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Overexpression of *Dd* PK2 protein kinase causes rapid development and affects the intracellular cAMP pathway of *Dictyostelium discoideum*

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Summary

The *Dd* PK2 gene codes for a putative protein of 648 amino acids with a C-terminal half sharing high homology with protein kinase A catalytic subunits from other organisms. In order to find out more about the physiological role of the *Dd* PK2 kinase, its gene, and a version having a frame shift mutation in the middle of the catalytic region, were overexpressed in developing *Dictyostelium* cells. Both the intact gene (K-) and the frame shift mutant (Kdel-) caused rapid development with spores formed in 16-18 hours compared to the 24 hours required by their parent. This result was confirmed by the pattern of expression of some developmentally regulated genes. Other rapid developing strains (*rde*) are activated in the cAMP second messenger system. Both K- and Kdel-containing strains have lower cAMP levels than the parental strain during late development, thus resembling *rde*C mutants. K-cells (but not Kdel-cells) produced bizarre fruiting bodies with many prostrate forms. The parallel with *rde* mutants was confirmed by demonstrating that K-cells are able to form spores in submerged monolayer culture. Furthermore, K-cells have about four times more protein kinase A (cAPK) activity than wild-type cells. These results indicate that the N-terminal domain of *Dd* PK2 is sufficient to influence cAMP levels and to provoke rapid development, whereas kinase activity seems to be required for the sporogenous phenotype. The association between elevated cAPK and *Dd* PK2 overexpression phenotype further indicates a role for cAPK in the formation of spores.

Key words: protein kinase, Dictyostelium, cAMP, development.

Introduction

cAMP has two distinct roles during *Dictyostelium discoideum* development. Its extracellular role as the chemoattractant during aggregation is well understood: cAMP is released by cells, detected by surface receptors linked to G proteins and then evokes three principal responses from the cells: chemotaxis towards cAMP, release of more cAMP and induction of the expression of a number of developmental genes (Sonneborn et al., 1963; Town et al., 1976; Sampson, 1977; Kessin, 1977).

An intracellular role for cAMP in development as a second messenger has also been suspected, principally because the cAMP-dependent protein kinase (cAPK) is developmentally regulated (Sampson, 1977; Rutherford et al., 1982; de Gunzburg et al., 1986). In *Dictyostelium*, this enzyme consists of single catalytic (C) and regulatory subunits (R). The R-subunit has been cloned and shown to be highly homologous to that from higher organisms (Mutzel et al., 1987), whereas the C-subunit is only known as a biochemical activity. Overexpression of the R-subunit, or of a mutant R-subunit unable to bind cAMP (effectively a dominant negative mutant of cAPK) blocks aggregation, indicating a role for cAPK in early development (Simon et al., 1989; Firtel and Chapman, 1990). A role for intracellular cAMP in spore maturation has been suggested by several lines of evidence. Addition of 8-bromo-cAMP (a cell-permeable analogue of cAMP) to submerged amoebae causes them to differentiate into spores, whereas cAMP itself only causes them to become prespores (Kay, 1989). A number of mutants able to form spores without 8-bromo-cAMP have been isolated (Sonneborn et al., 1963; Town et al., 1976; Kessin, 1977; Abe and Yanagisawa, 1983). These sporogenous and rapidly developing (*rde*) mutants develop rapidly and all form spores prematurely in development, so that the final “fruit” varies from a mound of stalk and spore cells (*rde*C mutants) to a stump with spores at the base (*rde*A mutants). Recently, the *rde*C locus has been shown to encode the R-subunit of cAPK (Simon et al., 1992). Since elimination of the R-subunit in the *rde*C mutants is expected to yield a constitutively active C-subunit, this result provides further strong evidence that activation of cAPK induces spore maturation. Finally, expression of an R-subunit of cAPK with mutated cAMP-binding sites driven by a prespore-specific promoter blocks the maturation of prespores into spores (Hopper, N., Harwood, A., Veron, M., Bouzid, S. and Williams, J.G., personal

communication). These results indicate a central role for cAPK during cell differentiation in *Dictyostelium.*

