

The cell adhesion molecule DdCAD-1 regulates morphogenesis through differential spatiotemporal expression in *Dictyostelium discoideum*

Shrivani Sriskanthadevan, Yingyue Zhu, Kumararaj Manoharan, Chunxia Yang and Chi-Hung Siu*

SUMMARY

During development of *Dictyostelium*, multiple cell types are formed and undergo a coordinated series of morphogenetic movements guided by their adhesive properties and other cellular factors. DdCAD-1 is a unique homophilic cell adhesion molecule encoded by the *cadA* gene. It is synthesized in the cytoplasm and transported to the plasma membrane by contractile vacuoles. In chimeras developed on soil plates, DdCAD-1-expressing cells showed greater propensity to develop into spores than did *cadA*-null cells. When development was performed on non-nutrient agar, wild-type cells sorted from the *cadA*-null cells and moved to the anterior zone. They differentiated mostly into stalk cells and eventually died, whereas the *cadA*-null cells survived as spores. To assess the role of DdCAD-1 in this novel behavior of wild-type and mutant cells, *cadA*-null cells were rescued by the ectopic expression of DdCAD-1-GFP. Morphological studies have revealed major spatiotemporal changes in the subcellular distribution of DdCAD-1 during development. Whereas DdCAD-1 became internalized in most cells in the post-aggregation stages, it was prominent in the contact regions of anterior cells. Cell sorting was also restored in *cadA*⁻ slugs by exogenous recombinant DdCAD-1. Remarkably, DdCAD-1 remained on the surface of anterior cells, whereas it was internalized in the posterior cells. Additionally, DdCAD-1-expressing cells migrated slower than *cadA*⁻ cells and sorted to the anterior region of chimeric slugs. These results show that DdCAD-1 influences the sorting behavior of cells in slugs by its differential distribution on the prestalk and prespore cells.

KEY WORDS: Cell-cell adhesion, Cell sorting, Pattern formation, *Dictyostelium*

INTRODUCTION

The development of ordered structures in multicellular organisms proceeds through a complex series of cellular interactions, in which cells sort out and associate into specific multicellular groups. Cells frequently migrate from one site to another and reorganize at their final destination to establish the various tissue primordia. Therefore, individual cells or groups of cells must continuously change their relative positions and mutual adhesiveness during morphogenesis. It has been proposed that proteins involved in cell adhesion, cell-cell signaling and cell differentiation are genetic tool kits for multicellularity (Vogel and Chothia, 2006; King et al., 2007; Abedin and King, 2010).

The search for the origin of multicellularity often begins with cell-cell adhesion molecules (Bowers-Morrow, 2004; Harwood and Coates, 2004). Recent studies of cell adhesion molecules (CAMs) show that, in addition to cell-cell adhesion, they play key roles in sensing environmental cues and in generating signals that regulate a diversity of cellular processes, including gene expression, cell proliferation, cell polarity, cell motility and apoptosis. Differential temporal and spatial expression of cell adhesion molecules in specific groups of cells or tissues is stringently regulated during development. The formation or dissolution of specific adhesion complexes frequently leads to signals that serve as a major driving force behind migration and cell sorting, as well as differentiation and tissue formation. Therefore, CAMs have been recognized as

major morphoregulators and signaling molecules (Edelman and Crossin, 1991; Gumbiner, 1996) because of their participation in many dynamic physiological and pathological processes, such as tissue architecture, organ regeneration and cancer metastasis.

The use of *Dictyostelium discoideum* to investigate the nature and function of CAMs was popularized by the pioneering work of Gerisch about half a century ago (Beug et al., 1970; Müller-Taubenberger and Bozzato, 2006). During development, cells transit from their solitary state of vegetative growth and embark on a social interactive phase. Cells undergo chemotaxis to form mound structures, which develop into slugs with clearly demarcated anterior and posterior zones. Slugs eventually culminate in the formation of fruiting bodies, which comprise a sorus of spores suspended by a filamentous stalk. The stalk cells display altruism and give up reproduction in order to benefit the spore cells by lifting them above the hazards of the soil, thus increasing their chances of dispersal to a more favorable environment. In addition, aggregative development exposes *D. discoideum* to chimerism, which includes cheating, whereby individuals have access to group benefits without contributing their fair share. This phenomenon raises the issues of how social cooperation persists in nature and what factors would permit individuals with a compromised phenotype to survive (Foster et al., 2004; Nowak, 2006; Gilbert et al., 2007). Among the many potential factors, cells tend to cooperate with genetically similar individuals and the cell adhesion genes *csaA* and *tgrCI* have been found to confer survival advantages to *D. discoideum* cells that carry the same or highly related genes (Queller et al., 2003; Benabentos et al., 2009).

Several adhesion systems are known to influence the behavior of *D. discoideum* cells. Mutations in these genes have major effects on aggregate formation and morphogenesis (Coates and Harwood,

The Banting and Best Department of Medical Research and Department of Biochemistry, University of Toronto, Toronto, ON M5S 1A8, Canada.

*Author for correspondence (chi.hung.siu@utoronto.ca)

2001; Siu et al., 2004). The Ca^{2+} -dependent cell-cell contact sites are mediated by the adhesion molecule DdCAD-1 (also known previously as gp24), which is encoded by the *cadA* gene and expressed soon after the initiation of development (Brar and Siu, 1993; Wong et al., 1996; Wong et al., 2002; Yang et al., 1997). The $\text{Ca}^{2+}/\text{Mg}^{2+}$ -independent contact sites are mediated by *csA* (gp80) (Harris and Siu, 2002; Kamboj et al., 1989; Siu et al., 1985; Stein and Gerisch, 1996) and *TgrC1* (LagC, gp150) (Dynes et al., 1994; Gao et al., 1992; Wang et al., 2000), and they contribute to the formation of stable cell-cell adhesion during the aggregation phase and the post-aggregation phase, respectively.

DdCAD-1 is unique among CAMs because it is synthesized as a soluble protein and transported to the plasma membrane by contractile vacuoles (Sesaki et al., 1997; Sriskanthadevan et al., 2009). DdCAD-1 contains two distinct domains with β -sandwich architecture. Whereas the N-terminal domain is involved in homophilic binding, the C-terminal domain tethers the protein to a membrane anchor (Lin et al., 2006). Studies on *cadA*⁻ cells have implicated DdCAD-1 in cell sorting and cell-type proportioning (Wong et al., 2002). Because DdCAD-1 is expressed transiently on the plasma membrane during cell aggregation (Sesaki and Siu, 1996), it is not known how DdCAD-1 regulates cell differentiation and cell sorting.

In this report, we examined chimeras consisting of *cadA*⁻ cells and the parental AX4 (*cadA*⁺) cells. Results show that the gene *cadA* elicited different social behavior under different environmental conditions. In chimeras of *cadA*⁺ and *cadA*⁻ cells, the *cadA*⁺ cells were favored for spore formation. However, on agar surfaces, *cadA*⁻ cells differentiated into spores whereas the stalk was made up of mostly *cadA*⁺ cells. Morphological studies of cells expressing DdCAD-1-GFP ectopically or treated with Alexa 488-conjugated recombinant DdCAD-1 have revealed remarkable changes in the temporal and spatial expression of DdCAD-1 during development. Whereas DdCAD-1 is abundant in the cell-cell contact regions in the anterior zone, it is present primarily in the cytoplasm of posterior cells. Cells that retain DdCAD-1 on the surface sort to the tip and differentiate into stalk cells. These data suggest that DdCAD-1 regulates the sorting behavior of cells through its differential subcellular distribution in prespore and prestalk cells.

