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# Ege A, a Novel C2 Domain Containing Protein, Is Essential for GPCR-Mediated Gene Expression in *Dictyostelium*

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**During early stages of development, expression of aggregative genes in *Dictyostelium* is regulated by G protein-linked signaling pathways. We have isolated an aggregation-deficient mutant from a restriction enzyme-mediated insertional mutagenesis screen and have obtained its cDNA. Since the mutant expresses prestarvation genes but fails to express early genes, such as cAR1 and GP80, during development, we designated it *early gene expression* A (*ege A*). Ege A, encoding a cytosolic protein of 26 kDa, along with Ege B, belongs to a novel C2 domain-containing gene family. While Ege A mRNA is expressed during the first 2 h of development, Ege B is expressed at later stages. Ege A is not directly required for either G protein-mediated actin polymerization or activation of adenylyl cyclase. Ege A overexpressing and *ege A*$^-$ cells display similar phenotypes, suggesting that an optimal level of Ege A is required for proper function. Constitutive expression of a fully functional cAR1-YFP enables *ege A*$^-$ cells to form loose aggregates, but cAR1-YFP/*ege A*$^-$ cells are still unable to express GP80, suggesting that losses of gene expression were not solely due to a lack of cAR1. Overexpression of PKAcat, the constitutively active subunit of PKA, does not rescue the *ege A*$^-$ phenotype, suggesting that PKA is not located downstream from Ege A in the signaling pathway. We propose that Ege A is a novel cytosolic component required by early gene expression.** 

***Key Words:*** **Ege A; *Dictyostelium*; aggregative gene expression; G protein; C2 domain.**

## INTRODUCTION

Seven-transmembrane receptors play an essential role in regulating the expression of developmental genes in metazoa. These receptors, such as Frizzled in *Drosophila* and cAMP receptors in *Dictyostelium*, can trigger both G protein-dependent and -independent signaling pathways (Mlodzik, 1999; Bejsovec, 1999; Devreotes, 1994; Liu *et al.*, 2001). Studies of the developmental program of *Dictyostelium* can provide insights into serpentine receptor-regulated gene expression. The program is initiated by nutrient depletion, and within 24 h, free-living amoebae, coordinated by sets of extracellular signaling molecules, aggregate and differentiate in a pattern into spore and stalk cells (Kay, 1997; Firtel, 1995; Devreotes, 1994; Gomer, 1997). During this process, a family of serpentine cAMP receptors regulate chemotaxis, morphogenesis, and cell differentiation (Firtel, 1995). Many elements of these pathways are conserved in higher organisms. For instance, the expression of cell type-specific genes requires a glycogen synthase kinase 3 (GSK3)/$\beta$-catenin pathway similar to the Wnt/Frizzled pathway in *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammals (Kim and Kimmel, 2000; Harwood, 2001).

The receptor-mediated responses are part of a repertoire of gene expression events that are tightly regulated at various developmental stages by different signaling pathways. At the earliest stages, the expression of prestarvation genes, such as Discoidin I and $\beta$-mannosidase, is regulated by a secreted peptide, prestarvation factor (PSF), through a G protein-independent pathway (Burdine and Clarke, 1995; Devine *et al.*, 1982). In addition, conditioned medium factor (CMF), another signaling peptide, helps to initiate the chemoattractant signaling relay system (Yuen *et al.*, 1995; Van Hasstert *et al.*, 1996). At the following stage, both cell aggregation and expression of aggregative genes are mediated by cAMP, acting on the chemoattractant receptor,

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cAR1 (Firtel, 1995). Previous research has identified multiple components in this pathway in addition to cAR1, including a specific heterotrimeric G protein ($G\alpha2\beta\gamma$), YakA, and protein kinase A (PKA) (Sun and Devreotes, 1991; Wu *et al.*, 1995; Schulkes and Schaap, 1995, Souza *et al.*, 1998; Van Es *et al.*, 2001; Mann *et al.*, 1991). Cells lacking $G\alpha2$, $G\beta$, YakA, or carrying dominant negative PKA do not induce aggregative genes (Kumagai *et al.*, 1991; Wu *et al.*, 1995; Schulkes and Schaap, 1995; Van Es, 2001; Firtel and Chapman, 1990; Harwood *et al.*, 1992). During later developmental stages, the expression of cell type-specific genes is orchestrated by differentiation inducing factor (DIF), cAMP, and other unidentified factors, acting on several other seven-transmembrane receptors, cAR2, cAR3, and cAR4 (Berks and Kay, 1990; Saxe *et al.*, 1996; Hadwiger and Firtel, 1992; Firtel, 1995; Thompson and Kay, 2000). Surprisingly, the existence of postaggregative gene expression in temperature-sensitive $g\beta^-$ cells indicates that G proteins are not required for these serpentine receptors to control differentiation (Jin *et al.*, 1998b). PKA has been shown to play an essential role in cell type-specific gene expression (Mann *et al.*, 1992).

Expression of aggregative genes, including glycoprotein 80 (GP80), cAMP phosphodiesterase (PDE), and cAR1, is critical for aggregation and later gene expression. GP80 is involved in cell adhesion, while PDE regulates extracellular cAMP concentration (Desbarats *et al.*, 1994; Hall *et al.*, 1993). Through coupling to $G\alpha2\beta\gamma$ proteins, cAR1 mediates a wide range of signaling events required for chemotaxis besides induction of gene expression, including activation of adenylyl cyclase A (ACA) to produce more cAMP, induction of actin polymerization, and activation of guanylyl cyclase (Parent and Devreotes, 1996). To study the cAR1-regulated aggregative gene expression, we carried out a restriction enzyme-mediated insertion (REMI) mutagenesis screen for mutants defective in early development. A mutant designated *early gene expression A null* (*ege* $A^-$) was isolated, and functional analysis traced the defect to the induction of GPCR-mediated early gene expression.

## MATERIALS AND METHODS

***Materials.*** 2′-Deoxyadenosine 3′:5′-cyclic monophosphate, cAMP, and TRICT phalloidin were obtained from Sigma; restriction enzymes from New England Biolabs; and *Taq* polymerase from Perkin-Elmer. Cyclic-AMP [$^3$H] and cyclic-GMP [$^3$H] assay systems were obtained from Amersham, and anti-HA antibody was purchased from Boehringer Mannheim. A rabbit anti-cAR1 polyclonal antibody was made in our laboratory. All other reagents were at least reagent grade and were obtained from standard suppliers.

***Cell culture and development.*** Cells were grown in suspension with shaking at 200 rpm (Watts and Ashworth, 1970), in 100-mm culture plates, or on SM plates with a *Klebsiella aerogenes* lawn (Kay, 1984). Cells were washed and developed in shaking suspension at $2 \times 10^7$/ml in developmental buffer (DB; 10 mM Na/K phosphate, 0.2 mM $MgSO_4$, 1 mM $CaCl_2$, pH 6.5) for 1 h, then pulsed with 75 nM cAMP at 6-min intervals for 5 h. Then, cells were preincubated with 3 mM caffeine for 30 min before use in biochemical assays.