We previously isolated a gene from *Dictyostelium discoideum* (*Dd* PK2) encoding a putative protein kinase of 648 amino acids whose C-terminal half is highly homologous (54% sequence identity) to the C-subunit of mammalian and yeast cAPKs (Bürki et al., 1991). Despite this homology, *Dd* PK2 seems unlikely to be the C-subunit of cAPK because the putative protein is too large and has an N-terminal domain with no homology to the C-subunit of cAPK. The *Dd* PK2 mRNA increases during development (Bürki et al., 1991) and an essential role for the protein in development is indicated by the lack of aggregate formation of a null mutant (D*dpk*3$^-$ in Mann and Firtel, 1991). To try to understand the function of *Dd* PK2, we overexpressed the complete protein or a truncated version with half the catalytic domain in *Dictyostelium.* Our results indicate a link between *Dd* PK2, rapid development and spore formation.

Materials and methods

Plasmid construction

An *Xba*I cassette containing the actin 15 promotor/Tn903/actin 15 terminal part of pDNeo2 (Witke et al., 1987) was ligated into *Xba*I linearized pGEM3 DNA (Promega) containing the 6 kb genomic fragment coding for *Dd* PK2 (Bürki et al., 1991). The resulting construct named K-Neo was linearized with *Kpn*I, blunt ended with T4 polymerase (Boehringer-Mannheim) in the presence of the four dNTPs (Sambrook et al., 1989) and recircularized with T4 DNA ligase (Boehringer-Mannheim). The resulting plasmid was named Kdel-Neo (Fig. 1).

Dictyostelium *transformation and culture*

K-Neo and Kdel-Neo plasmids were electroporated (0.67 kV, 3 mF in 0.2 cm cuvette) into AX2 in sterile water. Cells were plated on tissue culture plates (Falcon 25020) with 10 ml of HL5 broth (Ashworth and Watts, 1970). After 24 hours, selection was applied at 10 μg/ml of G418 (Gibco BRL) for 48 hours, followed by repetitive media changes (every 2 to 3 days) keeping a constant level of 5 μg/ml of G418. After 10-14 days, *Dictyostelium* clones were removed from plates with cloning rings and the rest of the cells harvested as a population. Once the cells were growing under shaking conditions (160 revs/minute) at 22°C, the G418 concentration was progressively raised to 50 μg/ml.

For filter development, cells were grown up to 3-6 $\times 10^6$/ml and harvested by centrifugation at 1000 ***g***. After resuspension in the same volume of PDF buffer (20 mM KCl, 1.2 mM $MgSO_4$, 6.7 mM K_2HPO_4, 13.3 mM KH_2PO_4), the cells were centrifuged again and resuspended at 10^8/ml in PDF. 100 μl of cell suspension was deposited on 25 mm nitrocellulose filters (Schleicher and Schuell AG) on a pad (Whatman no. 1003125) saturated with PDF containing 500 μg/ml streptomycin and incubated at 22°C in a humid chamber for the indicated times.

The sporogenous phenotype was detected as described previously (Kay, 1989). Briefly, cells were incubated at 5×10^5/ml in a 5 cm tissue culture dish (Sterilin) with 2 ml of 10 mM MES, 20 mM NaCl, 20 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 200 μg/ml streptomycin, 15 μg/ml tetracyclin, 5 μg/ml discadenine, with 5 mM cAMP, with or without 20 mM 8-Br-cAMP. Differentiation was scored by phase microscopy after 24 hours of incubation, and each condition was done in duplicate.

