxis. We have therefore analyzed the shape of CAP bsr cells during the aggregation phase in detail. At this developmental stage, cells of the parental strain AX2 elongate and form cell to cell contacts. Proteins like actin or the csA protein accumulate at polar regions of
the cells and at cell to cell contacts. CAP bsr cells also aggregated, although with a delay, and within these aggregates the cells were more rounded and did not exhibit the typical elongated shape. Taking the developmental delay of the CAP bsr mutant into account we performed the analysis between 9 to 15 hours after the start of development. The developmental stage was monitored by analyzing the presence of the contact site A protein, a developmentally regulated cell adhesion molecule which is expressed at the beginning of aggregation and disappears as soon as tight aggregates are formed (Noegel et al., 1986). Thus, this adhesion molecule is an excellent marker for comparing the developmental stage of CAP bsr and wild type strains. At all time points tested, the mutant cells did not polarize as well as wild type cells. We then supplied exogenous pulses of cAMP to enhance development (Noegel et al., 1985). Also under these conditions the mutant cells remained less polarized although expression of csA occurred earlier as in unpulsed cells (our unpublished results). In general, the data shown in this report all have been obtained with cells that developed without additional cAMP pulses unless indicated.

Figure 1 shows the typical migrating pattern of AX2 wild type and mutant cells after 6 h (AX2) or 12 h (CAP bsr) of starvation in shaking culture. Whereas the AX2 cells were elongated and migrated even without an external cAMP source in a rather directed fashion, the CAP mutants were rounded and moved only short distances. Speed, direction change and roundness reflect this behaviour. In contrast to wild type cells and despite a comparable developmental stage, the mutant cells have a strong tendency to extent pseudopods into all directions and to continuously change the angle of the migration track (Figure 2). As soon as strong cAMP gradients are present either due to the formation of large aggregates during development in submerged culture or by stimulating the cells with microcapillaries that are filled with $10^{-4}$ M cAMP, also the CAP bsr cells start to polarize and to migrate towards the cAMP source. This suggests a reduced sensitivity to chemoattractant which leads to a reduced cell polarization.
The expression of a GFP-tagged full length CAP reverted the cell polarity defect completely, and cells after 6 hours of starvation were highly elongated like wild type cells. It also restored the F-actin accumulation at leading edges, whereas in the mutant cells it was present in multiple patches in the cortical region (Figure 3). Furthermore, the developmental defect was reverted and expression of developmentally regulated proteins was as in wild type (our unpublished results).

**Signaling to the actin cytoskeleton is normal in the CAP mutant**

Upon application of cAMP, a characteristic pattern of actin polymerization and depolymerization is observed. After an initial increase of the F-actin concentration, the filaments depolymerize again followed by another phase of F-actin accumulation. We studied this response in wild type AX2 and CAP bsr mutant cells at comparable developmental stages. We found, that the pattern of F-actin accumulation in the mutant exhibited characteristics similar to the wild type pattern (Figure 4). We conclude from these data that in this process there occurs no involvement of CAP beyond the stimulation of the cAMP-receptor.

**The cAMP induced cGMP response is altered in CAP bsr**

The signal transduction chain leading from cell surface receptors to chemotactically induced cell polarization and pseudopod formation involves also cGMP. cGMP production is responsible for recruiting myosin to the actin cortex which finally leads to the elongated morphology of the cells. When we assayed cGMP production in response to a cAMP pulse we observed in AX2 cells a cGMP peak at 10 sec after stimulation. Basal levels were reached again after 45 sec. The mutant showed a similar pattern as AX2 wild type cells. However, the increase in cGMP was substantially lower and reached only about 20 percent of wild type levels (Figure 5). cGMP has been linked to myosin assembly (Liu and Newell, 1988) and a
reduced cGMP response might lead to changes in the actomyosin cortex. Stimulation of aggregation competent CAP bsr mutants with cAMP and determination of myosin assembly over the following 120 sec showed that the mutant altered the levels of polymerized myosin, however this happened in a highly irregular fashion. Whereas assembled myosin peaked in wild type cells after about 30 sec, the peaks in the mutant were smaller and occurred between 20 - 120 sec despite the inhibition of endogenous signal relay by treatment with caffeine. We conclude from these data that regulation of myosin assembly is disorganized in the mutant and might be the reason for the frequent formation of additional pseudopods in aggregation competent cells.