MATERIALS AND METHODS

Construction of DdCAD-1-GFP and mutant plasmids for cell transfection

PCR products were obtained from *cadA* cDNA and *cadA* genomic DNA cloned in pBluescript SK⁺ vectors. To construct the -631::*cadA*-GFP expression vector, the *cadA* promoter starting at -631 (GenBank accession number: AAFI02000079.1) was ligated to *cadA* cDNA and then inserted before GFP in the pIGFP vector, which contains the neomycin marker (kindly provided by Dr David Knecht, University of Connecticut, CT, USA) (see Fig. S1 in the supplementary material). Mutations in *cadA* were generated as described previously (Sriskanthadevan et al., 2009) and all constructs were sequenced from both ends. Plasmid DNAs were electroporated into *cadA*⁻ cells (Pang et al., 1999). Cells were collected at mid-log phase, washed twice in cold H50 buffer (20 mM Hepes, 50 mM KCl, 10 mM NaCl, 1 mM MgSO_4 , 5 mM NaHCO_3 , 1 mM NaH_2PO_4 , pH 7.0) and then suspended in H50 at 2×10^7 cells/ml. Cell samples (100 μl each) were mixed with $\sim 5 \mu\text{g}$ of plasmid DNA and incubated for 5 minutes on ice in 1-mm cuvettes. Electroporation was carried out by two consecutive pulses of 0.85 kV with a capacitance of 25 mF applied to the cuvette and with a 5-second recovery between pulses. After 5 minutes of incubation on ice, the cells from each cuvette were transferred to 3 ml of HL5 (0.5% Difco proteose peptone No. 2, 0.5% BBL Thiotone E peptone, 1% glucose, 0.5% yeast extract, 2.5 mM KH_2PO_4 , 2.5 mM Na_2HPO_4 , pH

6.7) in wells of a six-well tissue culture plate (Pang et al., 1999). Transfectants were selected by sequential incubation in 2, 5, 10 and 20 $\mu\text{g}/\text{ml}$ G418 (Sigma Chemical Co., St Louis, MO, USA) over a period of 3 weeks. The expression of wild-type or mutant DdCAD-1 was monitored by western blot analysis. Transfectants were maintained in 20 $\mu\text{g}/\text{ml}$ of G418.

Development on non-nutrient agar and soil plates

Dictyostelium strains, AX4 (parental strain of the *cadA*⁻ strain), *cadA*⁻ and various transfectants, were cultured either in association with *Klebsiella aerogenes* or in HL5 axenic medium (Sussman, 1987). Cells were grown and developed at 22°C. To determine the percentage of *cadA*⁻ spores in chimeric fruiting bodies, axenically cultured AX4 and *act15::GFP;cadA*⁻ cells were mixed to give a final concentration of 1×10^8 cells/ml in the developmental buffer DB (5 mM sodium phosphate buffer, pH 6.5, 1 mM CaCl_2 , 2 mM MgCl_2) (Fey et al., 2007). Cell mixtures at 20:80, 50:50 and 80:20 ratios were developed on either 2% non-nutrient agar buffered with KK-2 (2.2 g/L KH_2PO_4 and 0.7 g/L K_2HPO_4 , pH 6.4) or soil plates. Soil plates were prepared using commercially available garden soil (neutral pH) (All Treat Farm Ltd, Wellington North, ON, Canada) which was sieved to obtain particles of homogenous size (≤ 1 mm in diameter) and then autoclaved (Ponte et al., 1998). Aliquots of 20 g were distributed homogeneously on 90-mm Petri dishes, which were then moistened with 4 ml of sterile water. Aliquots of 0.25 ml (2.5×10^7 cells) of mixed cells were pipetted onto soil plates to cover an area of ~ 2 cm². Four samples were placed on the each dish and then incubated at room temperature in a moisture box covered with aluminum foil. The cells were developed for 48 hours. Pictures were taken from two random regions for each example using a dissecting microscope for the quantification of fruiting bodies. Spores were picked randomly, treated with 0.05% SDS in DB, and the percentage of fluorescent *cadA*⁻ spores was estimated.

Cell cohesion assay

Cells were collected for development in 17 mM phosphate buffer (7.35 mM KH_2PO_4 and 2.65 mM Na_2HPO_4 , pH 6.4) at 2×10^7 cells/ml on a platform shaken rotating at 180 rpm at 22°C. After 4 hours, cells were resuspended at $\sim 2.5 \times 10^6$ cells/ml. Cells in 200- μl samples were dispersed by vortexing for 15 seconds and then allowed to re-aggregate by shaking at 180 rpm. At regular intervals, the numbers of non-aggregated cells were scored using a hemocytometer.

Temporal and spatial expression pattern of DdCAD-1

Axenically grown -631::*cadA*-GFP transfectants were washed free of medium and resuspended in DB at 4×10^6 cells/ml. Aliquots of 0.5 ml were deposited on positively charged coverslips (Fisher Scientific, Pittsburgh, PA, USA) for 30-60 minutes and excess buffer was removed. Aggregates were fixed in 3.7% formaldehyde at different time points and permeabilized with 0.5% Triton X-100 for 15 minutes. Samples were incubated in 1% BSA in MCG buffer (50 mM MES, pH 6.4, 0.2 mM CaCl_2 , 2 mM MgCl_2) for 30 minutes. Then, samples were stained with phalloidin (1:400) to mark the cell periphery (Molecular Probes, Eugene, OR, USA) for 30 minutes. Coverslips were mounted in DAKO fluorescent mounting medium (DakoCytomation, Glostrup, Denmark). Confocal images were acquired using the Zeiss LSM 510 microscope.

Analysis of cell sorting in slugs

Cells were cultured either in association with *K. aerogenes* or axenically in HL5 medium. Cells were collected, washed and resuspended in 17 mM phosphate buffer at 10^7 cells/ml. The cells were stained in 0.05% Neutral Red for 5 minutes at room temperature (Weijer et al., 1987). Prestalk cells stain more strongly with Neutral Red, which accumulates in the large intracellular acidic vesicles (Gross, 1994). After washing, cells (2×10^8 cells/ml) were deposited on 1.5% non-nutrient agar (Eichinger et al., 2005). Development was carried out at 22°C in the dark. Slugs were photographed using a stereomicroscope at different time intervals. The distribution pattern of Neutral Red-stained cells within the slug was examined. The lengths of the whole slug (y) and the intensely stained anterior zone (x) were measured using Image J. The x/y ratio (R) was calculated and the frequencies of occurrence of the R values were determined. Experiments

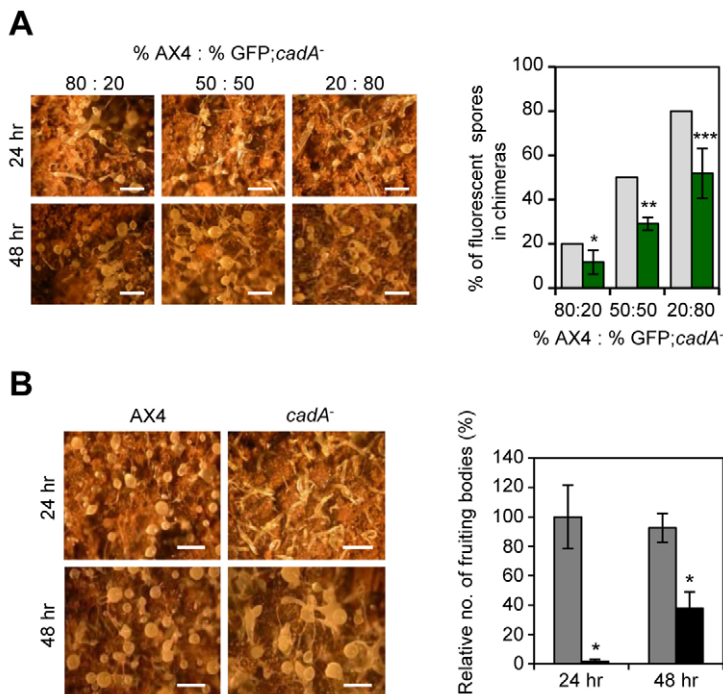


Fig. 1. Developmental phenotype of *cadA*⁻ cells on soil plates. (A) Spore formation by *act15::GFP;cadA*⁻ cells in the fruiting body of *Dictyostelium* chimeras developed on soil plates. AX4 cells were mixed with *act15::GFP;cadA*⁻ cells at different ratios and allowed to develop for 48 hours. Scale bars: 1 mm. The percentages of fluorescent spores in chimeric fruiting bodies were scored ($n=3$) (green bars). The data were compared with the theoretical values if both types of cells were distributed evenly in the sorus (gray bars). Data represent the mean \pm s.d. of three different experiments. (* $P>0.1$, ** $P<0.05$, *** $P<0.05$, Student's unpaired t -test). (B) Development of *cadA*⁻ cells and the parental AX4 cells (*cadA*⁺) on soil plates. Images were taken at 24 hours and 48 hours of development. Scale bars: 1 mm. The number of fruiting bodies formed by AX4 (gray bars) and *cadA*⁻ (black bars) cells were scored and normalized to that of AX4 cells at 24 hours of development. The *cadA*⁻ values were significantly different from their AX4 counterpart. Values represent the mean \pm s.d. ($n=8$; * $P<0.0001$, Student's unpaired t -test).

were repeated three times and 100-200 slugs were measured in each experiment. Data from all three experiments were pooled for the frequency calculations.