RNA analysis

Filters supporting developing cells were lysed in Eppendorf tubes by vortexing with 50 mM Tris pH 8.5, 0.1% SDS, 1 mM ribonucleotide vanadyl complex (Sigma). After removal of the filter, the cell lysate was extracted six times with phenol/chloroform (1:1 with 8-hydroxy-quinoline) and the RNA was ethanol-precipitated twice. 20 μg of RNA was loaded per lane on 1.2% agarose gels in 20 mM MES, pH 6.5 (Fluka) as described (Reymond et al., 1984), transferred under vacuum (Moretronic) to Genescreen membrane (NEN) and UV-crosslinked for 5 minutes. Blots were hybridised in Rapid Hybridisation Buffer (Amersham) with either random labelled (Boehringer-Mannheim kit) *Hin*dIII-*Kpn*I fragment of *Dd* PK2 or probes derived from CP2, 2H3, discoidin I and actin 6.

cAMP and cAPK measurements

For cAMP measurements, developing cells were harvested as

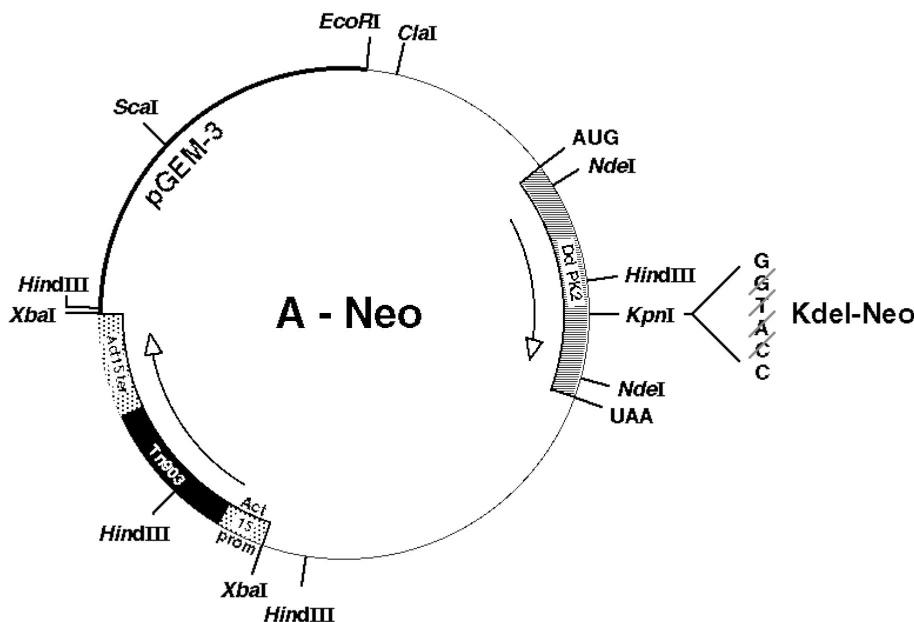

Fig. 1. Map of plasmid constructs K-Neo and Kdel-Neo. Arrows indicate direction and extent of transcription of the neo^R gene (Tn903) and *Dd* PK2. All indicated restriction sites were tested. The modification of the *Kpn*I site (Kdel-Neo construct) leading to premature stop of the *Dd* PK2 protein is shown on the right. Crossed-out bases were deleted by T4 DNA polymerase treatment.

described for RNA analysis, except that ice-cold PDF was used to collect and rinse cells. The cells were then lysed in 6% TCA (Merck) by three freeze/thaw cycles. TCA was extracted six times with water-saturated ether before cAMP was assayed by binding of [^{3}H]cyclic AMP to bovine muscle protein (kit from Amersham).

cAPK activities were determined using the Protein Kinase A Assay System from Gibco BRL. Essentially, 2×10^7 vegetative cells of the different strains were centrifuged at 1000 ***g*** for 5 minutes and resuspended in 200 µl of extraction buffer containing protease inhibitors (100-fold concentrated mix contains: 0.5 M benzamidine, 10^4 units/ml aprotinin, 5 mg/ml trypsin inhibitor, 1 mg/ml leupeptin, 2 mg/ml antipain, 100 mM TLCK). The cells were lysed by freezing in dry ice and rapid thawing at 20°C (water bath). After centrifugation at 10000 g for 2 minutes at 4°C, cAPK activity was measured at 20°C in 10 µl of each *Dictyostelium* extract as phosphorylation of Kemptide substrate (in the presence of 0.1 mM cAMP) which can be inhibited by PKI inhibitor, according to the instructions of the Kit. The linearity of the reaction was verified by using twofold dilutions of the same extracts.

