Identification of the catalytic subunit of cAMP-dependent protein kinase from the photosynthetic flagellate, *Euglena gracilis* Z[1]

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Abstract A gene named *epk2* that encodes the amino acid sequence of a protein kinase was identified from the photosynthetic flagellate, *Euglena gracilis* Z. Homology search and phylogenetic analysis revealed that the deduced amino acid sequence of *epk2* is most similar to that of the catalytic subunit of cAMP-dependent protein kinase (PKA). Northern blot analysis showed that *Euglena* cells express a 1.4-kb transcript of this gene. When the EPK2 protein was coexpressed with the rat regulatory subunit of PKA in cultured mammalian cells, these two proteins were coimmunoprecipitated. The association of EPK2 and the rat regulatory subunit of PKA was not detected in the cell lysate incubated with cAMP. EPK2 immunoprecipitated from the transfected cells phosphorylated Kemptide, a synthetic peptide substrate for PKA, and the phosphorylation was inhibited by PKI, a PKA-selective protein kinase inhibitor. These results indicate that EPK2 is a PKA homologue in the photosynthetic flagellate, and this is the first evidence for the occurrence of the PKA catalytic subunit in photosynthetic organisms.

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Key words: *Euglena gracilis*; Protein kinase A; Cyclic adenosine monophosphate; Molecular evolution; Photosynthetic flagellate

1. Introduction

cAMP is a second messenger found in a variety of organisms, and the major target of cAMP is cAMP-dependent protein kinase (PKA). The role of PKA has been investigated extensively in animal cells [1]. Recently, the PKA genes have been cloned from single-cell organisms such as *Saccharomyces* [2], *Schizosaccharomyces* [3], and from a protozoon, *Dictyostelium* [4]. In most organisms, PKA is a heterotetramer composed of two catalytic and two regulatory subunits [1]. In *Dictyostelium*, however, PKA is a heterodimer consisting of a catalytic and a regulatory subunit [5]. The protein kinase activity of the catalytic subunit is inhibited by the regulatory subunit, and the binding of cAMP to the regulatory subunit releases the catalytic subunit from the holoenzyme complex and allows it to phosphorylate the substrate proteins [1]. The catalytic domain of the protein kinases has a common structure which is divided into 12 subdomains [6]. The catalytic domains of the PKA catalytic subunit from different organisms have a conserved structure, which is closely related to that of cGMP-dependent protein kinase (PKG) and protein kinase C (PKC), and therefore these three protein kinases are collectively called 'the AGC family protein kinases' short for PK<u>A</u>, PK<u>G</u>, and PK<u>C</u> [7].

The following roles of cAMP have also been suggested in several photosynthetic organisms: (1) addition of cAMP to plant extracts stimulates phosphorylation of specific proteins [8], (2) Kemptide, a synthetic substrate for PKA, is phosphorylated by a putative protein kinase from petunia [9]. Carrie and Edmunds [10] have reported that cAMP oscillations mediate the phasing of the cell division cycle by the circadian clock in *Euglena*, and they detected a protein kinase activity which is dependent on cAMP in the homogenate of the algal flagellate [11]. Many attempts have been made to clone the PKA gene in photosynthetic organisms. Nevertheless, the presence of PKA genes in these photosynthetic organisms has not been reported yet. In this study, we isolated a clone that encodes a putative catalytic subunit of PKA from the photosynthetic flagellate *Euglena gracilis* by reverse transcriptase-polymerase chain reaction (RT-PCR) using oligonucleotide primers corresponding to the regions conserved among the AGC family protein kinases from different species. This is the first report of the identification of a member of the typical AGC protein kinase from the photosynthetic organisms.

2. Materials and methods

2.1. Cell culture

Euglena gracilis Z was cultured at 25°C in Koren-Hunter (KH) medium [12]. Mammalian COS (CV-1 origin-defective SV40) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2/95% air incubator.

2.2. Cloning

Total RNA was extracted from *Euglena gracilis* Z with the mRNA Extraction kit (Pharmacia), and then poly(A)$^+$ mRNA was purified by oligo(dT)-cellulose column chromatography with the mRNA Purification kit (Pharmacia). Poly(A)$^+$ RNA (1 μg) was reverse-transcribed with avian myeloblastosis virus reverse transcriptase using oligo(dT)20-M4 adapter primer and random 9-mers (RNA PCR Kit Ver.2, TaKaRa). Degenerate oligonucleotide primers were designed according to the sequences GK(R)GS(N)F(Y)G(A)K, FYAAEV(I), DF(Y)GM(L)CK, TFCGTP, and VDWWA(S)F(L), which are conserved in the subdomains I, VIa, VII, VIII, and IX of the AGC protein kinase family, respectively [6]. The 5′-sense primers used were 5′-GG(T/C/A/G)AA(A/G)GG(T/C/A/G)AA(T/C)TT(T/C)GG(T/C/A/G)AA-3′ and 5′-TT(T/C)TA(T/C)GC(T/C/A/G)GC(T/C/A/G)GA(A/G)(A/G)T-3′, and the 3′-antisense primers were 5′-TT(A/G)CA(T/C/A/G)A(T/A/G)(T/C/A/G)CC(A/G)(A/T)A(A/G)TC-

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[1] The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB021126.

