Adenylyl Cyclase G Is Activated by an Intramolecular Osmosensor

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Adenylyl cyclase G (ACG) is activated by high osmolality and mediates inhibition of spore germination by this stress factor. The catalytic domains of all eukaryote cyclases are active as dimers and dimerization often mediates activation. To investigate the role of dimerization in ACG activation, we coexpressed ACG with an ACG construct that lacked the catalytic domain (ACG Δ cat) and was driven by a UV-inducible promoter. After UV induction of ACG Δ cat, cAMP production by ACG was strongly inhibited, but osmostimulation was not reduced. Size fractionation of native ACG showed that dimers were formed between ACG molecules and between ACG and ACG Δ cat. However, high osmolality did not alter the dimer/monomer ratio. This indicates that ACG activity requires dimerization via a region outside the catalytic domain but that dimer formation does not mediate activation by high osmolality. To establish whether ACG required auxiliary sensors for osmostimulation, we expressed ACG cDNA in a yeast adenylyl cyclase null mutant. In yeast, cAMP production by ACG was similarly activated by high osmolality as in *Dictyostelium*. This strongly suggests that the ACG osmosensor is intramolecular, which would define ACG as the first characterized primary osmosensor in eukaryotes.

INTRODUCTION

Fluctuations in external osmolality are one of the most commonly encountered stress signals of living cells. In prokaryotes, osmotic up-shifts activate transporters, such as ProP, BetP, and OpuA, which increase cytosolic solute levels. In addition, dual-component histidine kinases, such as KdpK and EnvZ are activated, which trigger transcription of transporter genes. Osmotic down-shifts trigger opening of mechanosensitive channels, such as MscL, and release of solutes. All these proteins harbor intramolecular osmosensors that either detect changes in membrane tension or in cytosolic ion concentrations. Such changes are the consequences of the passive water fluxes that follow osmotic shifts (Blount *et al.*, 1996; Jung *et al.*, 2000, 2001; van der Heide and Poolman, 2000; Racher *et al.*, 2001; Rubenhagen *et al.*, 2001).

In eukaryotes, the yeast dual component histidine kinase SLN1 is the best characterized osmoregulated protein. Phosphorelay initiated by the SLN1 histidine kinase inhibits the mitogen-activated protein (MAP) kinase HOG1, which triggers synthesis of the solute glycerol. Osmotic up-shift inhibits SLN1 kinase activity, which then allows HOG1 activation and glycerol synthesis to occur (Maeda *et al.*, 1994). In *Arabidopsis*, the histidine kinase ATHK1 functions in a similar manner as SLN1 to activate a MAP kinase pathway after osmotic up-shift (Urao *et al.*, 1999). MAP kinase pathways also mediate osmotic stress responses in animals, but no primary osmosensors have yet been identified (Kultz and Burg, 1998). It is also not clear whether SLN1 or ATHK1 harbor an intramolecular osmosensor.

In the social amoeba *Dictyostelium discoideum*, osmotic up-shift increases cAMP levels by two separate pathways. In the

amoeba stage, osmotic up-shift reverts the histidine kinase DokA into a histidine phosphatase, which, similar to SLN1 and ATHK1, then acts as a phosphoryl group sink. This results in inactivation of the cAMP phosphodiesterase RegA and ultimately in fortification of the cell cortex (Schuster *et al.*, 1996; Zischka *et al.*, 1999; Ott *et al.*, 2000). DokA is a soluble protein that is indirectly regulated by osmotic up-shift through phosphorylation of a serine in the histidine kinase domain (Oehme and Schuster, 2001). In the spore stage, osmotic up-shift activates adenylyl cyclase G (ACG), an enzyme that is structurally homologous to the *Trypanosoma* receptor adenylyl cyclases (Ross *et al.*, 1991; Pitt *et al.*, 1992; Van Es *et al.*, 1996). High osmolality prevents premature germination of spores, while still in the fruiting body (Cotter and Raper, 1966; Viridy *et al.*, 1999), and this process is mediated by ACG acting on cAMP-dependent protein kinase (Van Es *et al.*, 1996).

The catalytic domains of eukaryote adenylyl- and guanylyl cyclases can only be active as dimers (Tesmer *et al.*, 1997; Zhang *et al.*, 1997). Although the catalytic domains can sometimes dimerize in isolation (Zhang *et al.*, 1997; Taylor *et al.*, 1999), the signaling processes that activate these enzymes usually act on regions elsewhere in the protein to either induce dimerization or to correctly juxtapose or stabilize the monomer partners (Hurley, 1998; Yu *et al.*, 1999).

We investigated the role of ACG dimerization in osmoregulation of enzyme activity, and we addressed the question whether the osmosensor is intrinsic to the ACG protein.

