cAMP-dependent protein kinase activity is essential for preaggregative gene expression in *Dictyostelium*

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Abstract Constitutive inhibition of cAMP-dependent protein kinase (PKA) in *Dictyostelium* cells blocks cell aggregation and development. We investigated the cause of the aggregation defect in transformants overexpressing dominant-negative PKA regulatory subunits (PKA-R_M) under an actin 15 promoter. These mutants could not relay pulses of the chemoattractant cAMP, due to a defect in expression of the aggregative adenylyl cyclase (ACA) gene. Unstimulated and cAMP pulse-induced expression of other aggregative genes encoding the cAMP receptor cAR1, adhesive contact sites A and cAMP-phosphodiesterase were also strongly reduced in the mutants. Additionally, the expression of the *discoidin I* gene, that is expressed early in development in response to cell density sensing factors, was almost completely absent. These data are in interesting contrast with observations that cAMP relay and aggregative gene expression are normal in null mutants for the PKA catalytic (C) subunit and suggest the presence of multiple C subunit genes in *Dictyostelium* and an almost universal requirement for PKA activity in developmental gene expression.

Key words: Adenylyl cyclase A; cAMP relay; cAMP-dependent protein kinase; Aggregative gene expression; *Dictyostelium discoideum*

1. Introduction

During *Dictyostelium* development, extracellular cAMP acts as a chemoattractant coordinating cell aggregation and multicellular morphogenesis, and as a hormone-like signal controlling the expression of almost all classes of developmentally regulated genes. cAMP is autonomously secreted in an pulsatile manner by a few cells in a starving cell population and pulses are propagated by a mechanism of signal relay. An autocatalytic feedback system consisting of an adenylyl cyclase (ACA), that is regulated by stimulatory and inhibitory G-proteins coupled to surface cAMP receptors (cARs) is responsible for signal initiation and relay. cARs also mediate the effects of cAMP on chemotaxis and gene regulation [see 1].

Phenotypical abberations resulting from manipulation of genes encoding the regulatory (R) and catalytic (C) subunits of cAMP-dependent protein kinase (PKA) suggests that intracellular cAMP plays an almost equally ubiquitous role as extracellular cAMP. Inhibition of PKA by either overexpression of dominant-negative R subunits lacking cAMP binding sites (R_M), or by disruption of the catalytic subunit (C) gene blocks cell aggregation and development [2–5]. When R_M is overexpressed under either prestalk or prespore promoters, cells do not differentiate into stalk and spore cells respectively [6,7]. Conversely, constitutive activation of PKA either by overexpression of C subunits or by disruption of the R subunit gene, accelerates cell aggregation and induces precocious spore differentiation [8,9]. Stalk cell differentiation is also prematurely induced, but only when extracellular cAMP is depleted ([10], unpub. data).

The cause of the block in stalk and spore differentiation by inhibition of PKA is obvious; here the transcription of prestalk and prespore specific genes is either completely absent or strongly reduced, or the transcripts are unstable [6,10,11]. It is less evident why PKA inhibition early in development should block the aggregation process and results obtained by different workers seem sometimes contradictory. PKA-C null mutants show normal aggregative gene expression on solid substratum and stimulation of gene expression by cAMP pulses. The cells aggregate in synergy with wild-type cells and relay cAMP signals normally [3]. Mutants overexpressing PKA-R_M cannot relay cAMP signals, but show normal chemotaxis and aggregation in synergy with wild-type cells [5].

In this study we investigated the cause of the aggregation defect and the discrepancy between the different sets of data. Using a new series of transformants, we confirm the data of Harwood et al. [5] that overexpression of R_M blocks the cAMP relay response. This block was not caused by any direct effect on the response itself, but to the fact that the *ACA* gene was not transcribed. Transcription of other aggregative genes and of the early *discoidin I* gene was also strongly reduced, suggesting that PKA activity is required for the earliest gene regulatory events in *Dictyostelium* development.

