Isolation and characterization of casein kinase I from *Dictyostelium discoideum*

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In the present study, the molecular cloning and characterization of a 49-kDa form of casein kinase (CK)I from *Dictyostelium discoideum* is reported. The predicted amino acid sequence shares 70 % identity with the catalytic domain of the mammalian δ and ϵ isoforms, *Drosophila* CKI ϵ and *Schizosaccharomyces pombe* Hhp1, and 63 % identity with Hrr25, a 57-kDa form of yeast CK involved in DNA repair. *D. discoideum* CKI (DdCKI) was expressed in vegetative asynchronous cells as well as in differentiated cells, as detected by Northern-blot analysis. The level of DdCKI expression did not change during the cell cycle. Antibodies raised against a truncated version of the protein recognized a 49-kDa protein from *D. discoideum* extracts. Protein expression

paralleled the pattern found for the RNA. The expression of DdCKI in *Escherichia coli* resulted in an active enzyme that autophosphorylated and phosphorylated casein. Immunofluorescence assays showed that DdCKI was localized in the cytoplasm and nuclei of *Dictyostelium* cells. The lack of disruptants of the CKI gene suggests that this protein is essential for the vegetative growth of *D. discoideum*. Overexpression of DdCKI resulted in cells with increased resistance to hydroxyurea, suggesting a potential role for this kinase in DNA repair.

Key words: casein kinase, cDNA cloning, immunofluorescence, protein expression.

INTRODUCTION

Casein kinase (CK)I was among the first protein kinases to be described. It is an ubiquitous monomeric enzyme ranging in size from 25 to 55 kDa and is present in the plasma membrane, nuclei, cytoplasm and cytoskeleton of eukaryotic cells [1]. Once considered to represent a single entity, it is now known to comprise a family, which represents a distinct branch of the eukaryotic protein kinase. CKI homologues have been identified in organisms ranging from yeasts to humans [2]. All members of the CKI family contain a catalytic domain in the N-terminal portion of the protein. This domain structurally resembles that of other protein kinases. The main differences are observed in subdomain VIII, in which the common peptide triplet Ala-Pro-Glu is replaced by Ser-Ile/Val-Asn, and in subdomain XI, in which a normally invariant arginine residue is absent.

The substrate specificity is determined by acidic or phosphoryl groups located three to four residues upstream of the target residue. Some CKI isoforms can be classified as dual-specificity protein kinases, because they can phosphorylate tyrosine as well as serine or threonine residues [3,4]. Several CKI substrates have been identified, such as glycogen synthase, RNA polymerases I and II, p53, spectrin and NF-AT4, among others [2]. However, only in a few instances has phosphorylation by CKI been shown to correlate with changes in substrate function, such as in the case of the SV40 T antigen [5] or the NF-AT4 transcription factor [6].

CKI has been implicated in several aspects of cell regulation. In mammals it has been found associated with the adaptor protein Nck [7], and it can phosphorylate the 14-3-3 protein [8],

suggesting a role in signal transduction. It has been shown recently, in *Xenopus* and *Caenorhabditis elegans*, that CKI is a component of the Wnt signaling pathway, which controls a variety of processes including cell growth and development [9]. A CKI gene (*Dbt*) has been cloned in *Drosophila* [10]. The DBT protein is essential for viability, and is involved in maintaining circadian rhythms. CKI homologues in budding and fission yeast may regulate aspects of cellular DNA metabolism and are involved in DNA repair [11,12]. *Saccharomyces cerevisiae* Hrr25 is involved in double-strand break repair, in sporulation and in the cell cycle, and it has also been shown recently to be involved in vesicle budding from the endoplasmic reticulum [13]. A pair of related CKI homologues, Hhp1 and Hhp2, play similar roles in DNA repair in *Schizosaccharomyces pombe*.

The *Sa. cerevisiae* *YCK1* and *YCK2* genes supply an activity essential for vegetative growth, and confer halotolerance when overexpressed [14], and *YCK2* also plays a role in cytokinesis [15]. Overexpression of *Cki2* in *Sch. pombe* leads to a severe growth defect and aberrant morphology [16]. Ultraviolet irradiation of early *Drosophila* embryos relocates CKI to the interphase nucleus, suggesting a possible requirement for this enzyme in mechanisms associated with DNA repair [17].

Dictyostelium discoideum is a simple eukaryote that shows, in the course of its life cycle, a period of cell differentiation and morphogenesis, which makes it an attractive system for addressing a wide variety of biological problems.

In this organism, development is triggered by the removal of the food source from vegetatively growing amoebae. Cells thereupon aggregate to form a multicellular mound, by means of chemotaxis to pulsatile cAMP signals acting through cell-surface

Abbreviations used: Bsr, blastocidin; CK, casein kinase; DdCKI, casein kinase I gene and gene products from *Dictyostelium discoideum*; IPTG, isopropyl- β -D-thiogalactoside; K38R, CKI mutation in which the lysine-38 residue is substituted with arginine.

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receptors [18]. cAMP is also involved in the expression of genes essential for aggregation, entry into the morphogenetic stage, pre-spore cell differentiation and culmination [19]. Cellular differentiation ultimately gives rise to the spore and stalk cells of the fruiting body. All the internal cAMP responses are mediated by cAMP-dependent protein kinase [20].

In *D. discoideum*, the unique *CKII* gene has been shown to be essential for vegetative growth [21]. This CKII, unlike its higher eukaryotic counterparts, lacks the β subunit [21,22]. Several CKI activities have been characterized on the basis of their biochemical and chromatographic properties [23].

In the present report we describe the isolation and characterization of a *CKI* gene from *D. discoideum* (*DdCKI*), the analysis of its expression in the life cycle of the organism, its intracellular localization and its genetic analysis.

MATERIALS AND METHODS

Growth and development of *D. discoideum*

AX-2 cells were cultured in exponentially modified HL-5 medium (peptone was substituted for tryptone) [24], and differentiated on nitrocellulose filters in Mes/PDF buffer (20 mM KCl, 1.2 mM MgSO_4 , 7.5 mM Mes, pH 6.5).

For the developmental time-course assays, cells were plated on to sterile 4.5-cm diam. plates (0.5×10^8 – 1×10^8 cells/plate). At different time points, the cells were rapidly scraped from the plate and frozen in liquid N_2 until further use. Synchronization experiments were performed as described previously [25].