MATERIALS AND METHODS

ACG Expression in Yeast

Preparation of ACG cDNA. An ACG cDNA was prepared from vector pBACG (gift from Peter N. Devreotes, Johns Hopkins University School of Medicine, Baltimore, MD), which contains a 3.44-kb genomic fragment with the complete ACG coding sequence, 51 nucleotides (nt) 5'-untranslated region (UTR) and 616 nt 3'UTR, cloned into *EcoRV*-*Clal* digested pBluescript SK. The

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Table 1. Oligonucleotides used in this study

Name	Sequence
cACG1	AGAGAGATCTTCAAAAC
cACG2	GACTCCATTCTAGAGGC
HindIII-pBS5'	CACAAGCTTCCCGGGCTGCAGGAATTTCG
SacI-ACGc3'	GACGGAGCTCATCACTTGGAAATGGATCATATGG
BamHI-PrA5'	CAAGGATCCGGAGCAGGGCGGGTGTC
XhoI-PrA3'	GAAGTCGAGCGAATTCGCGTCTACTTTCGGCGCG
BamHI-N-ACG	AAAGGATCCAAAACATTTGTAAAGATACTATC
XhoI-ACGΔCat	AACCTCGAGATCTAAAAAGAAGACACAAGC
XhoI-C-ACG	AACCTCGAGCTTTTTTGTATTCTACATTTTCGTC

XbaI site in the multiple cloning site of pBACG was eliminated by excision of the surrounding *SacI*/*BamHI* fragment, treatment with T4 DNA polymerase to blunt the 3' and 5' overhangs, and religation of the vector. The *BglIII*/*XbaI* fragment from the ACG gDNA, which contains the two introns (Pitt *et al.*, 1992), was then replaced by *BglIII*/*XbaI* digested cDNA, which was generated by reverse transcription-polymerase chain reaction of *D. discoideum* spore RNA with primers cACG1 and cACG2 (Table 1). This yielded vector pBcACG.

GAL1-ACG Construct and Yeast Transformation. An ACG cDNA fragment consisting of 51 nt of 5'UTR and 2049 base pairs of ACG open reading frame was amplified from pBcACG by using primers *HindIII*-pBS5' and *SacI*-ACGc3' (Table 1).

This amplified product lacks the last 528 base pairs of the C-terminal region, which contains highly AT-rich repetitive sequence and may be recombined or not properly translated in yeast. The *HindIII*-*SacI* digested product was fused to the yeast GAL1 promoter by subcloning into the *HindIII*-*SacI* digested yeast expression vector pYES2 (Invitrogen, San Diego, CA). The 430-base pair ZZ domain of protein A, obtained by amplification of vector pZZ-his5 (Rayner and Munro, 1998) with primers *BamHI*-PrA5' and *XhoI*-PrA3', was fused to the C terminus of ACG by cloning into the *BamHI*-*XhoI* sites of the pYES2 multiple cloning site for future protein purification. Construct integrity was verified by DNA sequencing. The construct (GAL1-ACG-ZZ) was transformed by means of the lithium acetate method (Gietz *et al.*, 1992) into the *Saccharomyces cerevisiae* *cyr1* null mutant TC41F2-1 (*MATa* *cyr1::ura3 cam1 cam2 cam3 leu2-3,112 his3-532 his4 ura3*) (Heideman *et al.*, 1990). Transformants were selected for growth in the absence of 1 mM cAMP. Standard methods and media for yeast culture were used (Burke *et al.*, 2000).

ACG Constructs and Transformation of Dictyostelium Cells

To generate a construct for spore-specific expression of truncated ACG (ACGΔcat), we amplified 1487 base pairs of genomic ACG DNA from vector pBACG with primers *BamHI*-N-ACG and *XhoI*-ACGΔCat (Table 1). When translated, this DNA fragment (which contains the two introns) will yield the first 401 amino acids of the ACG gene but will lack almost the entire catalytic domain and the low-complexity C terminus. The *BamHI*-*XhoI* digested fragment was used to replace the *BglIII*-*XhoI* *LacZ* insert from pSA-Neo-Gal (gift from Jeff Williams, University of Dundee, Dundee, United Kingdom). This placed ACGΔcat downstream of the pSA prespore promoter and start codon and yielded plasmid pSA-ACGΔcat. AX2 cells were transformed with the plasmid by electroporation, and transformed clones were selected for growth in the presence of 100 μg/ml G418.

To generate a construct for inducible expression of ACGΔcat, we first exchanged a *BamHI*-*HindIII* fragment from plasmid RNR-P that contained the neomycin selection cassette (Gaudet *et al.*, 2001) with a *BamHI*-*HindIII* fragment from PucBsr that carried the blasticidin selection cassette (Sutoh, 1993) to generate pBsr-RNR. The *BamHI*-*XhoI* digested ACGΔcat fragment was then fused to the RNR promoter by ligation into *BglIII*-*XhoI* digested pBsr-RNR to create vector RNR-ACGΔcat. *aca* cells (Pitt *et al.*, 1992) were cotransformed with RNR-ACGΔcat and vector pGSP1, which harbors the neomycin selection cassette and a gene fusion of the constitutive actin15 promoter and full-length ACG (Pitt *et al.*, 1992). Cells were selected for growth in the presence of 20 μg/ml G418 and 5 μg/ml blasticidin. pGSP1 was also singly transformed into *aca* cells and selected for growth with 20 μg/ml G418.