In vitro reconstitution of DdCAD-1 using recombinant proteins

Cells were developed for 4-6 hours before staining with Neutral Red. After staining, cells were incubated at 2×10^8 cells/ml with wild-type or mutant recombinant DdCAD-1 protein at 0.5 mg/ml or 3 mg/ml for 30 minutes. Then, 100 μ l of cells were placed in a straight line ~ 5 cm long on 1.5% non-nutrient agar plates for development until the slug stage. His-DdCAD-1 was prepared as described previously (Srikanthadevan et al., 2009) and labeled with sulfodichlorophenol esters (SDP) conjugated with Alexa Fluor 488 at a dye:protein ratio of 5:3 (Molecular Probes). Conjugated proteins were separated from un-reacted labeling reagent using Zeba desalt spin columns (Pierce, Rockford, IL, USA). The protein was resuspended at 6 mg/ml in 20 mM PIPES, pH 6.4. Then, *cadA*⁻ cells (2×10^6 cells) were developed for 4-6 hours and incubated with Alexa-488-conjugated His₆-DdCAD-1 proteins at 3 mg/ml for 12 hours on coverslips and then excess buffer was removed. Cells were developed until the culmination stage before fixation with 3.7% formaldehyde for confocal microscopy.

Antibody-induced cap formation

To induce DdCAD-1 cap formation, anti-GFP mAb (1:100; Sigma Chemical Co., St Louis, MO, USA) was added to 1.2×10^6 DdCAD-1-GFP-expressing cells suspended in 300 μ l of 50 mM MES buffer, pH 6.3, and incubated for 30 minutes at room temperature. Alexa-568-conjugated goat anti-mouse antibody (1:400) was added and cells were rotated at room temperature for another 30 minutes. After washing, aliquots of cells were deposited on coverslips and allowed to attach for 15 minutes. The coverslips were washed gently with MCG buffer, fixed in 3.7% formaldehyde, washed and mounted for fluorescence microscopy.

Flow cytometry

Axentially grown -631:: *cadA*-GFP transfectants were washed and resuspended at 2×10^8 cells/ml. Cells were deposited on 1.5% non-nutrient agar and development was carried out at 22°C in the dark. Cells at different stages were collected, dissociated and analyzed by flow cytometry (Becton Dickson LSRII) using excitation wavelengths of 488 nm and 603 nm. Data were analyzed using the WinMDI software.

Chemotactic cell migration assay

The chemotactic migration assay was performed according to Wallace and Frazier (Wallace and Frazier, 1979). Cells were collected at 4 hours of development and resuspended at 2.5×10^8 cells/ml in 17 mM sodium phosphate buffer. The cAMP stock solution (10 mM) was diluted to 100 μ M or 250 μ M. Non-nutrient 1.5% agar plates were prepared fresh and fully hydrated with buffer before the experiment. Four wells were created in the center of the quadrants of an agar plate. Then, the wells were filled with 30 μ l of cAMP and 1 μ l aliquots of the cell suspension were placed 5 mm away from the well. The plates were incubated at room temperature in a moisture box covered with aluminum foil. Cell migration was monitored using a dissecting microscope and the distance of migration was measured after 16 hours.

RESULTS

The *cadA* gene elicits different social behavior depending on environmental conditions

DdCAD-1 appears to function transiently at the aggregation stage of development as it redistributes from the cell-cell contacts when the cell adhesion protein csA moves into these regions (Sesaki and Siu, 1996). However, disruption of the *cadA* gene leads to aberrant cell sorting and defects in cell-type proportioning (Wong et al., 2002), suggesting a role for DdCAD-1 in the post-aggregation phase of development. Changes in the environmental conditions of development have been used successfully to investigate subtle functions of cell adhesion proteins (Ponte et al., 1998; Queller et al., 2003), we have taken a similar approach to investigate the role of DdCAD-1 in the post-aggregation stages of development. Chimeras of wild-type and mutant cells were analyzed under different environmental conditions. When the parental AX4 cells and GFP-expressing *cadA*⁻ cells were mixed for development on soil plates, disproportionate spore yields were observed in fruiting bodies collected at 48 hours of development (Fig. 1A). The yield of *cadA*⁻ spores was $\sim 40\%$ lower than the expected value if cells from both strains were equally represented in the chimeras,