2. Materials and methods

2.1. Cell lines and culture conditions

D. discoideum strain AX3 was transformed by calcium-phosphate precipitation with vector pA15R_M which harbours the actin6-NEO^R selection cassette [12] and a gene fusion of the actin15 promoter directing expression of PKA-R subunit gene in which both cAMP binding sites where inactivated by site-directed mutagenesis yielding the mutant R_M. Control cell lines (R_C) were transformed with vector pA15R_C which carries an additional mutation, that prevents interaction with the PKA-C subunit [5,6]. Transformants were selected for growth in the presence of 100 μg/ml G418 in axenic medium. For each transformation 24 individual clones were selected. All R_M clones could not aggregate, whereas all R_C clones developed normally into fruiting structures.

2.2. RNA isolation and analysis

Total RNA was isolated from 2×10^7 cells, purified by phenol/chloroform extraction and two ethanol and one LiCl precipitation step [13].

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Abbreviations: ACA, adenylylcyclase A; cAR, cAMP receptor; csA, contact site A; CMF, conditioned medium factor; PDE, cAMP-phosphodiesterase; PKA, cAMP dependent protein kinase; PKA-R, PKA regulatory subunit; PKA-C, PKA catalytic subunit; PSF, prestarvation factor; 2'HcAMP, 2'deoxy-3'5'adenosine monophosphate.

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RNA was then size fractionated on 1.5% agarose gels containing 2.2 M formaldehyde, transferred to Gene Screen membranes, and hybridized under conditions of high stringency to [α-^{32}P]dATP-labeled DNA probes according to standard procedures.

2.3. cAMP relay

Cells were harvested from growth medium and starved for 6 h on non-nutrient agar. Cells were resuspended in 10 mM Na/K phosphate buffer, pH 6.5 (PB) to 10^8 cells/ml and stimulated with 5 μM 2'deoxy-3'5'adenosine monophosphate (2'HcAMP) in the presence of 5 mM of the PDE inhibitor dithiothreitol. Reactions were terminated after 0 and 5 min of stimulation and cAMP levels were assayed by isotope dilution assay [14].

3. Results and discussion

Our initial objective was to elucidate the role of PKA in the activation of ACA during the cAMP relay response. AX3 cells were transformed with vectors containing gene fusions of the constitutively active actin15 promoter with either the dominant-negative PKA-R_M subunit or the control PKA-R_C subunit. We first measured cAMP accumulation induced by the cAR agonist 2'HcAMP in starved cells derived from 7 independent clonal isolates from each transformation. Fig. 1 shows that in all clones transformed with the control R_C vector, basal cAMP levels are elevated compared to clones transformed with R_M. The R_C cell lines furthermore show a considerable increase in cAMP levels after 5 min of stimulation. In all R_M cell lines, basal cAMP levels were very low and there is only a minor increase after stimulation with 2'HcAMP.

We tested whether the defect in cAMP relay was due to reduced expression of ACA and other early and aggregative genes. mRNA was isolated during the first 8 h of starvation of representative R_M and R_C clones and hybridized to an *ACA* DNA probe [15] and to DNA probes for a number of other genes expressed after the onset of starvation, such as the early gene *discoidin I*, that is induced by cell density sensing factors and repressed by cAMP [16–18], and the aggregative genes encoding cAMP-phosphodiesterase (*PDE*) [19], adhesive contact sites A (*csA*) [20] and the cAMP receptor *cAR1* [21]. The aggregative genes are expressed at a low level in response to cell density sensing factors and to a high level by stimulation with cAMP pulses [22]. Fig. 2 shows that in the control R_C line *ACA, cAR1* and *csA* are expressed to a low level and *PDE* to a moderate level during 8 h of starvation in buffer. Both *csA* and *cAR1* transcription increase strongly upon stimulation with 30 nM cAMP pulses; *ACA* and *PDE* transcription are only about 2-fold stimulated. Neither of these genes is expressed during starvation of R_M cells and cAMP pulses can only restore a very low level of *csA, cAR1* and *PDE* expression. The early gene *discoidin I* is also strongly affected by PKA inhibition. In control R_C cells, *discoidin I* mRNA levels increase during starvation in buffer and are repressed by cAMP pulses. In R_M cells, induction of *discoidin I* expression is almost absent; cAMP pulses seem to reduce the low levels of expression even further.