Spore Germination Assay

Cells were grown in axenic medium, harvested, and plated at 10⁶ cells/cm² on KK2 agar (1.5% agar in 10 mM K-phosphate, pH 6.2) until fruiting bodies had formed. Spores were harvested from 2-d-old fruiting bodies by shaving their spore heads with the edge of a glass slide. Spores were washed three times with KK2, resuspended to 10⁷ spores/ml, and either heat shocked for 30 min at 45°C or left at 22°C. Spore suspensions were then diluted 1:1 with

either KK2 or 0.5 M sucrose in KK2 and shaken for 12 h at 165 rpm and 23°C. The number of spores and germinated amoebae was counted every 2 h in a hemocytometer under a phase-contrast microscope (Cotter, 1981).

Assay for cAMP Accumulation in Yeast

Exponentially growing yeast cells were harvested from YPD medium (Burke *et al.*, 2000), washed, and resuspended in 5 mM MES buffer, pH 6.0, at 30 mg of wet weight per milliliter. Cells were preincubated for 10 min at 30°C and subsequently stimulated with NaCl or sorbitol at 30°C as indicated in the figure legends. At different time intervals, 2.5-ml aliquots of cell suspension were quenched by rapid submersion in 10 ml of 60% methanol, precooled to -40°C. Cells were pelleted by centrifuging for 5 min at 1560 × g and 1°C and resuspended in 0.5 ml of 1 M perchloric acid. The suspension was transferred to a Microfuge tube containing 0.5 ml of glass beads (size 425–600 μm), vortexed for 10 cycles of 30 s at 4°C, and centrifuged for 5 min at 16,000 × g. Then 20-μl aliquots of supernatant were neutralized with 8 μl of 100% saturated KHCO₃, and cAMP levels were measured by isotope dilution assay (Gilman, 1972; Thevelein *et al.*, 1987a).

Activation of the RNR Promoter and Assay for ACG Activity in Dictyostelium Cells

Cells grown to 5 × 10⁶ cells/ml in HL5 were irradiated with UV at 30 J/m² by using a Bio-Link UV cross linker (VWR, Poole, United Kingdom). Cells were then diluted 10-fold in HL5 and left for 1 h at 22°C to allow transcription from the RNR promoter (Gaudet *et al.*, 2001). The uninduced controls were treated in the same manner, except that UV irradiation was omitted. For assay of ACG activity, cells were washed free from HL5, resuspended to 5 × 10⁷ cells/ml in KK2, and shaken for 10 min at 165 rpm. Aliquots of 25 μl of cell suspension were incubated with different variables in a total volume of 30 μl. Reactions were started by addition of the phosphodiesterase (PDE) inhibitor dithiothreitol to a final concentration of 5 mM and terminated by the addition of 30 μl of 3.5% perchloric acid (vol/vol). Lysates were neutralized with 50% saturated KHCO₃ and cAMP levels were measured by isotope dilution assay.

Protein Fractionation

To extract total *Dictyostelium* proteins under denaturing conditions, cells were resuspended to 2 × 10⁷ cells/ml in KK2, mixed with an equal volume of 2× SDS-PAGE sample buffer, boiled for 5 min, and size fractionated on 10% SDS-PAA gels. The proteins were transferred to nitrocellulose membrane, and Western blots were incubated overnight at 4°C with a 1:2000 diluted αACG antibody. This antibody was raised in rabbit by Sigma Genosys (Pampisford, United Kingdom) against a cysteine-linked peptide SLNSNDLIDGSEYHD-DPFP in the C-terminal of ACG (aa 663–681). Detection was performed with the Supersignal chemoluminescence kit (Pierce Chemical, Rockford, IL) according to the manufacturer's instructions, by using 1:2000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Promega, Madison, WI) as secondary antibody.

To extract yeast proteins, 5 × 10⁸ cells were resuspended in 1.2 ml of 10 mM Tris-HCl, pH 7.4, containing 0.3 M sorbitol, 0.1 M NaCl, 5 mM MgCl₂, and 1× Complete protease inhibitor cocktail (Roche Diagnostics, Lewes, United Kingdom) and vortexed at 4°C for 15 cycles of 30 s with 0.7 g of glass beads. Lysates were centrifuged at 800 × g and 4°C for 10 min. Supernatants were mixed with an equal volume of 2× sample buffer and further treated as *Dictyostelium* total protein extracts.

