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Communication

Adenylyl Cyclase G, an Osmosensor Controlling Germination of *Dictyostelium* Spores*

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Dictyostelium **cells express a G-protein-coupled adenylyl cyclase, ACA, during aggregation and an atypical adenylyl cyclase, ACG, in mature spores. The *ACG* gene was disrupted by homologous recombination. *acg*$^-$ cells developed into normal fruiting bodies with viable spores, but spore germination was no longer inhibited by high osmolarity, a fairly universal constraint for spore and seed germination. ACG activity, measured in aca$^-$/*ACG* cells, was strongly stimulated by high osmolarity with optimal stimulation occurring at 200 milliosmolar. *Rde*C mutants, which display unrestrained protein kinase A (PKA) activity and a cell line, which overexpresses PKA under a prespore specific promoter, germinate very poorly, both at high and low osmolarity. These data indicate that ACG is an osmosensor controlling spore germination through activation of protein kinase A.**

In many organisms, hypo- or hyperosmolarity is encountered as an environmental stress factor. In plant seeds and spores of the slime mold *Dictyostelium discoideum*, high osmolarity functions as a signal to maintain dormancy. During *Dictyostelium* development, starving amoebae aggregate to form fruiting bodies consisting of a stalk and a globular mass of spores. The spores are embedded in a viscous fluid and remain dormant due to ambient high osmolarity and the presence of a cytokinin-type germination inhibitor, called discadenine (1, 2). Extracellular cAMP acts as chemoattractant controlling cell aggregation, but also induces the differentiation of spore cells (see Ref. 3). Two adenylyl cyclase genes have been cloned; the aggregation adenylyl cyclase (ACA)[1] harbors 12 putative membrane-spanning domains and is highly homologous to vertebrate adenylyl cyclases. This enzyme is activated through G-protein-coupled surface cAMP receptors and is responsible for producing the oscillatory cAMP signals that regulate cell movement and differentiation. The germination adenylyl cyclase (ACG) is a novel type of adenylyl cyclase that contains a single transmembrane domain. The *ACG* gene is only expressed in spores and its function was hitherto obscure (4).

In this paper we show that ACG mediates inhibition of spore germination by high osmolarity. Inactivation of the *ACG* gene by homologous recombination generates spores that have lost the ability to respond to high osmolarity. When *ACG* is expressed under a heterologous promoter during growth, the enzyme activity is strongly stimulated by high osmolarity. Spore germination is impaired in mutants that display unrestrained PKA activity, suggesting that PKA is the target for cAMP in the control of spore dormancy.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—The *Dictyostelium* cell lines *AX3, aca*$^-$*/ACG*, *acg*$^-$, and *Sp60-PKA* were cultured in axenic medium which was supplemented with 20 μg/ml G418 for *aca*$^-$*/ACG* and *Sp60-PKA* cells. NC4 and *rdeC* cells were grown on glucose/peptone agar in association with *Escherichia coli* 281.

ACG Gene Targeting—A 1.7-kb *Xba*I-*Bcl*I fragment containing the 3′-region of the *ACG* gene (4) was inserted into the *Xba*I and *Not*I sites of pBluescript KS. Subsequently, a 2.0 kb fragment, containing the 5′-untranslated region and the first 142 bases of the coding sequence, was ligated in the *Eco*RI-*Hin*dIII sites of the polylinker. The 2.2-kb actin6-NEO® cassette (5) was inserted into the *Xba*I site in between both *ACG* fragments. The ACG-knockout construct was isolated from pBluescript as a 6-kb *Kpn*I-*Sac*I fragment and transformed to wild-type AX3 cells. G418-resistant transformants were isolated and analyzed for disruption of the *ACG* gene by probing Southern blots of *Cla*I digested genomic DNA with appropriate *ACG* DNA fragments.

cAMP Accumulation by Intact Cells—aca$^-$*/ACG* cells were harvested from growth medium, resuspended to 10^7 cells/ml in 10 mM sodium/potassium phosphate buffer, pH 6.5 (PB), and shaken at 120 rpm for 1 h at 22 °C. Cells were collected, resuspended in PB, and aerated for 5 min. 25-μl aliquots of cell suspension were incubated with variables to a final volume of 30 μl in microtiter plate wells. Reactions were started by addition of 5 mM dithiotreitol (DTT) and terminated by addition of 30 μl of 3.5% perchloric acid. Lysates were neutralized with $KHCO_3$ and cAMP levels were determined by isotope dilution assay.