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3′, 5′-GG(T/C/A/G)GT(T/C/A/G)CC(A/G)CA(A/G)AA(T/C/A/G)G-T-3′, and 5′-A(T/A/G)(T/C/A/G)G(T/C/A)CCACCA(A/G)TC(T/C/A/G)AC-3′. PCR was carried out using *Euglena* cDNA as a template and the degenerate oligonucleotide primers described above. The PCR was run for 30 cycles (94°C for 1.5 min, 46°C for 2.5 min, 72°C for 2.5 min) followed by an extension at 72°C for 10 min. The PCR products were subcloned into pUC18 vector, and sequenced by the dideoxy chain termination method using a DNA Sequencing System Model 373A (Applied Biosystems). The *Euglena* cDNA library was constructed using the Uni-ZAP XR cloning kit and Gigapack III gold packaging extracts (Stratagene). Sequential plaque screening of the *Euglena* cDNA library was carried out using the PCR fragments labeled with [α-^{32}P]dCTP as the probes. The hybridization and washing were carried out as described [13]. Using the PCR fragment PK-2, cDNA clones were isolated, and the clone having the longest insert was named *epk2*. The alignment and similarity of the amino acid sequences were analyzed by BLAST and FASTA programs on the GenomeNet WWW Server [14,15]. The cluster analysis was carried out with the NJ (neighbor joining) method [16] of cluster w of GenomeNet WWW Server [17]. The drawing of the phylogenetic tree was done on the program TREEVIEW [18].

2.3. Northern blot

Poly(A)$^+$ mRNA (1 μg) was electrophoresed on a 1% agarose gel containing 18% formaldehyde, 40 mM MOPS (pH 7.0), 10 mM sodium acetate, and 2 mM EDTA, and then blotted onto a Hybond-N membrane (Amersham). The coding region of *epk2* was labeled with [α-^{32}P]dCTP and employed as a probe. Hybridization was performed at 65°C for 16 h in 0.25 M disodium hydrogen phosphate (pH 7.2) containing 1 mM EDTA and 7% SDS. The membrane was washed at 65°C for 30 min in 20 mM disodium hydrogen phosphate (pH 7.2) containing 1% SDS, and then at 65°C for 60 min in 0.2×SSC (1×SSC is 150 mM NaCl, 15 mM sodium citrate at pH 7.0) containing 0.1% SDS. Radioactivity in the hybridized band was analyzed by Bioimaging Analyzer BAS2000 (Fuji).

2.4. Expression plasmids and transfection

The cDNA fragments encoding the full-length sequence of *epk2* (amino acids 1–336) and the rat type Iα regulatory subunit of PKA (RIα subunit) donated by Dr. Takayoshi Kuno (Kobe University) were inserted into pTB701-HA and pTB701-FLAG vectors [19,20], respectively, to construct the expression vectors containing each epitope tag at the amino-terminal end. These vectors were named HA-*epk2* and FLAG-RIα, respectively. COS-7 cells were transfected with the expression vectors by electroporation using Gene Pulsar (Bio-Rad).

2.5. Regulatory subunit binding assay

COS-7 cells transfected with the expression plasmids of HA-*epk2* and FLAG-RIα were cultured at 37°C for 48 h. Cells were washed with phosphate-buffered saline, and lysed in 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, and 50 μg/ml phenylmethylsulfonyl fluoride (PMSF) (lysis buffer). After centrifugation for 10 min at 18 000×*g*, the supernatant (500–600 μg of protein) was incubated for 1 h with either an anti-FLAG monoclonal antibody (Kodak Scientific Imaging Systems) or an anti-HA monoclonal antibody (12CA5, Boehringer Mannheim), and then, protein A-Sepharose (Pharmacia) was added to the mixture and incubated for 30 min in the presence or absence of cAMP at 10 μM. The immunoprecipitates were collected by centrifugation and washed twice with 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 1% Triton X-100. The procedures above were carried out at 0–4°C. After boiling in SDS-sample buffer, immunoprecipitated materials were separated on SDS-PAGE and transferred onto an Immobilon P membrane (Millipore). Immunoblot analysis was carried out using either an anti-FLAG or an anti-HA monoclonal antibody as the primary antibody, an alkaline phosphatase-conjugated anti-mouse IgG antibody (Promega) as the secondary antibody, and the coloring reaction was carried out as described [21].