Plating assay for sensitivity to DNA-damaging agents

For viability assays, 2×10^6 cells/ml were cultured for 20 h in modified HL-5 medium with 100 or 200 mM hydroxyurea (Sigma) or 100 μM cisplatin (*cis*-diamine-dichloroplatinum II) (Sigma). Control cells were kept for a similar time without the addition of drugs. In all treatments, a volume, equivalent to 350 cells of untreated control cells, was plated on to SM plates with a lawn of *Klebsiella aerogenes* to determine viability. The surviving colonies were monitored after three or four days. The percentage of viability was calculated as the number of colonies obtained following the various treatments compared with the number of control cells.

D. discoideum extracts and S-Sepharose chromatography

For Western-blot experiments, *Dictyostelium* cells were lysed by sonication (vegetative or early differentiated cells) or with glass beads (late differentiated cells) in a buffer containing 50 mM Tris/HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA, 40 mM sodium pyrophosphate, 30 % (w/v) sucrose, 0.5 mg/ml leupeptin, 50 $\mu\text{g}/\text{ml}$ aprotinin, 0.1 mM tosyl-lysylchloromethane ('TLCK') and 0.1 mM Na_3VO_4 .

Cytosolic extracts, prepared in the same buffer without NaCl, were applied to an S-Sepharose (5 ml) column equilibrated in 50 mM Tris/HCl (pH 7.5), 2 mM EDTA, 5 mM 2-mercaptoethanol, 10 mM sodium pyrophosphate, 20 % (v/v) glycerol and 10 mM NaF. After washing with 10 volumes of equilibration buffer, the bound proteins were eluted with a linear gradient of 0–1 M NaCl, and 10 μl aliquots from each fraction were assayed for casein kinase activity using casein as substrate. Nuclei were prepared as described previously [26].

Southern- and Northern-blot hybridization analysis

Southern- and Northern-blot hybridization were done using standard protocols [27]. Hybridization was performed at high

stringency in buffer containing 50 % (v/v) formamide at 42 °C, or at 37 °C for low stringency conditions. DNA was obtained from vegetative cells as described in [28], and 1 μg was digested with various restriction enzymes, resolved on a 0.7 % (w/v) agarose gel and transferred to a nylon membrane for hybridization.

For Northern-blot analysis, total RNA from vegetative and differentiated cells was isolated by the method of Chomczynski et al. [29]. Poly(A)⁺ mRNA was obtained as in [30]. For reverse transcription-PCR, total RNA was prepared using Trizol (Gibco BRL), following the manufacturer's conditions.

Molecular cloning of *DdCKI*

PCR primers were designed using the yeast Yck2 sequence as a guide. A forward primer (5' CAACATATTCCTGACAGGGGA) and a reverse primer (5' ATCGTCTCTTCTGGATTGTTC) were used to amplify a 99-bp fragment from yeast genomic DNA. This PCR product was sequenced and used as a probe to screen a *Dictyostelium* cDNA library from 4-h differentiated cells cloned in $\lambda\text{gt}11$ (Clontech). Recombinant phages (160 000) were screened, in duplicate, with the yeast probe labelled with random primers and [α - ^{32}P]dATP (5000 Ci/mmol), Amersham). Hybridization was done under low stringency conditions, and positive clones were plaque purified. One of the positive clones (clone 1) was isolated and subcloned in pUC19. This clone contained a 1200-bp insert with a single open reading frame and a missing 5' initiation codon. The 5' initiation site was found by rescreening the cDNA library under stringent conditions using clone 1 cDNA as a probe (clone 2).

To obtain the complete gene (*pBS-CKI*), the *EcoRI/PstI* fragment of clone 1 was replaced with the same fragment of clone 2 (the *PstI* site in the clone 1 polylinker was previously eliminated by cutting, filling and ligation).

Recombinant *DdCKI* constructs

Three different constructs were obtained. First, in the pRSETc (Invitrogen) vector to express recombinant *CKI* in bacteria. For this construct, a *PvuII* site was engineered to clone the complete *CKI* coding region in-frame with the His₆ tag present in the vector. Secondly, a fusion with the maltose binding protein, to obtain antibodies against *CKI*. This construct was made with an *EcoRI/HindIII* fragment of the *CKI* gene. Thirdly, a construct to express the *CKI* gene constitutively under the Act-15 *D. discoideum* promoter. We used the pDXA-HC vector [31] that gives protein tagged with the c-myc epitope in the C-terminus. The *CKI* gene was amplified with primers (sense, 5' ACGTGGTACCGATTTAAGAATTGGTGG, and antisense, 5' AACACAACAGGCCACCAGCAAAGACTCGAGATCGG) in order to create *KpnI* and *XhoI* sites respectively (underlined) to clone into the corresponding sites of the pDXA-HC vector. Finally, for gene replacement, the 1.4-kb blasticidin (*BsrS*) cassette, obtained by digestion of pBsrS 2 [32] with *SmaI* and *HindIII*, was shunted and inserted into the *NcoI* site of the *CKI* gene. The 2.7 kb fragment of *DdCKI* harbouring the *BsrS* cassette was excised with *EcoRI* and used to transform *D. discoideum* cells.

D. discoideum transformation

Transformation was performed by electroporation, using 20–30 μg of the final constructs. Transformants were selected at 5 $\mu\text{g}/\text{ml}$ G418 or 5 $\mu\text{g}/\text{ml}$ *BsrS*, according to the construct used. Colonies became visible after about 15 days. The resistant clones

were plated on to a lawn of *K. aerogenes* grown on SM nutrient agar plates. The plates were incubated at 23 °C, and individual colonies were removed, grown in a HL5 medium with 10 µg/ml G418 or 10 µg/ml BsrS and analysed.

Purification of His₆-tagged CKI

Wild-type *Dictyostelium* cells or cells expressing His₆-CKI were cultured in modified HL5 medium and harvested at 4×10^6 cells/ml. After washing once with cold PBS, the cells were resuspended in lysis buffer A [20 mM Hepes (pH 7.5), 10 mM 2-glycerophosphate, 5 mM 2-mercaptoethanol, 1 mM Na₃VO₄, 20 mM imidazole, 0.1 % (v/v) Triton X-100] with 40 µg/ml aprotinin, 50 µg/ml leupeptin and 1 mM tosyl-lysylchloromethane. The cell extracts were centrifuged at 100 000 g for 30 min at 4 °C. Equal amount of protein from the supernatants were loaded on to Ni²⁺-agarose columns (2 ml) equilibrated with lysis buffer. After washing with 10 column volumes of the same buffer, the bound protein was eluted step-wise with 150 mM and 300 mM imidazole in the same buffer without Triton. The eluted fractions were used for Western-blotting and CK assays *in vitro*.

