An Unusual Catalytic Subunit for the cAMP-Dependent Protein Kinase of *Dictyostelium discoideum*†

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ABSTRACT: The cAMP-dependent protein kinase (cAPK) plays an essential role during differentiation and fruit morphogenenesis in *Dictyostelium discoideum*. The presence of an open reading frame on the gene, *pkaC* (previously named either *Dd PK2* or *Dd PK3* by different groups), predicts a 73-kDa polypeptide with 54% similarity to the catalytic subunits of cAPKs from other organisms. Using anti-peptide antibodies, we show that the *pkaC* gene product, PkaC, is a 73-kDa polypeptide. Despite the fact that PkaC is about twice the size of its mammalian counterparts, it possesses all of the properties required of a catalytic subunit. It is physically associated with the regulatory subunit, and this association results in an inhibition of the catalytic activity which is reverted by cAMP. PkaC copurifies with cAPK activity, and an increased cAPK activity is observed in cells overexpressing PkaC. We conclude that PkaC is a catalytic subunit of the *Dictyostelium discoideum* cAPK and discuss the unusual features of this protein with the highest molecular weight of known cAPKs.

A variety of hormonal stimuli are transmitted via intracellular cAMP[1] level changes. cAMP-dependent protein kinases (cAPK), found only in eukaryotic cells, are the main elements responding to such variations (Adams et al., 1991). cAPK holoenzymes are generally composed of two catalytic (C) and two regulatory (R) subunits (Taylor, 1989; Taylor et al., 1990). In higher eukaryotes, two types of R subunits (R_I and R_{II}) have been distinguished by their biochemical properties (Corbin et al., 1975), and four different genes (RIα, RIβ, RIIα, and RIIβ) have now been identified (Jahnsen et al., 1986; Skalhegg et al., 1992). Each R subunit carries two cAMP binding sites and inhibits one catalytic subunit by binding at its catalytic site (Beebe & Corbin, 1986). The presence of isozymes of the R subunit seems to be restricted to higher eukaryotes since only one type of R subunit is found in yeast (Sy & Roselle, 1982) or in *Dictyostelium discoideum* (De Gunzburg & Véron, 1982; Rutherford et al., 1982), which diverged at the earliest branch point leading to animal cells (Sogin et al., 1989). While only one type of catalytic subunits could be characterized by biochemical methods (Beebe & Corbin, 1986), the isolation of cDNAs allowed the distinction of several isozymes. In mammals, three genes encode Cα, Cβ, and Cγ, which are highly homologous but not identical (Showers et al., 1986; Uhler et al., 1986; Beebe et al., 1990). Isozymes of the C subunit are also found in lower eukaryotes such as yeast (Toda et al., 1987).

The simple eukaryote, *Dictyostelium discoideum*, is able to enter a developmental cycle upon food deprivation, leading to the morphogenesis of a stalk supporting a mass of spores (Loomis, 1982). cAPK activity is low in vegetatively growing *Dictyostelium* amoebae and increases during development at the time of aggregation (Leichtling et al., 1984; Part et al., 1985) to reach a maximum at culmination (Vaughan & Rutherford, 1987). Sequence comparison indicates that the only R subunit of *Dictyostelium* is of the R_I isotype (Mutzel et al., 1987). Contrary to its mammalian counterpart, this 37-kDa R subunit is a monomer, which associates with one C subunit to form an inactive RC holoenzyme (de Gunzburg et al., 1984; Mutzel et al., 1987).

Analysis of morphogenetic mutants and the use of reverse genetics have shown that cAPK plays an essential role during development. Overexpression of either wild-type R subunit (Simon et al., 1989; Firtel & Chapman, 1990) or mutants thereof unable to bind cAMP (Harwood et al., 1992a) prevents aggregation, presumably by inhibiting cAPK activity. A more specific role for cAPK in cell-type differentiation has been demonstrated by the finding that a mutation within the R subunit gene which impairs its interaction with the C subunit leads to rapid development and to a sporogenous phenotype (Simon et al., 1992). Furthermore, specific cell-type expression of a dominant inhibitory R mutant protein in prestalk or prespore cells prevents subsequent morphogenesis of the stalk (Harwood et al., 1992b) or spore maturation (Hopper et al., 1993).

Several groups (Haribabu & Dottin, 1991; Bürki et al., 1991; Mann & Firtel, 1991; J. G. Williams, personal communication; B. Wetterauer and M. Véron, unpublished results) have tried to clone *Dictyostelium* genes encoding protein kinases by taking advantage of the high conservation of the sequences during evolution (Hanks et al., 1988). We isolated the *Dd PK2* gene encoding a putative protein of 648 amino acids presenting about 54% sequence identity in its C-terminal half with mammalian and yeast cAPK catalytic subunits (Bürki et al., 1991). The same gene was also cloned independently under the name *Dd PK3* by others (Mann & Firtel, 1991). In order to avoid confusion in gene and/or protein names, from now on we shall call this gene *pkaC* and its product PkaC. Overexpression of *pkaC* in *Dictyostelium* results in increased cAPK activity and rapid development, as well as a sporogenous phenotype, thus suggesting that *pkaC*

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[1] Abbreviations: cAMP, cyclic adenosine monophosphate; cAPK, cAMP-dependent protein kinase; PkaC, catalytic subunit of *Dictyostelium* cAPK; *pkaC*, *Dictyostelium* gene encoding PkaC.