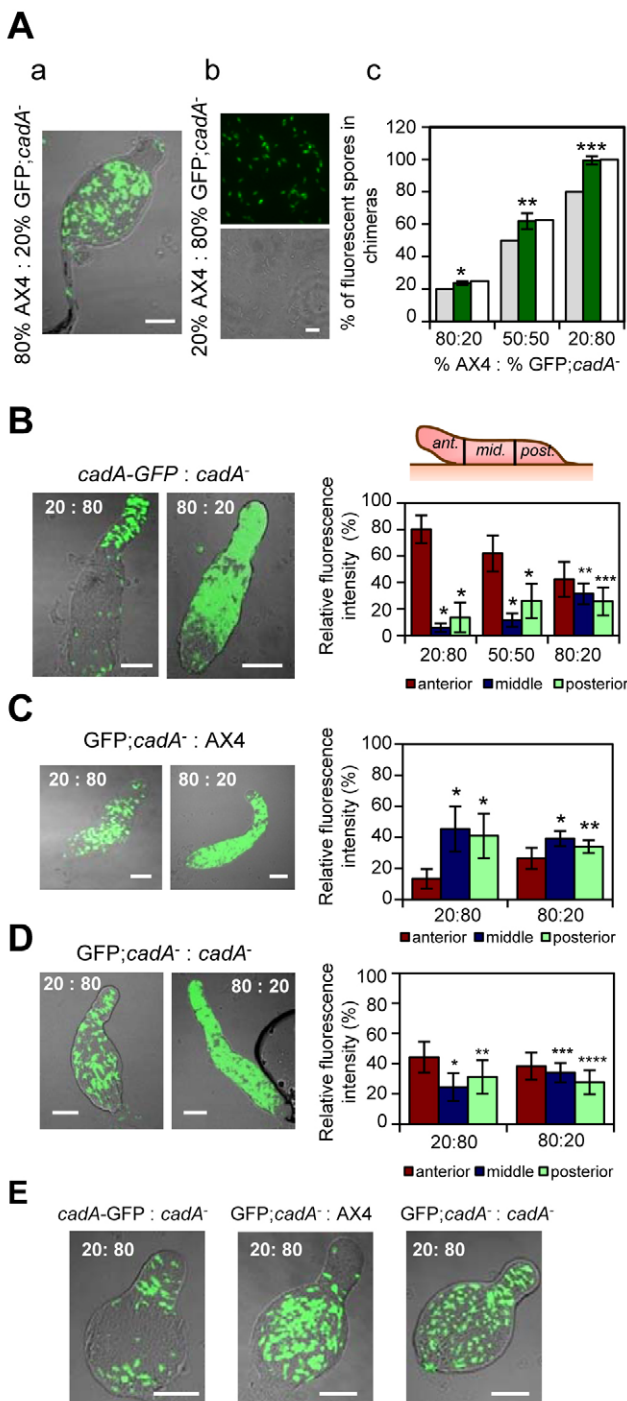


Fig. 2. The *cadA* gene confers different social behavior to *Dictyostelium* cells developed on agar plates. (A) Spore formation by *act15::GFP;cadA*⁻ cells in the fruiting body of chimeras developed on non-nutrient agar. (a) Confocal image showing the predominant presence of fluorescent spores inside the sorus of a chimeric fruiting body. Scale bar: 50 μm. (b) A pair of fluorescence and phase images of spores collected from a chimeric fruiting body made up of 20% AX4 and 80% *act15::GFP;cadA*⁻ cells. (c) Quantification of fluorescent spores in different chimeras ($n=3$) (green bars), which are compared with the theoretical values if both types of cells were distributed evenly throughout the sorus (gray bars) or if the *cadA*-null cells cheat and constitute to 100% of the spores (white bars). Data represent the mean \pm s.d. of three different experiments. (* $P<0.05$, ** $P<0.05$, *** $P<0.05$, Student's unpaired *t*-test). **(B)** The *act15::cadA*-GFP;*cadA*⁻ transfected cells were mixed at different ratios with *cadA*⁻ cells for development on coverslips. Developing structures were fixed for confocal microscopy. The micrographs show the spatial distribution of *act15::cadA*-GFP;*cadA*⁻ cells (green) in the slug structures of chimeras. Scale bars: 50 μm. Fluorescence intensity per unit area was quantified using Image J software (NIH) for the anterior, middle and posterior regions of the slug as shown in the schematic. The relative fluorescence intensities were calculated for each region of the different chimeras and the fluorescence values of the middle and posterior regions were compared with that of the anterior region. Data represent the mean \pm s.d. ($n=20-25$; * $P<0.0001$, ** $P<0.01$, *** $P<0.0005$, Student's *t*-test). **(C)** Confocal images of chimeric slugs containing *act15::GFP;cadA*⁻ cells and AX4 cells mixed at different ratios. Scale bars: 50 μm. The bar graph shows the relative fluorescence intensities of the three slug regions and values of the middle and posterior regions are significantly different from that of the anterior. Data represent the mean \pm s.d. ($n=20$; * $P<0.0001$, ** $P<0.001$, Student's *t*-test). **(D)** Confocal images of chimeric slugs containing *act15::GFP;cadA*⁻ and *cadA*⁻ cells mixed at different ratios. Scale bars: 50 μm. Relative fluorescence intensities were measured for each segment and compared with the anterior region. Data represent the mean \pm s.d. ($n=20$; * $P<0.001$, ** $P>0.01$, *** $P>0.05$, **** $P<0.001$, Student's *t*-test). **(E)** Confocal images of the different chimeras at the culmination stage. Scale bars: 50 μm.

(Fig. 1B). At 48 hours, the number of *cadA*⁻ fruiting bodies reached only 40% of that of the AX4 cells, indicating that the loss of DdCAD-1 expression hampered the aggregation process.

Interestingly, *cadA*⁻ cells displayed opposite behavior when chimeras were developed on non-nutrient agar plates. Contrary to the data obtained with soil plates, the *cadA*⁻ cells differentiated primarily into spores (Fig. 2Aa,b). Only a few of the fluorescent *cadA*⁻ cells were found occasionally in the stalk. The quantitative data showed that almost all of the *cadA*⁻ cells in the chimeras formed spores when chimeras were made up of 20% *cadA*⁻ cells (Fig. 2Ac). When chimeras were made up of 80% *cadA*⁻ cells, essentially all spores were derived from the *cadA*⁻ strain. The *cadA* gene allowed the *cadA*⁻ to cheat and survive as they preferentially differentiated into whereas the *cadA*⁺ cells differentiated into stalk cells and eventually died.

Preferential localization of *cadA*⁺ cells in prestalk region of chimeras during development

In order to investigate how DdCAD-1 exerts an influence on cell survival during development, *cadA*⁻ cells were transfected with plasmid DNA containing the *act15::cadA*-GFP construct (see Fig. S2A,B in the supplementary material) to ensure even expression of the fusion protein in cells (see Fig. S2C,D in the supplementary material). Normal sorting pattern was restored in slugs by the ectopic expression of DdCAD-1 (see Fig. S2E,F in the supplementary material), confirming our previous observation

consistent with the developmental phenotype of *cadA*⁻ cells (Wong et al., 2002). These results show that the *cadA*⁺ cells promoted the survival of other *cadA*⁺ cells, but not the *cadA*⁻ cells. It is possible that the presence of DdCAD-1 on the cell surface allows AX4 cells to adhere preferentially to each other and facilitates the recruitment of cells into aggregates. The presence of soil particles might have compromised further the ability of *cadA*⁻ cells to form aggregates. Indeed, when AX4 cells and *cadA*⁻ cells were developed separately on soil plates, *cadA*⁻ cells developed much more slowly than AX4 cells. Whereas most of the AX4 cells completed development by 24 hours, only a few fruiting bodies were observed for *cadA*⁻ cells

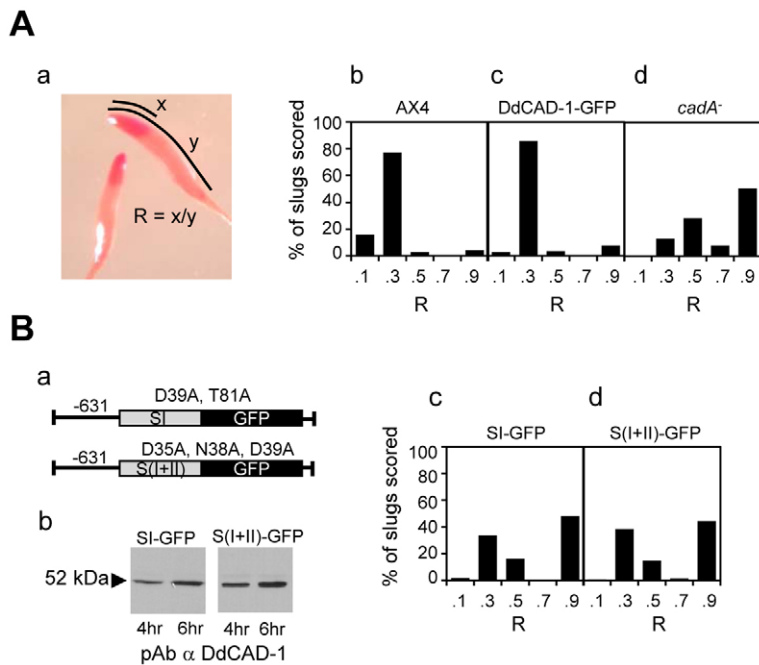


Fig. 3. Rescue of cell sorting in *cadA*⁻ slugs by DdCAD-1 depends on its Ca²⁺-binding capability. (A) Expression of DdCAD-1-GFP driven by its endogenous promoter restored normal cell sorting in *cadA*⁻ slugs. (a) A light micrograph showing Neutral Red-stained slugs formed by $-631::cadA-GFP;cadA^{-}$ transfected cells. The relative length of the stained anterior zone in 200–300 slugs was measured for each strain and the R values were plotted against the frequency of occurrence of the R values for AX4 slugs (b), $-631::cadA-GFP;cadA^{-}$ slugs (c) and *cadA*⁻ slugs (d). Slugs without a clear sorting pattern were given an R value of 0.9. **(B)** Aberrant cell sorting patterns in slugs expressing DdCAD-1 containing mutations in its Ca²⁺-binding sites. (a) Schematics depicting the mutant Ca²⁺-binding constructs of *cadA* [SI, S(I+II), with amino substitutions indicated on top of the schematic] fused to GFP. (b) Expression of mutant DdCAD-1-GFP in transfectants. Transfectants derived from *cadA*⁻ cells were cultured on a bacterial lawn and collected for development. Cell lysates were prepared from 4- and 6-hour cells for SDS-PAGE and the protein blots were probed with rabbit anti-DdCAD-1 antiserum. (c,d) Sorting patterns of Neutral Red-stained transfectants. The relative lengths of the anterior zones in slugs were measured and the R values were plotted against the frequency of occurrence.