***Plasmid construction and cell lines.*** The plasmid pBSR was a gift from Dr. W. F. Loomis (Department of Biology, University of California at San Diego). The plasmid pBSEGE was rescued from *ege* $A^-$ genomic DNA by circularization of a *Hin*dIII fragment. pBSEGE was then linearized and transformed into wild-type AX2 cells to disrupt *ege A*. The *ege* $A^-$ disruption was confirmed by phenotyping and Southern blotting. To make Ege A plasmid, a full-length Ege A open reading frame was cloned into the *Dictyostelium discoideum* extrachromosomal expression plasmid p88d1 (Hughes *et al.*, 1994). Ege A was then electroporated into *ege* $A^-$ or AX2 to make Ege A/*ege* $A^-$ or Ege A/AX2 cells. The plasmid HA-Ege A was made by fusing a HA-tag to the $NH_2$-terminal of Ege A expression vector, and the plasmid Ege A-GFP was made by fusing GFP to the COOH-terminal of Ege A expression vector. pPKAcat was a gift from Dr. A. Kuspa (Department of Biology, Baylor University) and was electroporated into *aca*- and *ege* $A^-$ to make PKAcat/*aca*- and PKAcat/*ege* $A^-$ cells. Cell transformation was carried out as described (Insall *et al.*, 1994). Plasmids were electroporated into cells by using a Bio-Rad gene pulser. *ege* $A^-$ was selected with 10 mg/ml blastcidin. Other cell lines were selected in 20 mg/ml G418.

***Immunofluorescent staining of discoidin Ic.*** The antiserum for discoidin-Ic was a gift from Dr. Margaret Clarke (Oklahoma Medical Research Foundation). The bacteria, *Klebsiella aerogenes*, were grown on SM agar plates at room temperature for 2 days and rinsed from the plates with 17 mM potassium phosphate buffer (pH 6.4). The final volume of the bacteria suspension was adjusted to $OD_{660nm} = 8$. Amoebae were mixed with freshly prepared bacteria suspension to a density of 1000 cells/ml and allowed to grow until reaching the desired density. Cells then were harvested and washed three times with DB. The immunofluorescent staining was carried out as described by Rathi and Clarke (1992).

***Restriction enzyme-mediated insertion screening and isolation of ege*** $A^-$**.** Restriction enzyme-mediated insertion screening was carried out as described (Kuspa and Loomis, 1994). Plasmid pBSR3 was linearized with *Bam*HI and electroporated into AX2 cells along with restriction enzyme *Dpn*II. About 25,000 independent transformants with random insertions were screened on a *Klebsiella aerogenes* lawn. Eighteen small plaque mutants were picked, *ege* $A^-$ among them.

We identified the *ege A* gene on the basis of its genomic sequence. The plasmid, along with 3 kb of flanking genomic sequence, was rescued from *ege* $A^-$ as a *Hin*dIII fragment. The flanking region was sequenced, and the sequence data were used in a BLAST search of GenBank. Several cDNA clones from the *Dictyostelium* cDNA project in Japan were found to encode the gene *ege A*, and clone SSII45 encoded its full-length cDNA (Morio *et al.*, 1998).

***Southern, Northern, and Western blotting.*** Southern analysis was carried out as described by Ausubel *et al.* (1987), with minor modifications: 10 $\mu$g of genomic DNA, digested with restriction enzyme *Hin*dIII, was loaded onto a 0.7% agarose gel, electrophoresed, and transferred onto a nylon membrane (Ausubel *et al.*, 1987). Ege A cDNA were used as templates for the synthesis of random-primed, labeled probes. The $^{32}$P-labeled probes were hybridized to the blot and visualized with a FUJIFILM BAS1500 phosphoimager. Northern blotting was also carried out as described by Ausubel *et al.* (1987), with a minor modification: 20 $\mu$g of total RNA were loaded onto each lane of a 1% formaldehyde gel, electrophoresed, and blotted to a nylon membrane (Ausubel *et al.*,

1987). The cDNAs of Ege A and Ege B were used as templates for the synthesis of corresponding probes. Ege B cDNA was a gift from Dr. R. Firtel (Center for Molecular Genetics, UCSD). Western analysis was carried out as described previously, with minor modifications: PVDF membranes were used, and transfer was performed at 300 mA at 4°C for 3 h. The secondary antibody was a horseradish peroxidase (HRP)-labeled anti-rabbit IgG conjugate (Sigma; Ausubel *et al.*, 1987). Band detection was achieved by using enhanced chemiluminescene (ECL).

***Adenylyl cyclase assays, cAMP-induced actin polymerization assays, cAMP-binding assays, and synag assays.*** All biochemical assays were carried out by using cells at 6 h of development. *In vivo* adenylyl cyclase assays were performed as described by Parent and Devreotes (1995). cAMP-induced actin polymerization assays were performed as described (Insall *et al.*, 1996). cAMP-binding assays were performed as described by Xiao *et al.* (1997a). Cell-autonomous mutation (Synag) analysis was performed as described by Jin *et al.* (1998a).

***Microscopic analysis.*** Ege A-GFP/AX2 or Ege A-GFP/*ege A*$^-$ cells were plated onto the glass surface of an observation chamber and allowed to adhere. DB buffer (2 ml) was added to the top. Fluorescent microscopy was performed on an inverted microscope (Axiovet 135 TV; Zeiss, Inc., Thornwood, NY) equipped with an HBO 100-W mercury lamp, a 40× oil neoflur objective lens, and an eFFP filter. Confocal microscopy was performed on a confocal laser scanning microscope (Noran OZ; Noran, Middletown, WI) at an excitation wavelength 488 nm from a Krypton–Argon multiline laser. A barrier filter of 500-550 nm cutoff was used. The fixation procedure was carried out in the observation chamber according to the protocol described by Xiao (1997b).

## RESULTS

### *Isolation of Ege Gene from a REMI Screen*

*D. discoideum* cells lacking components of the cAR1/G$\alpha 2\beta\gamma$ display a clear "aggregationless" developmental phenotype (Sun and Devreotes, 1991; Wu *et al.*, 1995; Kumagai *et al.*, 1991). To identify additional components involved in these pathways, we carried out a REMI screen of 25,000 independent insertions and isolated 18 mutants similar to *g*$\beta^-$ (Kuspa and Loomis, 1994). We rescued a genomic DNA fragment from one of these mutants; a BLAST search yielded SSI135 and SSB184 from the Japanese *Dictyostelium* cDNA project (Morio *et al.*, 1998). Sequencing analysis yielded identical stop codons in the 5′-untranslated region of both clones, suggesting they encoded a full-length open reading frame. Northern analysis revealed a single band, smaller than 1 kb, consistent with the predicted size of its open reading frame (Fig. 1D). The gene contained three exons separated by two short introns: The REMI vector was inserted into the beginning of the third exon (Fig. 1B), resulting in no mRNA production as indicated by Northern analysis (data not shown). The phenotype of the original REMI mutant was recapitulated by disrupting the gene by homologous recombination in wild-type cells. As shown in the Southern blot (Fig. 1A, lanes 2 and 3), the freshly disrupted cells had the same genotype and phenotype as that of the original isolate, suggesting that the defects had specifically resulted from the insertion at that locus. We designated the gene *early gene expression A*, or *ege A*, and the mutant clones as *ege A*$^-$. The newly disrupted cells were used for further characterization of the mutation.

