Development 120, 1997-2002 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994

Extracellular cAMP can restore development in *Dictyostelium* cells lacking one, but not two subtypes of early cAMP receptors (cARs).

Evidence for involvement of cAR1 in aggregative gene expression

Ron D. M. Soede[1], Robert H. Insall[2], Peter N. Devreotes[2] and Pauline Schaap[1,*]

[1]Cell Biology Unit, Institute of Molecular Plant Sciences, University of Leiden, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands
[2]Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA

*Author for correspondence

SUMMARY

Extracellular cAMP induces expression of several classes of developmentally regulated genes in *Dictyostelium*. Four highly homologous surface cAMP receptors (cARs) were identified earlier, but involvement of specific cARs in gene regulation has not been clarified. Cells lacking the chemotactic receptor, cAR1, neither aggregate nor express developmentally regulated genes. Expression of aggregative genes is in wild-type cells induced by nanomolar cAMP pulses and repressed by persistent micromolar cAMP stimuli, which induce expression of prespore and prestalk-enriched genes during the postaggregative stages of development. We show here that in cell lines carrying a *cAR1* gene disruption, nanomolar pulses cannot induce aggregative gene expression. Remarkably, micromolar cAMP can induce expression of aggregative genes in *car1*$^-$ cells as well as expression of prespore and prestalk-enriched genes, and furthermore restores their ability to form normal slugs and fruiting bodies. These data indicate that cAR1 mediates aggregative but not postaggregative gene expression and morphogenesis, and suggest that after gene disruption, its function is partially taken over by a lower affinity receptor that is not subjected to desensitization. The absence of another early cAMP receptor, cAR3, does not affect development. However, in a *car1*$^-$/*car3*$^-$ double mutant, cAMP stimulation cannot restore any developmental gene expression, indicating that cAR3 may have substituted for cAR1 in *car1*$^-$ cell lines.

Key words: *Dictyostelium discoideum*, cAMP receptor plasticity, gene disruption, developmental gene expression, adaptation

INTRODUCTION

During *Dictyostelium* development extracellular cAMP induces chemotaxis and regulates expression of several classes of genes. In preaggregative cells, nanomolar cAMP pulses enhance expression of aggregative genes, coding for, e.g., surface cAMP receptors (cARs), and adhesive contact sites A (csA) (Darmon et al., 1975; Gerisch et al., 1975; Klein et al., 1988). After aggregation, micromolar cAMP levels induce the expression of prespore genes and of a subclass of prestalk genes, independent of stimulus modulation (Kay, 1982; Barklis and Lodish, 1983; Mehdy et al., 1983; Schaap and Van Driel, 1985). Studies using cAMP derivatives have indicated that the effects of cAMP on chemotaxis and gene expression are mediated by cARs (Van Haastert and Kien, 1983; Schaap and Van Driel, 1985; Oyama and Blumberg, 1986; Schaap et al., 1993), although additional activation of cAMP-dependent protein kinase is also required for spore and stalk cell differentiation (Harwood et al., 1992; Mann et al., 1992; Hopper et al., 1993).

At present, genes coding for four highly homologous cARs have been characterized; (Klein et al., 1988; Saxe, 1991; Saxe et al., 1991). *cAR1* is expressed shortly after starvation and is responsible for virtually all cAMP binding activity at the aggregative phase of development. Expression of *cAR1* is directly followed by expression of *cAR3*, which is at its optimum at the mound stage. *cARs 2* and *4* are expressed during slug and fruiting body formation, and are only found in a subset of cells. In *Dictyostelium*, interaction of cAMP with cARs results in a transient activation of adenylyl cyclase (Roos et al., 1975; Dinauer et al., 1980), guanylyl cyclase (Mato et al., 1977; Würster et al., 1977) and phospholipase C (Europe-Finner and Newell, 1987; Van Haastert et al., 1989), as well as displacements of Ca^{2+}, H^+ and K^+ ions (Milne and Coukell, 1991; Malchow et al., 1978; Aeckerle et al., 1985).

To investigate the role of cAR1 in signal transduction and development, cAR1 antisense cells have been constructed (Sun et al., 1990). These cells do not differentiate and show neither chemotaxis nor second messenger accumulation in response to cAMP. Since the four cARs are highly homologous, a consequence of the cAR1 antisense mutagenesis may be the loss of other cAR mRNAs besides cAR1. To overcome this, the *cAR1* gene was disrupted by homologous recombination (Sun and Devreotes, 1991). The *car1*$^-$ cells display a similar phenotype

to the antisense lines; they neither aggregate nor develop into fruiting bodies. However, it is at present not clear if the block in development is due to defects in signal production or in transduction of the signal to gene regulation. We investigated whether *car1⁻* cells can express developmentally regulated genes in response to cAMP stimulation and present evidence that cAR1 mediates aggregative, but not postaggregative gene induction. Cells carrying a double disruption of both the *cAR1* and *cAR3* genes are completely unresponsive to cAMP as an inducer of gene expression.