Assay for Dimer Formation

To test for effects of osmotic up-shift on ACG dimer formation, cells were resuspended in KK2 and incubated for 5 min with or without 100 mM NaCl before being lysed by passage through nucleopore filters (pore size 3 μm). Lysates were prepared in the presence of 1× Complete protease inhibitor cocktail (Roche Diagnostics) and centrifuged for 30 min at 20,000 × g and 4°C. Pellets were washed in 5 mM glycine, pH 7.4, and centrifuged once more at

Figure 1. Schematic of the ACG expression constructs. The constructs and protein structural domains are drawn according to scale except for the promoters (block arrows). The position and sequence of the peptide used to generate the α ACG antibody is marked on the A15-ACG construct. The size of the proteins in amino acid residues is indicated.

20,000 $\times$ g. The membranes were resuspended in 0.5% Triton in 5 mM glycine, pH 7.4, and incubated for 1 h on ice to solubilize the membrane proteins. The suspension was cleared by centrifuging for 15 min at 16,000 $\times$ g, and the protein concentration of the supernatant fraction was measured using Bradford reagent (Bio-Rad, Hemel Hempstead, United Kingdom). Equal amounts of protein were directly loaded on nonreducing 8% SDS-PAA gels, transferred to nitrocellulose membranes after electrophoresis, and incubated with ACG antibody as described above.

RESULTS

Heterologous Expression of ACG

To investigate whether high osmolality acts directly on ACG or requires a separate sensor protein, we expressed ACG in the fungus *S. cerevisiae*, which is only distantly related to the mycetozoon *Dictyostelium* (Baldauf *et al.*, 2000). To avoid interference from the yeast adenylyl cyclase, we used strain TC41F2-1, which carries a null mutation in the *CYR1* gene, that encodes the single yeast adenylyl cyclase (Heideman *et al.*, 1990). *CYR1* is essential for growth and the *cyr1* null mutant can only grow in the presence of 1 mM cAMP. An ACG cDNA was prepared in which 175 aa of C-terminal low-complexity sequence was replaced by a 140-aa protein A ZZ tag. The construct was expressed in *cyr1* under control of the yeast *GAL1* promoter (GAL1-ACG-ZZ; Figure 1). Figure 2A shows rescue of the growth-deficient phenotype of *cyr1* in four independent transformed clones. This indicates that ACG is active in the *cyr1* mutant.

Figure 2. Expression of ACG in *S. cerevisiae*. (A) Growth of the yeast *cyr1* mutant TC41F2-1 (parent) and four clones (A1, A5, B1, and B5) transformed with the GAL1-ACG-ZZ fusion construct (*cyr1*/ACG) on standard yeast culture medium without cAMP. (B) Western blots of expressed ACG proteins. Lysates of *Dictyostelium acg* and *aca*/ACG cells, and of yeast *cyr1* and *cyr1*/ACG cells were size fractionated by SDS-PAGE and immunoblotted with α ACG antibodies. The size (in kilodaltons) of protein markers is indicated.

Figure 3. Osmoregulation of ACG in *S. cerevisiae*. (A) Time course of cAMP accumulation. *cyr1*/ACG cells were harvested from growth medium and resuspended in 5 mM MES, pH 6.0. Cells were stimulated with 1/10 volume of either 5 M NaCl or 1/5 volume of 5 M sorbitol. Reactions were quenched at the indicated time intervals by sixfold dilution in 60% methanol at -40°C . After cell rupture, cAMP levels were measured by isotope dilution assay. Means and SE of three experiments performed in triplicate are presented. (B) Dose-response curve. *cyr1*/ACG cells were stimulated with the indicated concentrations of NaCl. Reactions were quenched at 45 s after stimulation and assayed for cAMP. Means and SE of four experiments performed in triplicate are presented.

Expression of the ACG fusion construct was confirmed by immunoblotting of size-fractionated cell lysates with an α ACG antibody. The antibody was raised in rabbit against a 19-aa peptide in the C-terminal region of ACG (Figure 1) and was tested for specificity on *acg* null cells and *aca* null cells that constitutively express ACG (*aca*/ACG) (Pitt *et al.*, 1992). The antibody identified a single band of ~ 100 kDa on Western blots of *aca*/ACG cells, which agrees with the predicted size of 98 kDa for full-length ACG. The band was absent from *acg* null mutants, which indicates that the antibody is specific for ACG (Figure 2B). The yeast *cyr1* cells transformed with the GAL1-ACG-ZZ construct (*cyr1*/ACG) showed a somewhat smaller band, which was absent from the untransformed *cyr1* cells (Figure 2B). The difference in size is due to replacement of the C-terminal low-complexity region with the ZZ tag.