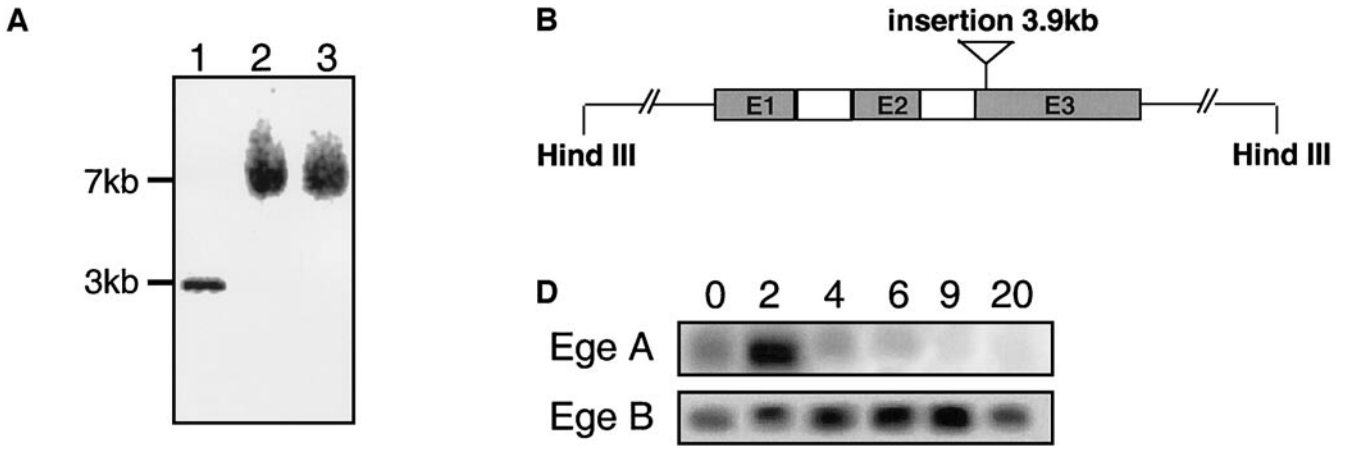
The Ege A cDNA encoded a protein of 254 amino acid residues, with no apparent transmembrane or catalytic domains (Fig. 1C). It contained a C2 domain, which spanned residues 40 to 120 (Nalefski and Falke, 1996; Sutton *et al.*, 1995). When Ege A cDNA was used in a GenBank BLAST search, another *D. discoideum* gene, R2005, was found to be highly homologous to Ege A (Fig. 1C). We suggest R2005 to be designated Ege B, even though it may not play a role in early gene expression. Ege B contained a 174-amino-acid extension at its $NH_2$ terminus. The aligned sequences of the two genes are 48% identical, excluding the Ege B $NH_2$-terminal extension. The high level of identity in the primary sequence of these two molecules suggests that they are also similar in structure and function.

Levels of Ege A and Ege B mRNA were tightly regulated during development. Ege A mRNA was expressed in vegetatively growing cells, reaching a maximum level at the 2-h stage, and decreasing sharply afterwards (Fig. 1D). In contrast, Ege B showed a relatively low level of expression in vegetative growth cells. Its mRNA level increased with development and peaked at the 6- to 9-h stage. The difference in the expression pattern of these two genes suggested that they might act at different stages of development, with Ege A functioning mainly at preaggregation stage and Ege B at later stages.

We further characterized the developmental phenotype of the *ege A*$^-$ cells. When single AX2 cells were plated on *Klebsiella aerogenes* lawns, they formed plaques and entered development, displaying aggregation territories then developing into spores and stalks (Fig. 2). The plaques of *ege A*$^-$ expanded more slowly than those of AX2, and the cells never entered the development phase. These defects were confirmed with nonnutrient agar plates: *ege A*$^-$ cells remained as a smooth monolayer, whereas AX2 cells formed aggregates within 6 h and spores on stalks in 30 h. These data indicated that Ege A was required before or during the aggregation stage.

### *The Deletion Mutation Impairs the Expression of Aggregative Genes*

Since cAR1 is known to play an essential role in aggregation, its expression in *ege A*$^-$ cells was examined. As shown in Fig. 3A, a basal level of cAR1 protein was present in vegetative-growth wild-type AX2 cells and this level increased 20-fold within 5 h, as measured by a cAMP-binding assay (Fig. 3B). Northern blotting demonstrated that this increase in cAR1 protein resulted from a sharp increase in the level of its mRNA (Fig. 3A). The vegetative *ege A*$^-$ cells had the same low expression of cAR1 as did the AX2 cells. However, we observed only a 2- to 3-fold increase in the cAR1 protein level in 5-h stage *ege A*$^-$ cells. This low level of protein reflected a low level of mRNA (Fig. 3A). The




C

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Ege A ---------- ---------- ---------- ---------- ----------
Ege B MGLEEEQNNNNNNNNN NNNNNNNNNNNNNNN NTSTINNNSANTTPL LEKRPNNSTITNIEI YPQQYDQQYYQQQQE

      ---------- ---------- ---------MMQAPM IAATNPNCPIAIVAN HKLSLPPGACPFVVN
      YYQTQPTQAILNGIL PTTTTTTTTTTSSSS SQYLIEPMLMIPTGA IATFDMNMNSKATIN FDSICVPDTCTFYCS

      -PNGNGKIRIRLISA SNLISADANGYSDPY IKVKSTSIDSMKVTK VIEKNLNPVWNEEVV LDISSVGRELFIFDV
      SPIGKGRLKVRIISA GNLIAADIGGTSDPY VKIKSSCLKSFKATR IVDKCLNPVWEETLA VEIDCVERELLMFDI

      YDSDKVGNDDLLGFV GVDLSLLPLGIEVLT CENLSFAKHGVLQIG ITALDFGLTNIPPNY LHAYTEWR-NTLPGH
      YDHDVVGCDDLLGYV GIDVSKLPLGIEVVT NENLSFAKHGTLKIA LTALDFGLTNLPQNY LTTYQEWRSHTLGGI

      QRKEFKKLKKHKDSC GPYANKVVH-EDYKV TNGYLKRKKSKREKT AKGFVKGLKIAGKVS LKVLDSATEDD  254
      TKKHFKSIKKSSK-I GPYVSKLTHPHDYKL VNGFVKRRKTRREKT ADGIITGLKVAGVVA LVAISS-----  425
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**FIG. 1.** Isolation of Ege. (A) Southern blotting of *ege A*$^-$. The blot was probed with Ege A cDNA. Lane 1, AX2 genomic DNA cut with *Hin*dIII. Lane 2, Restriction enzyme-mediated insertion *ege A*$^-$ genomic DNA cut with *Hin*dIII. Lane 3; Homologous recombinant *ege A*$^-$ genomic DNA cut with *Hin*dIII. (B) The genomic structure of gene Ege A and the position of REMI insertion. (C) Alignment of Ege A and Ege B protein sequences. The identical amino acid residues are bold. (D) Northern blot analysis of Ege A and EgeB mRNAs during development. At time 0, 2, 4, 6, 9, or 20 h, AX2 cells were harvested and 20 $\mu$g of total RNA was loaded in each lane. The blot was probed with Ege A or Ege B cDNA probes separately.

loss of expression was not limited to cAR1, since a similar defect was observed for another aggregative gene, GP80. There was a large increase in the amount of GP80 protein in 5-h stage AX2 cells, while only a 3-fold increase was observed in *ege A*$^-$ cells (Fig. 3C). The induction of aggregative gene, G$\alpha$2, was also impaired in *ege A*$^-$ cells (data not shown). However, different from cAR1 and GP80, a significant amount of G$\alpha$2 had been expressed when cell density was high, suggesting this gene may also be regulated by starvation (R. E. Gundersen, personal communication). The expression defects were also observed in mutant cells that were induced repeatedly with 75 nM cAMP stimulation at 6-min intervals for 5 h (data not shown). Thus, it appears that developmentally regulated aggregative gene expression requires Ege A.