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encodes a cAPK catalytic subunit (Anjard et al., 1992). This was confirmed by the demonstration that a truncated PkaC protein expressed in *Escherichia coli* showed specific cAPK activity which could be inhibited by PKI (Mann et al. 1992).

In this work, we address the question of PkaC activity in *Dictyostelium* extracts and its interaction with the R subunit. We show, using polyclonal antibodies against PkaC, that a polypeptide corresponding to the complete coding sequence of *pkaC* is present in *Dictyostelium* and that this protein has all of the biochemical properties defining a catalytic subunit of a cAMP-dependent protein kinase.

EXPERIMENTAL PROCEDURES

Polyclonal Antibody Purification and Western Blots. A peptide (PK2) corresponding to residues 329–339 of the PkaC sequence (Bürki et al., 1991), with a Tyr added at the N-terminus and MetAla added at the C-terminus, was synthesized: PK2 = NH_2-TyrArgGluArgLeuLysGluPheLys-GlnIleArgMetAla-COOH. The last two amino acids were cleaved by CNBr before a second immunization in order to lower the proportion of antibodies recognizing the C-terminus only. PK2 peptide (10 mg) was linked to 10 mg of KLH proteins using bis(diazobenzidine) in 100 mM borate buffer (pH 9.0). PK2 peptide (20 mg) was treated with 2-mercaptoethanol, purified on a G-25 column, lyophilized, and cleaved with 100 molar excess of cyanogen bromide for 12 h in the dark. After renewed lyophilization, 6 mg of PK2 peptide was linked to either 6 mg of KLH or 6 mg of BSA proteins using bis(diazobenzidine).

KLH–PK2 (2 mg) was emulsified with complete Freund's adjuvant (Sigma, cat. no. F-5581) and injected subcutaneously into three rabbits (L1, L8, and L16). A boost injection with 2 mg of CNBr-cleaved PK2 peptide linked to KLH in incomplete Freund's adjuvant was performed 26 days later, and sera were collected after 1–10 days.

Serum preparations were tested by ELISA (Harlow & Lane, 1992) using of 100 ng of CNBr-cleaved PK2 peptide linked to BSA per well. Sera were serially diluted from 1/2 to 1/5116 by steps of 2 in PBS. The presence of antibodies was detected using 100 ng of protein A–peroxidase (Sigma, cat. no. P-8651) per well and revealed using tetramethylbenzidine (Sigma, cat. no. T-2885).

IgGs were enriched using protein A–Sepharose CL-4B columns (Pharmacia, cat. no. 17-0780-01). Essentially 3–4 mL of serum was adjusted to pH 8.0 with 1/10 vol of 1 M Tris (pH 8.0) and passed over 2 mL of column. After a wash with 25 mL of 100 mM Tris (pH 8.0) and 25 mL of 10 mM Tris (pH 8.0), IgGs were eluted with 100 mM glycine (pH 2.5). Fractions of 1 mL were collected in 1.5-mL Eppendorf tubes containing 100 μL of 1 M Tris (pH 8.0). Elution was monitored by the Bradford spot test.

Anti-PkaC antibodies were purified by affinity chromatography. CNBr-cleaved PK2 peptide (8 mg) linked to BSA was coupled to 0.5 g of CNBr-activated Sepharose 4-B (Pharmacia, cat. no. 17-0430-01). Alternatively, we have successfully used the PK2 peptide alone coupled to Sepharose. Antibodies obtained after protein A columns were diluted 1/10 in 10 mM Tris (pH 8.0) and loaded 3 times on a 0.5-mL peptide affinity column. The columns were washed with 20 bed vol of 10 mM Tris (pH 8.0) and then with 20 bed vol of 10 mM Tris (pH 8.0)/500 mM NaCl. Columns were equilibrated with 5 mL of 10 mM phosphate buffer (pH 7.2) and eluted with 10 mM phosphate buffer (pH 7.2)/5 M LiCl. Fractions of 500 μL were collected. The presence of proteins in fractions was checked by absorbence at 280 nm. Protein-containing fractions were dialyzed against PBS.