2.6. Protein kinase assay

COS-7 cells transfected with HA-*epk2* were lysed and the HA epitope-tagged protein was immunoprecipitated as described above, and washed three times with 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 1% Triton X-100, followed by 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, and 50 μg/ml PMSF. The protein kinase activity of the immunoprecipitate was measured using Kemptide (Sigma) as a synthetic peptide substrate, as described [22]. The reaction mixture (50 μl) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 20 μM ATP, [γ-^{32}P]ATP (100 kBq/nmol), 50 μM Kemptide, and the immunoprecipitated HA-EPK2 protein. Where indicated, 10 μM cAMP and 1 μM protein kinase inhibitor (PKI, a synthetic peptide inhibitor of PKA, Sigma) were added to the reaction mixture [23]. The incubation was carried out at 30°C for 5 min. An aliquot (40 μl) of the reaction mixture was spotted onto a Whatman P81 paper and washed in 75 mM phosphoric acid, and incorporation of the radioactivity into the Kemptide was determined by Cerenkov counting.

3. Results

3.1. Isolation and characterization of EPK2 gene

The catalytic subunit of PKA was screened in *Euglena* by RT-PCR using the degenerate oligonucleotide primers, which are based on the conserved sequences of the subdomains I, VIa, VII, VIII, and IX in the AGC family protein kinases [7]. Five DNA fragments were obtained, which encode amino acid sequences homologous to the AGC family protein kinases

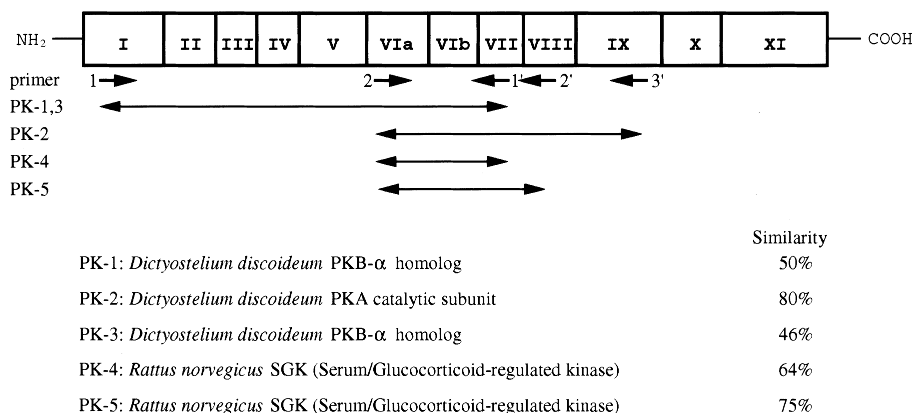

Fig. 1. Screening of the AGC family protein kinases from *E. gracilis* Z. A schematic structure of the catalytic domain of protein kinase is shown with the subdomains I to XI. The positions of the 5′-sense primers (1 and 2) and the 3′-antisense primers (1′, 2′, and 3′) are indicated by arrows. The obtained PCR fragments (PK-1 to PK-5) are shown by bidirectional arrows. The GenBank clones which showed the highest amino acid sequence similarity to each PCR fragment are listed together with the similarity percent.