Activation-trap Assay for Adenylyl Cyclase—aca$^-$*/ACG* cells were starved for 1 h, resuspended in 0.5 mM PB at 10^8 cells/ml, and preincubated for 10 min in the presence or absence of high osmolarity. Subsequently 0.1 volume of 20 mM $MgCl_2$ in 0.1 M Tris, pH 8, was added, and cells were rapidly lysed through nuclepore filters. Lysates were incubated at 23 °C for the indicated time periods with 0.5 mM ATP, 5 mM $MnCl_2$, and 10 mM DTT. The reaction was terminated by addition of EDTA to a final concentration of 30 mM. cAMP levels were assayed by isotope dilution assay.

RESULTS AND DISCUSSION

To investigate the function of ACG, *ACG* null mutants (*acg*$^-$) were generated by gene targeting. Wild-type cells were transformed with a knock-out construct, replacing an internal 1.6-kb fragment of the ACG gene with the actin6-NEO® cassette. The *acg*$^-$ mutants show normal development into fruiting bodies

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[1] The abbreviations used are: ACA, aggregation adenylyl cyclase; ACG, germination adenylyl cyclase; *Rde*, rapid developing; PKA, cAMP-dependent protein kinase; DTT, dithiothreitol; NEO^R, Tn5 aminoglycoside phosphotransferase; PDE, cAMP phosphodiesterase.

with viable spores. We investigated whether spore germination of acg$^-$ mutants displayed abnormalities. *Dictyostelium* spores can be induced to germinate by exposure to the amino acid component of the food source or by a short heat-shock. The germination process can be subdivided in four stages: activation, a post-activation lag phase that lasts about 1 h, followed by swelling and emergence. During post-activation lag, nonpermissive conditions such as high osmolarity will induce a return to the dormant state; once swelling has started, germination becomes irreversible (see Ref. 6). Fig. 1 shows that after heat-shock, wild-type spores germinate synchronously within 9 h, when incubated at low osmolarity. However, in the presence of 250 mM sucrose, almost all spores return to dormancy. *acg*$^-$ spores do not require a heat-shock to induce germination and furthermore germinate efficiently in the presence of 250 mM sucrose (Fig. 1). In wild-type spores, heat-shock induces a transient loss of ACG and unrelated mRNAs (data not shown), which is possibly the cause of spore activation by heat-shock. It should be noted that *acg*$^-$ spores do not germinate in the spore head, indicating that discadenine inhibition of spore germination is still functioning in these mutants.

We next investigated whether ACG is regulated by osmolarity. Since the spore stage is not very accessible to biochemical procedures, we used an ACA null mutant that constitutively expresses *ACG* under an actin15 promoter (*aca*$^-$/*ACG*). In this cell line, ACG activity can be measured in starving amoebae without interference by ACA (4). To measure cAMP accumulation, *aca*$^-$/*ACG* cells were incubated in phosphate buffer in the presence of the cAMP phosphodiesterase (PDE) inhibitor dithiothreitol. Cells accumulate cAMP under these conditions to about 40 pmol/10^7 cells (Fig. 2*A*). When either 100 mM NaCl or 200 mM sucrose is included in the buffer, a stimulation of cAMP accumulation to up to 120 pmol/10^7 cells is observed. Enhanced cAMP accumulation is already evident at 10 mM of NaCl or sucrose and reaches a maximum at 100 mM NaCl and 100–200 mM of sucrose (Fig. 2*B*). Other factors that inhibit spore germination, such as discadenine and stress factors as high temperature and high or low pH did not affect ACG activity (data not shown). After stimulation with either NaCl or sucrose, high ACG activity is retained for about 10 min and then decreases to basal levels (Fig. 2*C*).