Purification of CKI expressed in bacteria

Escherichia coli strain BL21(DE3) was transformed with the pRSETc construct. A single colony was removed and grown at 30 °C to a D_{600} of 0.8. Isopropyl-β-D-thiogalactoside (IPTG) was added to 0.4 mM, and the cells were incubated for 1 h at 37 °C. The induced cells were harvested, washed and resuspended in 5 vol. of lysis buffer B [20 mM Tris/HCl (pH 7.9), 0.5 M NaCl, 10 % (v/v) glycerol, 5 mM imidazole] containing 1 mM PMSF and 25 µg/ml each of leupeptin, pepstatin and aprotinin. The cell suspension was sonicated and the resulting lysate was cleared by centrifugation at 15 000 g for 20 min. The supernatant was loaded on to a 1 ml Ni²⁺-agarose column pre-equilibrated in lysis buffer B containing 0.05 % (v/v) Nonidet P-40. The column was washed with 15 vol of the same buffer, and the proteins were eluted with sequential 5 ml volumes of lysis buffer B containing 0.02 % (v/v) Nonidet P-40 and 50, 100, 200 or 500 mM imidazole. The fractions collected were analysed for protein by Western-blotting and protein kinase activity.

Production of antibodies against CKI

E. coli strain TG1 was transformed with pMal-NCKI. Cells containing the pMal-NCKI plasmid were grown at 37 °C to a D_{600} of 0.4–0.6. IPTG was added to 0.3 mM and the cells were incubated for 3 h. The induced cells were harvested, washed and resuspended in 5 vol of 10 mM Tris/HCl, pH 8, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, with 10 mM NaF and 25 µg/ml each of leupeptin and aprotinin. The cell suspension was sonicated and the resulting lysate was cleared by centrifugation at 15 000 g for 20 min. The recombinant protein was purified from the *E. coli* extracts by chromatography on an amylose column using standard protocols. To generate antibodies against DdCKI, rabbits were immunized with the purified maltose-binding-protein fusion protein, which had been excised and extracted from an SDS/polyacrylamide gel.

CK assays

Two different assays were used to measure CKI activity. In some assays p-81 paper was used to measure the ³²P-label incorporated into casein [23], and in others the radioactivity incorporated into casein was monitored by SDS/PAGE and autoradiography of

the gels. The assay was performed in a final volume of 30 µl containing 50 mM Tris/HCl (pH 7.4), 10 mM MgCl₂, 0.2 mM Na₃VO₄, 2–20 µM [γ -³²P]ATP (800–1000 c.p.m./pmol), 10 mg/ml casein and 10–30 µg of the protein fraction to be assayed.

Immunofluorescence analysis

For staining intact cells, the following procedure was used. Cells (10⁸) growing in modified HL-5 medium were spotted on to coverslips, which had been pre-treated with gelatin, and were centrifuged at 2000 g for 10 min in a Cyto-spin centrifuge. The harvested cells were washed once in PBS and fixed in 3.7 % (v/v) paraformaldehyde in PBS for 15 min, followed by incubation in 5 mM NH₄Cl in PBS for 5 min. After washing with PBS and permeabilization with 0.2 % (v/v) Triton for 8 min, the cells were washed once in PBS and blocked with 5 % (v/v) fetal-calf serum in PBS for 30 min. The coverslips were then incubated with affinity-purified CKI antibody in PBS for 1 h at room temperature. A pre-immune serum was used as a control. The coverslips were washed five times (10 min each) with PBS and then incubated for 1 h at room temperature with a fresh 1:200 dilution of Rhodamine-labelled goat anti-rabbit IgG (Dianova). Coverslips were washed as above and mounted in Mowiol. Some samples were stained with 0.1 µg/ml 4,6-diamidino-2-phenylindole ('DAPI') in PBS for 2–5 min.

Immunoblotting

Purified fusion proteins or 10–50 µg of protein from bacterial or *Dictyostelium* extracts were subjected to SDS/PAGE (10–12 % gels) and transferred to Immobilon filters (Millipore) in 12.5 mM Tris/HCl (pH 8.5), 86 mM glycine and 15 % methanol. Membranes were blocked in Tris-buffered saline containing 0.05 % (v/v) Tween-20 and 5 % (w/v) non-fat dried milk or, for the His₆-tagged proteins, 3 % (w/v) BSA. Primary and secondary antibodies were diluted in the same buffer. Bound antibodies were detected by enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

RESULTS

Characterization of a *D. discoideum* CK

The screening of a *D. discoideum* cDNA library with a 99-bp PCR product obtained from yeast yielded one positive clone. The cDNA insert of this clone (clone 1) was sequenced completely. Clone 1 represented a cDNA that encodes a sequence with high similarity to higher eukaryotes and yeast CKI, and did not contain the initiation site. The cDNA of clone 1 was used as probe to rescreen the cDNA library. Several additional clones were isolated, and three of them were analysed (clones 2, 3 and 4). The lengths of the clones were 1122, 1152 and 1140 nt respectively.

Clone 2 was truncated at the 3' end and contained the initiation site, 84 nt of 5'-untranslated sequence and 1012 nt overlapping the sequence of clone 1. Clone 3 was also 3' truncated and contained the initiation site, 26 nt of untranslated sequence and 1104 nt overlapping the sequence of clone 1 and clone 4 which was 5' truncated. The complete coding sequence (pBS-CKI) was obtained as described in the Materials and methods section.

The putative ATG initiation codon lies within a translation initiation consensus sequence defined by Kozak [33], and it is immediately preceded by a stop codon in the same reading frame. Moreover, the predicted start site is consistent with that of other CKI isoforms. The stop codon present in the cDNA of clones 1 and 4 includes the first nucleotide of the *Eco*RI cloning

Figure 1 Alignment of *D. discoideum* CKI sequences
Comparison of the primary structure of the kinase domains of *D. discoideum* (DdCk1), human (h) CKIε and CKIδ, yeast (y) kinases Hrr25, Hhp1 and Hhp2, and *Drosophila* CKIε (dDBT). Residues that are identical for all members of the CKI family are indicated with dots.

Figure 2 Southern- and Northern-blot analysis of DdCKI at different developmental stages
(A) Map of the *DdCKI* gene. Genomic DNA was digested with *Eco*RI (E), *Hind*III (H), *Pst*I (Ps), and *Bgl*II (B). (B) Total RNA (30 µg, lane 1) or poly (A)⁺ RNA (5 µg, lane 2) from 4 h-differentiated cells were resolved on an agarose/formaldehyde gel, transferred to a nylon membrane and probed with the insert of clone 1 labelled by random priming. (C) Top panel: total RNA (6 µg/lane) was prepared from samples taken at various times after cell starvation. V, vegetative cells. Bottom panel: rRNA is shown as an indicator of gel loading. (D) Top panel: Poly(A)⁺ RNA (5 µg/lane) was prepared from cells synchronized by the stationary phase release method. Aliquots of cells were taken after resuspension in fresh medium for the indicated time periods. S, stationary cells. Bottom panel: the same filter hybridized with a myosin probe as an indicator of gel loading.