We measured whether cAMP production by ACG in the *cyr1*/ACG cells was stimulated by high osmolality. *Dictyostelium* cells secrete most of the cAMP that they produce and cAMP production by ACG can be readily detected in the extracellular medium. Under those conditions solute concentrations of 200 milliosmolar induce a further two- to fourfold stimulation of cAMP accumulation (Van Es *et al.*, 1996). In contrast, yeast cells secrete very little cAMP and require rigorous procedures to break the cell wall before cAMP can be measured (Thevelein *et al.*, 1987a). *cyr1*/ACG cells and the yeast wild-type strain AY297 were stimulated with either 0.5 M NaCl or 1 M sorbitol or with low-osmolality buffer, and cAMP levels were measured at the indicated time intervals. Figure 3A shows that both 0.5 M NaCl and 1 M sorbitol induced a rapid increase of cAMP levels, which peaked at 45 s after stimulation. The osmolality required for maximal ACG stimulation in yeast was at 1 M rather higher than in *Dictyostelium* (Figure 3B). This could be due to dissimilarities in the phospholipid composition of *Dictyostelium* and yeast membranes, as was demonstrated for the bacterial osmosensor BetP (Rubenhagen *et al.*, 2000). In wild-type yeast, both NaCl and sorbitol induced a decrease in cAMP

Figure 4. Effects of a truncated ACG protein on spore germination. Wild-type cells were transformed with a fusion construct of the psA prepore promoter and an ACG truncation that lacks the catalytic domain (psA-ACGΔcat). Wild-type and transformed spores either received a 45°C heat shock for 30 min or were left at 22°C before being incubated for 11 h in the presence and absence of 250 mM sucrose. Every 2 h, the ratio of spores to emerged amoebae was counted in a sample size of 100 cells. Means and SD of four experiments are presented.

levels (Figure 3A). This demonstrates that the osmostimulation of cAMP production in *cyr1*/ACG cells must result from activation of ACG and not from, e.g., inactivation of a yeast phosphodiesterase.

The Role of Dimerization in Osmoregulation of Spore Dormancy

Truncated forms of the receptor guanylyl cyclases that lack the catalytic domain act as dominant-negative inhibitors because they sequester the native proteins by dimerization (Chinkers and Wilson, 1992). We used the same strategy to establish whether dimerization outside the catalytic domain is essential for ACG function during spore germination. Wild-type cells were transformed with a fusion construct of the psA prepore promoter and sequence encoding the N-terminal 401 amino acids of ACG. This construct (psA-ACGΔcat) lacks the cyclase domain and the low-complexity C-terminal region (Figure 1). The psA promoter directs expression of the construct in prepore and spore cells during normal development (Hopper *et al.*, 1993).

Dictyostelium spores are normally activated to germinate by exposure to food, but rapid synchronous activation can be achieved by a heat shock. After activation spores enter a lag phase of 1–2 h before swelling and emergence occur. Exposure to high osmolality during the lag phase will induce the spores to return to dormancy (Cotter and Raper, 1967; Viridy *et al.*, 1999). Figure 4A shows that after heat shock, wild-type spores germinated within 10 h at low osmolality, but not at all at high osmolality or in the absence of

heat shock. In contrast to wild-type spores, the psA-ACGΔcat spores did not require a heat shock to germinate and germination was not inhibited by high osmolality (Figure 4B). These data suggest that ACGΔcat inhibits ACG function during spore germination.

The Role of Dimerization in Osmoregulation of ACG Activity

Spores are inaccessible for direct measurement of ACG inhibition by ACGΔcat. We therefore coexpressed ACG and ACGΔcat in growing cells. Heterologous protein expression presents the problem that the absolute amount of expressed protein is determined by the copy number of the transformation vector. Results that depend on stoichiometric interaction between two heterologously expressed proteins will for this reason suffer from unpredictable clone-to-clone variability. To circumvent this problem, we coexpressed ACG under control of the constitutive actin15 promoter (A15-ACG) (Pitt *et al.*, 1992) with ACGΔcat under control of the UV-inducible ribonucleotide reductase promoter (RNR-ACGΔcat). The UV-inducible promoter allows modulation of the level of ACGΔcat in the same transformed cell line (Gaudet and Tsang, 1999; Gaudet *et al.*, 2001). The vectors that harbor the A15-ACG and RNR-ACGΔcat gene fusions carried cassettes for neomycin and blasticidin selection, respectively, to allow for simultaneous selection and were transformed into *aca* cells to prevent interference with cAMP production by ACA, which is expressed during early development (Pitt *et al.*, 1992). The RNR promoter was activated by exposure of growing cells to UV light, followed by further incubation for 1 h in growth medium to allow for optimal induction of RNR-ACGΔcat gene expression. Pilot experiments showed that in the absence of RNR-ACGΔcat, UV irradiation had no effect on ACG activity.