MATERIALS AND METHODS

Culture and incubation conditions

The *Dictyostelium discoideum* control cell lines AX3 and PJK1:AX3 as well as *car1⁻*cell line JS14, the *car1⁻/car3⁻* line RI-4 (Insall et al., 1994) and *aca⁻* cells (Pitt et al., 1992) were grown in HL-5 medium (Watts and Ashworth, 1970) and harvested at the late log phase of development. Cells were washed once with 10 mM Na/K phosphate buffer, pH 6.5 (PB) and subsequently shaken at 150 rpm in PB at 5×10^6 cells/ml and 22°C. Cells were challenged with different regimes of stimulation with cAMP or adenosine 3′:5′-monophosphorothioate, Sp-isomer (cAMPS) (Boehringer, FRG) as indicated in the figure legends.

RNA isolation and analysis

Total cellular RNA was isolated from 2.5×10^7 cells as described by Nellen et al. (1987), size fractionated on 1.5% agarose gels containing 2.2 M formaldehyde and transferred to Gene Screen membranes. Northern transfers were hybridized to [^{32}P]dATP-labeled DNA probes according to standard procedures and exposed to X-ray films. The optical density of specific mRNA bands was quantitated using an LKB Ultrascan densitometer.

Membrane preparation and western blotting

Membranes from *car1⁻* and AX3 control cell lines were prepared by the method of Klein et al. (1988) and dissolved in 100 μl SDS sample buffer. 30 μl aliquots of membrane preparation were size-fractionated on a 10% acrylamide gel, electroblotted onto nitrocellulose and probed with cAR3 specific antiserum. Antibody binding was visualised using an enhanced chemiluminescence (ECL) western blotting detection system (Amersham).

RESULTS

Induction of aggregative gene expression in *car1⁻* cells

During *Dictyostelium* development several classes of cAMP-regulated genes are expressed, which vary in requirements for signal modulation. Following starvation, cAMP pulses in the nanomolar range strongly accelerate the expression of aggregative genes, which include *cAR1* itself and the gene coding for contact sites A (csA), a glycoprotein mediating cell adhesion. We compared the effect of different concentrations of cAMP pulses on expression of the csA gene in wild-type and *car1⁻* cells. Fig. 1 shows that pulses of 30 nM cAMP at 6 minutes intervals optimally induce the synthesis of *csA* mRNA in the control cell line (AX3); at higher cAMP concentrations no further increase in *csA* mRNA levels is observed. In *car1⁻* cells, 30 nM pulses cannot induce *csA* gene expression, but increasingly higher cAMP concentrations induce a significant level of *csA* expression.

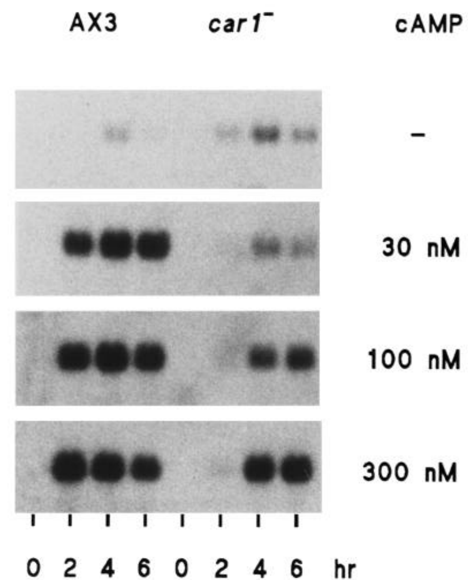

Fig. 1. Induction of aggregative gene expression in *car1⁻* cells. AX3 and *car1⁻* cells, harvested during growth phase, were incubated in PB and stimulated for 6 hours with pulses of either 30, 100 or 300 nM cAMP, added at 6 minute intervals. mRNA was isolated after 0, 2, 4 and 6 hours of incubation, and northern transfers were probed with a ^{32}P-labeled cDNA for the contact site A (*csA*) gene.