Western Blots. Dictyostelium cells (2×10^6) were pelleted by 1 min of centrifugation at 2500 rpm (Eppendorf centrifuge), boiled for 5 min in 20 mL of Laemli buffer (Sambrook et al., 1989), and loaded in 5-mm slots on an 8% SDS–polyacrylamide gel. The gels were run at 80–100 mA transferred onto nitrocellulose (Sartoblot II semidry from Sartorius), and the transferred proteins were visualized by Ponceau-S staining. Membranes were incubated for 1 h at room temperature in 3% skim milk/0.2% Tween-20 before the addition of anti-PK2 antibody. After an overnight incubation at 4 °C, the membranes were washed 3 times in PBS and incubated for 1 h with 100 ng/mL protein A–peroxidase in 3% skim milk/0.2% Tween-20. Membranes were washed 4 times in PBS, and PkaC localization was revealed using chemiluminescence (ECL, Amersham no. RPM 3004).

For competition experiments, 4×10^7 Ax2 vegetative cells were boiled in 200 μL of Laemli buffer and loaded in a 7-cm-long well on an 8% polyacrylamide gel. Running, transfer, and saturation were performed in the same manner as for the Western blot. The nitrocellulose membrane was cut in 5-mm-wide strips. Anti-PK2 antibody was preincubated for 1 h at 4 °C in 1 mL of 3% skim milk/0.2% Tween-20 with the indicated amount of PK2 peptide. Nitrocellulose strips were then reacted with antibody/PK2 solutions and processed as described above for Western blots.

Anti-R antibodies were raised in rabbits against purified R subunit from *Dictyostelium* (De Gunzburg et al., 1984).

Native Gel Electrophoresis. The method of Van Patten et al. (1986) was adapted for *Dictyostelium* cells. Cells (10^7) were lysed in 10 mM Tris-HCl (pH 6.2), 100 μM ATP, 400 μM $MgCl_2$, and protease inhibitors (Fasel et al., 1992) by three cycles of freezing and thawing. The extracts were centrifuged at 10000*g* for 5 min at 4°C, and the supernatant was brought to 8% glycerol and 0.02% Bromphenol blue before being loaded on a 7.5% acrylamide gel in 240 mM Tris-HCl (pH 7.3), with a stacking gel of 2.5% acrylamide in 70 mM Tris-HCl (pH 6.2). Electrophoresis was performed for 2–4 h at 150 V using 60 mM Tris–acetate (pH 7.1), 100 μM ATP, and 400 μM $MgCl_2$ as the upper buffer and 60 mM Tris–acetate (pH 6.0) as the lower buffer. Proteins were then transferred by semidry blotting to nitrocellulose and reacted overnight with either anti-R antibody diluted 1/500 or anti-PK2 1/100. Protein A conjugated to peroxidase was used to reveal the antibodies using Amersham ECL reagents. In experiments without ATP, ATP and $MgCl_2$ were omitted both in sample buffer and in upper buffer. About 0.1 μg of purified R subunit was subjected to the same separation conditions (lane R in Figure 2).

cAPK Activity. cAPK activities were determined using the protein kinase A–assay system from Gibco BRL, with 50 μM synthetic peptide LRRASLG (Kemptide) as substrate. cAMP was added up to 100 μM in all experiments, except when R subunit was added or when stated otherwise. The purified recombinant R subunit of cAPK from *Dictyostelium* (Simon et al., 1988) was used at a concentration of 2 μg/mL unless otherwise indicated. Either the PKI peptide fragment encompassing amino acids 6–22 (Amersham) or a synthetic PKI_{5-24} was used alternatively at 20 μM (unless otherwise stated) as the high-affinity inhibitor of cAPK (Scott et al., 1986). One unit of protein kinase activity corresponds to 1 pmol of ^{32}P incorporated on Kemptide per minute at 22 °C.

DEAE–Sephacel Chromatography. Dictyostelium strains K-P and P3 (Anjard et al., 1992) were grown at 22 °C in

