The *Crithidia fasciculata* CRK gene encodes a novel cdc2-related protein containing large inserts between highly conserved domains

Lisa Brown, Jane C.Hines and Dan S.Ray*

Molecular Biology Institute and Department of Biology, University of California, Los Angeles, CA 90024, USA

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ABSTRACT

A gene (CRK) encoding a cdc2-related protein has been identified in the trypanosomatid *Crithidia fasciculata*. CRK has a high degree of sequence identity with the human cdc2 gene and contains the sixteen amino acid PSTAIR motif, characteristic of p34^{cdc2} protein-serine/threonine kinases, with four amino acid substitutions in the motif. In addition, two inserts of more than sixty amino acids have been found between conserved domains of this putative protein-serine/threonine kinase. CRK is a single copy gene and is expressed on a 3.8 kb mRNA. Anti-CRK antibodies detect a 53kDa protein in extracts of *C.fasciculata* in agreement with the size predicted from the nucleotide sequence of the cloned gene. These antibodies also recognize proteins of 48 and 60 kDa in extracts of the trypanosomatid *Leishmania tarentolae*. Antibodies against the human PSTAIR peptide detect the p34^{cdc2} protein in human nuclear extracts but fail to detect a 34 kDa protein in *C.fasciculata* extracts. These results suggest that novel higher molecular weight forms of the cdc2 protein family may be involved in cell cycle control in trypanosomes.

INTRODUCTION

The mitochondrial DNA present in the single mitochondrion of kinetoplastid protozoa consists of a highly catenated network of circular DNAs (kinetoplast DNA). Replication of both the nucleus and kinetoplast occurs only once per cell division (see 1 for review) and a single copy of each organelle is segregated to each daughter cell. In the trypanosomatid *Crithidia fasciculata*, the S phases of the kinetoplast and nucleus initiate at approximately the same time in the cell cycle and are of nearly equal length (2). Nuclear and kinetoplast S phases in the procyclic form of the kinetoplastid protozoan *Trypanosoma brucei* are also periodic and initiate approximately in synchrony (3).

The mechanisms coordinating kinetoplast and nuclear replication are not known. One possible mechanism for coordination of S phase in the kinetoplast and nucleus of

trypanosomes might be the presence of a shared cell cycle regulator. The p34^{cdc2} protein kinase is a key regulator of cell cycle progression in eukaryotes. This 34 kDa protein-serine/threonine kinase is required both at the G₁/S and G₂/M phase transitions and is encoded by the cdc2 gene in the fission yeast *Schizosaccharomyces pombe* and by the CDC28 gene in the budding yeast *Saccharomyces cerevisiae* (for review see 4). In *Xenopus laevis*, the p34 protein kinase has been shown to be a component of maturation-promoting factor which regulates entry into mitosis (5, 6). The kinase activity of p34 varies dramatically during the cell cycle, in part as a consequence of interactions with cyclins (see 7 for review). Cyclins were identified initially in marine invertebrates by their periodic accumulation prior to each mitosis (8). cdc2 kinase activity at mitosis has been shown to require its association with a B-type cyclin in a variety of organisms (9, 10, 11, 12). In addition, a set of cyclins (the CLN genes) have been identified in *S.cerevisiae* (see 13 for review) that are required for the G₁/S phase transition (14, 15). Thus, the distinct cell cycle activities of cdc2 appear to require association with separate sets of cyclins.

Homologs of cdc2 have been identified in a wide range of eukaryotic organisms, including *Drosophila* (16), maize (17) and human cells (18). Characteristic features of p34^{cdc2} proteins include the association with cyclins that act as regulatory subunits and the presence of a conserved sixteen amino acid sequence referred to as the PSTAIR sequence (18). A closely related 33 kDa protein kinase has been identified in human cells (19) and in *Xenopus* (20). The p33 protein is also associated with cyclin A and contains the conserved PSTAIR sequence. These kinases have been named cyclin dependent kinases or cdk's and the gene encoding the p33 kinase is known as cdk2.

Several human proteins related to cdc2 but containing substitutions in the PSTAIR sequence and having amino- or carboxyl-terminal extensions have also been identified (19). It is not known whether these putative kinases require association with a cyclin or whether the amino- or carboxyl-extensions might relieve the requirement for association with cyclins. Other genes, more distantly related to cdc2, have also been identified, such as the yeast FUS3 gene (21) and the human gene CLK1 (22).