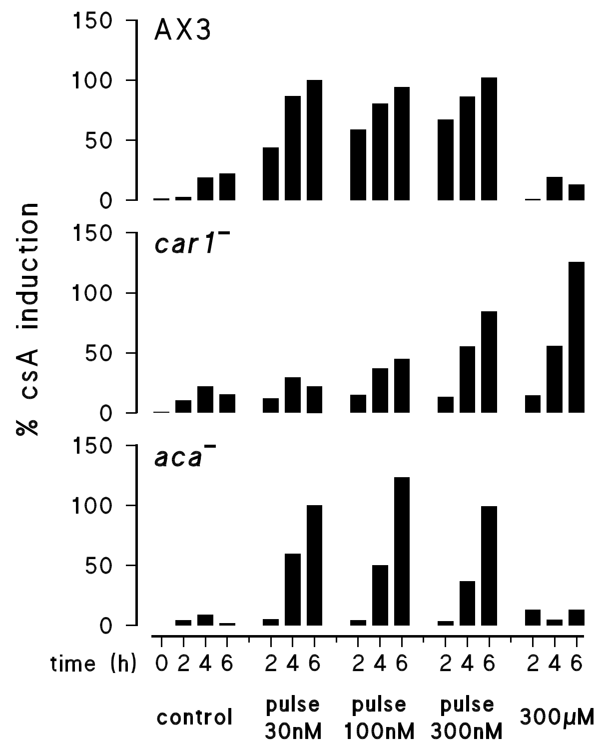

Fig. 2. Comparison of *csA* gene induction in wild-type, *car1⁻* and *aca⁻* cells. Cells were incubated as described for Fig. 1 and stimulated with either 30, 100, or 300 nM cAMP at 6 minute intervals, or with 300 μM cAMP added every hour. Northern blots were probed with *csA* cDNA and specific bands were quantitated by optical density scanning. Data are expressed as percentage of the OD of mRNA bands obtained from AX3 cells pulsed for 6 hours with 30 nM cAMP. The means of two experiments are presented.

Fig. 2 represents pooled data from two experiments. These data emphasize that whereas *csA* expression in AX3 cells is as efficiently induced by 30, 100 or 300 nM cAMP pulses, only 300 nM pulses induce an appreciable level of *csA* expression in *car1*$^{-}$cells. One possible explanation would be that low cAMP concentrations are amplified by relay in wild-type, but not *car1*$^{-}$ cells (Insall et al., 1994). However, 30 nM cAMP pulses can induce optimal *csA* expression in an adenylyl cyclase null mutant (*aca*$^{-}$), which also cannot amplify the cAMP signal (Pitt et al., 1992). It therefore appears that the requirement for high concentrations in *car1*$^{-}$ cells is not a consequence of defective signal amplification, but of defective signal perception. In AX3 cells, a continuous micromolar cAMP stimulus cannot induce csA gene expression, most likely because persistent stimulation causes desensitization of this response. Quite remarkably, this stimulation regime is highly effective in inducing *csA* expression in *car1*$^{-}$ cells. This suggests that in *car1*$^{-}$ cells the response is mediated by a lower affinity receptor, which is not subjected to desensitization mechanisms.

Induction of postaggregative gene expression and development in *car1*$^{-}$ cells

After cells have entered the aggregation phase of development, micromolar cAMP concentrations can induce expression of prespore genes and of a class of genes that are more strongly expressed in prestalk cells. We compared expression of the two prestalk-enriched genes, *D14* and *CP2* (Barklish and Lodish, 1983; Pears et al., 1985), and prespore genes *D19* and *SP96* (Barklis and Lodish, 1983; Fosnaugh and Loomis, 1989) in *car1*$^{-}$ and AX3 cells. Fig. 3 shows that both in *car1*$^{-}$ and AX3 cells, expression is absent or barely detectable in the absence of exogenous cAMP. In both cell lines the prestalk-enriched genes are induced within 2 hours after addition of cAMP, whereas the prespore genes gain optimal expression levels after 4 to 6 hours of stimulation. No significant difference between gene induction in AX3 and *car1*$^{-}$ cells was observed.

After 8 hours of stimulation with 300 μM cAMP, cells have formed tight aggregates in suspension. When these aggregates are placed on agar, they form tips, slugs and fruiting bodies in a similar fashion to wild-type cells. The same results are obtained when cells are stimulated with a single 5 μM dose of the non-hydrolysable cAMP derivative, adenosine 3′:5′-monophosphorothioate, Sp-isomer (cAMPS) (Fig. 4). The use of this derivative obviates the need for the nonphysiological, high concentrations of cAMP that are required to counteract degradation by phosphodiesterases.

To conclude, it appears that induction of postaggregative genes by micromolar cAMP concentrations occurs normally in *car1*$^{-}$ cells. Furthermore, once transcription of postaggregative genes has been induced in suspension, *car1*$^{-}$ cells have acquired full potency to complete all subsequent stages of development.

cAR3 expression in *car1*$^{-}$ cells

The data presented in Fig. 2 indicate that a lower affinity receptor may have taken over the function of cAR1 in aggregative gene expression. The most obvious candidate for a receptor substituting for cAR1 is cAR3, which is expressed somewhat later during development than cAR1. We first measured whether appreciable amounts of cAR3 protein are expressed during treatments inducing gene expression in *car1*$^{-}$ cells. AX3 cells and the *car1*$^{-}$ cell line JS14 were stimulated with 300 nM cAMP pulses or with a single dose of 5 μM cAMPS. A western blot of membrane proteins isolated during stimulation shows that both cAMP pulses and 5 μM cAMPS induce a detectable amount of cAR3 protein in *car1*$^{-}$ cells, although in both cases *cAR3* expression remains lower than in AX3 cells (Fig. 5).