To determine whether Ege A was required for the expression of other developmental genes, we used immunofluorescent staining to examine the expression of the prestarvation gene Discoidin Ic at the growth stage (Clarke *et al.*, 1987; Rathi *et al.*, 1992). Previous data have shown that Discoidin Ic expression is regulated by PSF concentration, which is proportional to the ratio of cell density vs bacteria (Burdine and Clarke, 1995). In our experiments, at a low cell density ($1 \times 10^5$ cells/ml), Discoidin Ic was not expressed (Fig. 3D). When the cells reached a higher density ($5 \times 10^6$ cells/ml) and PSF accumulated, Discoidin Ic, representing a group of prestarvation genes, was expressed (Burdine and Clarke, 1995; Clarke and Gomer, 1995). The *ege A*$^-$ cells displayed the same pattern of Discoidin Ic transcriptional regulation as did the wild-type AX2 cells. Thus, the regulation of prestarvation gene expression does not require Ege A.

Mutations in cAR1, G proteins, and PKA have been shown to impair various G-protein-mediated signal trans-

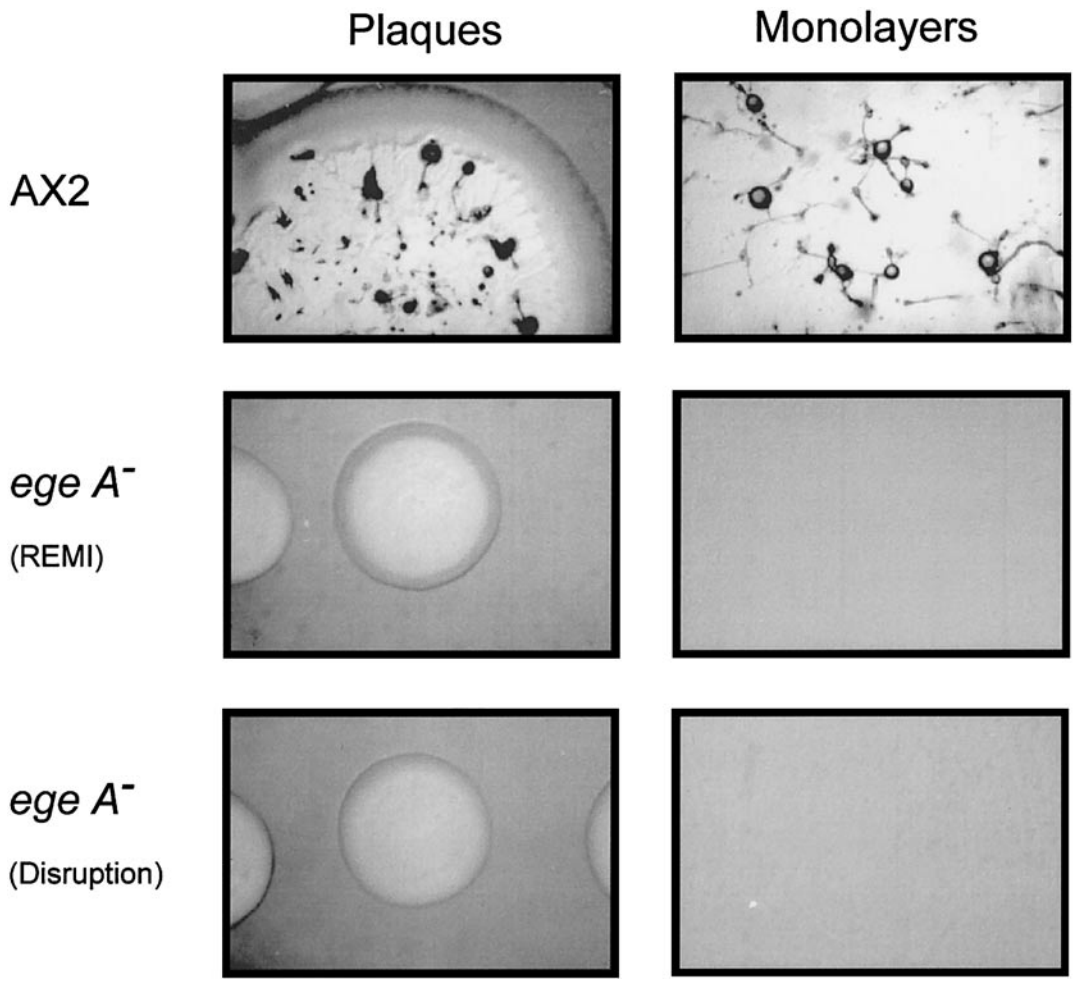


**FIG. 2.** The *ege A*$^-$ mutant displays a development defect. *ege A*$^-$ cells form small and aggregationless plaques on a SM bacterial lawn and fail to form aggregates on a nonnutrient agar plates. The development phenotypes of the wild-type cell, AX2, are shown as controls.

duction pathways, such as cAMP production and actin polymerization. These pathways were analyzed in *ege A*$^-$ cells. Upon adding cAMP stimuli, the level of F-actin formation in *ege A*$^-$ cells is comparable to that in wild-type cells (Fig. 4A). Previous data have shown that cAR1 exhibits two affinities, $K_d$ of 300 nM for 90% of receptors and $K_d$ of 10 nM for 10% of receptors, and 2 nM cAMP induces a full-scale actin response (Caterina *et al.*, 1995; our unpublished results). Thus, 4% of the receptors are sufficient to elicit a full actin polymerization response. *ege A*$^-$ cells are impaired in cAMP-induced adenylyl cyclase activation, probably due to low expression of cAR1 (Fig. 4B). Such a defect was rescued by overexpressing functional YFP-cAR1 in mutant cells (Fig. 4B).[2] When the G proteins in the cell lysate of *ege A*$^-$ cells were stimulated with GTPγS, adenylyl cyclase was activated, suggesting that the cAMP production pathway was functional (Fig. 4C).

### *The Defect in ege A*$^-$ *Is Cell Autonomous*

Cells need to secrete signaling molecules as well as respond to them. Mutants that are defective in generating or regulating extracellular signaling molecules, such as PSF, CMF, or cAMP, can be synergistically rescued when they are mixed with wild-type cells, which supply the missing components. To test whether the *ege A*$^-$ developmental phenotype could be rescued, *ege A*$^-$ and AX2 cells were mixed in equal ratios and allowed to develop into spores. The spores were then harvested and screened for possible rescued *ege A*$^-$ on a bacterial lawn. Of 150 plaques screened, no *ege A*$^-$ plaques were observed, suggesting that *ege A*$^-$ was a cell-autonomous defect. To further test the possibility that AX2 might rescue some of the gene expres-

[2] Based on its ability to rescue *car1-/car3-* mutants, YFP-cAR1 can be used as a functional equivalent of cAR1, with the additional advantage of having a yellow fluorescent protein label (data not shown).