**CAP is required for cAMP relay**

So far a direct link between CAP and adenylyl cyclase from organisms other than yeast has not been made. We have performed a series of experiments that were designed to unravel a cross talk between both proteins. First we did cAMP relay experiments. For this we used cells starved for 3 or 6 hours either in the presence or absence of exogenous cAMP pulses. In AX2 cells, which had been starved for 3 hours, cAMP pulsing induced a strong relay response. In CAP bsr cells the relay response is much lower. Although it increases in cells after cAMP pulsing, the kinetics of the cAMP relay response is different from the one in AX2 (Figure 6A,B). The cAMP relay response after 6 hours in the presence or absence of cAMP also differs between wild type and mutant. In AX2 the relay response of unpulsed cells is higher than that of pulsed cells (Figure 6C) and the response of pulsed cells after 6 hours is lower than that after 3 hours of pulsing. Pulsed CAP bsr cells exhibit a different response. After 6 hours of pulsing the cAMP production is higher than after 3 hours of pulsing, and CAP bsr and AX2 are similar in their relay response both with regard to magnitude and kinetics (Figure 6C,D, closed symbols). It is noteworthy, that we never observed such a rapid and
dramatic rise in cAMP production in the mutant as is seen in 3 hours pulsed AX2 cells. Non pulsed mutant cells at 6 hours show a small relay response very similar to the 3 hour result.

These findings were supported by results from darkfield wave measurements with which one can analyze the cAMP relay (Dormann et al., 2000). In these experiments the waves appeared at comparable stages of development in AX2 and CAP bsr. However, the waves produced by the mutant were larger as compared to AX2, and the slightly slower oscillation frequency in the mutant reflects changes in the cyclase activity as a result of the feedback in cAMP production (Figure 6E).

We also analyzed the level of ACA directly by western blot analysis followed by a quantitation of the blots to ensure that the differences we observed were not due to lower amounts of the protein. In AX2, the levels of ACA increase during development, while pulsing produces much higher levels of protein. In CAP, bsr there is less of an increase during development in unstimulated cells, but after cAMP pulsing ACA expression increases at least to similar amounts as observed in pulsed AX2 cells (Figure 7). This shows that CAP bsr cells can express ACA to normal levels when presented with appropriate cAMP signals.

Finally we measured ACA activity in cell lysates of AX2 wild type and CAP bsr mutant after 6 hours of starvation in the presence of Mg$^{2+}$, Mn$^{2+}$ and GTP$_{\gamma}$S. We found that cyclase activity in CAP bsr can be stimulated by GTP$_{\gamma}$S in vitro which might be an indication of ACA activation by G$\beta\gamma$. The absolute activity after GTP$_{\gamma}$S stimulation is, however, much lower in the mutant than in AX2. This is also true for the basal activity which is measured in the presence of Mg$^{2+}$ and for the unregulated adenylyl cyclase activity measured in the presence of Mn$^{2+}$. The lower absolute activation of adenylyl cyclase is probably due to the fact that the cells were not pulsed. However, the degree of stimulation measured as the activity in the presence of GTP$_{\gamma}$S compared to the basal activity measured in the presence of Mg$^{2+}$ is very similar in the mutant compared to AX2 (Figure 8). From the data we conclude
that adenylyl cyclase is present in the mutant cells and is functional. The alteration in the cAMP relay response however suggests that CAP/ASP56 participates in this pathway.

**Does CAP physically interact with ACA?**

To further support these data we tested the interaction between CAP and ACA directly. In yeast a physical interaction had been shown between adenylyl cyclase and CAP. The interaction domain in CAP was localized to a short N-terminal stretch which is highly conserved in CAP from other species. In the adenylyl cyclase the binding site is located at the C-terminus (Nishida et al., 1998). This sequence is also conserved in the *Dictyostelium* protein. While we could not detect an interaction when we investigated the corresponding domains of the *Dictyostelium* proteins in the yeast two hybrid system, we observed an effect in an adenylyl cyclase mutant, aca, when moderately overexpressing GFP-tagged full length CAP.