The most important conclusion that can be drawn from these observations is that inhibition of PKA by overexpression of R_M during early development blocks the transcription of the *discoidin I* gene and of all aggregative genes that were tested. This sufficiently explains why cells cannot aggregate and relay cAMP signals. Remarkably, the expression of almost the first gene to be transcribed upon starvation is already affected. This gene, *discoidin I*, is induced in response to the factors PSF

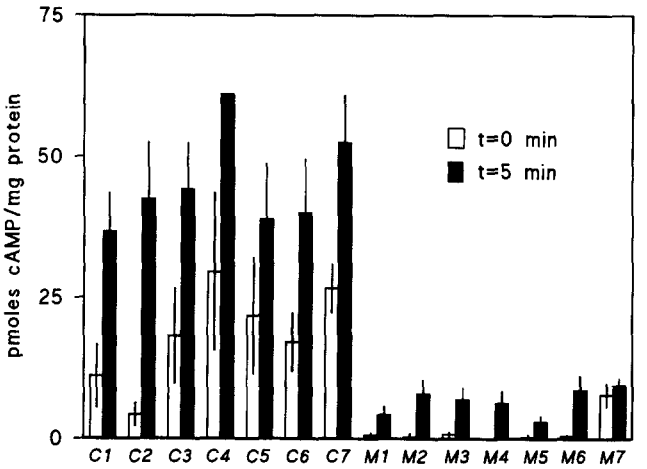

Fig. 1. cAMP relay in PKA-R_C and PKA-R_M transformants. Seven independent clonal isolates from AX3 cells transformed of with either the dominant-negative actin15-R_M (M1 to M7) or the control actin15-R_C (C1 to C7) constructs were starved for 6 h on non-nutrient agar, resuspended in PB to 10^8 cells/ml and stimulated for 0 (open bars) or 5 min (closed bars) with 5 μM 2'HcAMP in the presence of 5 mM DTT. Total cAMP levels were measured by isotope dilution assay and standardized on the protein content of the cell suspension. Means and S.D. of 2 experiments performed in triplicate are presented.

(prestarvation factor) and CMF (conditioned medium factor) that cells secrete during growth and early starvation respectively, and are used to sense whether the cell density is sufficient to proceed with aggregation [17,18]. Possibly PKA activation is part of the sensory transduction mechanism of these signals. CMF and/or PSF are also required for basal expression of aggregative genes as *cAR1, csA* and *PDE* [22,23]. Since basal expression of these genes is completely absent in R_M cells, while a low level of cAMP-pulse induced transcription is retained; it seems that also here PKA may be required for basal induction by CMF or PSF, rather than for pulse-induced expression. This is supported by findings that pulse-induced gene expression is normal in ACA null mutants [24]. PSF has not yet been characterized, but CMF encodes an 80 kDA glycoprotein. Expression of CMF antisense mRNA blocks aggregation, indicating that this signal is essential for the initiation of development [25].

Our results are in interesting contrast to results obtained with mutants harbouring a *PKA-C* gene disruption. These mutants, though blocked in development, show normal pulse-induced gene expression and cAMP relay [3]. This discrepancy suggests the possibility of two *PKA-C* genes. Overexpression of PKA-R_M would inactivate the proteins from both genes and its effect on development could therefore be more severe. There is some evidence for the existence of multiple *PKA-C* genes. The PKA catalytic subunit was purified to homogeneity by Veron and coworkers and was of similar size as mammalian PKA-C subunits with a molecular weight of 40 kDa [26]. The cloned *PKA-C* gene translates into a protein of 73 kDa, which is not sensitive to proteolytic cleavage [27]. Furthermore the cloned gene is equally expressed during the entire course of development [3,28], whereas expression of the 40 kDa protein increased sharply after aggregation [29]. The purified C-subunit showed rather unusual sensitivity to regulation by pH [26]; it would be of interest to determine whether the cloned PKA-C shows the