(Wong et al., 2002). Transfected cells were mixed with *cadA*⁻ cells at different ratios and the location of the DdCAD-1-GFP-expressing cells in the chimeras was monitored at different stages. The green cells appeared mostly in the periphery at the mound stage. Most of them were present in the anterior prestalk region by the slug stage. To quantify the distribution of green cells, images of slugs were collected and divided into three segments for the measurement of fluorescence intensity (Fig. 2B). The data confirmed the preferential localization of green cells in the anterior zone of slugs. Spore yield was also identical to that of AX4 cells in similar chimeras.

As a control, *cadA*⁻ cells were transfected with an *act15::GFP* construct. The GFP;*cadA*⁻ cells were mixed with the AX4 (*cadA*⁺) cells for development. Most of the GFP;*cadA*⁻ cells were found in the middle and posterior regions of the slug, whereas the anterior region was occupied primarily by the AX4 cells (Fig. 2C). Even within the 80:20 chimeras, a distinctly ‘dark’ area was evident at the tip region. In a second control, GFP;*cadA*⁻ cells were mixed with *cadA*⁻ cells. As expected, the green cells distributed more or less evenly along the whole length of the slug (Fig. 2D). At the early culmination stage, all three types of chimeras showed patterns of green cell distribution similar to those observed at their respective slug stage (Fig. 2E). These results suggest that *cadA*⁺ cells preferentially sort to the anterior zone of chimeric slugs and differentiate into prestalk cells, whereas *cadA*⁻ cells occupy primarily the posterior two-third of slugs and eventually differentiate into spores.

Rescue of *cadA*⁻ cells using wild-type and mutant constructs of DdCAD-1

The notion that DdCAD-1 plays an important role in the sorting of prestalk cells from prespore cells would predict the presence of DdCAD-1 on the cell surface during morphogenesis. To investigate how DdCAD-1 might be involved in cell sorting during development, *cadA* cDNA was fused to GFP and the fusion construct was ligated to the endogenous *cadA* promoter -631 (Yang et al., 1997) (see Fig. S3A in the supplementary material) for

transfection into *cadA*⁻ cells. The use of the endogenous *cadA* promoter ensured that expression occurred according to the normal temporal program of development. G418-resistant clones were screened by direct visualization under a fluorescence microscope. The proper temporal expression of DdCAD-1-GFP in transfectants was confirmed by probing protein blots with antibodies against DdCAD-1 and GFP (see Fig. S3B in the supplementary material). Whereas both transfectants and the parental *cadA*⁻ cells expressed the EDTA-sensitive or EGTA-resistant adhesion sites, cell cohesion assays showed that the EDTA/EGTA-sensitive or Ca²⁺-dependent cell-cell adhesion sites were observed only in the transfectants (see Fig. S3C in the supplementary material).

As DdCAD-1-mediated cell-cell adhesion requires Ca²⁺, we tested whether the Ca²⁺-binding capability of DdCAD-1 is required for proper cell adhesion and cell sorting during development. *cadA*⁻ cells were transfected with two constructs of DdCAD-1 that contained mutations in the Ca²⁺-binding site I and S(I+II). Expression of the mutant proteins were confirmed with western blots (Fig. 3Bb). When cell sorting experiments were performed using Neutral Red-stained cells, *cadA*⁻ cells expressing DdCAD-1-GFP ectopically showed the normal sorting pattern similar to AX4 cells (Fig. 3Ab-d). However, ectopic expression of DdCAD-1 containing mutated Ca²⁺-binding sites failed to rescue the cell sorting defects of the *cadA*⁻ cells. Most of the mutant slugs showed non-sorted patterns (Fig. 3Bc,d). These results indicate that the Ca²⁺-binding capability of DdCAD-1 is required for the proper sorting process of prestalk and prespore cells at the slug stage.

Dynamic changes in the temporal and spatial distribution of DdCAD-1 during development

Transfectants that expressed DdCAD-1-GFP at levels comparable to the DdCAD-1 level in AX4 cells were selected for development and the temporal and spatial expression of DdCAD-1-GFP was monitored by confocal microscopy. Developmental structures were fixed and F-actin was stained with phalloidin to demarcate the cell boundaries (Fig. 4). Confocal microscopy revealed that DdCAD-1-GFP was synthesized inside the cytoplasm shortly after the

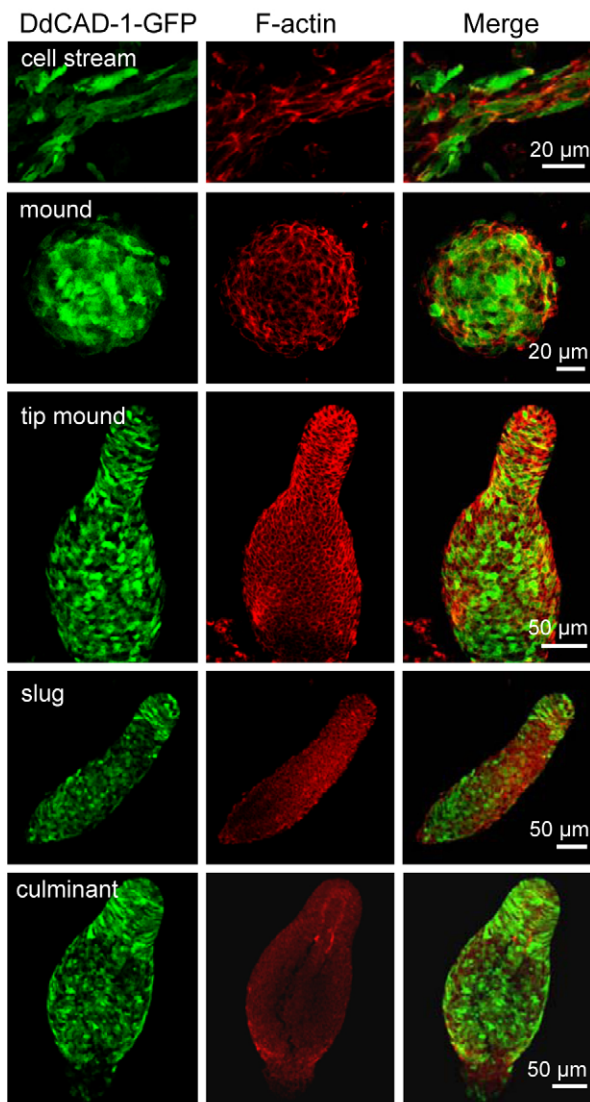


Fig. 4. Changes in the pattern of DdCAD-1 expression during development. The $-631::cadA-GFP;cadA^-$ transfected cells were developed on coverslips for 10 to 22 hours. The developing structures were fixed and permeabilized with 0.5% Triton X-100 before staining with phalloidin (red). Confocal images show changes in the temporal and spatial expression of DdCAD-1-GFP (green) at different stages of development.

initiation of development and became associated with the plasma membrane and filopodial structures as previously reported (Sesaki and Siu, 1996). At the mound stage, DdCAD-1-GFP was present mostly in the cytoplasm whereas enrichment in the cell-cell contact regions was not observed. A similar pattern of DdCAD-1 expression was evident at the tipped mound stage. Additionally, DdCAD-1 appeared to be absent from a subset of cells scattered throughout the aggregate. At the migrating slug stage, cells with higher levels of DdCAD-1 appeared in the anterior region. This difference in DdCAD-1 expression between the anterior cells and the posterior cells became more obvious in culminants.

When cells were subjected to fluorescence-activated cell sorting (FACS) analysis, a single group of fluorescent cells was observed at the mound stage (see Fig. S4 in the supplementary material).

However, two groups of fluorescent cells became evident at the culmination stage. About 30% of the cells had a much reduced level of DdCAD-1, whereas the other group showed a broad range of higher fluorescence intensity. Taken together, the data indicate heterogeneity in DdCAD-1 expression as development progressed and that cells with a higher level of DdCAD-1 expression had a propensity to differentiate into prestalk cells.