We first tested the protein levels in the aca mutant and found that they were comparable to AX2 wild type. At the immunofluorescence level we detected the protein in the cytosol and at the plasma membrane as has been reported for the wild type (Gottwald et al., 1996). Moreover, GFP-CAP in the aca mutant showed similar dynamics as in wild type. It relocalized during phagocytosis and pinocytosis to the phagocytic or pinocytic cup, respectively, and was enriched in pseudopods (our unpublished results). The levels of the GFP-fusion protein were similar to the ones of the endogenous protein (our unpublished results). In further analysis we observed that the developmental phenotype of aca cells expressing the GFP-CAP was altered. Normally, aca mutant cells can not undergo development. In contrast, aca cells expressing GFP-CAP formed aggregates when starved in suspension (our unpublished results). The aggregates were dissociated when EDTA was added which is indicative of the formation of EDTA-sensitive cell contacts mediated by the
adhesion molecule DdCAD-1 (Wong et al., 1996). When we tested the GFP-tagged domains of CAP we found that N-CAP-Pro-GFP had a similar effect. From these results it appears that the block in early development of aca mutant cells can be overcome by increased levels of CAP.

**Distinct CAP domains restore cell morphology and development**

Having shown that expression of full length CAP in CAP bsr cells restores polarity and reverts the developmental defect we extended this analysis to the individual CAP domains which were expressed as GFP fusion proteins. For full length CAP and Pro-C-CAP the levels of the GFP fusion proteins were nearly comparable to wild type CAP levels; N-CAP, N-CAP-Pro and C-CAP showed higher levels of expression (our unpublished results). We found that all domains restored the cell polarity defect and cells were elongated when they aggregated (Figure 9 (top), shown for N-CAP-Pro and C-CAP). They corrected the developmental defect and csA expression exhibited the same pattern as in AX2 wild type (Figure 9 (bottom), shown for t9 and t12; Table 1).

We also tested if further defects in the mutant like cell morphology, cell size and the cytokinesis defect could be rescued by any of the domains. CAP bsr cells are heterogeneous in cell size with their diameters being shifted to 15 to 20 µm from 10 to 12 µm in AX2 wild type. Furthermore, mutant cells have 3 and more nuclei, whereas wild type cells are mostly mono- and binucleated. N-CAP expression in the mutant did not affect multinuclearity and cell size. The presence of the proline rich region in either N-CAP or C-CAP GFP fusion proteins led to the occurrence of mostly mononucleated cells and to a reduction in cell size. C-CAP expressing mutant cells resembled AX2 wild type with regard to nuclei number
(Table 1), in contrast, cell size was not reduced to normal. We rather observed a broad distribution of cell sizes (our unpublished results). The data from this analysis suggest that the proline rich region regulates the cell size and nuclei number, whereas the C-terminal domain on its own affects cytokinesis.

**CAP is indispensable for correct phototaxis**

The slug stage of development is important for the survival of *Dictyostelium* in its natural surroundings. Slugs migrate with great sensitivity towards light i.e. the soil surface from where the spores can be dispersed. Several phototaxis mutants have been described. In some of them the underlying defect resides in genes encoding cytoskeletal proteins (Fisher et al., 1998; Stocker et al., 1999), another mutant was defective in a Ras gene (Wilkins et al., 2000).

CAP mutant cells show a general delay in development, they do however complete the developmental cycle. When we studied the multicellular slug stage we noted a defect in phototaxis. Wild type slugs migrate almost directly towards a lateral light source whereas CAP bsr slugs do not orient correctly and do not migrate as far as wild type slugs in the same period of time (Figure 10A). That they do not have a general defect in light sensing was revealed in experiments where we changed the position of the light source after the slugs had migrated for 28 hours which was followed by another migration period of 24 hours. CAP bsr slugs showed a turn in the direction of migration as did wild type slugs (our unpublished results). CAP bsr slugs expressing N-CAP showed an improved orientation. They migrated in an angle of approximately 40 degrees towards the light source as compared to 20 degrees for wild type slugs and nearly 60 degrees for CAP bsr (Figure 10B). C-CAP expression led to some improvement in the migratory behaviour since these slugs traveled over longer distances. The presence of the proline rich region in the N- or the C-terminal domain had an
inhibitory effect on the distance traveled (Figure 10C and Table 1). CAP therefore seems to be involved in two aspects of slug phototaxis, migration and orientation.