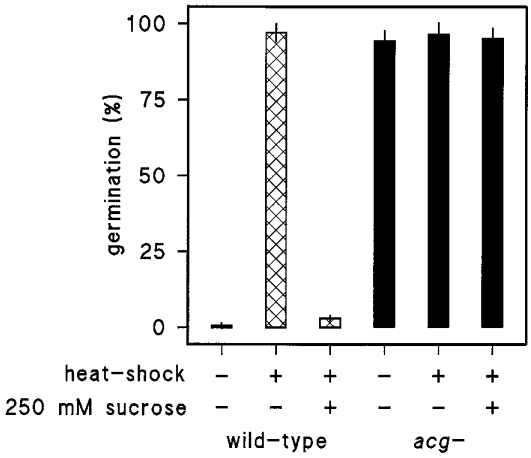

FIG. 1. **Osmoregulation of spore germination in ACG null mutants.** ACG null mutants (*acg*$^-$) and NC4 wild-type spores were harvested from 3-day-old fruiting bodies and resuspended in PB at 2×10^7 spores/ml. One batch of spores was heat-shocked for 30 min at 45 °C, the other batch was left at 23 °C. Both batches were then shaken at 350 rpm and 23 °C in the presence and absence of 250 mM sucrose. After 9 h of incubation, the percentage of emerged amoebae was determined. Means and S.E. of five experiments are presented.

In theory, osmolarity-induced cAMP accumulation by intact cells could also be due to inhibition of an intracellular PDE activity. An activation-trap assay was used to demonstrate that enhanced cAMP production was caused by activation of ACG. Intact cells were preincubated for 10 min at high and low osmolarity, rapidly lysed through Nuclepore® filters and assayed for ACG activity. Fig. 3 shows that in lysates made from unstimulated cells, a low level of ACG activity can be detected. After prestimulation with 100 mM NaCl, a 4-fold stimulation of ACG activity is observed, indicating that enhanced cAMP accumulation in the presence of high osmolarity is indeed due to stimulation of ACG activity.

Inhibition of germination by ACG is most likely mediated by PKA. Single genes for the regulatory and catalytic subunit of PKA have been isolated in *Dictyostelium* (7–9). PKA activation is critically important for both preaggregative development as well as spore and stalk cell differentiation (9–14). Thus far it has remained obscure how PKA is activated, since *aca*$^-$ cells, which are defective in oscillatory cAMP signaling, show normal developmental gene expression, provided that surface cAMP receptors are activated (15). The rapid developing rdeC mutant HTY217 lacks a functional PKA regulatory subunit and displays unrestrained PKA activity. The mutant forms aberrant fruiting bodies, in which spores have differentiated precociously (16). We tested the capacity of these spores to germinate. Table I shows that after activation by heat-shock, the *rde*C mutant spores germinate very poorly both at low and high osmolarity, suggesting that unrestrained PKA activity keeps

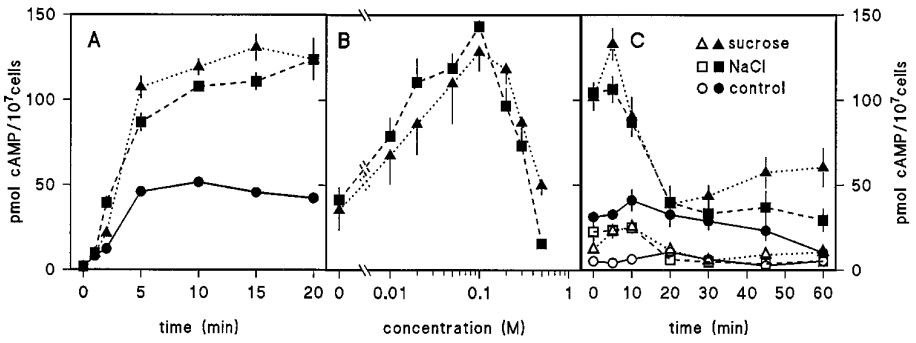

FIG. 2. **Effect of osmolarity on ACG activity in postvegetative *aca*$^-$/*ACG* cells.** *A*, time course of cAMP accumulation by ACG. *aca*$^-$/*ACG* cells were starved for 1 h, resuspended to 10^8 cells/ml in PB, and incubated with 5 mM of the PDE inhibitor DTT and either 100 mM NaCl (■), 200 mM sucrose (▲), or no further addition (●). Cells were assayed for total accumulated cAMP levels. *B*, dose response curve for the effect of osmolarity on ACG activity. *aca*$^-$/*ACG* cells were incubated for 20 min with the indicated concentrations of NaCl or sucrose and assayed for total accumulated cAMP levels. *C*, recovery of basal cAMP accumulation after prestimulation with high osmolarity. *aca*$^-$/*ACG* cells were preincubated for 15 min at 5×10^8 cells/ml with 100 mM NaCl, 200 mM sucrose, or H_2O and then diluted 10-fold with PB to reduce the osmotic stimulus. At the indicated time periods after dilution, cells were incubated for 0 (*open symbols*) and 7 min (*closed symbols*) with 5 mM DTT, and cAMP levels were determined. Means and S.E. of two to three experiments performed in triplicate are presented.