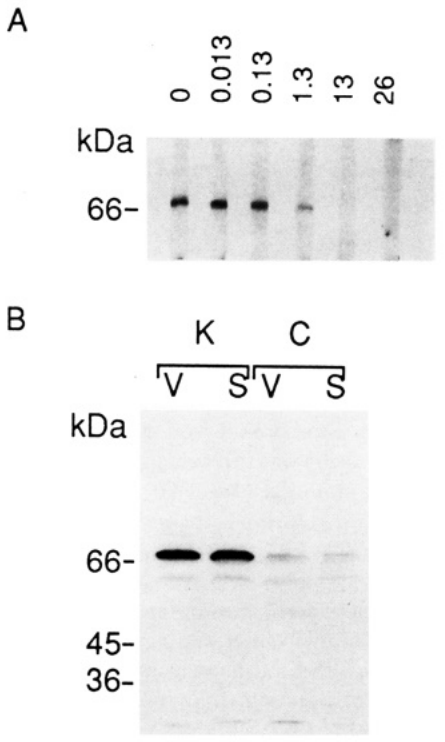

FIGURE 1: Specificity of polyclonal anti-PK2 antibodies. (A) Proteins from 5×10^7 *Dictyostelium* cells were separated by SDS–PAGE on a 10-cm-wide gel. After transfer, membrane strips were cut and reacted separately with anti-PK2 antibodies purified by affinity (anti-PK2), which had been preincubated with the indicated amounts of PK2 peptide. Protein standards were run in parallel as size markers: 66 kDa, bovine albumin; 45 kDa, ovalbumin; 36 kDa, glyceraldehyde-3-phosphate dehydrogenase.(B) Proteins from 2×10^6 cells either grown vegetatively (V) or starved for 6 h (S) were separated by SDS–PAGE, blotted onto nitrocellulose, and reacted with anti-PK2. K represents cells overexpressing *pkaC* [K-P in Anjard et al. (1992)], whereas C stands for control cells containing the vector alone [P3 in Anjard et al. (1992)]. Size markers were as indicated for A above.

HL-5 broth containing 20 μg/mL G418. Cell extracts were prepared by freeze–thawing 5×10^8 cells in 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 0.1% triton, 50 μg/mL TLCK, 50 μg/mL antipain, 2 mM benzamidine, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 1 mM PMSF (buffer A). The lysates were centrifuged at 13000*g* for 10 min, and the supernatants were applied onto 5-mL DEAE–Sephacel columns previously equilibrated with the same buffer. The columns were then washed with 25 mL of buffer A, and elution was performed with a linear gradient of 0–0.3 M NaCl in 30 mL of buffer A (flow rate 30 mL/h). Fractions (0.5 mL) were collected and assayed for cAPK activity. The procedure was carried out at 4 °C.

RESULTS

pkaC Is Expressed as a 73-kDa Protein. The sequence of the putative protein encoded by the *pkaC* gene contains three potentially highly antigenic sequences as determined by computer analysis. The region just preceding the beginning of the putative catalytic domain (Bürki et al., 1991) was chosen to synthesize the peptide PK2, which was used to immunize rabbits (see the Experimental Procedures). The best sera (rabbits L1 and L16) scored positive by ELISA up to dilution of 1/5000, but they reacted with multiple protein bands when analyzed on Western blots using *Dictyostelium* extracts (data not shown). When IgGs were affinity-purified on a PK2 peptide–Sepharose column, a single major protein band was detected (Figure 1A). The specificity of the serum was further verified by competition with increasing amounts of PK2 peptide. Affinity-purified antibodies from L8 and L16 rabbits detected the same band (data not shown). To further test antibody specificity, we analyzed *Dictyostelium* extracts overexpressing the *pkaC* gene (Anjard et al., 1992). As shown in Figure 1B, the increased signal (about 10-fold) matches the increased expression of the *pkaC* gene. We conclude that PkaC is expressed in the cell as a 73-kDa polypeptide which is specifically recognized by anti-PK2 antibodies.

PkaC Forms Oligomeric Complexes with the R Subunit. The ability of PkaC to associate with the *Dictyostelium* R subunit was tested. For this, we took advantage of the large difference in isoelectric points between the holoenzyme and the free catalytic subunit: Indeed, cAPK holoenzymes from other species have an acidic isoelectric point and migrate inside native gels (Van Patten et al., 1986), whereas isolated catalytic subunits migrate in the opposite direction. The theoretical isoelectric point of PkaC calculated from its amino acid sequence is 7.99, from which we can predict that the protein should not enter native gels prepared at pH 7.3. In contrast, the R subunit has a calculated isoelectric point of 5.53, thus leading to a net negative charge at this pH. Accordingly, purified R migrated inside the gel (Figure 2A, lane R, dotted arrow). When crude extracts from vegetative *Dictyostelium* cells were separated on native gels and stained with anti-R serum, a predominant band with a slower migration than that of the free R subunit was observed (Figure 2A, arrow). A faint band corresponding to the migration of pure R subunit was also present, indicating the presence of some free R subunit in these extracts.

Using anti-PK2 antibody, we detected a single band which migrated at the position of the major band detected with anti-R serum, indicating the presence of both R and PkaC epitopes on the same protein complex. Extracts from cells overexpressing PkaC showed about the same amount of the complex (Figure 2A, lane K), indicating that the R subunit is limiting for the formation of the holoenzyme. Indeed, a measure of cAMP binding in total cell extracts indicated the same amount of R subunit in Ax2 and K cells (M.-N. Simon & M. Véron, unpublished results). These results indicate that an R–PkaC complex is present and that overexpression of *pkaC* does not lead to an increase in the R subunit level.