Gene expression and development in *car1*$^{-}$/*car3*$^{-}$ cells

To investigate whether cAR3 can substitute for cAR1 in development and gene induction experiments, a double *car1*$^{-}$/*car3*$^{-}$ cell line was constructed (Insall et al., 1994). Similar to *car1*$^{-}$ cells, *car1*$^{-}$/*car3*$^{-}$ cells neither aggregate nor differentiate, when deposited on solid substratum. In a first attempt to test whether the developmental defect in the *car1*$^{-}$/*car3*$^{-}$ cells is

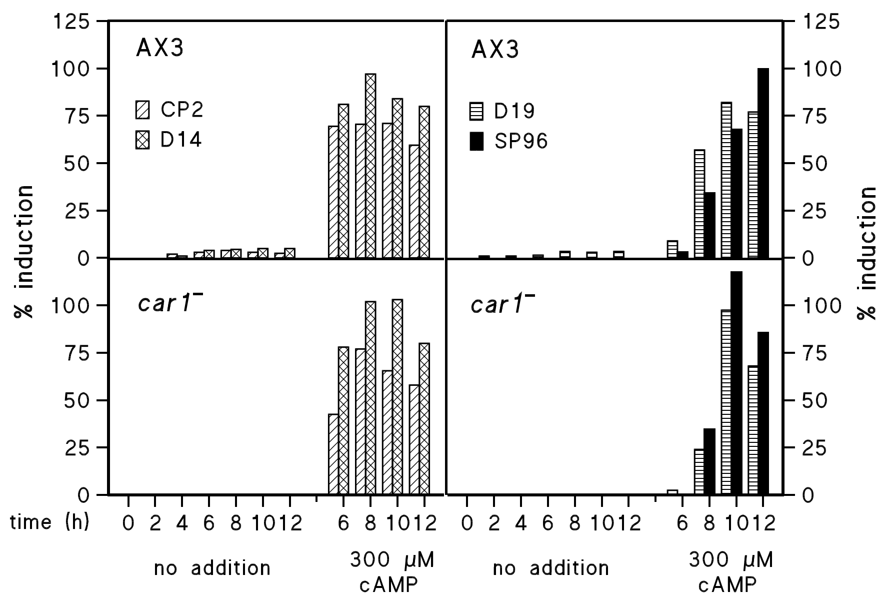

Fig. 3. Induction of postaggregative gene expression in *car1*$^{-}$ cells. Cells harvested in growth phase were first shaken in PB for 4 hours and subsequently incubated in the presence and absence of 300 μM cAMP, added every hour. mRNA was isolated at 2 hour intervals and northern transfers were probed with ^{32}P-labeled cDNAs of the prestalk-enriched *CP2* and *D14* genes and the prespore genes *D19* and *Sp96*. Means of optical density scans of two experiments are presented. Data are expressed as percentage of mRNA levels in AX3 cells stimulated for 4 hours (*CP2, D14*) or 8 hours (*D19, Sp96*) with 300 μM cAMP.

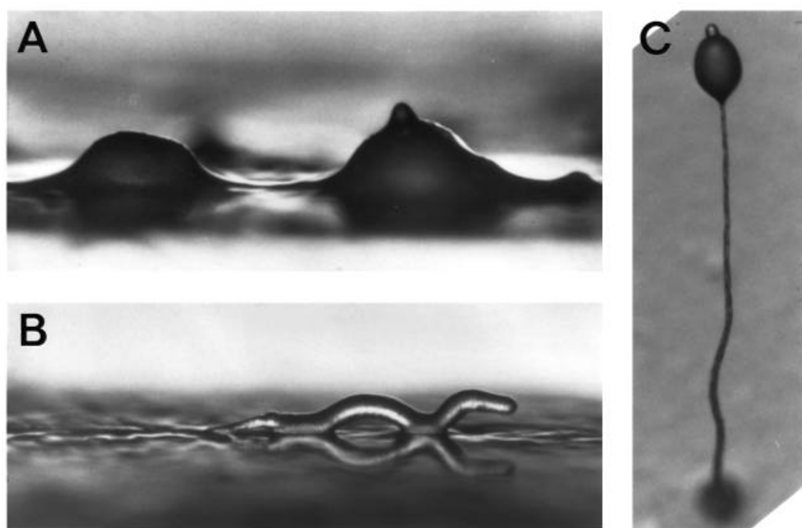

Fig. 4. Development of *car1⁻* cells after cAMPS stimulation. *car1⁻* cells were shaken in PB for 8 hours with 5 µM cAMPS. At this stage, cells had formed tight aggregates, which were deposited on non-nutrient agar. The aggregates sequentially formed tipped mounds (A), migrating slugs (B), and fruiting bodies (C) of normal size and morphology.