Figure 5A shows that cAMP accumulation in the parent *aca* strain is very low. Cells cotransformed with A15-ACG and RNR-ACGΔcat accumulate ~40 pmol of cAMP/10⁷ cells over a 10-min period in the absence of UV induction of ACGΔcat expression. Hyperosmotic conditions, created by 100 mM NaCl induced a twofold stimulation of cAMP accumulation (Figure 5B). After induction of RNR-ACGΔcat expression by UV, cAMP production by A15ACG was ~50% inhibited, but 100 mM NaCl still induced a twofold stimulation (Figure 5C). These data show that expression of ACGΔcat reduces the absolute level of ACG activity but does not prevent stimulation of the enzyme by high osmolality. The inhibitory effect of ACGΔcat seems rather moderate in comparison with its complete block of osmoregulation of spore germination as shown in Figure 4. However, it should be realized that in the spore germination experiment only the endogenous ACG, with low expression levels (Pitt *et al.*, 1992) needs to be inactivated, whereas in the double-

Figure 5. Effect of conditional ACGΔcat expression on osmoregulation of ACG. *aca* cells were double transformed with constructs A15-ACG and RNR-ACGΔcat. The A15 and RNR promoters in these constructs direct constitutive and UV-inducible transcription, respectively. Effects of 100 mM NaCl on cAMP accumulation were measured in untransformed *aca* cells (A), uninduced transformants (B), and UV-induced transformants (C). Means and SE of three experiments performed in triplicate are presented.

Figure 6. Effects of high osmolality on ACG and ACG Δ cat homo- and heterodimerization. *aca* cells, either single transformed with A15-ACG or double transformed with A15-ACG and RNR-ACG Δ cat, were UV induced as indicated and subsequently treated with and without 100 mM NaCl for 5 min and lysed. The untransformed *aca* parent was lysed without pretreatment. Membranes were purified and membrane proteins were solubilized in 0.5% Triton X-100. For one sample of double-transformed cells, the membrane proteins were denatured (DN) by boiling in sample buffer containing 2% SDS. Equal amounts of native protein and denatured protein were loaded on nonreducing SDS-PAGE gels and immunoblotted with α ACG antibodies. The position of protein size markers is indicated.

transformed cells ACG Δ cat competes with ACG expressed from the strong actin15 promoter.

Immunological Detection of ACG Dimerization

To demonstrate that ACG and ACG Δ cat form homo- and heterodimers, we isolated the native protein complexes. Cells transformed with both the A15-ACG and RNR-ACG Δ cat constructs were exposed to UV to induce ACG Δ cat expression and both UV-treated and untreated cells were stimulated for 5 min with 100 mM NaCl and lysed. As controls, we also included the *aca* parent strain and *aca* cells that were transformed with A15-ACG alone. Membrane proteins were solubilized, and native proteins were size fractionated by gel electrophoresis and immunoblotted with α ACG antibody. The antibody can detect full-length ACG but not ACG Δ cat because the epitope is in the truncated region (Figure 1).

Figure 6 shows that in *aca* cells transformed with A15-ACG and RNR-ACG Δ cat, that were not exposed to UV, the ACG antibody detected bands around 100 and 200 kDa in a roughly 70:30% ratio. These bands most likely represent monomers and dimers of full-length ACG, which are 98 and 196 kDa, respectively. Membrane proteins that were denatured and therefore monomerized by boiling in SDS sample buffer (DN) did not show the dimer band at 200 kDa, which indicates that this band does not represent nonspecific reactivity of the ACG antibody with a 200-kDa protein. This is further confirmed by the absence of any bands in native preparations from untransformed *aca* cells. After UV-induction of RNR-ACG Δ cat expression, the 200-kDa bands largely disappeared and a novel band of $\sim$ 140 kDa occurred. This band most likely represents the heterodimer of full-length ACG (98 kDa) and ACG Δ cat (46 kDa). The appearance of the 140-kDa band is not an artifact of the UV exposure, because cells that only contained the A15-ACG construct showed the

100- and 200-kDa bands in the presence and absence of UV exposure. In both UV-induced and uninduced cells the monomer/dimer ratio was not altered by stimulation with 100 mM NaCl, which suggests that high osmolality does not promote dimer formation.