Figure 2B shows the results obtained when the experiment was performed in absence of Mg^{2+}ATP in both gel and electrophoresis buffers. While no band could be detected with anti-PK2, as expected from the p*I* of free PkaC, anti-R serum stained mainly a band migrating at the position of the free R subunit (dotted arrow). These results demonstrate an Mg^{2+}ATP dependence of the R–PkaC complex, which is typical of type R_I isozymes (Van Patten et al., 1986).

Inhibition of cAPK Activity by Purified R Subunit. In 10000*g* supernatants of wild-type *Dictyostelium* vegetative cells, about 60 pmol of phosphate is incorporated per milligram of protein per minute (Figure 3, panel A). From these, only about 15 pmol is incorporated by a specific cAPK activity, as judged from the addition of either PKI peptide or purified R subunit. Full original activity can be restored by the addition of cAMP, further showing cAPK specificity. The background phosphorylation of about 40 pmol/min/mg present in these crude extracts with or without Kemptide (data not shown) cannot be inhibited by PKI peptide and probably corresponds to other types of protein kinases and/or phosphotransferases acting on endogenous substrates.

In *pkaC* overexpressers (Anjard et al., 1992), we observed a 3–5-fold increase in cAPK catalytic activity as defined by

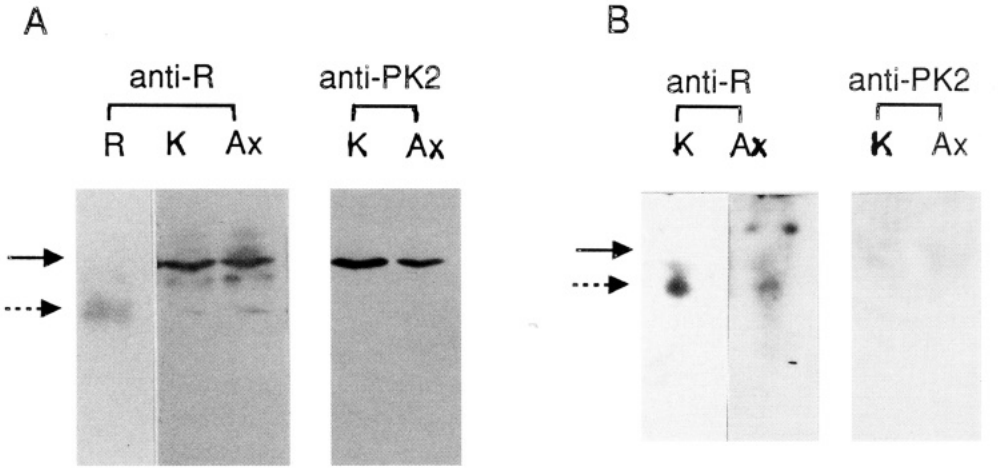

FIGURE 2: Native gels. (A) Wild-type Ax2 cells (Ax) (10^7) or cells overexpressing *pkaC* (K) were lysed by freezing and thawing and separated under nondenaturing conditions in the continuous presence of Mg^{2+}ATP. Purified R subunit was separated by electrophoresis under the same conditions on a separate gel (lane R). After transfer, one-half of the gel was reacted with anti-R serum (anti-R), whereas the second half was reacted with anti-PK2 antibody (anti-PK2). (B) Crude extracts of both K (K) and Ax2 (Ax) cells were separated and analyzed as in A, except that Mg^{2+}ATP was omitted in the buffers. The K lane is a lower exposure of the same blot to correct for the artifactually high amount of protein loaded in this lane. The solid arrow indicates the migration of the complex R–PkaC. The dotted arrow indicates the migration of free R subunit.

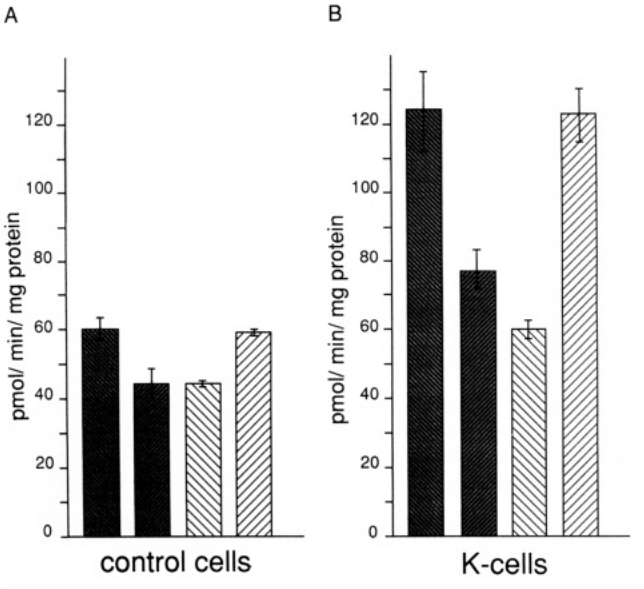

FIGURE 3: cAMP-dependent protein kinase activities. Control cells (10^7) (A) or cells overexpressing PkaC (B) were lysed by freezing and thawing and assayed for Kemptide phosphorylating activity in the presence of either cAMP alone (far left), PKI peptide inhibitor and cAMP (middle left), purified R subunit (middle right), or purified R subunit and cAMP (far right). See the Experimental Procedures for concentrations.

its ability to be inhibited by PKI peptide (Figure 3B). When purified R subunit was used, an even more potent inhibition was observed, which was fully relieved by adding cAMP. Thus, the protein kinase activity encoded by *pkaC* has the specificity and the biochemical properties characteristic of a C subunit of a cAPK.