cell autonomous, we measured whether these cells can go through development in synergy with wild-type cells. 10^7 cells of either the G418 resistant control cell line pJK1:AX3, the *car1⁻* cell line or the *car1⁻/car3⁻* cell line were mixed with an equal amount of AX3 cells and allowed to develop. After 4 days, spore heads were picked into HL5 medium and serial dilutions were plated in duplicate. After allowing 2 days for spores to hatch, the medium in one duplicate set was replaced with medium supplemented with 20 µg/ml G418. 10 days later, after each spore had given rise to a colony of cells, the number of G418 resistant colonies was compared with the total number in the unselected plates. From the AX3-pJK1:AX3 mixture, 2400 out of 8700 (28%) spores were G418 resistant; out of 26000 spores from the *car1⁻*-AX3 mix, 250 spores (1%) were resistant and from the *car1⁻/car3⁻*-AX3 mix 0 out of 20000 spores were G418 resistant. The *car1⁻* cells already show very poor efficiency to synergize with wild-type cells as was also reported by Sun and Devreotes (1991), which is most likely due to their chemotactic defect. *car1⁻/car3⁻* cells cannot synergize at all, suggesting that they are completely insensitive to signals emitted by the wild-type cells.

We next measured the effects of cAMP on expression of aggregative, prestalk-enriched and prespore genes in *car1⁻/car3⁻* cells. Fig. 6 shows that neither pulses of 30, 100 or 300 nM cAMP nor high doses of 300 µM cAMP can increase expression of the aggregative csA gene in *car1⁻/car3⁻* cells. In both AX3 and *car1⁻/car3⁻* cells a low level of csA mRNA is detectable without cAMP stimulation, which is probably induced by CMF, an 80×10^3 M_r glycoprotein (Mann et al., 1988, 1989; Gomer et al., 1991).

Micromolar concentrations of cAMP effectively induce expression of the prespore gene *D19* and the prestalk-enriched gene *CP2* in AX3 cells, but no expression is observed in *car1⁻/car3⁻* cells (Fig. 7). These data show that disruption of both the *cAR1* and the *cAR3* genes completely obliterates cAMP-induced gene expression.

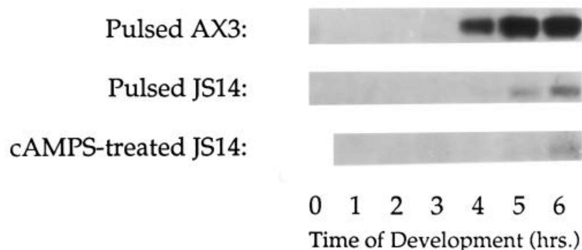

Fig. 5. Expression of *cAR3* in *car1⁻* cells. AX3 cells and *car1⁻* cell line JS14 were stimulated with either 300 nM cAMP pulses at 6 minute intervals or with a single dose of 5 µM cAMPS. Membrane proteins were isolated from cells at 60 minute intervals, size fractionated and probed with cAR3-specific antibodies.

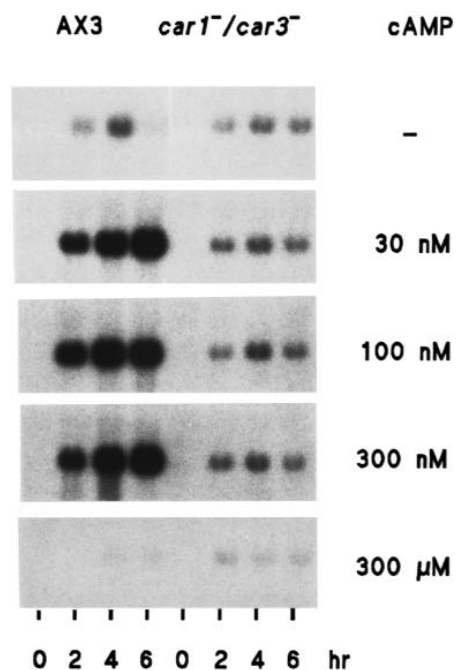

Fig. 6. Induction of aggregative gene expression in *car1⁻/car3⁻* cells. AX3 and *car1⁻/car3⁻* cells were challenged with different cAMP stimulation regimes as described in the legend of Fig. 2. RNA was isolated and probed with *csA* cDNA.