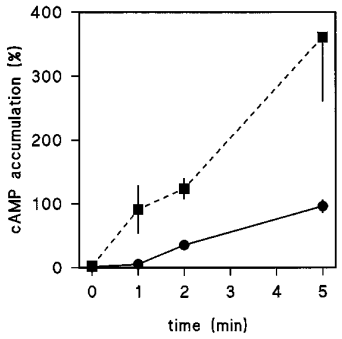

FIG. 3. **ACG activity in cell lysates after prestimulation with high osmolarity.** aca$^-$/*ACG* cells were prestimulated for 10 min with water or 100 mM NaCl. Cells were lysed, and lysates were incubated for the indicated time periods with 0.5 mM ATP, 5 mM $MnCl_2$, and 10 mM DTT. Total accumulated cAMP levels were determined. All data represent the means and S.E. of two to three experiments performed in triplicate.

TABLE I
Spore germination in PKA mutants

The *Rde*C mutant HTY217 lacks a functional PKA regulatory subunit, while mutant *Sp60-PKA* overexpresses PKA catalytic subunit under control of the prespore promoter Sp60. Mutant and wild-type spores were harvested from 3-day-old fruiting bodies and incubated in the presence and absence of high osmolarity after an initial heat shock. The percentage of newly emerged amoebae was measured after 9 h of incubation.

Strain	250 mM sucrose	Germination, mean ± S.E. (n = 5)
		%
Wild-type NC4	−	97.0 ± 3.0
Wild-type NC4	+	3.0 ± 1.0
*rde*C HTY 217	−	8.3 ± 2.3
*rde*C HTY 217	+	3.5 ± 1.0
Sp60-PKA	−	20.6 ± 5.1
Sp60-PKA	+	9.2 ± 2.9

Dictyostelium spores in the dormant state. We also measured spore germination in a *Sp60-PKA* cells, that overexpress the catalytic subunit of PKA under control of the promoter of the prespore gene *Sp60* (17). Spore germination in this cell line is also impaired, but not as strongly as in the *rdeC* mutant, presumably because here the regulatory subunit is still present. Inhibition of spore germination by overexpression of PKA points to a straightforward mechanism for control of spore germination. High osmolarity elevates intracellular cAMP levels through activation of ACG. This in turn results in activation of PKA and inhibition of germination (Fig. 4). In *Dictyostelium*, this is the first example of a clearly resolved mechanism for activation of PKA.

At present it is not clear whether ACG has intrinsic osmosensing activity. This is suggested by the fact that osmoregulation is retained when the gene is expressed at other developmental stages, but could also indicate constitutive expression of

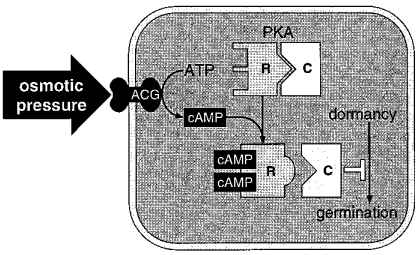

FIG. 4. **Signal transduction pathway for osmoregulation of spore germination.**

an auxiliary osmosensor. There is no obvious sequence homology between ACG and histidine-kinase coupled osmosensors like *E. coli* EnvZ and BarA and *Saccharomyces cerevisiae* SLN1 (18–20). High osmolarity is a universal constraint for germination of plant seeds (21) and is one of the factors that keep seeds dormant in fleshy fruits (22). Osmotolerant germination mutants of *Arabidopsis* have been isolated that germinate at high osmolarity, but are still inhibited to germinate by abscissic acid, an important hormone controlling seed dormancy in plants (23). This situation is similar to dormancy control in *acg*$^-$ mutants, where inhibition by high osmolarity is lost, while inhibition by discadenine is retained. It would be of great interest and potential importance for crop protection (*e.g.* prevention of preharvest sprouting) if plants would use the same mechanism for osmosensing as the slime mold *Dictyostelium*.

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