Discussion

Our studies show that the cyclase associated protein CAP determines cell polarity and affects development. We have also provided evidence that it is required for adenylyl cyclase activity by studying the cAMP relay response in Dictyostelium. Moreover, moderate overexpression of CAP in an adenylyl cyclase mutant led to induction of early developmental stages. Further progression into development did however not occur.

CAP in cell polarity

Mutation of CAP in the Drosophila eye (acu) leads to a premature differentiation of photoreceptors (Benlali et al., 2000). This is thought to be due to the inability of the cells to undergo shape changes which are required for proper hedgehog signaling. Associated with this inability to change shapes was an accumulation of F-actin. The Dictyostelium mutant which we have described shows defects in many properties and cellular reactions. Most notable is the deficiency of the cells to polarize properly. They share this defect with the Drosophila mutant cap in which oocyte polarity is disrupted and F-actin organization altered (Baum et al., 2000). For Drosophila it was concluded that CAP is required for the correct spatial regulation of actin polymerization and that normal actin organization is required for proper polarization of the oocyte. The central role of the actin cytoskeleton is certainly important for proper polarization of D. discoideum cells as well. Especially the inhibition of pseudopod formation at the sides of elongated cells requires a very strong acto-myosin cortex and favors the motility at the front and rear areas. CAP plays apparently two roles in cell polarization and migration: (i) As was shown previously, CAP accumulated in actin-rich regions at moving fronts which favors polarization and might be a function of the actin-
binding domain in the CAP C-terminus (Gottwald et al., 1996). (ii) The additional interaction with the cyclase via its N-terminus guarantees correct signaling activities. The reduced levels of cGMP in the CAP bsr mutant are sufficient to impair the recruitment of myosin to the actin cortex which leads to a soft actin meshwork and the formation of additional pseudopods at the sides of polarized cells thus disturbing overall elongation and orientation (see Figure 2). The reduced sensitivity to chemotactic signals from outside and the altered relay response in the mutant contribute to the poor polarization behaviour.

The data we have collected by performing rescue experiments with separate domains also indicate that CAP does not solely act as actin regulatory protein. We could clearly attribute a role to the proline rich domain since both the N- and the C-terminal polypeptides containing this stretch corrected the increase in cell size and led to a reduction in nuclei number. Moreover, these polypeptides had an adverse effect on phototaxis and inhibited phototactic migration, whereas the N-domain alone improved the orientation during phototaxis, and the C-domain on its own improved the slug migration. For the yeast protein it has been shown that the proline rich domain binds SH3-domain containing proteins and is responsible for CAP's localization at the cortical cytoskeleton (Lila and Drubin, 1997). In contrast, in CAP from Dictyostelium it is a N-terminal stretch that mediates correct localization (Noegel et al., 1999). Surprisingly, all four constructs rescued the developmental defect and led to formation of polarized cells. This confirms the dual function of CAP, rendering -similar to the findings in yeast- the N-terminus as being responsible for proper signal transduction and the C-terminus as being involved in cytoskeletal dynamics at moving fronts. The reexpression of any of the functional domains therefore is sufficient to overcome the defects of the mutant under laboratory conditions. We can however not exclude that also the N-terminus influences the cytoskeleton in an indirect fashion as suggested by Moriyama and Yahara (2002). They found that both domains independently affect F-actin polymerization, whereby the C-domain directly interacts with F-actin, and the N-domain interacted with an actin-cofilin complex.
Our results from the rescue experiments suggest a role for the proline rich domain as well which might act in combination with the N- or C-domain.

**CAP in cAMP signaling**

*Dictyostelium* is unique in its ability to use cAMP for initiation and progression through development. The chemotactic aggregation of starving cells is controlled by propagating waves of cAMP. The cAMP signals are periodically initiated by cells in the aggregation centers and relayed by surrounding cells. This results in outward propagating waves of cAMP which induce inward movement of the cells. The process of cAMP signaling has been studied in depth and the cAMP receptors and the cAMP synthesizing enzymes are well characterized. *Dictyostelium* harbors three adenylyl cyclases, an aggregation specific cyclase, ACA, a germination specific cyclase, ACG, and ACB, a more recently discovered adenylyl cyclase which has characteristics different from ACA and presumably acts in a G-protein independent way (Kim *et al.*, 1998; Pitt *et al.*, 1992). Responsible for the cAMP relay is the aggregation specific cyclase ACA. In CAP bsr cells the protein was present in unaltered amounts and its activity could be assayed in cell homogenates. However, the cAMP relay was altered and the cAMP induced secretion of cAMP did not occur with the same characteristics as in wild type. The data do however not imply that CAP is essential for adenylyl cyclase activity. The situation rather resembles the one in yeast where only one aspect of cyclase activation, the Ras-mediated activation, is affected.