* To whom correspondence should be addressed

These *cdc2*-like genes share significant homology to *cdc2*, but are unlikely to be functional homologs.

In order to investigate cell cycle regulation of kinetoplast and nuclear replication in trypanosomes, we have used PCR methods to isolate *cdc2*-related genes from *C. fasciculata*. Here we report the cloning and molecular characterization of a novel *cdc2*-related gene, *Cfa CRK*, and its protein product.

MATERIALS AND METHODS

Nucleic acid isolation

Genomic DNA, whole cell RNA and polyadenylated mRNA were isolated from *C. fasciculata* cells as previously described (23).

PCR

A polymerase chain reaction was performed using reaction conditions described (24). *C. fasciculata* genomic DNA was used as template for PCR amplification using degenerate oligodeoxynucleotides encoding the conserved sequence EGVPTAI and the sequence complementary to HRDLKP. A 270 bp product was cloned into the *Sma*I-digested phagemid vector pGEM-7Zf(+) (Promega) for sequencing by the chain termination method (25) with modified T7 DNA polymerase (Sequenase 2.0, United States Biochemical Corp.).

Construction and screening of λ GEM11 genomic library

A *C. fasciculata* genomic library in the vector λ GEM11 (Promega) was constructed as previously described (23). Using the 270 bp PCR product as probe, five positive clones were plaque purified from an initial screen of 120,000 plaques. Plaque lifts were done using Duralon-UV filters according to the manufacturer's specifications (Stratagene). Blots were hybridized in 50% formamide, 5 $\times$ SSC (1 $\times$ SSC is 150 mM sodium chloride/15 mM sodium citrate, pH 7.0)/0.1% SDS/5 $\times$ Denhardt's reagent (1 $\times$ Denhardt's reagent is 0.02% ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/25 mM potassium phosphate, pH 7.5, 100 μ g/ml sonicated salmon sperm DNA/50 μ g/ml yeast tRNA at 42°C with probe radiolabeled by random priming to a specific activity of at least 1 $\times$ 10⁸ cpm/ μ g. Plaque lifts were washed to a final stringency of 0.1 $\times$ SSC/0.1% SDS at room temperature. Phage DNA was isolated as previously described (26).

Construction and sequencing of pGCD2

Of the five positive clones obtained from the screen of *C. fasciculata* genomic DNA, one (λ 2) was chosen on the basis of Southern analysis and polymerase chain reactions done on recombinant λ GEM11 DNA, to be the most likely candidate for a *cdc2*-like gene. To sequence the *Cfa CRK* gene, a 3.0 kb *Eco*RI fragment of λ 2 was subcloned into the *Eco*RI site of pGEM-7zf(+) (Promega) creating the plasmid pGCD2. Nested deletions were constructed using the exonuclease III/S1 nuclease method (Erase-a-Base, Promega) after digestion of pGCD2 with either *Sph*I/*Xho*I or *Bst*XI/*Cla*I. Sequencing of both strands was performed using double stranded DNA as described above. The other λ GEM11 clones have not been characterized further.

Northern and Southern blots

For Northern blots, whole cell and polyadenylated RNA was electrophoresed in 1.4% agarose-formaldehyde gels and

transferred to Nytran (Schleicher and Schuell) as described (27). Northern blots were hybridized at 42°C in 50% formamide/5 $\times$ Denhardt's reagent/100 μ g/ml sonicated salmon sperm DNA/50 mM potassium phosphate, pH 6.8/5 $\times$ SSC/0.1% SDS/0.1 g/ml dextran sulfate. For Southern blots, 2 to 5 μ g of genomic DNA was digested with the indicated enzymes and fractionated in a 0.8% agarose gel in 0.5 $\times$ TBE buffer (1 $\times$ TBE is 89 mM Tris-base/89 mM boric acid/2.5 mM EDTA at pH 8.3) and transferred as described (27) to Nytran membrane. Southern blots were hybridized at 42°C in 50% formamide/5 $\times$ Denhardt's reagent/0.5% SDS/100 μ g/ml sonicated salmon sperm DNA/6 $\times$ SSC. Probes for Northern and Southern blots were radiolabeled by random priming of DNA fragments to a specific activity of greater than 1 $\times$ 10⁸ cpm/ μ g. Both blots were washed to a final stringency of 0.1 $\times$ SSC/0.1% SDS at 42°C.