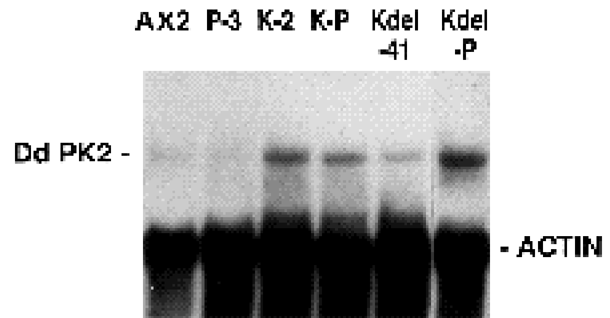

Fig. 2. Levels of *Dd* PK2 RNA in the indicated strains after 6 hours of starvation. Northern blotted RNAs were hybridized with a labelled *Hin*dIII-*Kpn*I fragment from *Dd* PK2 together with 10-fold less actin 6 probe. AX2, wild-type parental strain; P-3, cells containing pDNeo 2 (Witke et al., 1987); populations of cells containing K-Neo and Kdel-Neo are called K-P and Kdel-P respectively; K-2 and Kdel-41 are single clones.

Results

In order to obtain overexpression of *Dd* PK2, we ligated a neo^R cassette (Knecht et al., 1986) into the *Dd* PK2 genomic clone A7 (Bürki et al., 1991) in pGEM3 (Fig. 1). The genomic DNA fragment of about 6 kb, present in clone A7, contains the complete *Dd* PK2 coding region in its center and thus probably contains promoter and termination sequences as well. The unique K-Neo clone obtained showed an internal deletion of about 800 bp located 170 nucleotides upstream of the putative AUG start codon. This deletion within the promoter region does not seem, however, to prevent overexpression of the RNA (see below). We deleted the *Kpn*I internal site of *Dd* PK2 (Fig. 1). The resulting construct, Kdel-Neo, has a frame-shift mutation resulting in premature termination in the middle of the putative catalytic C-terminal domain (loss of the last 151 amino acids). The encoded protein contains the complete N-terminal half of *Dd* PK2 as well as the phosphate anchor site (Knighton et al., 1991), but lacks peptide-binding sites and catalytic loop. The presence of the introduced mutation was verified by the disappearance of the *Kpn*I site (data not shown).

K-Neo and Kdel-Neo DNAs were electroporated into *Dictyostelium discoideum* (Howard et al., 1988). We isolated a few individual clones (e.g. K-2 and Kdel-41) from the 40 to 50 obtained and kept the rest of the cells together as populations (K-P, Kdel-P). The pDNeo 2 (Witke et al., 1987) vector containing only the neo^R cassette was electroporated in parallel into *Dictyostelium* as control and called P-3.

The presence of 20 to 100 copies of the constructs in *Dictyostelium* cells was shown by hybridization of genomic blots with a *Dd* PK2 probe (data not shown). RNA was isolated from cells starved for 6 hours, since at that time *Dd* PK2 is fully expressed (Bürki et al., 1991) and analysed by hybridization with a *Hin*dIII-*Kpn*I probe from *Dd* PK2 (Fig. 2). Scanning the autoradiogram indicated a 3- to 18-fold overexpression of *Dd* PK2 in K-Neo- and Kdel-Neo-containing cells (K- and Kdel-cells respectively), as compared to P-3 control cells. An actin probe was included in the hybridizations to allow standardization of RNA levels (Fig. 2).

We then observed development of K- and Kdel-cells. Both formed fruits after 16-18 hours on filters, whereas either P-3 or the parental strain AX2 required about 24 hours. The timing of aggregation was unchanged but development was accelerated thereafter. In addition K-cells had broader stalks resulting in the formation of shorter fruits on filters (Fig. 3). When plated at high density on agar, K-cells had even shorter fruits with enlarged stalks and some prostrate forms. Kdel-cells produced relatively short fruiting bodies but otherwise resembled AX2 wild-type (Fig. 3). No difference was observed between population of cells (K-P, Kdel-P) and the single clones (K-2, Kdel-41), indicating that the observed phenotype was due to the reintroduced DNA and not to a peculiarity of clonal isolates (data not shown).