The activity of the *Dictyostelium* ACA is regulated by the Gβγ complex (Chen *et al.*, 1996) and other factors such as CRAC, pianissimo, aimless, a RasGEF, and ERK1, a MAP kinase (Chen *et al.*, 1997; Insall et al, 1994; Insall *et al.*, 1996; Segall *et al.*, 1995). Cells lacking these factors are very similar in their phenotypes and all have been isolated because they
failed to aggregate. These mutants are not only similar amongst each other with regard to their developmental phenotype, they are also specifically defective in the receptor/G-protein mediated activation of ACA since GTPγS did no longer stimulate ACA activity in cell lysates. The effect of CAP on ACA activity is distinctly different as CAP mutants can aggregate, although with a delay, and as GTPγS stimulation is still effective. Although our results from the yeast two-hybrid analysis were negative, the question is still open whether the CAP-ACA interaction is a direct one or whether it requires one or more proteins linking CAP and cAMP signaling.

**CAP in late development**

The phototactic defect which we have observed can also be linked to CAP's effect on the cAMP relay. Previous work showed that cAMP waves organize the slug (Dormann et al., 1997; 2001), and Miura and Siegert (2000) reported that cAMP mediates cell-cell signaling and chemotaxis of the cells in a slug. cAMP waves are generated in the anterior prestalk zone in response to light and are relayed to the posterior zone. In fact, we have observed a defect in signaling in the mutant during late stages of development and found in dark field measurements that wave formation was altered (our unpublished results). In the phototaxis assay we noted that CAP acts both on the orientation as well as on migration and that both components of phototaxis can be separated. The rescue experiments showed an impact of the N-domain on the efficiency of phototaxis by improving the angle of migration in the direction of the light, whereas the C-domain caused the slugs to migrate over longer distances. The first effect could be due to the activity of the N-domain in signaling, whereas the second one might require CAP's activity as actin associated protein. Taking the results from our analysis together, we conclude that the evolutionarily conserved protein CAP may play a critical part in cell polarity and movement in a diversity of organisms.
ACKNOWLEDGMENTS

We thank Daniela Rieger, Marc Borath and Berthold Gassen for cell culture and technical assistance. The work was supported by grants from the DFG, the Fonds der Chemischen Industrie and Köln Fortune. The work in C. J. Weijer's lab is supported by a Wellcome Trust Program Grant.
References


Figure Legends

Figure 1. Cell migration of AX2 wild type and CAP bsr mutant cells. After development in shaking culture for 6 h (AX2) or 12 h (CAP bsr) cells were harvested, washed, plated onto glass surfaces and monitored over 100 frames at intervals of 10 sec. The top panels show the cells and their outlines in the first frame, the lower panels the tracks of migration over time. The calculated numbers show that speed of migration, direction change and roundness of the cells are drastically altered in the CAP mutant.

Figure 2. Analysis of cell polarization. Top: Shape changes of a single CAP bsr cell after 12 h of starvation in a submerged culture were recorded at high magnification over 10 min at a rate of 10 sec per frame. The outlines were traced manually and the changes of direction (arrows) calculated using the DIAS image analysis software. The panel shows every third frame. The cell shapes suggest a lack of polarity and a continuous change of direction. Bottom: For comparison, polarization and directed migration of an AX2 wild type cell at the same stage of development. Window widths at top panels are 32.25 µm, at the bottom panels 42.8 µm.

Figure 3. (A) F-actin distribution in the CAP bsr mutant and (B) in CAP bsr cells expressing a full length GFP-CAP fusion. Cells after 6 hours of starvation were fixed with paraformaldehyde/picric acid. F-actin was detected by TRITC-phalloidin. The mutant cells had a more rounded cell shape and F-actin was present in multiple patches at the cortex, whereas cells expressing full length GFP-CAP were elongated and showed a thin F-actin rim along the boundaries of the cell and an enrichment at cell-cell contacts.
**Figure 4.** F-actin polymerization response upon cAMP stimulation is normal in the CAP bsr mutant. The F-actin content was determined using TRITC-phalloidin staining of cells fixed at various times (in sec) after stimulation with cAMP ($10^{-7}$ M). The amount of F-actin was normalized relative to the F-actin level of unstimulated cells (0 sec). Open symbols, CAP bsr, closed symbols, AX2. The results from a typical experiment are shown.