Partial Purification of PkaC from Vegetative Cells. Extracts from vegetatively growing *Dictyostelium* cells were fractionated on DEAE–Sephacel (Figure 4). The fractions were assayed for the presence of cAPK activity before analysis by Western blotting to detect both R subunit and PkaC. All fractions containing protein kinase activity were fully inhibited by PKI peptide. The peak of Kemptide phosphorylating activity was composed of two different components: while the fractions eluting at lower ionic strength were not stimulated by cAMP, stimulation did occur in the fractions eluting at higher salt concentrations (Figure 4). Western blots showed that the 73-kDa band corresponding to PkaC coeluted precisely with protein kinase activity, while the R subunit was present only in those fractions where protein kinase stimulation was observed. When the same column chromatography was performed in the presence of cAMP, cAPK activity eluted in a single symmetrical peak corresponding to PkaC as detected by Western blots in the column fractions (results not shown). This shows that the heterogeneity of the protein kinase activity peak in Figure 4 is due only to differential association with the R subunit and not to the presence of several types of C subunits.

The partially purified PkaC activity eluting from the DEAE column was characterized further. It was stable for at least 2 months at –20 °C in the presence of 0.5 mg/mL BSA. The apparent K_m for Kemptide determined in the presence of 0.5 mM ATP was 12.5 ± 1.7 μM, a value very close to that of the free mammalian C subunit. Half-maximal inhibition by PKI peptide was achieved at 0.7 μM peptide in the presence of 10 μM Kemptide and 0.5 mM ATP, which is close to the K_i of 0.4 μM determined earlier (Simon et al., 1992). This value is about 50-fold higher than the K_i of mammalian catalytic subunits for the same peptide (Scott et al., 1986) (see the Discussion).

A similar DEAE profile was obtained using K cells overexpressing PkaC (not shown). The estimated 10-fold partially purified cAPK activity eluting from the column was inhibited by PKI peptide (Table I). Here again, in contrast to crude extracts, the basal activity was low, thus indicating that most phosphorylation activity in these purified samples was due to cAPK. Addition of purified R subunit reduced phosphorylation activity, although to a lesser extent, possibly due to the presence of cAMP in the preparation. Addition of a saturating cAMP concentration restored full activity.

The match between the presence of PkaC and cAPK activity upon partial purification demonstrates that PkaC is a catalytic subunit of cAPK.

DISCUSSION

Although the presence of cAPK activity in *Dictyostelium* had been known for a long time (De Gunzburg & Véron, 1982), characterization of its catalytic moiety remained elusive.