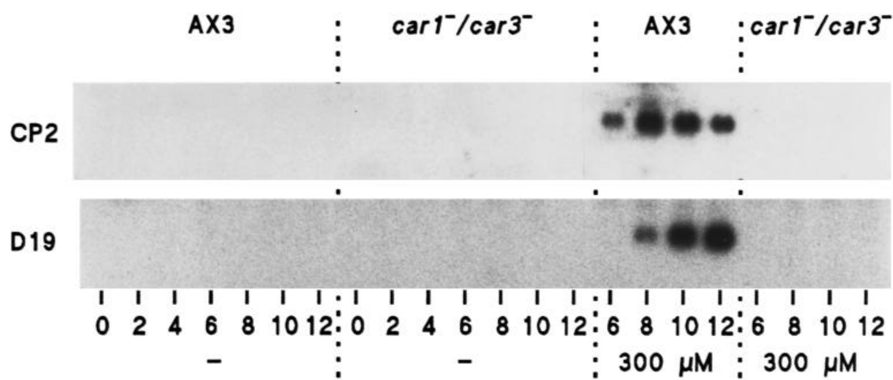

Fig. 7. Induction of postaggregative gene expression in *car1*⁻/*car3*⁻ cells. AX3 and *car1*⁻/*car3*⁻ cells were starved for 4 hours in PB and subsequently stimulated for 8 hours with 300 μM cAMP. Northern transfers were probed with *CP2* and *D19* cDNAs.

DISCUSSION

The surface cAMP receptor cAR1 has traditionally been associated with chemotaxis and chemotactic signaling. However, disruption of the *cAR1* gene blocks cell differentiation as well as aggregation. We investigated whether the developmental defect is due to the fact that cells cannot produce extracellular cAMP required for gene induction, or whether cAR1 itself mediates gene induction. A signifant outcome of the present study is that postaggregative gene expression and fruiting body formation can be completely restored by stimulating *car1*⁻ cells with exogenous cAMP.

car1⁻ cells cannot express aggregative genes in response to cAMP pulses of 30 nM, which is optimal for gene induction in wild-type cells. Pulses of 300 nM cAMP induce a low level of expression, but very remarkably, a continuous micromolar cAMP stimulus is highly effective to induce aggregative gene expression in *car1*⁻ cells, while inhibiting expression in wild-type cells. This strongly suggests that cAR1 mediates both activation and desensitization of pulse-induced gene expression. Apparently, in the absence of cAR1, a lower affinity cAR can restore the stimulatory but not the inhibitory component of this response. Similar results have been reported for adaptation of adenylylcyclase (ACA) activation (Pupillo et al., 1992). Whereas in wild-type cells a 5 minutes preincubation with 10 μM cAMP strongly reduces subsequent activation of ACA by GTPγS measured in lysates, preincubation with cAMP does not affect ACA activation in *car1*⁻ cells, suggesting that *car1*⁻ cells are defective in adaptation.

We investigated whether cAR3, the only presently known cAR that is expressed at the aggregation stage of development, has substituted for the function of cAR1. *car1*⁻/*car3*⁻ double mutants show no cAMP-induced gene expression, either to nanomolar cAMP pulses or to micromolar cAMP concentrations and cannot be induced to develop by prolonged stimulation with cAMP, as is the case for *car1*⁻ cells. Postaggregative genes also cannot be induced in *car1*⁻/*car3*⁻ cells, which would seem to suggest that either cAR may mediate this response and the double mutation has blocked transduction by both the primary and the substituting transducer. However, this cannot be stated with certainty, since postaggregative gene expression in general requires achievement of a state of differentiation competence, which may not be reached in the *car1*⁻/*car3*⁻ mutant. Involvement of cAR1 in postaggregative gene expression is unlikely, because the latter response requires high cAMP concentrations and is insensitive to adaptation.

The function of cAR3 in wild-type cells is thus far obscure; *car3*⁻ cells show no aberrant phenotype and display normal expression of developmentally regulated genes. This suggests that either cAR3 has no obvious function in gene regulation and signaling, or its functions can be completely replaced by other receptors. Curiously, also the other two cARs, cAR2 and cAR4, which are expressed later in development in the prestalk region of slugs and fruiting bodies, have no obvious function in the regulation of cAMP-induced prespore or prestalk-enriched genes. *cAR2* and *cAR4* null mutants appear to be defective in regulation of the DIF-induced prestalk genes and if anything, to overexpress prespore genes (Saxe et al., 1993). So at present, either none of the cloned cARs mediates prespore and prestalk-enriched gene expression, or groups of cAR subtypes show plasticity to replace each others function whenever required; single *cAR* gene disruptions may be insufficient to implicate functions of specific cARs, without careful analysis of the kinetic properties of the response.