**Figure 5.** cGMP response upon stimulation with cAMP. AX2 wild type and CAP bsr mutants at comparable developmental stages are distinguishable in their cAMP induced transient increase of intracellular cGMP concentrations. The curve for AX2 represents a single typical experiments. The curve for CAP bsr represents the data from three independent assays. The error bars were too narrow to be visible.

**Figure 6.** cAMP relay is altered in CAP bsr. cAMP relay was measured in wild type AX2 and CAP bsr cells that had been starved for 3 or 6 hours either in the absence or presence of cAMP pulsing. (A) cAMP relay in AX2 cells starved for three hours in the absence (open squares) or presence (closed squares) of cAMP pulsing. cAMP pulsing induces a much stronger relay response. (B) cAMP relay in CAP bsr cells starved for 3 hours in the absence (open squares) or presence (closed squares) of cAMP pulsing. The relay response is much lower than in AX2 in both cases. It is noticeable that the kinetics of the cAMP relay response is also considerably slower in CAP bsr as compared to AX2. (C) cAMP relay after 6 hours in AX2 in the presence (closed diamonds) or absence (open diamonds) of cAMP pulsing. The relay response of unpulsed cells is higher than that of unpulsed cells after 3 hours, however the relay response of pulsed cells after 6 hours is lower than after 3 hours of pulsing (A). There is little difference in relay response between pulsed and non pulsed cells after 6 hours. (D) cAMP relay after 6 hours in CAP bsr cells in the presence (closed diamonds) or absence (open diamonds) of cAMP pulsing. Non pulsed cells show a small relay response just as cells
after 3 hours of starvation (B), while the cells that have been pulsed for 6 hours show a slightly stronger relay response than CAP bsr cells that have been pulsed for 3 hrs. The magnitude and kinetics of relay in CAP bsr and AX2 cells that have been pulsed for 6 hours is indistinguishable (C, D). The data shown are the means and standard deviations of 4 independent experiments performed on different days. (E) Measurement of darkfield waves. Due to a slower oscillation frequency the waves of the mutant are slightly larger than the waves in AX2 wild type. The measurements of 75 (AX2) and 78 (CAP bsr) wave periods from three independent experiments showed with 270 sec (+/- 37, AX2) and 306 sec (+/- 38, CAP bsr) a statistically significant (P < 0.001) difference.

**Figure 7.** CAP bsr has normal amounts of ACA at the protein level. Cells were allowed to develop at 10^7/ml in DB buffer for various times with or without cAMP pulsing. Samples were collected and solubilized in Laemmli sample buffer and 10^6 cells were subjected to SDS polyacrylamide gel electrophoresis on 8\% gels. Immunoblotting was performed on nitrocellulose membranes using a peptide antibody directed against the last 15 amino acids of ACA (Parent and Devreotes, 1995). The antiserum was diluted 1:3.000 in TBST and detection was performed by chemiluminescence using horseradish peroxidase coupled sheep-anti-rabbit IgG. Chemiluminescence was recorded and quantitated using a Fujifilm LAS 1000 image reader.

**Figure 8.** Stimulation of ACA activity in cellular extracts of CAP bsr is normal. The ACA activity of cell lysates was measured for 2 minutes in the presence of Mg^{2+} (circles), Mn^{2+} (triangles) or GTP\_\gamma\_S (stars) in AX2 (A) and CAP bsr cells (B) starved for 6 hours without pulsing. It is seen that ACA activity can be stimulated in the mutant by GTP\_\gamma\_S. The degree of stimulation measured as the activity in the presence of GTP\_\gamma\_S compared to the basal activity
measured in the presence of Mg\(^{2+}\) is very similar in the mutant compared to AX2, however absolute activity after GTP\(\gamma\)S stimulation is much lower in the mutant than in AX2. This is also true for the unregulated adenylyl cyclase activity measured in the presence of Mn\(^{2+}\).