The cell-cell contact regions of anterior cells are enriched with DdCAD-1

A role for DdCAD-1 in cell sorting would imply the presence of DdCAD-1 on the cell surface. However, the high level of cytoplasmic DdCAD-1-GFP prevented the morphological analysis of membrane-associated DdCAD-1. Therefore, transfectants at the slug stage were treated with detergent for a longer period of time to allow intracellular DdCAD-1-GFP to leak out from cells. Confocal images of these cells revealed differential association of DdCAD-1-GFP with the plasma membrane along the long axis of the slug. An abundance of DdCAD-1-GFP was observed in cell-cell contact regions in the anterior zone, with close coincidence with the phalloidin-staining pattern, whereas the intracellular green fluorescence was relatively low (Fig. 5A). However, DdCAD-1 was present mostly in the cytoplasm in the posterior zone where membrane association of DdCAD-1 became less evident (Fig. 5B). During culmination, DdCAD-1 was present predominantly in cell-cell contacts in the upper cup region surrounding the descending stalk tube (Fig. 5C). In fruiting bodies, cells expressing high levels of DdCAD-1-GFP were found concentrated in the upper cup and lower cup regions of the sorus (Fig. 5D).

To determine whether DdCAD-1-GFP was associated with the surface of anterior cells, the anterior and posterior zones of slugs were cut and the cells were dissociated for further analysis. Cell-cell contacts of anterior cells, but not posterior cells, were found to be enriched with DdCAD-1-GFP (Fig. 5E). When antibody-induced clustering of DdCAD-1 was performed on living cells, DdCAD-1 ‘caps’ were observed on the elongated anterior cells, indicating that DdCAD-1 was present on the cell surface (Fig. 5Fa). By contrast, antibody treatment of posterior cells failed to induce cap formation (Fig. 5Fb), confirming that DdCAD-1-GFP was present at a very low level or absent from the cell surface.

Rescue of *cadA*⁻ phenotype by in vitro reconstitution of DdCAD-1

DdCAD-1 is synthesized as a soluble protein, which adheres to the cell surface via interaction with an as yet unidentified membrane anchoring protein (Lin et al., 2006). As this membrane anchoring protein is expected to be present in *cadA*⁻ cells, exogenously added DdCAD-1 should bind to *cadA*⁻ cells. We therefore examined whether exogenously added recombinant DdCAD-1 could rescue the mutant phenotype of *cadA*⁻ cells. Recombinant His₆-DdCAD-1 (wild type) and His₆-S(I+II) DdCAD-1 (containing mutations in the Ca²⁺-binding pockets SI and SII) were purified from *Escherichia coli* (Fig. 6A). Protein blots showed that both proteins bound to *cadA*⁻ cells with similar efficiency (Fig. 6B). To monitor the cell sorting phenomenon, cells were incubated with Neutral Red for 5 minutes before the addition of recombinant protein. The wild-type His₆-DdCAD-1 protein rescued the cell sorting defect in *cadA*⁻ cells, whereas the mutant protein failed to do so (Fig. 6C). It became evident that exogenously applied DdCAD-1 was sufficient to restore the wild-type cell sorting pattern in null cells and its rescue function was dependent on Ca²⁺.

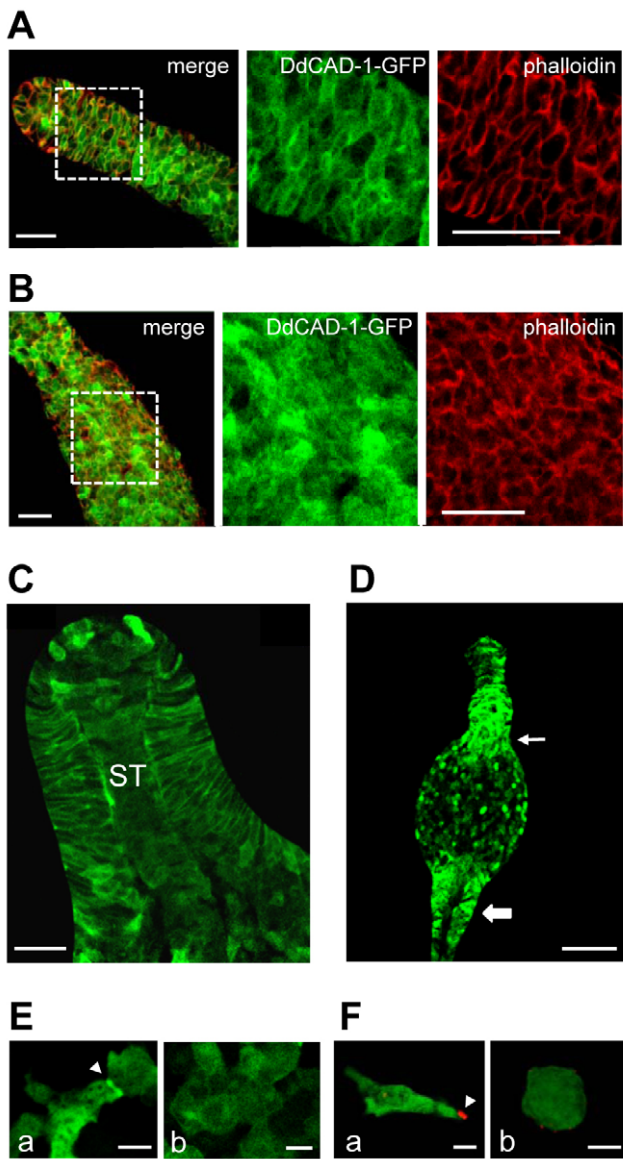


Fig. 5. Enrichment of cell-cell contacts in the anterior zone of slugs and culminants with DdCAD-1-GFP. (A, B) Differences in the subcellular localization of DdCAD-1-GFP in slugs. The *act15::cadA-GFP;cadA⁻* transfectants were developed on coverslips for 18 to 20 hours. DdCAD-1-GFP was abundant in cell-cell contacts in the anterior zone of the slug (A), whereas DdCAD-1-GFP was present mostly in the cytoplasm of cells in the posterior zone (B). The areas boxed by dashed lines are shown at higher magnification in the two adjacent panels. Scale bars: 20 μ m. (C) Confocal image showing abundance of DdCAD-1-GFP in cell-cell contact regions in the anterior zone of *-631::cadA-GFP;cadA⁻* culminant. ST, stalk tube. Scale bar: 20 μ m. (D) Confocal image of the sorus of an unfixed fruiting body showing an abundance of DdCAD-1-expressing cells in the upper cup (thin arrow) and lower cup (thick arrow) regions. (E) Surface localization of DdCAD-1-GFP in the anterior cells of *act15::cadA-GFP;cadA⁻* slugs: (a) cells dissociated from the anterior zone, showing that the cell-cell contact region was enriched with DdCAD-1-GFP (arrowhead); (b) cells dissociated from the posterior region of slugs. Scale bars: 5 μ m. (F) Capping of DdCAD-1-GFP in *act15::cadA-GFP;cadA⁻* cells. Capping was induced by incubating cells with mouse anti-GFP mAb followed by Alexa-568-conjugated secondary antibody (red, arrowhead): (a) cells derived from the anterior zone; (b) cells derived from the posterior zone. Scale bars: 5 μ m.

To monitor the fate of the bound recombinant DdCAD-1 during development, DdCAD-1 was conjugated with the fluorescent probe Alexa-488 before adding to cells and the distribution patterns of the labeled protein was followed by confocal microscopy. At the aggregation stage, DdCAD-1 was associated primarily with the cell membrane (Fig. 6Da,b). The honeycomb patterns of early mound and tight mound structures showed that the recombinant DdCAD-1 was present primarily in the cell-cell contact regions (Fig. 6Dc,d). However, in the core region of the tight mound (Fig. 6Dd), diffuse fluorescence in the cytoplasm became visible, providing the first sign of DdCAD-1 internalization. Also, cells in the outermost layer of the mound structure showed a higher level of fluorescence intensity associated with the cell membrane. At the slug stage, a lot of cells began to show fluorescence in their cytoplasm, indicative of protein internalization (Fig. 6De). Also, cells in the anterior zone appeared to be more elongated, suggesting that they might be moving toward the tip region. At the early culmination stage, the contact regions among cells in the anterior upper cup region were highly enriched with DdCAD-1, whereas DdCAD-1 was mostly in the cytoplasm in the posterior cells (Fig. 6Df). These results confirmed the extracellular role of DdCAD-1 in cell sorting. Additionally, membrane-bound DdCAD-1 was either shed or internalized by many cells at the slug stage.