We are grateful to Drs A. Noegel, J. G. Williams and W. F. Loomis for their kind gifts of *csA*, *CP2* and *SP96* DNA probes respectively, and to Dr R. L. Johnson for his gift of cAR3-specific antibody. This research was supported by GB-MW grant 900-546-077 from the Netherlands Organization for Scientific Research, by NIH grant GM34933 and by a SERC/NATO grant to R. H. I.

REFERENCES

Aeckerle, S., Wurster, B. and Malchow. D. (1985). Oscillations and cyclic AMP induced changes of the K^+ concentration in *Dictyostelium discoideum. EMBO J.* **4,** 39-43.

Barklis, E. and Lodish, H. F. (1983). Regulation of *Dictyostelium discoideum* mRNAs specific for prespore or prestalk cells. *Cell* **32,** 1139-1148.

Darmon, M., Brachet, P. and Pereira da Silva, L. H. (1975). Chemotactic signals induce cell differentiation in *Dictyostelium discoideum. Proc. Nat. Acad. Sci. USA* **72,** 3163-3166.

Dinauer, M. C., MacKay, S. A. and Devreotes, P. N. (1980). Cyclic 3′:5′-AMP relay in *Dictyostelium discoideum.* III. The relationship of cAMP synthesis and secretion during the cAMP signaling response. *J. Cell Biol.* **86,** 537-544.

Europe-Finner, G. N. and Newell, P. C. (1987). Cyclic AMP stimulates accumulation of inositol 1,4,5-trisphosphate in *Dictyostelium. J. Cell Sci.* **87,** 221-229.

Fosnaugh, K. L. and Loomis, W. F. (1989). Sequence of the *Dictyostelium discoideum* spore coat gene SP96. *Nucl. Acids Res.* **17,** 9489.

Gerisch, G., Fromm, H., Huesgen, A. and Wick, U. (1975). Control of cell-contact sites by cyclic AMP pulses in differentiating *Dictyostelium* cells. *Nature* **255,** 342-357.

Gomer, R. H., Yuen, I. S. and Firtel, R. A. (1991). A secreted 80×10^3 M_r

protein mediates sensing of cell density and the onset of development in *Dictyostelium. Development* **112,** 269-278.

Harwood, A. J. Hopper, N. A., Simon, M. -N., Driscoll, D. M., Véron, M. and J. J. Williams. (1992). Culmination in *Dictyostelium* is regulated by the cAMP-dependent protein kinase. *Cell* **69,** 615-624.

Hopper, N. N., Harwood, A. J., Bouzid, S., Véron, M. and Williams, J. G. (1993). Activation of the prespore and spore cell pathway of *Dictyostelium* differentiation by cAMP dependent protein kinase and evidence for its upstream regulation by ammonia. *EMBO J.* **12,** 2459-2466.

Insall, R. H., Soede, R. D. M., Schaap, P. and Devreotes, P. N. (1994). Two cAMP receptors activate common signalling pathways in *Dictyostelium. Mol. Biol. Cell* (in press).

Kay, R. R. (1982). cAMP and spore differentiation in *Dictyostelium discoideum. Proc. Nat. Acad. Sci. USA* **79,** 3228-3231.

Klein, P. S., Sun, J., Saxe III, C. L., Kimmel, A. R., Johnson, R. L. and Devreotes, P. N. (1988). A chemoattractant receptor controls development in *Dictyostelium discoideum. Science* **241,** 1467-1472.

Malchow, D., Nanjundiah, V. Wurster, B. Eckstein, F. and Gerisch, G. (1978). Cyclic AMP-induced pH changes in *Dictyostelium discoideum* and their control by calcium. *Biochim. Biophys. Acta* **538,** 473-480.

Mann, S. K. O. and Firtel, R. A. (1989). Two-phase regulatory pathways controls cAMP receptor-mediated expression of early genes in *Dictyostelium. Proc. Nat. Acad. Sci. USA* **4,** 2348-2351.

Mann, S. K. O., Pinko, C. and Firtel, R. A. (1988). cAMP regulation of early gene expression in signal transduction mutants of *Dictyostelium. Dev. Biol.* **130,** 294-303.

Mann, S. K. O., Yonemoto, W. M., Taylor, S. S. and Firtel, R. A. (1992). DdPK3, which plays essential roles during *Dictyostelium* development, encodes the catalytic subunit of cAMP-dependent protein kinase. *Proc. Nat. Acad. Sci. USA* **89,** 10701-10705.

Mato, J. M., Krens, F. A., Van Haastert, P. J. M. and Konijn, T. M. (1977). 3′:5′-Cyclic AMP-dependent 3′:5′-cyclic GMP accumulation in *Dictyostelium discoideum. Proc. Nat. Acad. Sci. USA* **74,** 2348-2351.