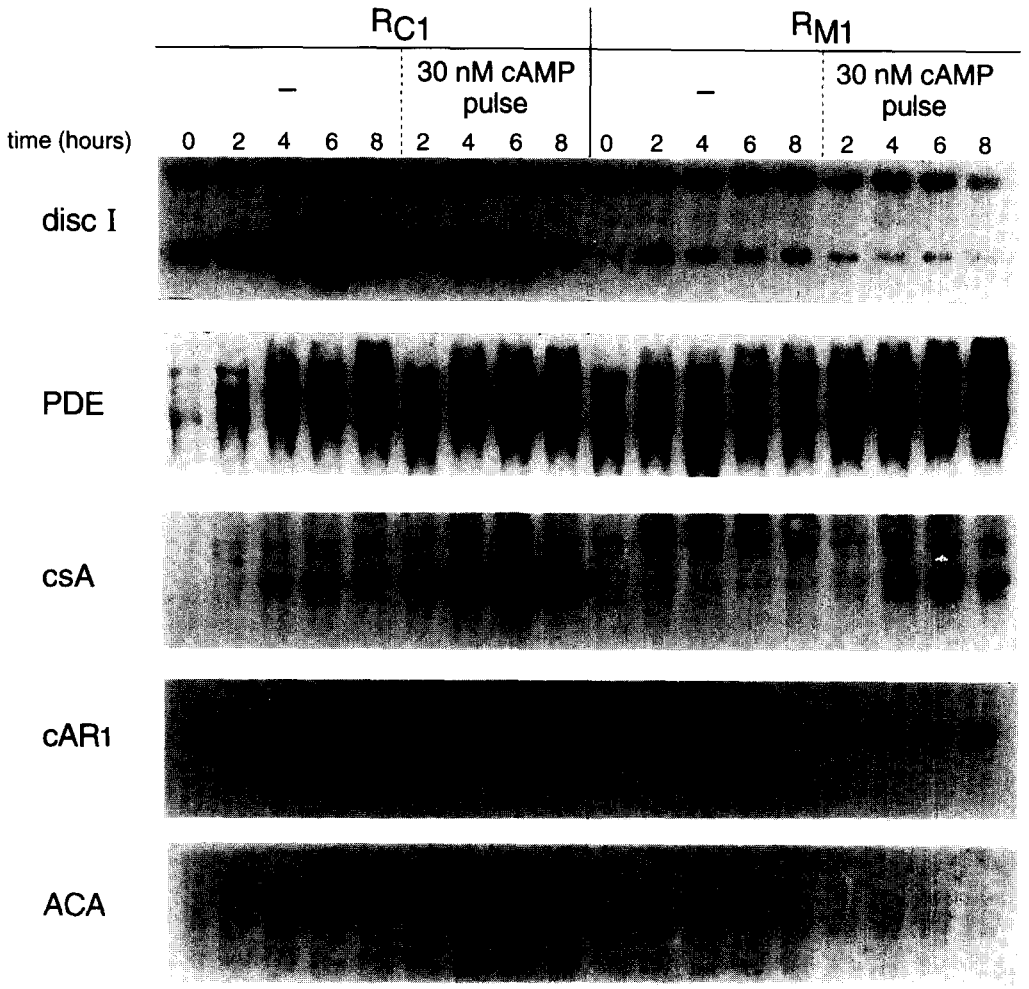

Fig. 2. Preaggregative gene expression in PKA-R_C and PKA-R_M transformants. Clonal isolates PKA-R_{M1} and PKA-R_{C1} were harvested during exponential growth, resuspended in PB to 10^7 cells/ml and shaken at 150 rpm and 22°C in the presence and absence of 30 nM cAMP pulses delivered at 6 min intervals. mRNA was isolated after 0, 2, 4, 6 and 8 h of incubation and Northern transfers were probed with ^{32}P-labeled *discoidin I* (*discI*), *PDE, csA, cAR1* and *ACA* DNA probes. The experiment was repeated with clonal isolates PKA-R_{M2} and PKA-R_{C5} with identical results.

same biochemical properties and if not, to intensify the search for a second PKA-C gene.

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