Distinct chemotactic response of AX4 and *cadA⁻* cells to cAMP

Differential chemotactic response to cAMP has been implicated in the sorting of prestalk from prespore cells (Jiang et al., 1998). When AX4 cells and *cadA⁻* cells were subjected to the chemotaxis assay, AX4 cells migrated at a rate \sim 20% slower than *cadA⁻* cells (Fig. 7A). These results would predict that AX4 cells took more time to reach the aggregation center than *cadA⁻* cells. Indeed, when *cadA⁺* and *cadA⁻* cells were mixed at equal numbers for development on agar, the *cadA⁺* cells were found primarily in the periphery of the mound structure (Fig. 7B).

DISCUSSION

Despite the previous finding that DdCAD-1 appears only transiently during early cell aggregation and becomes hardly detectable on the cell surface in the post-aggregation stages of development (Sesaki and Siu, 1996), studies presented in this paper have provided evidence that DdCAD-1 can exert opposite influences on the social behavior of *Dictyostelium* depending on the environment encountered by the organism. When development is carried out on soil plates, *cadA* promotes the survival of cells that express the same gene product on the cell surface. However, on non-nutrient agar plates, *cadA* confers opposite behavior on cells, allowing the *cadA⁻* cells to behave as cheaters and survive while the *cadA⁺* cells undergo terminal differentiation to form stalk cells and eventually die.

DdCAD-1 is expressed at high levels at the onset of development. During cell streaming, DdCAD-1 is abundant especially on membrane protrusions such as lamellipodia and filopodia, which are dynamic structures that are known to mediate initial contacts among *Dictyostelium* cells (Choi and Siu, 1987; Sesaki and Siu, 1996) as well as mammalian cells (Sandig et al., 1997; Vasioukhin et al., 2000). After entry into the cell stream, DdCAD-1 redistributes from the contact regions but remains associated with membranes on the outer edge of the stream to make contacts with incoming cells (Sesaki and Siu, 1996). The loss of DdCAD-1 would hinder the recruitment of cells into streams owing to compromised cell-cell adhesion. Development of *cadA⁻* cells is

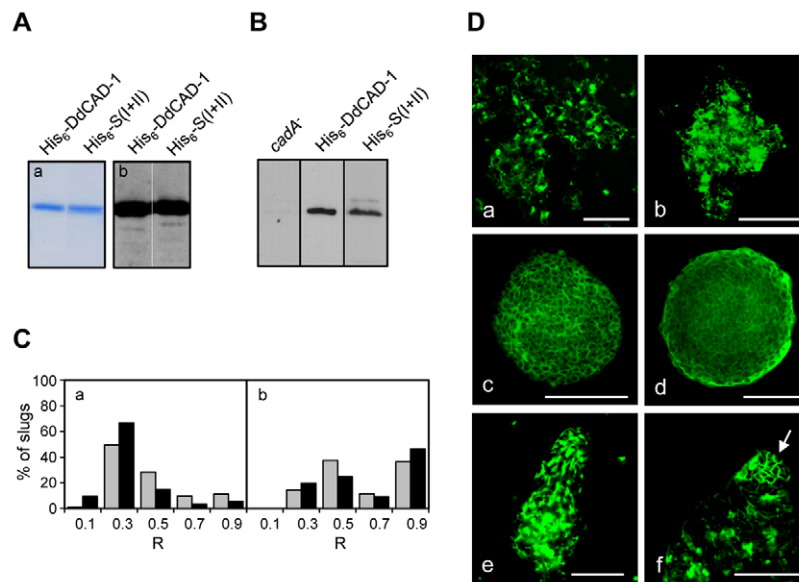


Fig. 6. Rescue of the cell sorting defects in *cadA*⁻ cells by DdCAD-1 recombinant protein. (A) Coomassie-stained gel profiles (a) and western blots (b) of purified His₆-DdCAD-1 and His₆-S(I+II) recombinant proteins (~1.25 μg per lane). (B) Lysates of *cadA*⁻ cells (25 μg) derived from slugs previously incubated with DdCAD-1 recombinant proteins at 3 μg/μl were prepared for SDS-PAGE and protein blots were probed with rabbit antiserum against DdCAD-1. (C) Cell sorting in the *cadA*⁻ slugs was examined in the presence of exogenous recombinant proteins. Cells were stained with Neutral Red and the length of the anterior zone of slugs was measured and the R values were determined. (a) *cadA*⁻ slugs developed in presence of 0.5 μg/μl (gray bars) and 3 μg/μl (black bars) of His₆-DdCAD-1 protein in 100 μl of cell suspension. (b) *cadA*⁻ slugs developed in the presence of 0.5 μg/μl (gray bars) and 3 μg/μl (black bars) of His₆-S(I+II) protein. In each case, 300-500 slugs were scored in three experiments. (D) *cadA*⁻ cells were mixed with 3 μg/μl of Alexa-488-conjugated His₆-DdCAD-1 (green) in 100 μl of cell suspension for development on coverslips. At 12 hours, excess buffer was removed and cells were developed for another 8 hours. Slugs at different stages of development were prepared for confocal microscopy: (a) early aggregation stage, (b) late aggregation stage, (c) early mound stage, (d) late mound stage, (e) early slug stage, (f) early culminant stage. An abundance of recombinant DdCAD-1 was observed in the cell-cell contacts of anterior cells (arrow). Scale bars: 50 μm.

known to be delayed for 5-6 hours and their ability to form spores is compromised (Wong et al., 2002). These defects are exacerbated by the presence of the rough and uneven surfaces of soil particles, resulting in the further delay of cell aggregation and subsequent developmental processes (Fig. 1). By contrast, wild-type cells complete development in 24 hours on soil plates, just as efficiently as development on agar surfaces. As the ability of the knockout cells to enter cell streams is compromised on soil plates, chimeric cell aggregates would contain mostly wild-type cells, resulting in a significant reduction of *cadA*⁻ spores. In this way, the *cadA* gene favors the survival of individuals that carry the same gene.

However, *cadA* switches to the opposite behavior in chimeras developed on agar surfaces and promote the survival of the knockout cells. An explanation of this unusual effect would involve two intertwining developmental processes, cell sorting and cell differentiation, both of which are defective in the *cadA*⁻ cells (Wong et al., 2002). Cells in the center of an aggregate are known to have a greater propensity to differentiate into prespore cells (Huang et al., 1997). In order that the *cadA*⁻ cells may form spores, the wild-type cells have to sort out from the knockout cells to occupy the anterior zone and differentiate into stalk cells. Both differential cell cohesiveness and chemotactic migration have been implied in the cell sorting process (Jiang et al., 1998). Our results show that the *cadA*⁻ cells undergo faster chemotactic migration than do wild-type cells. Although the difference is small, it is significant and should allow the knockout cells to reach the aggregation center earlier than the wild-type cells (Fig. 7). This might account for the abundance of wild-type cells in the periphery of chimeric aggregates, as illustrated in the schematics in Fig. 8.

Normally, cells that show greater cohesiveness for one another would stay in the core and the less cohesive ones would sort to the periphery in chimeric aggregates (Steinberg, 1975; Steinberg and Gilbert, 2004; Steinberg and Takeichi, 1994). But, how could the knockout cells remain in the core of the chimeric mounds? Interestingly, a similar sorting pattern has been observed when wild-type cells and *cadA*⁻ cells are mixed and gyrate in suspension cultures (Wong et al., 2002). Further analysis by these authors has

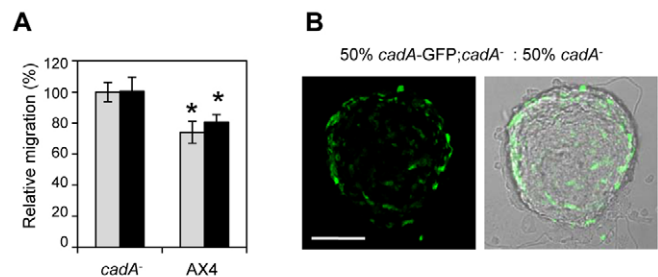


Fig. 7. AX4 and *cadA*⁻ cells show distinct chemotactic responses to cAMP. (A) Relative chemotactic migration of *cadA*⁻ and AX4 cells towards 100 μM (gray bars) or 250 μM (black bars) of cAMP. Data represent the mean ± s.d. (n=14; *P<0.05, Student's *t*-test). (B) The *act15::cadA-GFP;cadA*⁻ transfected cells were mixed at a 1:1 ratio with *cadA*⁻ cells for development on coverslips. Developmental structures were fixed for confocal microscopy. The pair of micrographs show the spatial distribution of *act15::cadA-GFP;cadA*⁻ cells (green) in a chimeric mound structure. Scale bar: 50 μm.