Figure 3 Immunoblot analysis of CKI

(A) pMal-CKI was grown in *E. coli* strain TG1 and the cells were induced with IPTG (+) or left untreated (–). Cell extracts, purified by amylose chromatography, were resolved on SDS/PAGE (10% gel) and the proteins were transferred to Immobilon membranes and incubated with preimmune (1) or immune (2) serum produced against recombinant CKI. Molecular mass markers (in kDa) are shown on the left, and the mass of the recombinant protein detected by the antibody is shown on the right. (B) Soluble *Dictyostelium* extracts (5 µg), prepared as described in the Materials and methods section were resolved on SDS/PAGE (10% gel), and a Western blot analysis was performed as described for (A); 1, preimmune serum; 2, immune serum. (C) CK activity assay in fractions eluted from S-Sepharose, performed as indicated in the Materials and methods section. The upper part of the Figure shows a Western blot of the various fractions, using the DdCKI antibody. (D) Soluble *Dictyostelium* extracts (10 µg) from cells differentiated for 4, 8, 18 or 20 h were used for Western-blot analysis as in (A). V, vegetative asynchronously growing cells. (E) Soluble *Dictyostelium* extracts (10 µg) from synchronized vegetative cells were used for Western-blot analysis as described above. S, stationary cells. Time (hrs), time after resuspension in fresh axenic medium at which the samples were removed.

site. Translation of the complete 1284 nt open reading frame predicts a 426-amino-acid polypeptide with a molecular mass of 48800. The predicted polypeptide has a calculated pI of 9.7.

The DdCKI contained the 12 conserved subdomains of protein kinases described by Hanks et al. [34]. The N-terminal 300 amino acids of DdCKI show more than 50% identity with other CKI family members (Figure 1). The greatest similarity, when the catalytic domains are compared, has been found in *Drosophila* CKI ϵ (70%), human CKI ϵ and δ (71%) and yeast Hhp1 (65%).

The hydrophilic segment, located downstream of the catalytic domain, was difficult to align and corresponded to a poorly conserved region among the different family members. The DdCKI C-terminal region was extremely rich in glutamine, as is the case of yeast Yck1 and Yck2 and Hhp1. However, stretches of glutamine and asparagine are very frequent in *D. discoideum* proteins [35].

Analysis of the *DdCKI* gene

Southern-blot analysis, performed using the cDNA of clone 1 as probe under stringent conditions, gave single bands, suggesting that a single gene encodes *DdCKI*. Only when the hybridization was performed at low stringency conditions were several bands observed, which may correspond to other related genes in *Dictyostelium* (results not shown). A physical map of the gene

has been constructed using genomic DNA and a combination of restriction enzymes (Figure 2A). The enzymes selected did not excise or excised only once within the cDNA sequence. As can be seen in Figure 2(B), the results obtained from the Southern-blot analysis indicate the presence of an *EcoRI* site at the end of the coding region.

Analysis of *DdCKI* mRNA expression

DdCKI expression was analysed with total or poly(A)⁺ RNA from the different developmental stages of *D. discoideum*, using the cDNA of clone 1 as a probe. As shown in Figure 2(B), there was a major message at 2.2 kb. The expression level of the mRNA did not change during the developmental programme (Figure 2C).

To determine if the DdCKI mRNA levels were regulated in the different phases of the cell cycle, synchronized cells were used. As shown in Figure 2(D), there were no significant changes in DdCKI mRNA expression during the cell cycle.

Antibody to DdCKI recognizes a 49-kDa *D. discoideum* protein

In Western-blot assays the polyclonal antibody raised against the N-terminal fragment of DdCKI recognized the recombinant

Figure 4 Expression and activity assay of DdCKI

(A) *E. coli* BL21(DE3) containing plasmid pLys was transformed with the expression vector pRSETc-CKI, as described in the Materials and methods section. After induction for 1 h with IPTG, cells were treated with 0.1 mg/ml rifampicin for the indicated time periods and labelled with 0.5 μ Ci of [35 S]methionine (Amersham) for 5 min. The cells were pelleted by centrifugation, lysed in sample buffer and analysed by SDS/PAGE and autoradiography. Molecular-mass markers (in kDa) are shown on the left, the molecular masses of the recombinant protein and of the major degradation products are shown on the right. (B) Recombinant DdCKI containing the His₆ tag was produced purified with Ni²⁺-agarose chromatography, as described in the Materials and methods section, and prepared for SDS/PAGE and Western blotting. 1, Coomassie Blue staining of SDS/polyacrylamide gel; 2, immunoblot of sample 1 probed with anti-His₆-tag antibody; 3, immunoblot of sample 1 probed with anti-CKI antibody; 4, autoradiogram of sample 1 incubated in the presence of Mg²⁺ and [γ -³²P]ATP. (C) Phosphorylation of casein by DdCKI. Recombinant purified CKI was incubated with [γ -³²P]ATP with 5 μ g of each putative protein substrate in kinase assay buffer. MBP, myelin basic protein. The phosphorylated proteins were resolved by SDS/PAGE (14% gel) and analysed by autoradiography.

protein induced and purified from bacteria as a maltose-binding-protein fusion (Figure 3A). This antibody also recognized a 49-kDa protein band in *Dictyostelium* extracts present in vegetative cells as well as in late differentiated cells (Figure 3B). Moreover, when *Dictyostelium* protein extracts were separated on S-Sepharose, the CK activity eluting at 0.25 M NaCl co-migrated with the 49-kDa band (Figure 3C).

Changes in protein levels during differentiation were not detected (Figure 3D). The protein level profile obtained for synchronized cells showed a slight decrease in the amount of protein after duplication of the cell numbers (Figure 3E).

Expression of DdCKI in *E. coli*

It appears that the CKI enzymes may interfere with bacterial cellular functions, as it was difficult to achieve high levels of DdCKI expression. Two different induction temperatures, 25 °C

and 37 °C, were used and no differences in expression were found with either. Short inductions were performed, because the protein was proteolysed at longer induction times, giving rise to several defined proteolytic products (Figure 4A).