**Figure 9.** Top: The cell polarization defect of the CAP bsr mutant can be rescued by expression of individual domains. Cells harvested after six hours of starvation in shaken suspension were allowed to settle on coverslips, fixed with cold methanol (-20°C) and stained with actin specific monoclonal antibody act1 followed by treatment with a secondary Cy3-labeled antibody. Actin staining is mainly seen around the cell boundaries and is enriched in the front and in the cell-cell contact regions. AX2 and mutant cells expressing N-CAP-Pro or C-CAP GFP fusion proteins are elongated and actin is enriched in the polar regions, whereas CAP bsr cells do not polarize properly at any time during development independent of the expression of developmental markers. The arrow in the lower right panel points to a cell cell contact region. Bottom: The developmental delay of CAP bsr is rescued by expression of the CAP domains. csA levels were used to monitor the developmental stage. In wild type cells csA is present after six hours of starvation in shaken suspension, in CAP bsr mutants the protein is strongly expressed after 12 hours. At t9 AX2 and all CAP bsr transformants expressing N- or C-terminal domains of CAP show maximal accumulation of the csA protein. Total cell homogenates derived from 5 x 10\(^5\) cells after 9 and 12 hours of starvation were subjected to SDS-PAGE (10 % acrylamide) and the resulting western blots were probed with mAb 33-294-17. The star indicates an incompletely processed form of the csA glycoprotein. The sizes in kDa of the molecular mass marker are given at the left.

**Figure 10.** The CAP bsr mutant has a phototaxis defect. (A) The phototactic behavior of the mutant is altered in comparison to AX2 wild type. Cells were incubated for 28 h in a
phototaxis chamber with a narrow light source (arrowhead at the bottom). After this time the chamber was rotated by 90 degrees (arrowhead at the left) and the cells incubated for additional 24 h. Subsequently, trails and cell material were blotted and stained with Amido Black. In the lower panels the migration tracks are highlighted for better visibility. AX2 slugs migrate directly towards the light source, whereas the mutant is slower and shows only poor directionality. (B) The angle of deviation during slug phototaxis is increased for all mutant strains as compared to AX2. (C) CAP bsr slugs expressing C-CAP-GFP can migrate nearly as far as wild type whereas the presence of the proline rich region in any of the domains has a negative effect on the distance traveled. The data are collected from between 6 and 30 experiments per strain.
Table 1. Rescue activities of individual CAP domains. For rescue experiments GFP tagged domains of CAP were used. The N-domain is visualized by dark grey, the C-domain by light grey and the proline rich region by a black bar. A plus sign (+) indicates rescue, i, indicates an inhibitory effect. For N-CAP-Pro and Pro-C-CAP we observed the presence of mostly mononucleated cells in the cytokinesis assay (+*).
<table>
<thead>
<tr>
<th></th>
<th>CELL SIZE</th>
<th>CYTOKINESIS</th>
<th>CELL POLARITY</th>
<th>DEVELOPMENT</th>
<th>SLUG TRAIL LENGTH</th>
<th>PHOTOTAXIS ANGLE</th>
<th>CAP DOMAINS</th>
</tr>
</thead>
<tbody>
<tr>
<td>fl CAP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>N-CAP</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>N-CAP-PRO</td>
<td>+</td>
<td>+*</td>
<td>+</td>
<td>+</td>
<td>i</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PRO-C-CAP</td>
<td>+</td>
<td>+*</td>
<td>+</td>
<td>+</td>
<td>i</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C-CAP</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Noegel et al.
Table 1
cell migration and cell shape (6 frames per minute / 100 frames)

speed:
- AX2 wild type: 7.7 μm/min (+/- 1.7)
- CAP bsr mutant: 2.7 μm/min (+/- 1.4)

direction change:
- AX2 wild type: 45 deg (+/- 8.5)
- CAP bsr mutant: 55 deg (+/- 9.9)

roundness
- AX2 wild type: 69% (+/- 9.5)
- CAP bsr mutant: 92% (+/- 3.7)

Noegel et al.
Figure 1
CAP bsr mutant

Window width = 32.25 um

AX2 wild type

Window width = 42.8 um

Noegel et al.
Figure 2
Noegel et al.
Figure 4
Noegel et al.
Figure 5
Noegel et al.
Figure 6
Noegel et al.
Figure 7
Noegel et al.
Figure 8
Noegel et al.
Figure 9
Noegel et al.
Figure 10