Probe 3, which encodes domain B sequence (See Figure 3) was generated by a polymerase chain reaction using linear pGCD2 DNA with the following oligodeoxynucleotide primers: 5'-GACTTCGCCGCCATGACCGCGTTGC-3' and 5'-GATAAGCTGATTCGGTGCCTCTG-3'. The 198 bp product of the polymerase chain reaction was gel purified and used to probe a Northern blot.

Antibody production

To obtain protein for use in antibody production, the 180 bp *Bgl*II fragment of pGCD2 was cloned into the *Bam*HI site of pGEX-3X (28), which produces proteins as a fusion with glutathione S-transferase, creating the plasmid pGBII. The lysis of DH5 α cells containing pGBII was performed as previously described (28) except that IPTG was added to a final concentration of 0.5 mM and the lysis buffer used was 1 $\times$ PBS, 2mM EDTA and 0.25 mM PMSF. The insoluble fusion protein (100 μ g) was electrophoresed in a 12% SDS polyacrylamide gel, cut out and used to immunize a New Zealand white rabbit (29).

Affinity purification of antibody

To obtain sufficient amounts of the peptide encoded by the *Bgl*II fragment of pGCD2 for affinity purification of the antibodies, the plasmid pGHBII was constructed. A linker adding 5 histidines to pGEX-2T (28) was constructed by hybridizing the oligodeoxynucleotide 5'-GATCTCATCACCACCATC-ACGG-3' with 5'-GATCCCGTGATGGTGATGTGATGA-3' as previously described (30). The linker was then cloned into the *Bam*HI site of pGEX-2T. The 180 bp *Bgl*II fragment of pGCD2 was cloned into the *Bam*HI site of this construct creating pGHBII. DH5 α cells containing pGHBII were induced with 0.5 mM IPTG and the fusion protein was purified on a Ni²⁺-NTA-agarose column (Qiagen) as described (31). Purified fusion protein precipitated after dialysis against 1 $\times$ TBS (1 $\times$ TBS is 137 mM NaCl, 2.7 mM KCl, 25 mM Tris pH 8.0). The precipitate was resuspended in 1 $\times$ TBS + 0.1%SDS. This solution was brought to 1% Triton X-100, 2.5 mM CaCl₂ and thrombin (Sigma) was added at a level of 1% of that of the fusion protein on a weight basis. The reaction was incubated at 37°C for at least five hours. The digested products were electrophoresed in a 12% SDS polyacrylamide gel, transferred to nitrocellulose and stained in 0.2% (w/v) Ponceau S in 3% trichloroacetic acid, 3% sulfosalicylic acid. The 9 kDa protein encoded by the *Bgl*II fragment was cut out and used for affinity purification. Strips were blocked with 3% BSA for 1 hr prior to incubation with antiserum and subsequent washes with 0.5% NP40 and 0.5%

Figure 1. Multiple sequence alignment of the deduced amino acid sequence of CRK with those of the mouse *cdc2* homolog (37), the human *cdc2* homolog (18), *S.pombe* *cdc2* (36) and the human gene CLK1 (22). The amino acid sequences were aligned using the computer program Clustal (33). Amino acid sequences of CRK and CLK were compared to amino acid sequences found in all three *cdc2* homologs (see boxes).

Figure 2. Schematic representation of CRK conserved and novel domains. Domain A, amino acids 1–143; Domain B amino acids 144–209; Domain C amino acids 210–300; Domain D, amino acids 301–379; Domain E, amino acids 380–474. Conserved domains A, C and E contain the protein kinase catalytic domain sequences (40) as follows: A (I–VIb), C (VII–X) and E (XI). Novel domains B and D are represented as inserts between the conserved domains.

Table I. Identity matrix of various *cdc2* homologs with Cf CRK

Protein ¹	% Identity with Cf CRK Domain		
	A ²	C ³	E ⁴
Hs <i>cdc2</i>	45	61	33
Zm <i>cdc2</i>	42	50	32
Dm <i>cdc2</i>	46	59	25
Sc CDC28	44	49	41

¹*cdc2* homologs from: *Homo sapiens*(18); *Zea mays*(17); *Drosophila melanogaster*(16); *Saccharomyces cerevisiae*(52)

²Domain A compared to Hs (1–141), Zm (1–141), Dm (1–141), Sc (1–148).