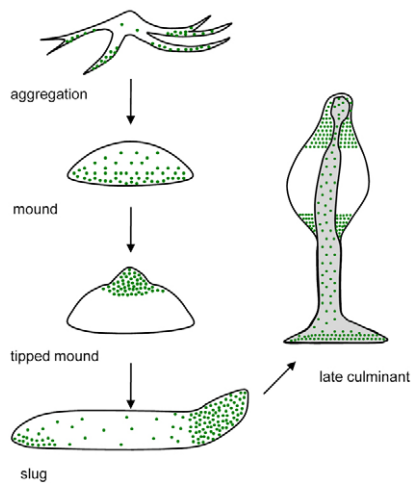


Fig. 8. Schematic drawings depicting the distribution of DdCAD-1-expressing cells in chimeras during development. During aggregation, DdCAD-1-expressing cells (green) migrate slower than the knockout cells on agar surface. Consequently, the *cadA*⁺ cells (green dots) reach the aggregation center later than *cadA*⁻ cells and become localized primarily in the periphery of the mound. In tipped mounds, most DdCAD-1-expressing cells sort out from the *cadA*⁻ cells and become concentrated in the tip region. Migrating slugs show a similar pattern as the tipped mound with the anterior zone becoming enriched with DdCAD-1-expressing cells. However, a small number of cells remain in the posterior zone of the slug, and they might eventually differentiate into the basal disc cells. Finally, the upper cup, lower cup and basal disc regions are enriched with DdCAD-1-expressing cells at the late culminant stage. These cells will eventually die whereas cells lacking surface expression of DdCAD-1, whether of wild-type or knockout origin, will differentiate preferentially into spores.

revealed that a higher level of *csA* is expressed at the later stages of cell aggregation by the knockout cells. The increased level of *csA* compensates for the loss of DdCAD-1 expression, allowing the knockout cells to stay in the aggregation core owing to higher mutual adhesion. However, wild-type cells inside the core of the mound structure eventually sort out to the periphery owing to their lower level of *csA* expression. Cells in the periphery of the mound have a propensity to differentiate into prestalk cells and migrate to the anterior zone of the slug (Siu et al., 1983). Eventually, the *cadA*⁺ cells undergo terminal differentiation and die in order to confer altruistic benefits to the knockout cells. The influence of the *cadA* gene on cell behavior in chimeras appears to rely on an intricate balance in the expression of the various cell adhesion molecules. As the loss of *csA* expression elicits the precocious expression of TgrC1 in *csaA*-knockout cells (Wang et al., 2000), it is conceivable that this phenomenon might also account for the behavior of the *csaA* gene in chimeras of *csaA*⁻ cells and wild-type cells (Queller et al., 2003).

The influence of *cadA* on cell sorting is also corroborated by rescue studies. Ectopically expressed DdCAD-1 rescues the cell sorting defect observed in the knockout cells. The sorting defect can also be rescued by exogenous recombinant DdCAD-1. Rescued cells display the normal ratio in the anterior-posterior pattern of slugs. In both types of experiments, the cell sorting function of DdCAD-1 is dependent on the integrity of its Ca²⁺-binding sites.

Several mechanisms have been proposed for the formation of the anterior-posterior pattern in *Dictyostelium*. This pattern might arise by the sorting out of prestalk cells primarily to the anterior zone

and the prespore cells to the posterior zone of the slug as a consequence of differential cell adhesiveness (Araki et al., 1994; Leach et al., 1973; Siu et al., 1983; Steinberg, 1975; Steinberg and Gilbert, 2004). Studies from several laboratories have suggested that prestalk and prespore cells arise in a spatially random manner throughout the aggregate and that sorting results from cell-type specific alterations in adhesion (Abe et al., 1994; Nicol et al., 1999). However, it has also been proposed that pattern can be generated by position-dependent differentiation of the different cell types (Krefft et al., 1984; Wolpert, 1981). The positional information model states that the value related to a position in a coordinate system determines cell differentiation (Wolpert, 1989; Wolpert and Szathmary, 2002). During *Dictyostelium* development, cells that are starved in different cell cycle phases tend to reach the aggregate at different times and therefore occupy different positions within the aggregate. Cells in the periphery, which are usually starved at S phase or early G2 phase, differentiate mostly into prestalk cells, suggesting that a combination of cell cycle stage and positional information dictates cell fate (Gomer and Firtel, 1987; Maeda et al., 2003; Weijer, 2009; Weijer et al., 1984; Zimmermann and Weijer, 1993). Although both wild-type and *cadA*⁻ cells are capable of differentiating into the different cell types required for fruiting body formation, the *cadA*⁻ cells achieve 100% cheating and form spores exclusively in chimeras. These results are consistent with position-dependent differentiation during development.

In addition to cell cycle position, a variety of factors are known to control cell fate determination. The differentiation-inducing factors (DIFs) are a family of small chlorinated diffusible molecules that induce stalk cell differentiation but suppress spore differentiation (Kay and Jermyn, 1983; Kay et al., 1999; Thompson and Kay, 2000). Recently, the nutritional state of cells has also been found to influence cell fate. Cells that starve first and have lower energy reserves tend to form the stalk, whereas the better-fed ones migrate faster to the aggregate center and differentiate into spores (Kuzdzal-Fick et al., 2010).

Evidently, a number of factors can influence the peripheral localization of cells and subsequent differentiation. Results shown in this paper have revealed heterogeneity in the temporal and spatial expression of DdCAD-1. Whereas most of the cells have internalized or shed their membrane DdCAD-1, a distinct subset of cells retain the expression of DdCAD-1 on the surface whether DdCAD-1 is expressed ectopically or added exogenously. These cells are localized first in the periphery of the aggregate and eventually move to the anterior zone occupying the tip region of the slug. Peripheral cells usually follow a circular path to move around the mound to the tip region (Nicol et al., 1999; Siegert and Weijer, 1995). In chimeras, the presence of DdCAD-1 on the surface of peripherally located wild-type cells would help them pull other DdCAD-1-expressing cells out towards them during the rotational movement, whereas prespore cells in the core would re-establish contact and continue to rotate uninterrupted.

In chimeras, cells in the anterior zone with high levels of DdCAD-1 on the cell surface display altruism and differentiate into stalk cells and die, whereas the posterior cells with DdCAD-1 localized mainly in the cytoplasm survive as spores. Remarkably, similar social behavior has been observed during development of wild-type cells. Additionally, *in vitro* rescue experiments have demonstrated that cells that retain DdCAD-1 in the contact region move into the anterior zone and differentiate into prestalk cells. These observations thus explain why genetically compromised cells might be able to survive and behave as cheaters in chimeras.

Our results also provide evidence for a direct extracellular role of DdCAD-1 in cell sorting, which in turn might account for the social behavior conferred by the *cadA* gene. Cell sorting during development is achieved by differential subcellular distribution of DdCAD-1, although the mechanism involved and its relationship with the differentiation signal remain obscure. *D. discoideum* cells express several adhesion systems to govern the social behavior of cells during morphogenesis. Future studies on the mechanisms that regulate the spatiotemporal expression of these proteins should lead to a better understanding of how cells communicate and influence behavior, as well as providing insights into the complexity of development.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.060129/-DC1>

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