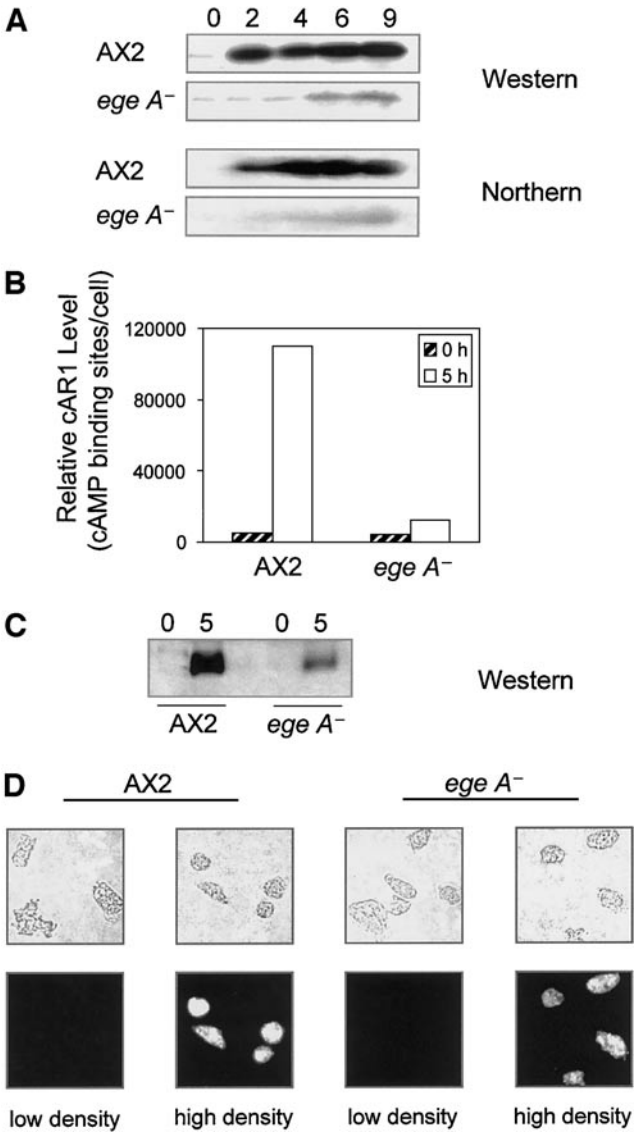


**FIG. 3.** GPCR-mediated cAR1 and GP80 expressions in *ege A*$^-$ cells. (A) The *ege A*$^-$ and wild-type, as a control, cells in suspension were stimulated with cAMP at 6-min intervals. The levels of cAR1 protein and cAR1 mRNA were determined at various time points by Western blot (upper panel) and Nothern blot (lower panel) analyses. (B) cAMP binding sites on the surface of *ege A*$^-$ and wild-type cells. Relative cAR1 level on the cell surface was determined by cAMP binding assay on the cells harvested from 0 and 5 h in suspension. (C) Western blot analysis shows the protein levels of GP80 at 0 and 5 h following cAMP treatment in suspension of the cells. (D) Immunofluorescent staining analysis showed the regulated expression of discoidin-Ic in both AX2 and *ege A*$^-$ cells. The upper panels showed phase contrast images and the lower panels showed fluorescent images of the same stained cells. Cells were examined at both low and high cell density: $1 \times 10^5$ and $5 \times 10^6$ cells/ml.

sion defects of *ege A*$^-$ by inducing cAR1 expression, we examined cAR1 expression in the same assay. An equal ratio mixture of AX2 and *ege A*$^-$ cells was allowed to develop for 6 h and assayed for cAMP binding. In controls, AX2 and *ege A*$^-$ cells were allowed to develop separately for 6 h, then mixed and assayed. The levels of cAMP binding sites were $4.1 \pm 0.2 \times 10^4$ and $4.3 \pm 0.2 \times 10^4$, respectively. The fact that the control mixed cells had the same level of cAMP-binding sites indicated that cAR1 was not expressed in *ege A*$^-$ cells when synergistically mixed with AX2 cells. These data are consistent with the observation that mutant cells were defective in expressing cAR1 even after treated with cAMP pulsing for 5 h. Since extracellular signaling molecules from wild-type cells could not rescue *ege A*$^-$, the Ege A protein must be required for individual cells to respond to signaling molecules.

### *HA-PKAcat Cannot Rescue ege A*$^-$

Previous studies have shown that PKA is involved in the regulation of aggregative genes as well as cell type-specific genes (Firtel and Chapmann, 1990; Harwood *et al.*, 1992; Mann *et al.*, 1992). Overexpression of a constitutively active subunit of PKA, PKAcat, can partially rescue many upstream aggregation defective mutants, such as *aca*$^-$, *crac*$^-$, and *car1*$^-$ (Wang and Kuspa, 1997; Reymond C., personal communication). We reasoned that cAR1 expression in *ege A*$^-$ would be enhanced by expressing PKAcat if PKA were located downstream of Ege A. HA-PKAcat was expressed in *ege A*$^-$ cells at the same level as in control *aca*$^-$ cells (Fig. 5A). Since expression of PKAcat in *aca*$^-$ suppresses the *aca*$^-$ phenotype, PKAcat/*aca*$^-$ cells aggregated and expression of cAR1 was as high as in wild-type cells (Fig. 5B). In contrast, PKAcat/*ege A*$^-$ cells failed to aggregate, and the expression of cAR1 remained low. These observations suggest that PKA does not function downstream of Ege A.

### *YFP-cAR1 Can Partially Rescue Aggregation*

Since signaling through cAR1 induces its own expression as well as the expression of other aggregative genes, the gene expression defect in *ege A*$^-$ might reflect a defect in the feedback signal for cAR1 expression. To assess this possibility, we transformed YFP-cAR1 into *ege A*$^-$.[2] Expression of YFP-cAR1 resulted in a partial rescue of *ege A*$^-$, with YFP-cAR1/*ege A*$^-$ cells forming loose mounds (Fig. 6A). Receptor-mediated adenylyl cyclase activation is recovered (Fig. 4B). However, Western blotting revealed that the expression of the aggregative gene, GP80, was still impaired in these cells (Fig. 6B). The same partial aggregation and GP80 expression defect were observed in YFP-cAR1/*ege A*$^-$ cells that were repeatedly stimulated with 75 nM cAMP at 6-min intervals for 5 h. The data indicated that constitutive expression of cAR1 was able to mediate a certain level of aggregation in *ege A*$^-$ cells, but not sufficient to fully induce aggregative gene expression. Thus, the defect is not solely due to a lack of feedback signal from cAR1.