³Domain C compared to Hs (142–231), Zm (142–231), Dm (142–231), Sc (149–239).

⁴Domain E compared to Hs (232–297), Zm (232–294), Dm (232–297), Sc (240–298).

referred to as the PSTAIR motif which is a hallmark of *cdc2* homologs (18).

One of the clones obtained in the polymerase chain reaction contained homology to *cdc2* and was used to screen a *C. fasciculata* genomic library to obtain the recombinant phage λ 2. The partial sequence of λ 2, which contains Cfa CRK (*Crithidia fasciculata* *cdc2*-related kinase), has been deposited in the EMBL Data Library (accession number Z12149). Cfa CRK encodes a protein (CRK) of predicted molecular mass of 53 kDa. An alignment of CRK with *S. pombe* *cdc2* (36) and *cdc2* homologs from mouse (37) and human cells (18) is shown in Figure 1. CRK contains significant homology to the *cdc2* family of protein kinases. CRK is most similar to the mouse homolog of *cdc2*, to which it is 47% identical and 67% similar. CRK contains the PSTAIR region, although with four amino acid changes in the sixteen residue motif. Also, in many places in the CRK sequence, a methionine is substituted for a conserved hydrophobic residue which is present in the various *cdc2* homologs.

As shown in Figure 1, CRK contains two insertions, of 66 and 79 amino acids (domain B and D), interspersed between three conserved domains (domains A, C and E; See Figure 1 and 2). Domain B is enriched in the amino acids alanine and proline; while domain D is enriched in the amino acids glutamine and serine. These large insertions are not found in other *cdc2*-like proteins identified to date. An alignment of CRK with the amino acid sequence encoded by the human gene CLK is also shown here since the CLK gene also contains an insert corresponding to domain B of CRK (22). CLK encodes a protein of predicted molecular mass of 54 kDa and contains significant homology to the *cdc2* family of protein kinases.

Domains A and C of CRK are highly conserved (Table 1), containing 46% identity to the *Drosophila* homolog of *cdc2* in

domain A and 60% identity to the human homolog of *cdc2* in domain C. The C-terminus is less conserved, as has been observed for *cdc2* homologs (38). CLK contains smaller amino acid insertions in regions similar to those of CRK. There is no significant similarity between the insertions in CRK and those found in CLK, however. A search of the GenBank database using the insert sequences B and D did not find any alignments of significance.

Genomic organization of Cfa CRK

To confirm that the sequence organization of the genomic clone λ 2 reflects the genomic organization of Cfa CRK and to determine whether or not the CRK gene is present as a single copy, Southern blot analysis of *C. fasciculata* genomic DNA was performed using the 373 bp Kpn I/Bgl II fragment of pGCD2 (see Materials and Methods) as a probe. A 2.2 kb Kpn I fragment and a 1.9 kb MluI fragment hybridized to the labeled probe, consistent with the CRK gene being present in a single copy (Fig. 3). In double digests the probe sequence hybridized with fragments of 0.37 kb (Bgl II/Kpn I), 0.62 kb (Mlu I/Kpn I) and 1.65 kb (Mlu I/Bgl II) in agreement with the restriction map of the λ 2 clone. These results indicate that the sequence domains B and D are present in the genomic DNA of *C. fasciculata* and did not arise as a cloning artifact.

Cfa CRK is expressed on a 3.8 kb polyadenylated transcript

Although no trypanosome gene has been found to contain introns, the presence of large inserts in the CRK genomic sequence, which otherwise has a high degree of sequence identity with *cdc2* genes, suggested that these inserts might represent introns. To examine this possibility, Northern blots of *C. fasciculata* whole cell and polyadenylated RNA were probed with an 896 bp MluI/Acc I fragment (Probe 2) and with a fragment produced by PCR amplification of domain B alone (Probe 3). Both probes hybridized with a polyadenylated transcript of 3.8 kb (Fig. 3b and c). Thus, Cfa CRK is expressed and domain B, in particular, is present in the mRNA of Cfa CRK. Although the presence of domain D in the CRK mRNA was not specifically examined here, results to be presented are consistent with the presence of both domains B and D in the CRK mRNA. The size of the CRK mRNA (3.8 kb) is larger than expected for the transcript of Cfa CRK, which contains a coding sequence of approximately 1.4 kb.