Mehdy, M. C., Ratner, D. and Firtel, R. A. (1983). Induction and modulation of cell type specific gene expression in *Dictyostelium. Cell* **32,** 763-771.

Milne, J. L. and Coukell, M. B. (1991). A Ca^{2+} transport system associated with the plasma membrane of *Dictyostelium discoideum* is activated by different chemoattractant receptors. *J. Cell. Biol.* **112,** 103-110.

Nellen, W., Datta, S., Reymond, C., Sivertsen, A., Mann, S., Crowley, T. and Firtel, R. A. (1987). Molecular biology in *Dictyostelium*: tools and applications. *Methods Cell Biol.* **28,** 67-100.

Oyama, M. and Blumberg, D. D. (1986). Interaction of cAMP with the cell-surface receptor induces cell-type specific mRNA accumulation in *Dictyostelium discoideum. Proc. Nat. Acad. Sci. USA* **83,** 4819-4823.

Pears, C. J., Mahbubani, H. M. and Williams, J. G. (1985). Characterization of two highly diverged but developmentally co-regulated cysteine proteinase genes in *Dictyostelium discoideum. Nucl. Acids Res.* **13,** 8853-8866.

Pitt, G. S., Milona, N., Borleis, J., Lin, K. C., Reed, R. R. and Devreotes, P. N. (1992). Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. *Cell* **69,** 305-315.

Pupillo, M., Insall, R., Pitt, G. S. and Devreotes, P. N. (1992). Multiple cyclic AMP receptors are linked to adenylyl cyclase in *Dictyostelium. Mol. Biol. Cell* **3,** 1229-1234.

Roos, W., Nanjundiah, V., Malchow, D. and Gerisch, G. (1975). Amplification of cyclic-AMP signals in aggregating cells of *Dictyostelium discoideum. FEBS Lett.* **53,** 139-142.

Saxe III, C. L., Ginsburg, G. T., Louis, J. M., Johnson, R., Devreotes, P. N. and Kimmel, A. R. (1993). CAR2, a prestalk cAMP receptor required for normal tip formation and late development of *Dictyostelium discoideum. Genes Dev.* **7,** 262-272.

Saxe III, C. L., Johnson, R. L., Devreotes, P. N. and Kimmel, A. R. (1991). Expression of a cAMP receptor gene of *Dictyostelium* and evidence for a multigene family. *Genes Dev.* **5,** 1-8.

Saxe III, C. L. (1991). Multiple cell-surface, receptor genes in *Dictyostelium. Dev. Genet.* **12,** 6-13.

Schaap, P. and Van Driel, R. (1985). Induction of post-aggregative differentiation in *Dictyostelium discoideum* by cAMP. Evidence of involvement of the cell surface cAMP receptor. *Exp. Cell Res.* **159,** 388-398.

Schaap, P., Van Ments-Cohen, M., Soede, R. D. M., Brandt, R., Firtel, R. A., Dostmann, W., Genieser, H.-G., Jastorff, B. and Van Haastert, P. J. M. (1993). Cell-permeable non-hydrolysable cAMP derivatives as tools for analysis of signaling pathways controlling gene regulation in *Dictyostelium. J. Biol. Chem.* **268,** 6323-6331.

Sun, T. J. and Devreotes, P. N. (1991). Gene targeting of the aggregation stage cAMP receptor cAR1 in *Dictyostelium. Genes Dev.* **5,** 572-582.

Sun, T. J., Van Haastert, P. J. M. and Devreotes, P. N. (1990). Surface cAMP receptors mediate multiple responses during development in *Dictyostelium*: Evidence by antisense mutagenesis. *J. Cell Biol.* **110,** 1549-1554.

Van Haastert, P. J. M., De Vries, M. J., Penning, L. C., Roovers, E., Van der Kaay, J., Erneux, C. and Van Lookeren Campagne, M. M. (1989). Chemoattractant and guanosine 5′-[γ-thio]triphosphate induce the accumulation of inositol 1,4,5-trisphosphate in *Dictyostelium* cells that are labelled with $[^3H]$inositol by electroporation. *Biochem. J.* **258,** 577-586.

Van Haastert, P. J. M. and Kien, E. (1983). Binding of cAMP derivatives to *Dictyostelium discoideum* cells. Activation mechanism of the cell surface cAMP receptor. *J. Biol. Chem.* **258,** 9636-9642.

Watts, D. J. and Ashworth, J. M. (1970). Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *Biochem. J.* **119,** 171-174.

Würster, B., Schubiger, K., Wick, U. and Gerisch. G. (1977). Cyclic GMP in *Dictyostelium discoideum*: oscillations and pulses in response to folic acid and cyclic AMP signals. *FEBS Lett.* **76,** 141-144.

(Accepted 18 April 1994)