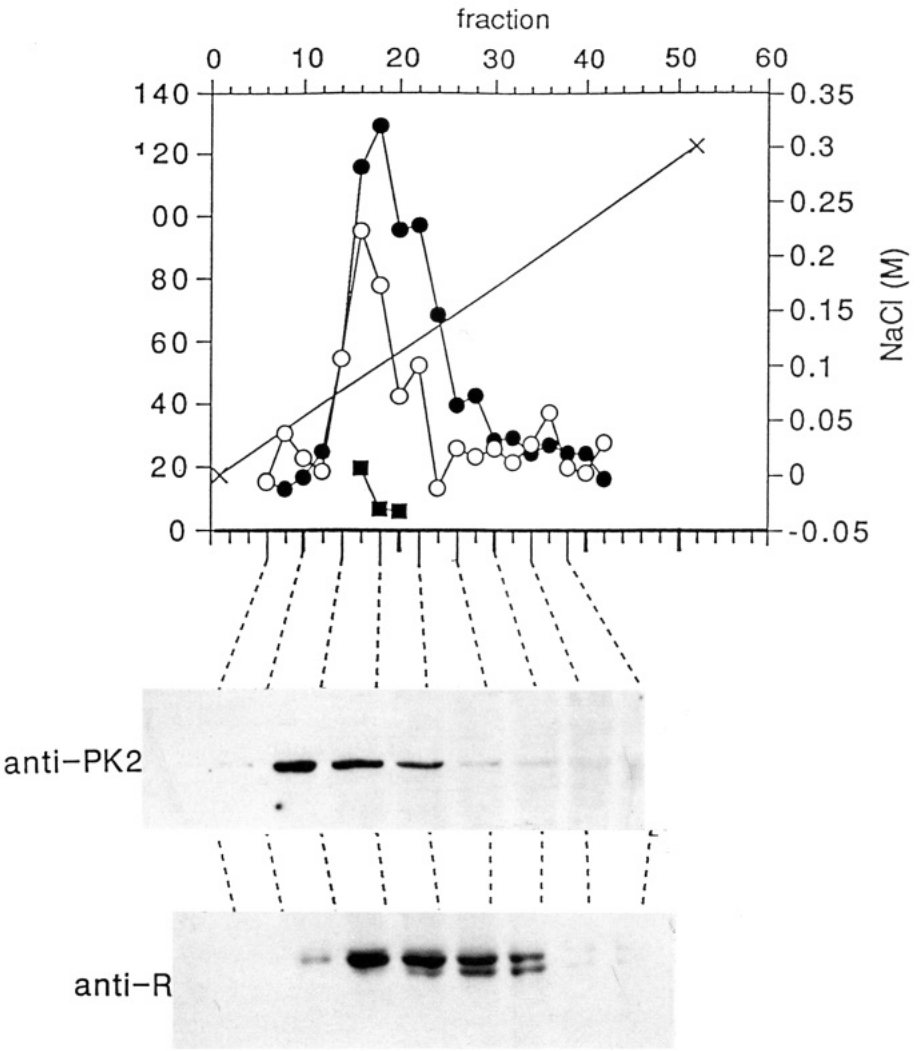

FIGURE 4: DEAE–Sephacel chromatography of the soluble fraction of vegetative *Dictyostelium* cells. Supernatant (13000*g*) in buffer A (67 mg of protein/mL) was applied onto a DEAE–Sephacel column (1.5 × 2.8 cm) at a flow rate of 18 mL/h. No enzyme activity was found in the flow-through fractions. The column was washed with buffer A, and elution was performed with a linear 0–0.3 M NaCl gradient. Fractions were assayed for Kemptide phosphorylating activity in the presence (●) or absence (○) of 50 μM cAMP. PKI peptide (■) was added to aliquots of the peak fractions. Proteins from fractions 6 to 38 were separated by SDS–PAGE,

Table I: cAPK Activity in K Cell Extracts after DEAE–Sephacel Chromatography

additions	activity[a]
none	100 ± 20
50 μM cAMP	154 ± 30
20 μM PKI peptide	6 ± 3
10 μg/mL R subunit	50 ± 13
cAMP + R subunit[b]	125 ± 27

[a] Enzymatic activity is expressed in percent and is the mean of three independent experiments. Values were normalized for activity without any addition. Standard deviations are indicated after ± signs. [b] At 50 μM and 10 μg/mL, respectively.

A developmentally regulated cAPK activity was identified (Leichtling et al., 1984), and a 41-kDa catalytic subunit was partially purified which was shown to associate with the monomeric R subunit to form a dimeric holoenzyme (de Gunzburg et al., 1984). Antibodies against mammalian C subunits also detected a protein of 41 kDa in extracts of differentiating *Dictyostelium* cells (Chevalier et al., 1986). However, further attempts to purify this cAPK activity were unsuccessful (M.-N. Simon, O. Pellegrini, and M. Véron, unpublished results), precluding the possibility of isolating its cDNA.

Several laboratories have recently tried to isolate genes encoding protein kinases from *Dictyostelium discoideum* on the basis of the conservation of specific motifs in this enzyme family (Hanks et al., 1988). While it did result in the identification of a number of genes clearly encoding protein kinases (Haribabu & Dottin, 1991; Bürki et al., 1991; Mann & Firtel, 1991; J. G. Williams, personal communication; M.-N. Simon, personal communication), this approach did not permit their precise assignment to a subclass of the superfamily. *Dictyostelium* diverged at a very early time from the branch leading to higher eukaryotes (Sogin et al., 1989). Thus, when one compares a sequence motif of a given *Dictyostelium* protein kinase with the consensus sequence of a subfamily (for example, with protein kinase C or with the catalytic subunit of cAPK), it is often impossible to decide whether a given divergence of a *Dictyostelium* sequence is due to phylogenetic distance within the subfamily or due to the fact that it belongs to a distinct subfamily. Therefore, functional characterization of the product of a protein kinase gene is needed for its precise identification.