cdc2-related proteins in *C. fasciculata* and *Leishmania tarentolae*

Expression of the open reading frame containing the CRK gene including the inserted domains B and D would be expected to yield a protein with a molecular mass of 53 kDa. To examine this possibility and to detect proteins containing the 16 residue PSTAIR region, Western blots of *C. fasciculata* whole cell extract were incubated with either affinity purified antibody to Cfa CRK (anti-CRK, see Material and Methods) or antibodies raised against the PSTAIR region of *cdc2* (Figure 4). Anti-CRK detected a protein of 53 kDa in *C. fasciculata* whole cell extract. In addition, both the PSTAIR antibody and anti-CRK weakly detected a protein of 48 kDa in *C. fasciculata* whole cell extract. This result suggests that *C. fasciculata* may also contain a species with a more highly conserved PSTAIR motif that, like CRK, is of a higher molecular mass than p34^{cdc2}. The PSTAIR antibody does not detect any protein in *C. fasciculata* of 34 kDa, suggesting that *C. fasciculata* does not contain a p34^{cdc2} homolog with a perfectly conserved PSTAIR motif. The inability of the anti-PSTAIR antibody to recognize the 53 kDa CRK protein suggests

Figure 3. Southern analysis of *C. fasciculata* genomic DNA and Northern blot analysis of Cfa CRK transcript. (A) 5 µg of *C. fasciculata* genomic DNA was digested with the indicated enzymes, electrophoresed through a 0.8% agarose gel, blotted to a nylon filter and probed with the 373 bp BglII/KpnI fragment of pGCD2 (Probe 1) (see Materials and Methods). (B) and (C) *C. fasciculata* whole cell (wc) and polyadenylated (polyA+) RNA was fractionated on a 1.4% formaldehyde gel, blotted and probed with the 896 bp MluI/ACCI fragment of pGCD2 (B; Probe 2) or Domain II sequence (C; Probe 3) (see Materials and Methods). (D) Partial genomic restriction map of Cf CRK. The 3.0 kb EcoRI fragment defines the fragment subcloned for sequence analysis in pGCD2.

that a 34 kDa cdc2 homolog in *C. fasciculata* containing the same substitutions in the PSTAIR sequence as CRK might not be detected by the anti-PSTAIR antibody.

Whole cell extracts of the trypanosomatid *L. tarentolae* and HeLa cell nuclear extracts were also incubated with anti-CRK antibody (see figure 4, Hs, lane 1, and Lt, lane 1). Anti-CRK antibody detects two proteins, of approximately 60 and 48 kDa, in *L. tarentolae* whole cell extract, suggesting that trypanosomes may contain a family of higher molecular mass cdc2-related proteins. Anti-CRK does not detect any proteins in HeLa cell nuclear extract. Whole cell extracts of *L. tarentolae* and HeLa nuclear extracts were also incubated with anti-PSTAIR antibody (see Figure 4, Hs Lane 2 and Lt Lane 2). In HeLa nuclear extract incubated with anti-PSTAIR, p34^{cdc2} is detected as well as a slightly slower migrating species which possibly represents the phosphorylated form of cdc2. In *L. tarentolae* whole cell extract proteins of 60, 48 and 34 kDa are recognized. This result suggests that *L. tarentolae* contains a 34 kDa protein with a more highly conserved PSTAIR motif than is present in *C. fasciculata* as well as higher molecular weight species.

Figure 4. Western blot analyses. *Crithidia fasciculata* whole cell extract (Cf), HeLa cell nuclear extract (Hs) and *Leishmania tarentolae* (Lt) whole cell extract were incubated with antibodies to CRK (Lane 1) or antibodies to the PSTAIR motif (Lane 2) as described in Materials and Methods. The numbers on the left indicate the positions of molecular weight markers.