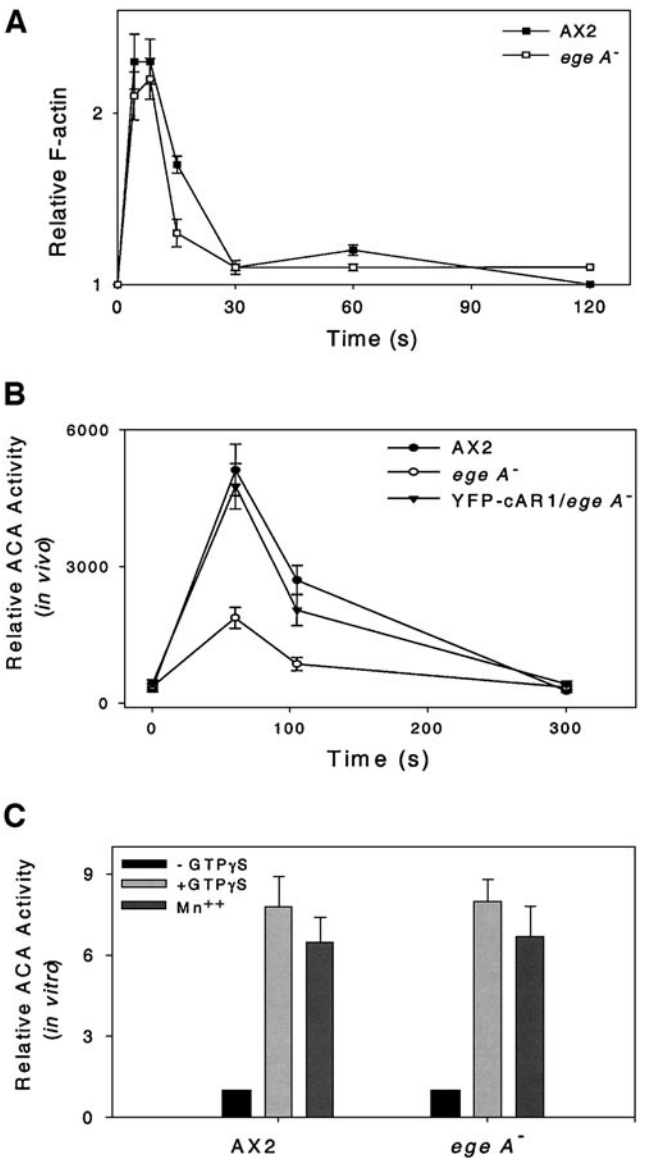


**FIG. 4.** G protein-mediated activation of adenylyl cyclase and actin polymerization in *ege A*$^-$ mutant. (A) cAMP-induced actin polymerization assay: 1 $\mu$M cAMP was added, and relative F-actin was quantified with TRICT-phalloidin at 0, 4, 8, 15, 30, 60, or 120 sec. (B) Chemoattractant-induced adenylyl cyclase activation: 1 $\mu$M cAMP was added, cells were lysed at 0, 60, 105, or 300 sec, and adenylyl cyclase activity was assayed. (C) Adenylyl cyclase A (ACA) activation assay. The plot shows the GTP$\gamma$S-induced ACA activation in AX2 and *ege A*$^-$ cells. (All plots represent the average of three independent experiments.)

### *Overexpression of Ege A Causes a Dominant Negative Effect on cAR1 Expression*

In order to test the effects of Ege A overexpression, a full-length Ege A was transformed into both AX2 and *ege A*$^-$ cells. Surprisingly, both Ege A/*ege A*$^-$ and Ege A/AX2 transformants failed to aggregate. Instead, they formed "smooth plaques" on *Klebsiella aerogenes* SM plates (Fig. 7A). To investigate the cause of these defects in aggregation, we examined cAR1 protein levels in each cell line. At 5 h of development, in both cell lines, cAR1 levels were slightly higher than that of *ege A*$^-$ cells, but much lower than that in AX2 cells (Fig. 7B). The expression of COOH-terminal green fluorescent protein-tagged Ege A (Ege A-GFP) or $NH_2$-terminal HA-tagged Ege A in wild-type cells also impaired the expression of cAR1 (data not shown). To demonstrate that the dominant negative effects were due to expression of Ege A, Ege A-GFP/AX2 cells were cultured in G418-free media for 5 weeks to cause a loss of expression of Ege A-GFP. Western blot showed that Ege A-GFP/AX2 cells lost 70% Ege A-GFP expression in drug-free media (Fig. 7C). The phenotypes of individual plaques from this culture were then examined. About half of plaques had reversed phenotype and aggregated as did wild-type cells. Furthermore, among examined clones, Western analysis showed that aggregative clones exhibited much lower expression of Ege A-GFP than those of "aggregationless" ones (data not shown).

We next investigated the distribution of Ege A-GFP in *ege A*$^-$ and AX2 cells. Fluorescent microscope imaging data showed that Ege A-GFP was uniformly distributed throughout cytosol in living Ege A-GFP/AX2 cells (Fig. 8A). Confocal imaging analysis further confirmed this observation (Fig. 8B). However, in fixed cells, confocal microscopic analysis revealed a small percentage of Ege A-GFP associated with plasma membrane and the Golgi apparatus (Fig. 8C). Other studies have shown that the fixation procedure enhances membrane signals perhaps by partially removing the cytosolic fluorescent signals (C. Manahan, personal communication). Thus, it is possible that there is a small amount of membrane and Golgi-associated Ege A-GFP in living cells. We are testing whether the membrane associa-

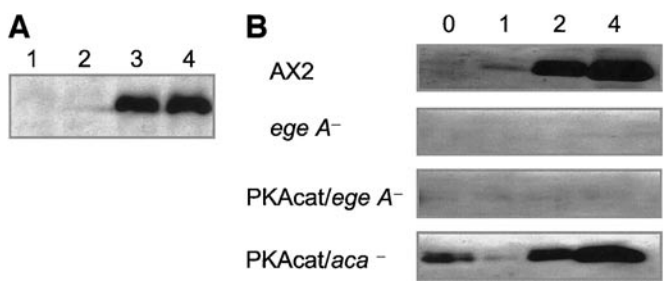


**FIG. 5.** Failure of PKAcat to rescue *ege A*$^-$. (A) Expression of HA-PKAcat was visualized by Western blot analysis with anti-HA antibody in AX2 (lane 1), *ege A*$^-$ (lane 2), PKAcat/*ege A*$^-$ (lane 3), and PKAcat/*aca*- cells (lane 4). (B) Western blot analysis of cAR1 expression in the four cell lines during development. Cells were harvested at time 0, 1, 2, or 4 h for SDS–PAGE. Each lane was loaded with $2 \times 10^6$ cell equivalents of extract, and the blot was probed with anti-cAR1 antibody.