DISCUSSION

ACG Harbors an Intramolecular Osmosensor

ACG is predominantly expressed in the spore stage (Pitt *et al.*, 1992), where it mediates inhibition of spore germination by high osmolality (Van Es *et al.*, 1996). However, when expressed from a constitutive promoter in the amoeba stage, the enzyme was also activated by high osmolality, which suggested that the osmosensor could be intramolecular (Van Es *et al.*, 1996). Because we could not rule out that an accessory sensor protein was expressed throughout development, it was essential to measure ACG activity in a system that was unlikely to express such a sensor. The choice of organism was limited to protists, because metazoan cells require culture at high osmolality. Preferably, the organism of choice should not display any adenylyl cyclase activity, which could interfere with the assay. We chose a mutant strain of the fungus *S. cerevisiae* that lacks the single adenylyl cyclase gene *CYR1*. In contrast to ACG, yeast *CYR1* is not a transmembrane protein (Mitts *et al.*, 1990); it is stimulated by glucose and requires both the heterotrimeric G protein Gpa2 and the small G protein Ras2 for activity (Thevelein *et al.*, 1987b; Colombo *et al.*, 1998). Because ACG is not regulated by G proteins (Pitt *et al.*, 1992), it is unlikely that the mechanisms that activate *CYR1* would act on ACG. In both yeast and *Dictyostelium*, high osmolality modulates histidine kinase activity (Maeda *et al.*, 1994; Schuster *et al.*, 1996), but the *Dictyostelium* histidine kinase DokA plays no role in regulating ACG (Ott *et al.*, 2000) and the yeast enzyme SLN1, which targets the HOG1 MAP kinase pathway, is therefore also unlikely to do so. In fact, responses to osmotic stress are very different in the two organisms. Yeast cells respond to high osmolality with synthesis of the compatible osmolyte glycerol, a process that is mediated by the HOG1 pathway (Maeda *et al.*, 1994). *Dictyostelium* cells do not accumulate compatible osmolytes and respond instead by remodeling of the cell cortex, a process that involves phosphorylation of actin and myosin (Zischka *et al.*, 1999). It is therefore unlikely that any component of the osmostimulated HOG1 pathway, including glycerol, would activate ACG.

Dictyostelium ACG complemented the growth defect of the yeast *cyr1* null mutant (Figure 2) and similar to *Dictyostelium* cells, cAMP production in the *cyr1*/ACG cells was stimulated by high osmolality. However, higher solute concentrations were required and the stimulated cAMP accumulation in yeast was more transient than in *Dictyostelium*.

The transient kinetics of cAMP accumulation in yeast is probably due to the fact that the yeast cAMP phosphodiesterase PDE1 is strongly stimulated by cAMP through activation of cAMP-dependent protein kinase. This rapidly neutralizes glucose-induced cAMP accumulation in wild-type yeast after 45 s of stimulation (Ma *et al.*, 1999), which agrees exactly with the kinetics of high osmolality-induced cAMP accumulation in *cyr1*/ACG cells (Figure 3A). This suggests that the transient kinetics of cAMP accumulation in yeast is a function of PDE1, rather than of either *CYR1* or ACG. The pronounced effect of PDE1 on cAMP levels in yeast raises the possibility that high osmolality could have increased cAMP by inhibiting PDE1 (or PDE2), instead of by activating ACG. However, this cannot be the case because in wild-type

yeast both NaCl and sorbitol decreased cAMP levels (Figure 3A). We cannot completely exclude that a currently unknown response to osmotic up-shift in yeast has led to activation of ACG, but direct stimulation of ACG by high osmolality is by far the most likely explanation. This implies that the osmosensor is intrinsic to the ACG protein.

The difference in concentration dependence between ACG expressed in *Dictyostelium* or in yeast may relate to the question of what is actually being detected by ACG. Apart from the increase in extracellular solute concentration, osmotic up-shift causes passive water efflux from cells, which in turn induces many physicochemical changes, such as cell shrinkage, increased intracellular solute and ion concentration, macromolecular crowding, and increased membrane curvature (Wood, 1999). Many of these parameters can potentially affect protein function. Elegant studies of reconstituted purified bacterial osmosensors in liposomes have shown that the ABC transporter OpuA and the channel protein MscL respond to alterations in membrane tension (Blount *et al.*, 1996; van der Heide and Poolman, 2000). The dual component histidine kinases KdpK and EnvZ sense the cytosolic increases of respectively ionic strength and K^+ ions that result from water efflux (Jung *et al.*, 2000, 2001). The Na^+ /betaine symport transporter BetP is also activated by K^+ ions (Rubenhagen *et al.*, 2001). If ACG activation is similarly dependent on the secondary effects of passive water efflux, the higher solute concentration dependence of ACG expressed in yeast may simply reflect higher resistance of yeast cells to those effects. The different membrane composition of yeast cells may also play a role, because it was demonstrated that the dose dependence for osmostimulation of the bacterial osmosensor BetP was strongly influenced by the phospholipid composition of the membrane (Rubenhagen *et al.*, 2000). Our next challenge will be to purify and reconstitute ACG in liposomes that mimic the *Dictyostelium* membrane lipid composition (Weeks and Herring, 1980), to identify the actual parameter for activation of ACG.