The high degree of similarity between DdCKI and other CKI isoforms strongly suggested that DdCKI encoded a CKI. To confirm this, a recombinant protein with a His₆ tag was obtained, as described in the Materials and methods section. After IPTG induction, affinity-purified extracts of cells carrying the DdCKI construct contained a 60-kDa protein that reacted with the His₆-tag antibody and with the anti-DdCKI antibody (Figure 4B).

When the purified recombinant DdCKI protein was incubated in the presence of [γ -³²P]ATP, the protein band identified by the CKI antibody appeared to be phosphorylated (Figure 4B). We then tested several CKI substrates (Figure 4C), and it was found that DdCKI phosphorylated casein but not myelin basic protein or histone, as was expected. Phosphorylation of the tyrosine-containing polymer poly-E₄Y₁ was not detected. The effect of heparin and polylysine, with casein as substrate, was tested in different experimental conditions. The effect of these two polycations varied depending on the NaCl concentration in the assay; when the NaCl concentration was high (0.1 M), heparin and polylysine behaved as slight activators, but when low (10 mM) NaCl concentrations were used, heparin inhibited CKI activity and polylysine modified the specificity of CKI for phosphorylation of different casein subunits.

Localization of DdCKI by immunofluorescence

Affinity-purified CKI antibodies were used to study the intracellular distribution of DdCKI in vegetative *D. discoideum* cells. The labelling specificity was confirmed in experiments where immunofluorescent staining was performed with a pre-immune serum. Cells treated with secondary antibodies alone showed no staining (results not shown). As shown in Figure 5 both cytoplasm and nucleus were stained by the antibodies, indicating that DdCKI is localized in both cellular compartments.

Genetic analysis of DdCKI function

The gene-expression data suggest that the CKI protein might be involved in different processes throughout the life cycle of *Dictyostelium*. We have attempted to determine the function of the gene in *Dictyostelium* by either disrupting or over-expressing the CKI gene.

To create a null strain for CKI, *Dictyostelium* cells were transfected with a gene replacement vector containing a BsrS cassette, as described in the Materials and methods section. Several attempts to obtain blasticidine-resistant clones with the CKI gene disrupted were unsuccessful.

In two independent experiments, seven blasticidine-resistant clones were obtained, and no homologous recombination was found when analysed by Southern blotting and PCR. Southern-blot analysis of one blasticidine-resistant clone (Figure 6A), showed that all the bands corresponding to the CKI wild-type gene were present (marked with crosses), indicating that the blasticidine-cassette containing construct had been inserted at different places of the genome. As can be seen in Figure 6(B), the clones obtained expressed the blasticidine-resistance gene, as well as the endogenous CKI gene.

A different strategy was adopted to study CKI gene function. The complete coding region of CKI downstream of the actin promoter was cloned, using the pDXA-HC vector. A similar construct was also used with the mutation Lys³⁸ → Arg (K38R).

Figure 5 Immunolocalization of DdCKI in untransformed (AX-2) cells

Asynchronous cells were prepared as described in the Materials and methods section. (a) Cells stained with preimmune serum; (b) cells stained only with affinity-purified anti-CKI antibody; (c) cells stained with affinity-purified anti-CKI antibody; (d) the same field of cells as in (c) stained with 4,6-diamidino-2-phenylindole. The arrowheads indicate the cells that show nuclear staining for CKI. Magnification $\times 63$.

Lysine-38 is required for activity in other members of the CKI family, and has been shown to act as a dominant-negative form of the protein.

Cells resistant to G418 were selected, as described in the Materials and methods section. In order to check that the transfected constructs were expressed in the G418-resistant cells, total RNA was prepared and reverse transcription-PCR was performed. For the PCR, a sense oligonucleotide designed according to the CKI sequence, and an antisense oligonucleotide designed according to the vector sequence was used. Several clones expressed the construct containing the wild-type sequence, but none was obtained containing the K38R mutation after two independent transfection experiments (results not shown). The clones containing the wild-type cDNA of CKI were verified by Northern-blot analysis using cDNA of CKI as a probe (Figure 7A).

In order to analyse if a functional protein was being produced, cytoplasmic extracts were made from vegetative growing untransformed cells and cells containing clone 4 expressing the His₆-CKI-Myc tagged protein. Extracts containing an equal amount of protein were separated on Ni²⁺-agarose, as described in the Materials and methods section. The presence of the recombinant protein was assayed using a human Myc antibody. As can be seen in Figure 7(B), a protein band of the expected mobility was clearly detected in the eluates from the CKI-expressing strain but not from the wild-type cells. The ability of the expressed protein to phosphorylate casein was assayed by incubating an aliquot of these fractions with casein and [γ -³²P]ATP; the products were

separated by SDS/PAGE and detected by autoradiography (Figure 7B). The 150 mM imidazole fractions showed casein phosphorylating activity which was higher in eluates from the His₆-CKI-expressing cells than in the control. This result indicates that the CKI expressed in the transformed cells is a functional protein. Nuclei were prepared from untransformed cells and two different transformants and, as can be seen in Figure 7(C), the overexpressed protein was also found in the nuclear fraction of the cell.

When the phenotype of the cells overexpressing *DdCKI* was examined, no growth or developmental phenotype was observed, nor was any difference found in the migration of the slugs towards a luminous source.

The response to DNA-damaging agents, such as cisplatin and hydroxyurea, was also examined under conditions known to decrease the viability of *Dictyostelium* cells [36,37]. The transfected cells showed more resistance than untransfected control cells to treatment with hydroxyurea (Figure 7D); no difference was found after the cisplatin treatment.

DISCUSSION

Using a PCR strategy, we have cloned the first *DdCKI*. Several cDNA clones were isolated and analysed from a cDNA library. Sequence analysis of this cDNA indicated that it encodes a single reading frame comprising 426 amino acids. Two of the clones analysed have a stop codon in the *EcoRI* cloning site. We believe

Figure 6 Southern- and Northern-blot analysis of blasticidine-resistant clones

(A) Genomic DNA from blasticidine-resistant clone 2 was cleaved with various restriction enzymes and probed with DdCKI cDNA. *EcoRI* (E), *HindIII* (H), *EcoRI* + *HindIII* (E + H), *BglII* (B), *EcoRI* + *BglII* (E + B), *PstI* (Ps) and *EcoRI* + *PstI* (E + Ps). Mass markers (in kb) are shown on the left. (B) Top panels: total RNA (6 μ g/lane), prepared as indicated in the Materials and methods section from various Bsr-resistant clones or from untransformed cells (AX2), was resolved on an agarose/formaldehyde gel, transferred to a nylon membrane and probed with the cDNA of the BsrS cassette or the cDNA of DdCKI labelled by random priming. Bottom panels: rRNA is shown as an indicator of gel loading.

that this *EcoRI* site is a constituent of the gene for two reasons. First, Southern-blot analysis indicates that the *EcoRI* site located in the 3'-coding region is a true site and not a cloning artifact. Secondly, the size of the protein detected by the antibody matches the predicted molecular mass of the coded protein.