DISCUSSION

We have identified a novel cdc2-related gene from *C. fasciculata*. Although the 53 kDa CRK protein contains two large insertions, it is highly homologous to the cdc2-like protein kinases and contains structural features which place it in the cdc2 family of protein kinases. The PSTAIR motif, characteristic of cdc2 genes, is present in CRK, although there are 4 amino acid changes in this sixteen residue motif. This amino acid sequence has been implicated in cdc2 regulation (6), perhaps affecting cdc2 association with cyclins (39). It will be of interest to determine whether the CRK protein associates with a cyclin or whether the inserted domains might replace the requirement for association with a cyclin for kinase activity.

CRK contains residues which are highly conserved in protein-serine/threonine kinases. To define conserved features of such kinases, Hanks and Quinn (40) aligned the amino acid sequences from the catalytic domain of 75 protein-serine/threonine kinases and eleven conserved domains (subdomains I-XI) were identified. In the eleven domains, fifteen amino acid positions are highly conserved in the 75 amino acid sequences (40). CRK contains fourteen of the fifteen conserved amino acids and contains such features as the consensus motif Gly-X-Gly-X-X-Gly (amino acids 14 to 19) and the invariant lysine (Lys36 in CRK) in subdomain II, which is apparently involved in the phosphotransfer reaction (41). A glutamic acid found in subdomain VIII of all protein kinases examined is replaced by the conservative substitution of an aspartic acid (Asp242) in CRK. Also, the amino acid sequence between the invariant Asp131 and Asn136 is a highly conserved indicator of serine/threonine kinases with the Gln135 being particularly characteristic of cdc2 kinases. The only difference in this region between CRK and previously identified members of the cdc2 gene family is the substitution of Met132 in place of a conserved leucine residue. Elsewhere in the CRK protein Met frequently replaces conserved Leu, Val or Ileu residues present in cdc2 genes.

Unlike the 34 kDa cdc2 kinases, CRK contains insertions (domains B and D) between subdomains VI and VII, and X and XI, respectively. Other protein kinases also contain large insertions (greater than 60 amino acids), including the CDC7 gene from *S. cerevisiae* (42, 43). CDC7 contains insertions between subdomains VII and VIII, and X and XI (35). Also the putative

protein kinase CLK (22) contains insertions in the same locations as CRK. Since these insertions occur between conserved domains, they are unlikely to disrupt kinase function. The sequences of Domain B and D are quite hydrophilic, and therefore, are likely to be on the protein surface. Although the importance of these domains are not known, they may confer substrate specificity or be involved in the regulation or localization of CRK in the cell.

Phosphorylation is important in the regulation of cdc2 kinases. In *S.pombe*, a tyrosine (Tyr15) in the ATP binding site is phosphorylated in a cell cycle dependent manner (44). Phosphorylation of this tyrosine inhibits cdc2 kinase bound to cyclin (45). In higher eukaryotes, this tyrosine is phosphorylated along with a threonine (Thr14) also in the ATP binding site (46, 47). Mutations in these residues result in premature activation of cdc2 kinase (47, 48). Both threonine (Thr17) and tyrosine (Tyr18) residues are present in the putative ATP binding site of CRK and these amino acids may also be sites of CRK phosphorylation and regulation. Threonine phosphorylation has also been implicated in association of cdc2 with cyclin in *S.pombe* at Thr167 (49) and in human cells at the corresponding position (39). CRK contains a serine residue at this position which may be a target for a serine-threonine protein kinase.

The cellular role of CRK is unknown. However, other cdc2-related genes from *S.cerevisiae* are involved in cell proliferation. Examples of these cdc2-related genes include FUS3, which is required for arrest of cells in G1 and promotion of conjugation (21), KSS1, which is involved in recovery from pheromone-induced growth arrest (50) and KIN28, which is essential for cell proliferation (51). Anti-CRK antibodies recognize at least two proteins in *L.tarentolae* having molecular weights greater than the 34 kDa size typical of cdc2 genes in other eukaryotes. In *L.tarentolae* 34, 48 and 60 kDa proteins are weakly detected by anti-PSTAIR antibodies as compared to the much stronger signals generated by the 48 and 60 kDa proteins when anti-CRK antibodies are used. These results together with the lack of detection of a 34 kDa protein with either anti-CRK or anti-PSTAIR antibodies in *C.fasciculata* extracts suggests that a family of higher molecular weight cdc2-related proteins are present in trypanosomes and that the protein-serine/threonine kinase(s) involved in cell cycle control are likely to have diverged somewhat in the sixteen amino acid PSTAIR motif.

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