In a further construct, we removed most of the *Dd* PK2 coding region by deleting the internal *Nde*I fragment (Fig. 1). After electroporation into AX2 cells, the integrity of vector sequences was verified by genomic Southern blot hybridization (data not shown). Such *Dictyostelium* cells showed wild-type development, strongly suggesting that rapid development results from overexpression of either complete or truncated *Dd* PK2 protein and not from any other sequence present on the vector (data not shown).

In order to analyse the pattern of gene expression during development, cells were starved on filters and RNA was extracted at time intervals (Fig. 4). *Dd* PK2 RNA is overexpressed throughout development with a relatively high level between 6 and 10 hours both in K- and K-del cells. We do not observe the shift in RNA size between 3 and 6 hours as in wild-type AX2 (Bürki et al., 1991), probably because of the internal 800 bp deletion within the upstream region in both K- and Kdel-Neo constructs. The presence of early and prespore genes was detected by hybridization with discoidin I and 2H3 probes, respectively. Even though CP2 is expressed both in prestalk and prespore cells (Pears et al., 1985), its timing of expression coincides with the appearance of prestalk cells. The P-3 control pattern corresponds to wild-type expression, with discoidin I expressed early, CP2 after about 14 hours (slug stage) and 2H3 after about 22 hours (culmination). We observed discoidin I expression in vegetative cells since they were grown axenically (Vauti et al., 1990). In K- and Kdel-cells, the expression of discoidin I was