The cDNA described in the present work belongs to the CKI protein family. The members of this family share several well-conserved motifs, such as LLGPSLEDLF (residues 86–95), HIPXR (residues 166–170), EXSRDD (residues 191–197) and LPWQGLKA (residues 213–220). Furthermore, this cDNA lacks the APE sequence in subdomain VIII, which is replaced by SLN

(residues 181–183). All these features are specific for CKI, and are probably important for protein–protein interactions.

On the basis of sequence identity, DdCKI is very close to the mammalian δ and ϵ isoforms and to the yeast Hhp1, Hhp2 and Hrr25, which are assumed to be located in the nucleus. This nuclear localization is postulated because of the sequence identity and because these isoforms contain a putative TTK/RQKY nuclear localization signal similar to the sequence thought to be involved in targeting other proteins, such as histone 2B, to the nucleus [38].

The C-terminal portion of DdCKI is rich in glutamine and proline residues. In *D. discoideum* a repetitive element, composed of the codon AAC, is found in developmentally regulated transcripts; these AAC sequences can be translated in all three reading frames in the proteins, giving rise to asparagine (ACC), threonine (ACA) or glutamine (CAA) [35]. Other proteins rich in glutamine and proline residues are a number of CKIs, such as Hrr25, Hhp1, Yck1 and Yck2, and transcription factors such as Sp1, PU1 or TAFII [39,40]. The function of these regions has been analysed in greater detail in transcription factors, where they have been shown to be involved in transcription transactivation through interaction with regulatory proteins [39].

Our Southern-blot hybridization data suggest that CKI-related isoforms might be present in *Dictyostelium*. This is in agreement with our previous biochemical studies, based mainly on the chromatographic behaviour, which revealed the presence of several CKI isoforms in this organism [23].

CKI is expressed throughout the whole life cycle of *Dictyostelium*

The results of the Northern-blot analysis performed with RNA from differentiated cells show a major 2.2-kb transcript. Two species have been detected in the rabbit, one of 3.5 kb, which is detected in most tissues, and one of 1.9 kb with a high level of expression in testis and heart [41]. Several such messages are also found in human cells [42]. No changes were detected either throughout differentiation or during the cell cycle of *Dictyostelium*, suggesting that CKI may have a role in the development of the slime mould. In parallel with the expression of DdCKI, Western-blot analysis showed that the protein was present throughout the differentiation programme of *Dictyostelium*. The rat and rabbit CKI δ , which are the CKs most similar to DdCKI, have a widespread distribution and have been detected in all tissues examined [41].

Antibodies generated against the N-terminal region of DdCKI recognized a 49-kDa protein, which was close to the predicted molecular mass based on the cDNA. A protein with the same molecular mass was detected when *Dictyostelium* cell extracts were prepared in the presence of high leupeptin concentrations or when cells were directly resuspended in sample buffer for Western-blot analysis, which suggests that this 49-kDa protein was not a proteolytic product. A wide range of molecular masses, ranging from 30 to 60 kDa, has been described for CKI isoforms. High-molecular-mass forms have been isolated from nuclei. However, the protein mass is not indicative of localization within the cell, as the largest CKI isoforms, yeast Yck1 and Yck2 [14] and Cki1 and Cki2 [16], are cytoplasmic membrane-associated proteins, while the human 34-kDa CKI α has been found associated to kinetochore fibres in mitosis [43].

DdCKI expressed in bacteria has CK activity

DdCKI has been expressed in bacteria, and the recombinant protein was very sensitive to proteolysis. The purified recom-

Figure 7 Expression of recombinant DdCKI in untransformed (AX2) cells

(A) Northern-blot analysis of RNAs (10 μ g) isolated from untransformed vegetative cells (AX2) or from three geneticin-resistant clones. cDNA from DdCKI was used as a probe. (B) Wild-type strain (AX2) and one Act15-His₆ CKI-Myc overexpressor (clone 4) were harvested at 4×10^6 cells/ml and lysed by sonication. Soluble extracts containing equal amounts of protein were purified by Ni²⁺-agarose as indicated in the Materials and methods section. Samples of the 150 mM or 300 mM fractions were used for immunoblotting or *in vitro* assay. Top panels: immunoblot probed with anti-Myc antibody. The mass of the recombinant protein detected by the antibody is shown on the left. Bottom panels: samples of the same fractions were used for *in vitro* phosphorylation of casein. (C) Nuclei from the wild-type strain (AX2) or from two clones (4 and 15) expressing ectopical DdCKI were prepared as indicated in the Materials and methods section. Nuclear extracts containing equal amounts of protein were used for Western blotting and probed with anti-Myc antibody. (D) Viability assay performed with 100 or 200 mM hydroxyurea (HU) or 100 μ M cisplatin (CIS), as indicated in the Materials and methods section, using untransformed (AX2) or clone 4 cells. The data are the means ($\pm$ S.E.M.) of triplicates for each treatment, determined by one-way analysis of variance ('ANOVA') and Tukey post-test. Control untreated cells = 100% viability. **, significantly different from AX2 cells, $P < 0.01$.

binant DdCKI autophosphorylated and phosphorylated casein, as expected, and did not phosphorylate basic proteins, such as myelin basic protein or histone H1. We have not detected phosphorylation of poly-E₄Y₁ under our assay conditions, in contrary to the recombinants Hhp1 and Hhp2 expressed in bacteria [3].

Studies on the effect of heparin on the activity of CKI from different sources have yielded inconsistent results. Inhibition was observed in some cases [44], whereas in others either no effect or activation was reported [45]. This also applies to the effect of polylysine on CKI activity; polylysine stimulates recombinant CKI α when assayed with D4 peptide, but no effect was detected when phosvitin was used as substrate [46].

When the effect of heparin on CKI activity was tested using casein as the substrate, we observed inhibition or activation depending on the salt concentration in the assay; a differential effect of polylysine at different salt concentrations was also observed. The experiments of Graves and Roach [47], using a truncated version of CKI δ , led them to postulate that the C-terminus of CKI δ was critical for the action of heparin, because the full-length enzyme was activated by heparin, whereas the mutant lacking the C-terminus was insensitive. Based on this observation, we suggest that the salt concentration probably

affects the structure of the C-terminal portion of the enzyme, although a change in substrate structure cannot be ruled out.