Using the approach described above, the same gene encoding a *Dictyostelium* protein kinase was isolated in two different laboratories under the respective names *Dd PK2* and *Dd PK3* (Bürki et al., 1991; Mann & Firtel, 1991). The C-terminal

half of the protein encoded by this gene showed significant similarity with the catalytic subunits of mammalian cAPKs (about 54% sequence identity). Since then, genetic and biochemical evidence has indicated that the gene encodes a protein kinase with the functional properties of a catalytic subunit of cAPK: (i) Cells lacking the gene were unable to aggregate (Mann & Firtel, 1991) and showed no cAPK activity. On the contrary, cells overexpressing it (Anjard et al., 1992; Mann et al., 1992) displayed a phenotype analogous to that of mutants with a nonfunctional R subunit (Simon et al., 1992). (ii) Recently, it was shown that a fragment of this gene expressed in recombinant bacteria encoded a protein kinase with the functional properties of a catalytic subunit of a cAPK (Mann et al., 1992). Accordingly, we have renamed the *Dd PK2* gene *pkaC* according to the three-letter nomenclature prevailing for *Dictyostelium* (Loomis, 1982), and PkaC indicates the encoded protein.

We provide here a further demonstration that *pkaC* encodes a *Dictyostelium* catalytic subunit by showing that PkaC can functionally interact with the *Dictyostelium* R subunit and that this interaction is relieved by cAMP addition. Moreover, a physical association between the R subunit and PkaC was demonstrated using native gel electrophoresis. The requirement of Mg^{2+}ATP for the formation of a stable holoenzyme is characteristic of a type I isotype (Van Patten et al., 1986) and thus is in full agreement with the classification of the *Dictyostelium* R subunit based on its sequence (Mutzel et al., 1987). In that respect, the *Dictyostelium* R subunit differs from those of other lower eukaryotes, such as yeast (Sy & Roselle, 1982) or the primitive fungus *Blastocladiella emersonii* (Valle Marques & Gomes, 1992), which are R_{II} isotypes.

The crystallographic structures of the binary complex of mouse recombinant cAPK catalytic subunit in the presence of an inhibitor peptide (Knighton et al., 1991) and the ternary complex in the presence Mg^{2+}ATP (Zheng et al., 1993) have been determined at high resolution. The 11 amino acids involved in ATP binding (Gly_{50}-X-Gly_{52}-X-X-Gly_{55}-X-Val_{57} from the P-loop, Lys_{72}, Glu_{91}, Asn_{171}, Asp_{184}, Glu_{208}, Asp_{220}, and Arg_{280}),[2] as well as the Asp_{166} in the "catalytic loop" (Taylor et al., 1992), are present in PkaC. The crystal structure of the Mn^{2+}ATP complex revealed further residues involved in ATP binding (Bossemeyer et al., 1993). These residues are present in PkaC, except for the conservative replacement of Ser_{53} by a threonine. Residues playing an important role in the R–C interaction (Gibbs et al., 1992) are also conserved (Lys_{189} and the couple Lys_{213}–Lys_{218}). However, the *Dictyostelium* holoenzyme is less stable than its mammalian counterpart (De Gunzburg et al., 1984), and functional differences may be found when the physicochemistry of the *Dictyostelium* R–C interactions are studied in more detail. Residues known to interact with the PKI inhibitor (Glass et al., 1992) are also identical, except for a Phe to Tyr change at position 239. Phe_{239} is known to be in a hydrophobic interaction with the Phe in position P_{-10} of PKI (Knighton et al., 1991). The pair Asp_{329}–Glu_{331} is changed to Met_{622}–Asp_{624}. These changes could account for the reduced affinity of PKI for the *Dictyostelium* catalytic subunit, as already proposed in yeast (Glass et al., 1992).

pkaC has an open reading frame of 648 amino acids as compared to about 350 residues for the mammalian catalytic subunits corresponding to polypeptides of 73 and 41 kDa, respectively. Using a specific peptide antibody, we show that the PkaC gene product indeed has an apparent molecular weight of 73 kDa, demonstrating the presence of a catalytic subunit of uniquely large size in *Dictyostelium*. This invalidates our previous hypothesis of a proteolytic process to yield a catalytic subunit with the typical 41-kDa size (Anjard et al., 1992). The large size of PkaC is unusual not only when compared to higher eukaryotes but also in comparison with C subunits of cAPKs from lower phyla. In *Drosophila* a catalytic subunit of 350 amino acids has been reported (Foster et al., 1984), and even in yeast where the three different C subunit genes encode significantly larger polypeptides their sizes do not exceed 398 residues (Toda et al., 1987). The question then arises: does the long amino-terminal extension in PkaC carry a specific function? Inspection of its sequence reveals that it contains long stretches of poly(glutamine) along with poly(threonine) and poly(asparagine). This might simply result from the presence of CAA repeats in *pkaC* with no particular functional significance. Analogous CAA repeats have been described in other *Dictyostelium* genes (Ennis et al., 1991). However, overexpression of this N-terminal domain results in rapid development (Anjard et al., 1992). The functional significance of the amino-terminal domain in *Dictyostelium pkaC* remains to be further documented.

[2] Numbers correspond to amino acid residues of the mammalian C subunits. Add 293 to obtain the corresponding residues in *Dictyostelium* PkaC, due to the long N-terminus.

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