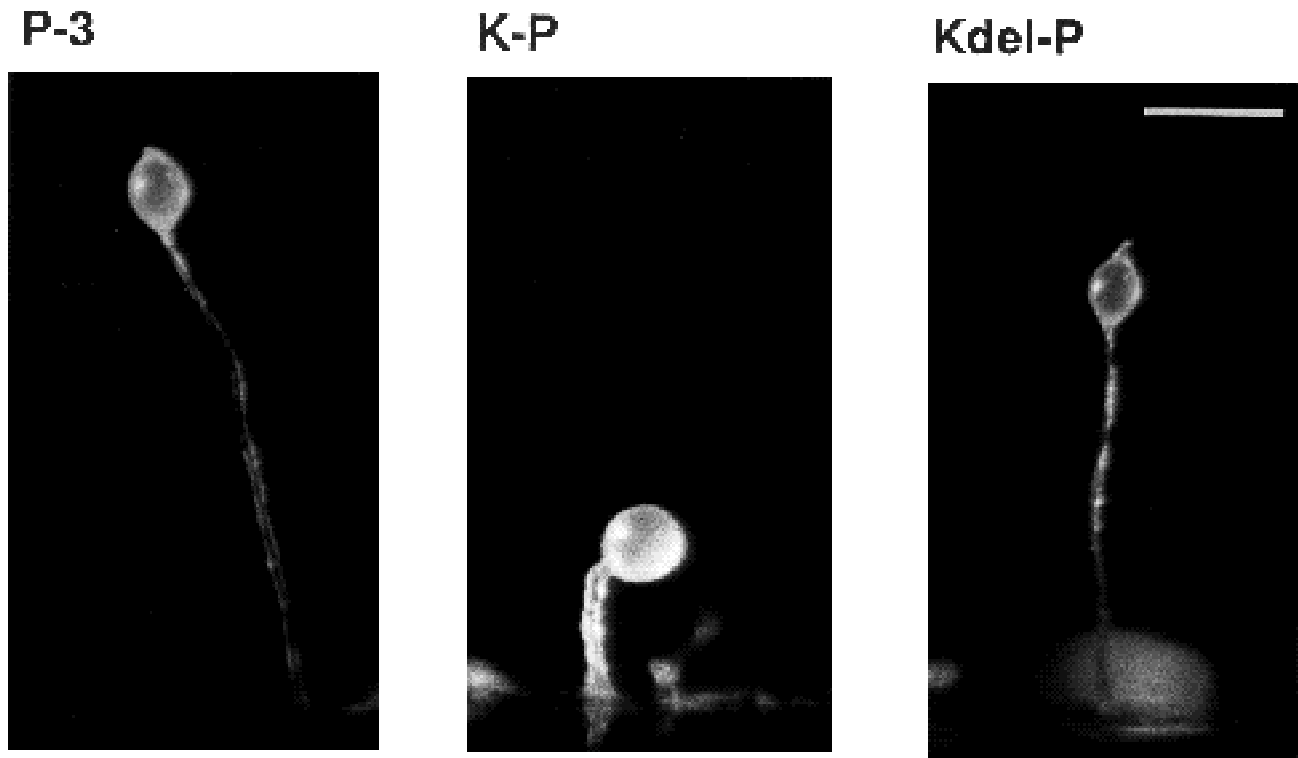

Fig. 3. Filter development of the indicated strains. 10^7 cells were allowed to develop on black, 2.5 cm diameter nitrocellulose filters (Schleicher and Schuell AG) for more than 24 hours. Portions of the filters were cut and photographed from the side. Fruits from the P-3 vector control show wild-type morphology, whereas fruiting bodies from both K-P are shorter and show enlarged stalks. When plating cells at lower densities, the stalks have a tendency to become thinner, whereas when plating cells on agar, the phenotype is more severe, showing many prostrate forms without stalk. Kdel-P fruits are smaller than P-3 on average.

brought forward by about 3 hours only. In Kdel-cells the expression of both CP2 and 2H3 was brought forward by about 8 hours, but they retained their wild-type order of appearance, as in *rde*A or C mutant development (Saxe and Firtel, 1986). K-cells also express CP2 earlier than wild type. However, most striking is the expression of 2H3 in K-cells which, unexpectedly, was advanced by about 12 hours and was expressed simultaneously with CP2.

The rapid development of K- and Kdel-strains resembled previously described rapid developing (*rde*) mutants which are affected in the intracellular cAMP signal transdution pathway (Abe and Yanagisawa, 1983). We therefore measured cAMP levels in the transformed strains (Fig. 5). The level of intracellular cAMP in P-3 cells during late development (up to about 6.5 pmol cAMP/mg of protein) is in good agreement with results from others (Abe and Yanagisawa, 1983) on wild-type cells. Both K- and Kdel-cells showed a lower level of intracellular cAMP after aggregation (10 hours) and for the rest of development compared to P-3. The levels measured at 0 and 5 hours are close to the limit of detection and do not differ significantly between the strains. The low level of intracellular cAMP observed in K- and Kdel-cells during late development resembles *rde*C rather than *rde*A mutants.

*rde*C mutants are sporogenous, that is they are able to form spore cells when the amoebae are incubated in submerged culture in buffered salts containing cAMP (Kay, 1989). Under the same conditions, wild-type cells form only prespores. K-cells are sporogenous by this criterion whereas their parents, P-3 and Kdel-P are not (Table 1). However, spores can be induced in both strains P-3 and Kdel-P by 8-Br-cAMP, an analogue that is able to penetrate cells and activate cAMP-dependent protein kinase (cAPK). This shows that these two strains have kept the potential to form spores and that the phenotypic effects of overexpression of the *Dd* PK2 gene mimics a treatment activating cAPK.

cAPK levels were measured directly in K-P, Kdel-P and P-3 cells. The basal level of cAPK in crude extracts varied from sample to sample, even from cells of the same strain, possibly due to activation of the enzyme by varying levels of con-

Table 1. *Differentiation of single starved cells*

		% cell type*			
		cAMP†		8-Br-cAMP‡	
Experiment	Strain	stalk	spore	stalk	spore
1	P-3	0	0	0.3	20.9
	K-P	0.7	12.3	0.5	24.5
	Kdel-P	0	0	0	0.5
2	P-3	0	0	0	2.8
	K-P	0.8	30.8	0.3	57.6
	Kdel-P	0	0	0	6.8
3	P-3	0	0	0	2.5
	K-P	0	40.6	0.3	51.1
	Kdel-P	0	0	0	2.5

*Differentiation was scored by phase-contrast microscopy after 24 hours of starvation.
†5 mM cAMP.
‡ 20 mM 8-Br-cAMP in addition to the 5 mM cAMP.