Immunolocalization of DdCKI

During its vegetative phase, the cellular slime mould *D. discoideum* grows as unicellular, haploid eukaryotic amoebae. Our immunofluorescence experiments indicate that the endogenous CKI is distributed in the cytoplasm and the nuclei of the vegetative cells. The overexpressed CKI is also detected in both the cytoplasmic and nuclear fractions, suggesting that this CKI might be playing a nuclear role, as has been proposed for some CKIs of other organisms [10,17,48,49].

Genetic analysis of DdCKI

Our genetic analysis suggests that DdCKI may be an essential gene for growth. We were unable to disrupt the gene by homologous recombination, and also to express the CKI K38R mutant using the same vector that allowed the expression of the wild-type protein. This suggests that CKI K38R might indeed be acting as a dominant negative, and lends support to the proposition that CKI might be essential for *Dictyostelium*. DdCKI

could be required to support the vegetative growth of the cells, in the same way that Hrr25 protein kinase is important for normal growth in yeast [11].

The observation that the cells from the overexpressing clone were more resistant to hydroxyurea treatment than the wild-type ones is consistent with a potential role of this protein in DNA repair mechanisms, as is the case in *Sch. pombe* [12] and *Sa. cerevisiae* [11].

One possible role for CKI would be in the Wnt signalling pathway, since CKI ϵ (72% homology with DdCKI in the catalytic domain) has been shown recently to be a conserved component of this pathway [9], and is involved in a variety of processes, including cell growth and development. Glycogen synthase kinase 3, a component of this pathway in metazoa, also regulates cell fate during *Dictyostelium* development.

We are at present preparing constructs of dominant negative and constitutively active versions of *DdCKI* under the promoters specific for stalk and spore genes, with the aim of obtaining further information on the potential role of this kinase in cell differentiation.

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REFERENCES

- Issinger, O. G. (1993) Casein kinases: pleiotropic mediators of cellular regulation. *Pharmacol. Ther.* **59**, 1–30.
- Gross, S. D. and Anderson, R. A. (1998) Casein kinase I: spatial organization and positioning of a multifunctional protein kinase family. *Cell. Signal.* **10**, 699–711.
- Hoekstra, M. F., Dhillon, N., Carmel, G., DeMaggio, A. J., Lindberg, R. A., Hunter, T. and Kuret, J. (1994) Budding and fission yeast casein kinase I isoforms have dual-specificity protein kinase activity. *Mol. Biol. Cell* **5**, 877–886.
- Pulgar, V., Tapia, C., Vignolo, P., Santos, J. and Sunkel, C. E. (1996) The recombinant a isoform of protein kinase CK1 from *Xenopus laevis* can phosphorylate tyrosine in synthetic substrates. *Eur. J. Biochem.* **242**, 519–528.
- Cegielska, A. and Virshup, D. M. (1993) Control of simian virus 40 DNA replication by the HeLa cell nuclear kinase casein kinase. *Mol. Cell. Biol.* **13**, 1202–1211.
- Zhu, J., Shibasaki, F., Price, R., Guillemot, J. C., Yano, T., Dötsch, V., Wagner, G., Ferrera, P. and McKeon, F. (1998) Intramolecular masking of nuclear import signal on NF-AT4 by casein kinase I and MEKK1. *Cell* **93**, 851–861.
- Lussier, G. and Larose, L. (1997) A casein kinase I activity is constitutively associated with Nck. *J. Biol. Chem.* **272**, 2688–2694.
- Dubois, T., Rommel, C., Howell, S., Steinhilber, U., Soneji, Y., Morrice, N., Moelling, K. and Aitken, A. (1997) 14-3-3 is phosphorylated by casein kinase I on residue 233. Phosphorylation at this site *in vivo* regulates Raf/14-3-3 interaction. *J. Biol. Chem.* **272**, 28882–28888.
- Peters, J. M., McKay, R. M., Mackay, J. P. and Graft, J. M. (1999) Casein kinase I transduces Wnt signals. *Nature (London)* **401**, 345–350.
- Kloss, B., Price, J. L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C. S. and Young, W. (1998) The *Drosophila* clock gene double-time encodes a protein closely related to human casein kinase I ϵ . *Cell* **94**, 97–107.
- Hoekstra, M. F., Liskay, R. M., Ou, A. C., DeMaggio, A. J., Burbee, D. G. and Heffron, F. (1991) HRR25, a putative protein kinase from budding yeast: association with repair of damaged DNA. *Science* **253**, 1031–1034.
- Dhillon, N. and Hoekstra, M. F. (1994) Characterization of two protein kinases from *Schizosaccharomyces pombe* involved in the regulation of DNA repair. *EMBO J.* **13**, 2777–2788.
- Murakami, A., Kimura, K. and Nakano, A. (1999) The inactive form of a yeast casein kinase I suppresses the secretory defect of the sec12 mutant. Implication of negative regulation by the Hrr25 kinase in the vesicle budding from the endoplasmic reticulum. *J. Biol. Chem.* **274**, 3804–3810.
- Robinson, L. C., Menold, M. M., Garret, S. and Culbertson, M. R. (1993) Casein kinase I-like protein kinases encoded by Yck1 and Yck2 are required for yeast morphogenesis. *Mol. Cell. Biol.* **13**, 2870–2881.
- Robinson, L. C., Bradley, C., Bryan, J. D., Jerome, A., Kweon, Y. and Panek, H. R. (1999) The Yck2 yeast casein kinase 1 isoform shows cell cycle-specific localization to sites of polarized growth and is required for proper septin organization. *Mol. Biol. Cell* **10**, 1077–1092.
- Wang, P. C., Vancura, A., Desai, A., Carmel, G. and Kuret, J. (1994) Cytoplasmic forms of fission yeast casein kinase-1 associate primarily with the particulate fraction of the cell. *J. Biol. Chem.* **269**, 12014–12023.
- Santos, J. A., Logarinho, E., Tapia, C., Allende, C. C. and Allende, J. E. (1996) The casein kinase 1 α gene of *Drosophila melanogaster* is developmentally regulated and the kinase activity of the protein induced by DNA damage. *J. Cell Sci.* **109**, 1847–1856.
- Firtel, R. A. (1991) Signal transduction pathways controlling multicellular development in *Dictyostelium*. *Trends Genet.* **7**, 14875–14883.
- Williams, J. G. (1991) Regulation of cellular differentiation during *Dictyostelium* morphogenesis. *Curr. Opin. Genet. Dev.* **1**, 358–362.
- Wang, B. and Kuspa, A. (1997) *Dictyostelium* development in the absence of cAMP. *Science* **277**, 251–254.
- Kikkawa, U., Mann, S. K., Firtel, R. A. and Hunter, T. (1992) Molecular cloning of casein kinase IIa subunit from *Dictyostelium discoideum* and its expression in the life cycle. *Mol. Cell. Biol.* **12**, 5711–5723.
- Ospina, B., Nuñez, A. and Fernández-Renart, M. (1992) Purification of a soluble casein kinase II from *Dictyostelium discoideum* lacking the β subunit: regulation during proliferation and differentiation. *Mol. Cell. Biochem.* **118**, 49–60.
- Ospina, B. and Fernández-Renart, M. (1990) Characterization of three casein kinases type I from *Dictyostelium discoideum*. *Biochim. Biophys. Acta* **1052**, 483–488.
- Watts, D. J. and Asworth, J. M. (1970) Growth of *Myxamoebae* of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *Biochem. J.* **119**, 171–174.
- Weijer, C. J., Duschl, G. and David, C. D. (1984) A revision of the *Dictyostelium discoideum* cell cycle. *J. Cell Sci.* **70**, 111–131.
- Knetsch, M. L., Epskamp, J. P., Schenk, P. W., Wang, Y., Segall, J. E. and Snaar-Jagalska, B. E. (1996) Dual role of cAMP and involvement of both G-proteins and ras in regulation of ERK2 in *Dictyostelium discoideum*. *EMBO J.* **15**, 3361–3368.
- Sambrook, J. D., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Nellen, W., Data, 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.
- Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.
- Vennström, B. and Bishop, J. M. (1982) Isolation and characterization of chicken DNA homologous to the two putative oncogenes of avian erythroblastosis virus. *Cell* **28**, 135–143.
- Manstein, D. J., Schuster, H.-P., Morandini, P. and Hunt, D. M. (1995) Cloning vectors for the production of proteins in *Dictyostelium discoideum*. *Gene* **162**, 129–134.
- Adachi, H., Hasebe, T., Voshnaga, K., Ohta, T. and Sutoh, K. (1994) Isolation of *Dictyostelium discoideum* cytokinesis mutants by restriction enzyme-mediated integration of the blasticidin S resistance marker. *Biochem. Biophys. Res. Commun.* **205**, 1808–1814.
- Kozak, M. (1987) An analysis of 5' non-coding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15**, 8125–8130.
- Hanks, S. K., Quinn, A. M. and Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domain. *Science* **241**, 42–51.
- Shaw, D. R., Ichter, H., Giorda, R., Ohmachi, T. and Ennis, H. L. (1989) Nucleotide sequences of *Dictyostelium discoideum* developmentally regulated cDNAs rich in (AAC) imply proteins that contain clusters of asparagine, glutamine or threonine. *Mol. Gen. Genet.* **218**, 453–459.
- Yu, S.-L., Lee, S.-K., Alexander, H. and Alexander, S. (1998) Rapid changes of nucleotide excision repair gene expression following UV-irradiation and cisplatin treatment of *Dictyostelium discoideum*. *Nucleic Acids Res.* **26**, 3397–3403.
- Freeland, T. M., Guyer, R. B., Lind, A. Z. and Deering, R. A. (1996) Apurinic/aprimidinic (AP) endonuclease from *Dictyostelium discoideum* cloning, nucleotide sequence and induction by sublethal levels of DNA damaging agents. *Nucleic Acids Res.* **24**, 1950–1953.
- Moreland, R. B., Laugevin, G. L., Singer, R. H., Garces, R. L. and Herford, L. M. (1987) Amino acid sequences that determine the nuclear localization of yeast histone 2B. *Mol. Cell. Biol.* **7**, 4048–4057.
- Fisher, R. C., Olson, M. C., Pongubala, J. M. R., Perkel, J. M., Atchinson, M. L., Scott, E. W. and Simon, M. C. (1998) Normal myeloid development requires both the glutamine-rich transactivation domain and the PEST region of transcription factor PU1 but not the potent acidic transactivation domain. *Mol. Cell. Biol.* **18**, 4347–4353.