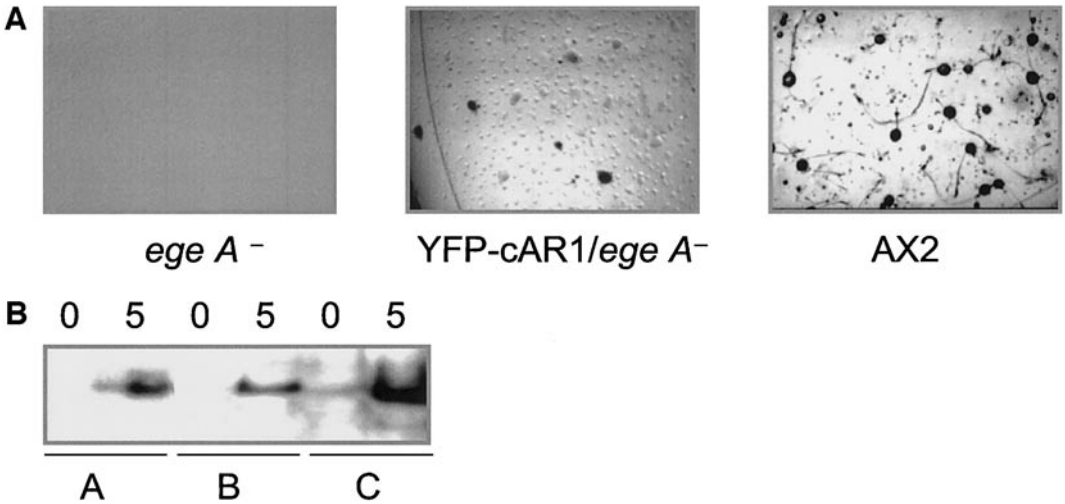


**FIG. 6.** Partial rescue of *ege A*$^-$ by expression of YFP-cAR1. (A) The developmental phenotype of *ege A*$^-$, AX2, and YFP-cAR1/ *ege A*$^-$ was assayed on nonnutrient agar plates. (B) Expression of GP80 in *ege A*$^-$ (A), YFP-cAR1/*ege A*$^-$ (B), and AX2 (C) cells was examined by using Western blot analysis with anti-GP80 antibody.

tion of Ege A-GFP that can be visualized only by fixation depends on the C2-motif in Ege A. Ege A-GFP has the same subcellular distribution in *ege A*$^-$ cells as in AX2 cells (data not shown).

## DISCUSSION

Our findings indicate that Ege A is required for aggregative gene expression. Western and Northern analysis showed defective expression of cAR1 and GP80 in *ege A*$^-$ cells, suggesting the requirement of Ege A in the GPCR-mediated early gene expression. Ege A mRNA is only found during the first 2 h of development, consistent with a proposed role as a regulator of early gene expression. Typically, proteins appear in a program during the times they are required. Aside from the defects in induction of aggregative gene expression, *ege A*$^-$ cells are similar to wild type cells in the regulation of prestarvation gene expression, the activation of adenylyl cyclase and guanylyl cyclase, and the cAMP-induced formation of actin filaments. Thus, Ege A does not appear to be a modulator of receptor or G protein function; instead, it may act downstream in one specific GPCR-linked pathway.

A C2 domain was the only motif found in Ege A. C2 domains, first discovered in protein kinase C and later in many other proteins, are $Ca^{2+}$ -dependent lipid-binding domains (Nalefski and Falke, 1996; Sutton *et al.*, 1995). Proteins containing C2 domains can be classified into two groups: One contains catalytic domains in addition to C2, with the C2 domain serving to redistribute the enzyme following a change in $Ca^{2+}$ concentration (Nalefski and Falke, 1996). The other is mainly composed of vesicle fusion proteins. In these proteins, C2 is directly involved in vesicle trafficking. We first examined the possibility that Ege A might be involved in vesicle trafficking, which targeted cAR1 and GP80 to plasma membrane. In *ege A*$^-$ cells, loss of such a targeting mechanism might induce a negative feedback signal to inhibit the expression of aggregative genes. However, ectopically expressed YFP-cAR1 molecules were found uniformly distributed on the membrane surface, the same as that in wild-type cells (data not shown; Xiao *et al.*, 1997). Thus, Ege A does not regulate the transport of cAR1 to membrane. A small quantity of Ege A-GFP was found on cytoplasmic membrane and Golgi apparatus of fixed cells, suggesting that its C2 motif have a low affinity to lipids *in vivo*. The C2 motif may mediate subcellular redistribution of cytosolic Ege A to membranes in responding to signals, which in turn leads to the induction of aggregative gene expression. Further careful investigation is needed to understand the function of the C2 domain in Ege A. G protein-dependent $Ca^{2+}$ release has been found in a Wnt/Frizzled pathway (Sheldahl *et al.*, 1999; Slusarski *et al.*, 1997). It will be interesting to investigate whether a C2 domain containing protein, regulated by $Ca^{2+}$ signal, is involved in Wnt/Frizzled signaling.

Expression of aggregative genes is mediated by a G protein-linked signaling pathway (Parent and Devreotes, 1996). In the absence of G protein signaling, starvation induces only a two to three fold increase in gene expression (Firtel, 1995; Jin *et al.*, 1998b). This small increase in gene expression is retained in *ege A*$^-$ cells, indicating that Ege A is not involved in the starvation-induced G protein-independent pathway. Interestingly, *ege A*$^-$ cells display a similar defective developmental phenotype to mutants lacking components in G protein-linked pathways, such as