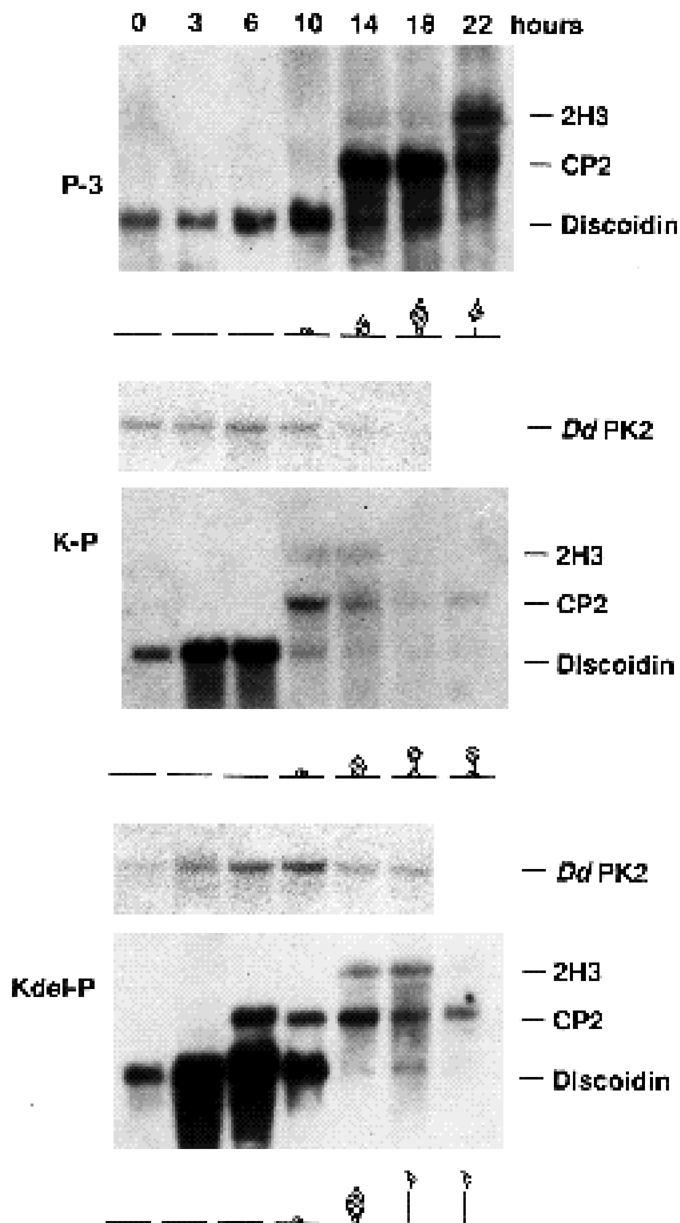

Fig. 4. Expression of developmentaly regulated genes in the indicated strains. RNA was isolated from cells developed on filters and lysed at the indicated times. *Dd* PK2 expression was detected using the *Hin*dIII-*Kpn*I fragment from the coding region presented in Fig. 1. 2H3 is prespore-specific, CP2 is co-expressed with prestalk-specific genes and discoidin I is expressed early during development. The amount of 2H3 probe varied in the hybridizations, leading to the difference observed between P-3, K-P and Kdel-P signal intensities.

taminating cAMP (data not shown). More reliable results were obtained after addition of saturating amounts of cAMP to the extracts (Table 2). We therefore measured cAPK activity as kemptide phosphorylation that was inhibitable by PKI, a specific inhibitor of cAPK. P-3 control and Kdel-P cAPK activities could not be statistically distinguished, whereas K- cells showed about 4-fold more activity. These results indicate that *Dictyostelium* cells, overexpressing the whole *Dd* PK2, possess increased cAPK activity.