-
- 40 Saluja, D., Vassallo, M. F. and Tanese, N. (1998) Distinct subdomains of human TAFII 130 are required for interactions with glutamine-rich transcriptional activators. *Mol. Cell. Biol.* **18**, 5734–5743
- 41 Graves, P. R., Haas, D. W., Hagedorn, C. H., DePaoli-Roach, A. and Roach, P. J. (1993) Molecular cloning, expression, and characterization of a 49-Kilodalton casein kinase I isoform from rat testis. *J. Biol. Chem.* **268**, 6394–9401
- 42 Rowles, J., Slaughter, C., Moomaw, C., Hsu, J. and Cobb, M. H. (1991) Purification of casein kinase I and isolation of cDNAs encoding multiple casein kinase I-like enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9548–9552
- 43 Brockman, J. L., Gross, S. D., Sussman, M. R. and Anderson, R. A. (1992) Cell cycle-dependent localization of casein kinase I to mitotic spindles. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9454–9458
- 44 Singh, T. J. (1988) Polyamines stimulate the activity of glycogen kinase and different rat tissues. *Arch. Biochem. Biophys.* **267**, 167–175
- 45 Floyd, C. C., Grant, P., Gallant, P. E. and Pant, H. C. (1991) Principal neurofilament-associated protein kinase in squid axoplasm is related to casein kinase I. *J. Biol. Chem.* **266**, 4987–4994
- 46 Zhai, L., Graves, P. R., Longenecker, K. L., DePaoli-Roach, A. A. and Roach, P. J. (1992) Recombinant rabbit muscle casein kinase I α is inhibited by heparin and activated by polylysine. *Biochem. Biophys. Res. Commun.* **189**, 944–949
- 47 Graves, P. R. and Roach, P. J. (1995) Role of COOH-terminal phosphorylation in the regulation of casein kinase I δ . *J. Biol. Chem.* **270**, 21689–21694
- 48 Vancura, A., Sessler, A., Leichus, B. and Kuret, J. (1994) A prenylation motif is required for plasma membrane localization and biochemical function of casein kinase I in budding yeast. *J. Biol. Chem.* **269**, 19271–19278
- 49 Brockman, J. L. and Anderson, R. A. (1991) Casein kinase I is regulated by phosphatidylinositol 4,5 bisphosphate in native membranes. *J. Biol. Chem.* **266**, 2508–2512
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