Role of Dimerization

A wide range of signal transduction proteins ranging from growth factor receptors (Schlessinger, 2002), dual component histidine kinases (Bilwes *et al.*, 1999), and STAT transcription factors (Imada and Leonard, 2000) to adenylyl cyclases (Tesmer *et al.*, 1997; Zhang *et al.*, 1997) requires dimerization for activity, and in many cases, dimer formation mediates activation of these proteins. Eukaryote adenylyl- and guanylyl cyclases can only bind substrate when two catalytic domains are combined in an antiparallel wreath-like structure (Liu *et al.*, 1997). In the case of the G protein-regulated adenylyl cyclases, which harbor two intramolecular catalytic domains, the monomers are already joined and activating factors such as G_{α} or forskolin promote the catalytically optimal juxtaposition of the two monomers (Hurley, 1998). However, for the single transmembrane cyclases, which like ACG harbor a single catalytic domain, dimerization can mediate activation. This is the case for the retinal guanylyl cyclase RetGC, which is dependent on guanylyl cyclase activator proteins (GCAPs) for dimer formation. Each RetGC monomer binds to a GCAP monomer. The GCAP monomers dimerize in the absence of cytosolic Ca^{2+} , and this also brings the RetGC monomers together (Olshchanskaya *et al.*, 1999; Yu *et al.*, 1999). In the receptor guanylyl cyclases, such as the atrial natriuretic peptide (ANP) receptor, activation is due to a change in relative orientation of the dimers, rather than in dimer formation itself. In the basal state the ANP receptor forms an inactive dimer by

Figure 7. Domain architecture of receptor adenylyl- and guanylyl cyclases. All sequences were retrieved from GenBank and the domain architecture was determined with the SMART program (Schultz *et al.*, 1998). The size of the proteins in aa residues is indicated. The cyclase catalytic domains (AC or GC), kinase homology domain (KHD), ANP binding domain (ANP-R), coiled-coil, and transmembrane domains are drawn according to scale. Accession numbers are *Homo sapiens* ANPR: XM_113360; *D. discoideum* ACG: M87278; *Trypanosoma brucei* ESAG4: Q26721; *T. brucei* GRESAG4.3: X52121; *T. brucei* GRESAG4.4B: AF228602; *Trypanosoma cruzi* ADC1: AJ012096; *T. cruzi* zAC: AF040382; and *Leishmania donovani* RAC-A: U17042.

interactions between the intracellular coiled-coil and kinase homology domains. The ligand ANP binds simultaneously to the extracellular regions of the two monomers, which triggers closure of their membrane proximal domains. This causes a change in alignment of the intracellular domains, which ultimately results in activation of guanylyl cyclase (Wilson and Chinkers, 1995; He *et al.*, 2001; Labrecque *et al.*, 2001).

ACG and the *Leishmania* and *Trypanosoma* adenylyl cyclases show a superficial structural similarity to the ANP receptor, but no kinase homology domain or coiled-coil domains are present (Figure 7). There is no significant sequence similarity between the extracellular domains of ANP-R, ACG, and the parasite cyclases. The isolated catalytic domains of the *T. brucei* enzymes ESAG4, GRESAG4.1, and GRESAG4.3 show a weak tendency to form catalytically competent dimers (Bieger and Essen, 2001). The catalytic domains of both GRESAG4.4B and TczAC can form stable dimers, although the full-length TczAC protein shows much higher catalytic activity (D'Angelo *et al.*, 2002). Enforced dimer formation by fusion of a leucine zipper to the GRESAG4.4B catalytic domains also greatly enhanced activity (Naula *et al.*, 2001). In the case of ACG, the isolated catalytic domains showed no catalytic activity and did not dimerize (our unpublished data). This group of enzymes is therefore likely to either require additional proteins or additional regions within the protein to enforce the dimerized state.

We show here that the ACG Δ cat construct without the catalytic domain can dimerize with the full-length protein (Figure 6) and acts as a dominant-negative inhibitor of enzyme activity (Figures 4 and 5). This indicates that in addition to the catalytic domain, ACG has a separate dimerizing region. Unlike ANP-R, neither ACG nor the parasite adenylyl cyclases have a significant juxtamembrane intracellular region that could mediate dimer formation (Figure 7), and dimerization therefore most likely requires the extracellular domain of the protein.

Our data do not support a role for osmotic up-shift as direct trigger for dimerization. Overexpression of ACG Δ cat in spores blocked the inhibition of spore dormancy by high osmolality, but the spores also no longer required a heat shock to activate the germination process (Figure 4). Spores contain 10 times higher cAMP concentrations than amoebae, most of which is produced by ACG (Virdy *et al.*, 1999). Shortly after heat shock cAMP levels decrease dramatically, but they transiently increase again during the postactivation lag phase. This increase is more pronounced when heat-activated spores are exposed to high osmolality. Heat shock induces a transient loss of ACG mRNA, which may cause the decrease of cAMP levels and activation of the germination process (Virdy *et al.*, 1999). The fact that ACG Δ cat both obviated the requirement for heat shock and prevented osmoregulation of germination suggests that it inhibits both basal and osmostimulated ACG activity. This was confirmed by measurement of ACG activity in the presence and absence of the ACG Δ cat. Even though ACG Δ cat strongly reduced ACG activity, it did not prevent activation of the residual activity by high osmolality (Figure 5). Most importantly, high osmolality did not increase the ACG dimer to monomer ratio (Figure 6). We therefore conclude that high osmolality does not activate ACG by causing it to dimerize and that osmoregulation of ACG involves a novel mode of regulation of this important class of enzymes.

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