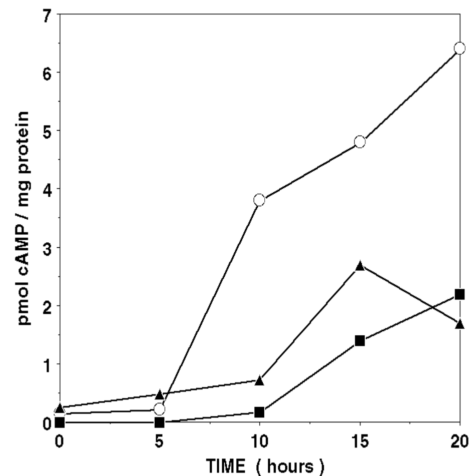

Fig. 5. Intracellular cAMP level during development. The cAMP level was measured using bovine muscle cAMP-binding protein in cells developed on filters, and collected at the indicated time (in hours) after starvation. Open circles, P-3 cells; triangles and squares, K-P and Kdel-P respectively.

Table 2. *cAMP-dependent protein kinase activity*

Strain	Activity* (pmol/min/10^8cells) 1	2	3	4	Mean value±s.d.
P-3	140	203	79	198	155±58
K-P	740	716	834	732	756±53
Kdel-P	124	164	414	294	249±132

*Cells were harvested as described in Table 1. 0.1 mM cAMP was added to the extracts. Activity is expressed as pmoles of phosphorylated kemptide inhibited by PKI. Results of 4 experiments.
s.d., standard deviation.

Discussion

Overexpression of the *Dd* PK2 mRNA in *Dictyostelium* cells (K-cells) modifies their phenotype. Development after aggregation is speeded up and aberrant fruiting bodies formed. On nitrocellulose filters, squat fruiting bodies with enlarged stalks are seen and on agar, at high cell density, many prostrate lumps also form. K-cells are also sporogenous, that is they can form spores when developed as submerged monolayers in the presence of cAMP. In these respects K-cells resemble members of the rapid developing/sporogenous class of mutants (*rde*) (Kessin, 1977; Abe and Yanagisawa, 1983). However, K-cells differ from *rde* mutants (Saxe and Firtel, 1986) in that a marker of prespore differentiation (2H3) is expressed at the same stage of development as the CP2 mRNA. Furthermore the fruit morphology of K-cells differs slightly from *rde*A mutants which show pyramid-shaped fruits and from *rde*C which form hemispheric mounds. The sporogenous phenotype and the premature expression of 2H3 both indicate that differentiation of spores is facilitated in K-cells.

The sporogenous phenotype has been shown previously to be associated with activation of the intracellular cAMP signal transduction pathway (Abe et al., 1981; Kay, 1989). This was confirmed recently by showing that the sporogenous

*rde*C mutants have a defective regulatory subunit of cAPK (Simon et al., 1992). Overexpression of the *Dd* PK2 gene also activates the intracellular cAMP signal transduction pathway, as evidenced by the high level of cAPK enzymatic activity found in K-cell lysates. The low level of intracellular cAMP measured in K-cells could be due to a negative feedback mechanism between cAPK and adenylyl cyclase as proposed earlier (Simon et al., 1992).

The phenotype of the Kdel-cells indicates that rapid development can be dissociated from spore formation in submerged monolayers. The presence of the N-terminal domain together with the ATP binding region (phosphate anchor, Knighton et al., 1991) is sufficient to confer rapid development without the sporogenous phenotype. The respective roles of the N-terminal part and ATP-binding site in the acceleration of development remain to be determined.

Although the *Dd* PK2 gene strongly resembles the catalytic subunit of cAPK (Knighton et al., 1991) in its C-terminal domain, the N-terminal half of the protein differs from any known sequence (Bürki et al., 1991). It therefore seems unlikely that this gene does encode the *Dictyostelium* catalytic subunit of cAPK, unless the protein is processed to remove the N-terminal domain. Furthermore, the absence of cAMP-binding site(s) and pseudosubstrate sequence within the N-terminal domain renders unlikely a regulation of *Dd* PK2 catalytic activity analogous to protein kinase C. The *Dd* PK2 gene, thus, resembles the SCH9 protein kinase, which has been found to substitute functionally for cAPK activity in yeast (Toda et al., 1988). Biochemical characterization of the *Dd* PK2 should clarify its relationship to cAPK.

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