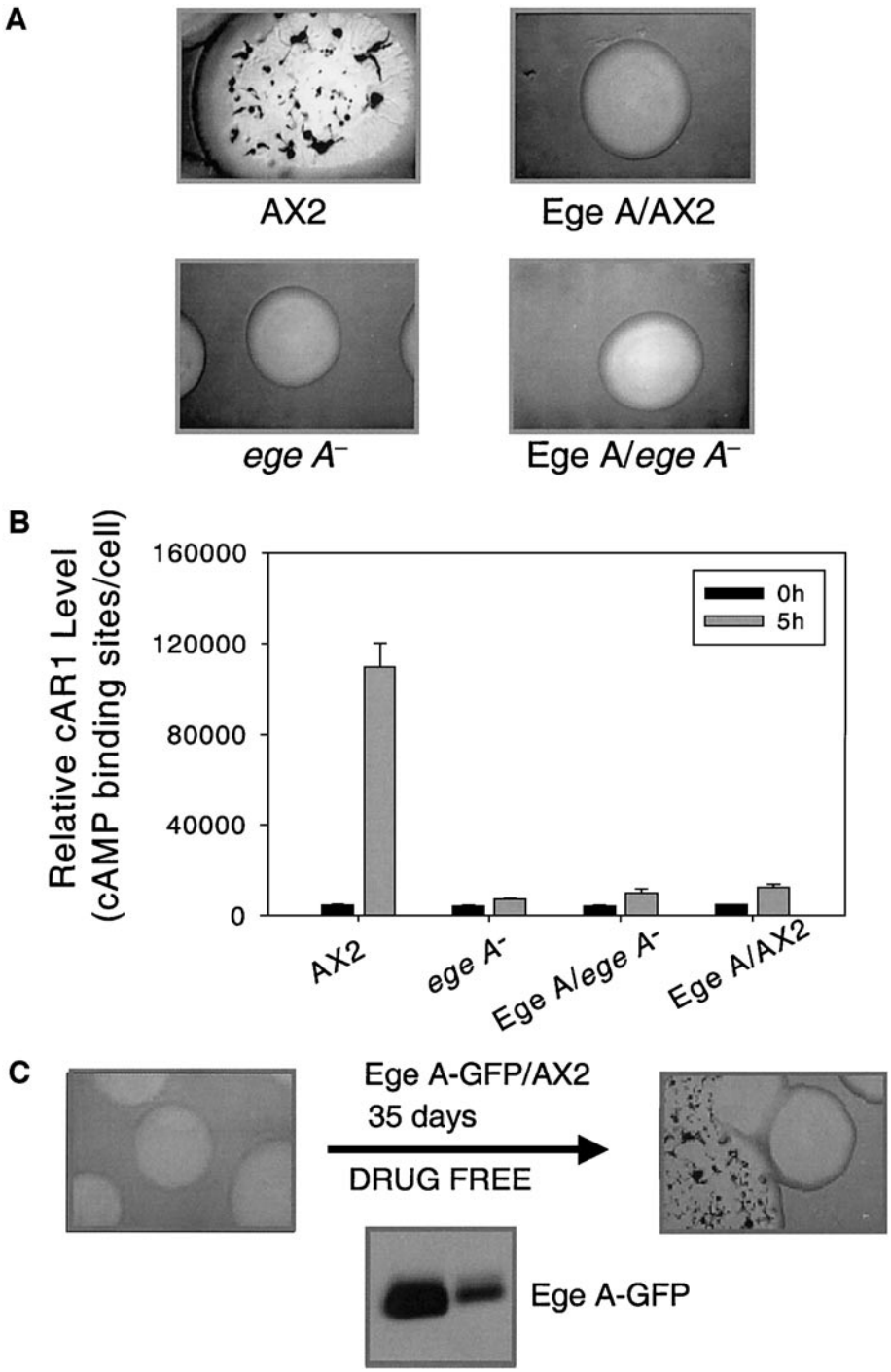


**FIG. 7.** Dominant negative effect of Ege A overexpression. (A) Developmental phenotypes of AX2, *ege A*$^-$, Ege A/*ege A*$^-$, and Ege A/AX2 cells were assayed on SM bacterial lawns. (B) Expression of cAR1 in the four cell lines was quantified by using a cAMP-binding assay. (C) Ege A/AX2 cells lost 70% of Ege A-GFP after growing in G418-free medium for 35 days. The left panel shows the Ege A/AX2 phenotype before removing the drug. After removing drug for 35 days, half of the cells carried out normal development as shown in the right panel. The bottom panel shows the Western blotting analysis of GP80 before and after drug removal.

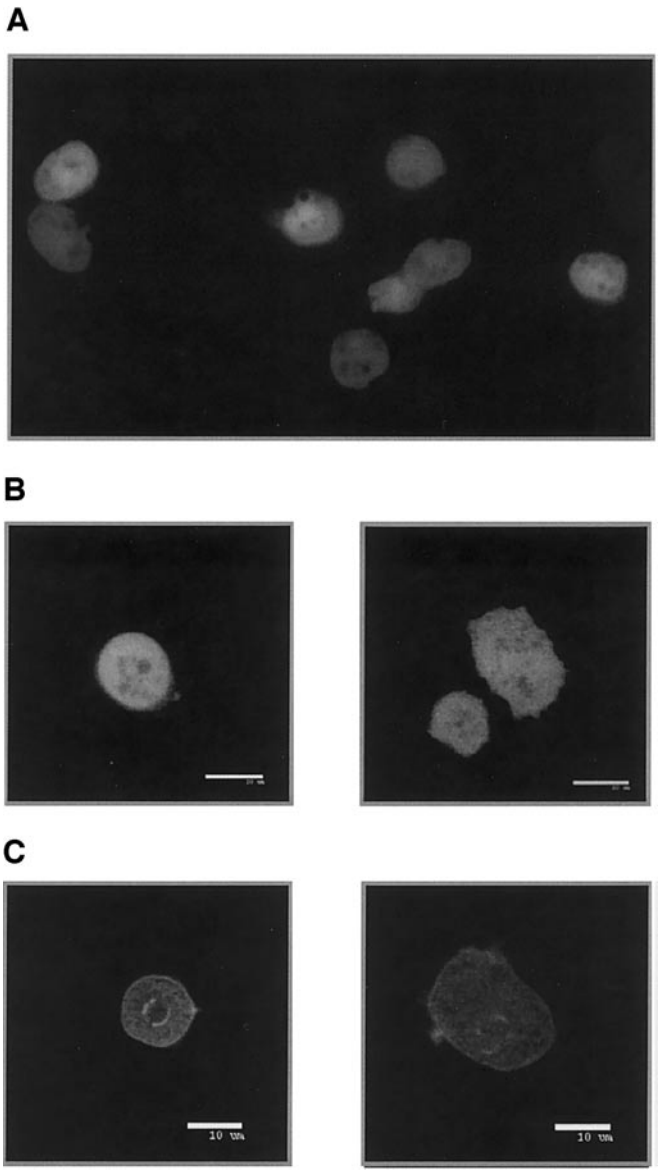


**FIG. 8.** Subcellular distribution of Ege A-GFP in AX2 cells. (A) Images of living Ege A-GFP/AX2 cells under Zeiss fluorescent microscope. (B) Confocal images of living Ege A-GFP/AX2 cells. (C) Confocal images of fixed Ege A-GFP/AX2 cells.

*car1*$^-$, *gβ*$^-$, *gα2*$^-$, and *yakA*$^-$ cells. The *ege A*$^-$ cells lose the induction of gene expression in a cell-autonomous manner, indicating that Ege A is required to respond to extracellular signals. Two Gα2βγ-mediated signaling pathways, cAMP-induced actin polymerization and GTPγS-induced adenylyl cyclase activation, can be fully activated in *ege A*$^-$ cells, suggesting that Ege A does not function at the level of G protein or cAR1. Constitutive expression of YFP-cAR1 partially rescued G protein-mediated aggregation in *ege A*$^-$ cells, but failed to rescue GP80 expression, showing that early gene expression in *ege A*$^-$ cells is still defective in the presence of functional receptors and G proteins. Thus, the defect is downstream of Gα2βγ. YakA is involved in multiple G protein-mediated signaling pathways, suggesting that it is located upstream of the branchpoint of different pathways (Van Es *et al.*, 2001). In contrast, Ege A is only required for GPCR-mediated gene expression, suggesting it is positioned after this branchpoint. The fact that PKAcat can rescue many mutants lacking upstream components, but fails to rescue the *ege A*$^-$ phenotype suggests that Ege A may be located downstream of PKA or in a parallel pathway. Based on the observations listed above, we propose a simple model for the regulation of aggregative gene expression: binding of extracellular cAMP enables cAR1 to activate G protein; Gβγ dissociates from Gα and activates downstream pathways through YakA; in one such pathway, Ege A mediates the expression of aggregative genes.

The isolation of Ege A provides a useful tool to study the GPCR-mediated aggregative gene expression. Ege A appears to be involved only in gene expression while other known components in this pathway, cAR1, G protein and Yak A, mediate a spectrum of signal transduction pathways. Such specificity of Ege A can be used to identify new components that regulate the aggregative gene expression. A REMI screen could be performed to isolate loss-of-function mutants that suppress the aggregation defects of *ege A*$^-$; or a cDNA library can be expressed in *ege A*$^-$ cells to identify the genes that can rescue the phenotype. Furthermore, the specificity of Ege A can be utilized to isolate potential interacting proteins by yeast two-hybrid technique or by biochemical copurification. A better understanding of aggregative gene expression may provide clues for the GPCR-mediated gene expression in higher organisms.

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