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                                                          TTTTTTTTTTTT   -61
     TCGGATCGGGGTGGTTCAGCCACACCGTACAGACCAGGCAAAATTGAGGAATCAACTAAT   -1
     ATGGCGCAGACCATCCCGGACCAGACCGCTTTGCCAGACACAACCAACTGGCAATTGTCG   60
  1   M  A  Q  T  I  P  D  Q  T  A  L  P  D  T  T  N  W  Q  L  S
     GACTTGACTCTCAAGGAGACTTTAGGGACAGGAACGTTTGGTCGAGTGCGACTCTGTCTG  120
 21   D  L  T  L  K  E  T  L  G  T  G  T  F  G  R  V  R  L  C  L
     CATAAGTCATCTGGAAATTACTATGCAATCAAGTGTCTGAAAAAGTCTGAAGTGCTGAGA  180
 41   H  K  S  S  G  N  Y  Y  A  I  K  C  L  K  K  S  E  V  L  R
     ATGAAGCAGGTGGAGCACATTCTTGCTGAAGCATCAATTCTTGGGTCAATCCGTCATCCT  240
 61   M  K  Q  V  E  H  I  L  A  E  A  S  I  L  G  S  I  R  H  P
     TTTATCGTGAATATGTTGAAGACTTTTCAGGATGATAAGCGGCTCTACATTGTCCTGGAG  300
 81   F  I  V  N  M  L  K  T  F  Q  D  D  K  R  L  Y  I  V  L  E
     TATGTGGTGGGAGGAGAGCTTTTCTCTCATCTGAGGAAGGCTGGTAAATTTCCGAACGAC  360
101   Y  V  V  G  G  E  L  F  S  H  L  R  K  A  G  K  F  P  N  D
     GTTGCCAAGTTTTATGCAGCTGAAGTGATTCTGGCCTTTGAGTACATCCATTCCATGGAT  420
121   V  A  K  F  Y  A  A  E  V  I  L  A  F  E  Y  I  H  S  M  D
     ATTTTGTACCGTGACTTGAAACCAGAGAACCTGTTGTTGGATGTTGGAGGACACATAAAG  480
141   I  L  Y  R  D  L  K  P  E  N  L  L  L  D  V  G  G  H  I  K
     ATCACGGACTTTGGTTTTGCAAAAAAGGTCCCAGAGCGAACGTTCACATTGTGTGGGACA  540
161   I  T  D  F  G  F  A  K  K  V  P  E  R  T  F  T  L  C  G  T
     CCCGAATACCTTGCTCCAGAAATCATTCAGTCGAAGGGACACGGAAAAGCCGTGGATTGG  600
181   P  E  Y  L  A  P  E  I  I  Q  S  K  G  H  G  K  A  V  D  W
     TGGGCTCTTGGGATCTTGACTTACGAAATGCTGGTTGGGTACCCACCTTTCTTTGATGAG  660
201   W  A  L  G  I  L  T  Y  E  M  L  V  G  Y  P  P  F  F  D  E
     TCGCCTTTCCGAATTTATGAAAAGATTTTGGAAGGGAAAGTGCAGTTTCCCAAGTGGGTT  720
221   S  P  F  R  I  Y  E  K  I  L  E  G  K  V  Q  F  P  K  W  V
     GATGGACGTGCCAAGGATCTTATCAAAGGGTTGCTCACAACCGATCACACAAAGCGGCTT  780
241   D  G  R  A  K  D  L  I  K  G  L  L  T  T  D  H  T  K  R  L
     GGAACATTGAAGAGAGGAGTGACTGACATCAAGAAGCACAAATGGTTTTATGGCGTGGAC  840
261   G  T  L  K  R  G  V  T  D  I  K  K  H  K  W  F  Y  G  V  D
     TGGGATATGTTGTTGGCACGGAAGATTCCTGCTCCCATTCCGGTGAAGGTCACCACCCCA  900
281   W  D  M  L  L  A  R  K  I  P  A  P  I  P  V  K  V  T  T  P
     GGAGATTCACGGTATTTCGACCGTTATCCTGAGAGCAAGGAGGACAAGTCGCAACCTTTG  960
301   G  D  S  R  Y  F  D  R  Y  P  E  S  K  E  D  K  S  Q  P  L
     ACTCCCGCACAACAAGAGTTATTCAAAGGGTTTGGGCCGTATTCGACGTAATCGCTTCCC 1020
321   T  P  A  Q  Q  E  L  F  K  G  F  G  P  Y  S  T  *
     TCTGCAAATTGCGGTGGTTGCGAATCTGTTCTGTGTTTGGTTTACACCCTGACCAGGTTT 1080
     TGTATAGTGCTGAGCTCTCATGTGTATTTGGCTTTGCTTTTGTGGGACAAGGCACGCAAC 1140
     CCTCCATTCTCCGCGTTGTAGACATTATGTGCTTGGGGTGGGGGAGGAGAGGGTTCGCTC 1200
     ACCTCCAAATTTGACCTCACCTAGCTTCCCTTGCTGTTCACCCTGCAAACCAAAGGACAC 1260
     AGGTTTGCTCAGTGACCTCCTTCGAGTTCAGCAACTGACCTTTCTTCTGTGTGATTATGT 1320
     TTGTCACTTCTGCATGTACATTTCGGGGATTAGGCACCTCTTTGCAGAAGAATGGGCGTG 1380
     CTGTTTTG                                                     1388
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Fig. 2. Nucleotide and deduced amino acid sequences of *epk2*. Nucleotide residues are numbered from the first ATG triplet encoding the putative initiating methionine and indicated at the right. Deduced amino acid residues are given under the nucleotide sequence and numbered at the left. The asterisk indicates the stop codon. The fragment obtained by PCR is underlined.

(Fig. 1). The deduced amino acid sequence of PK-2 containing a 245-bp fragment was most similar to that of the PKA catalytic subunit of *Dictyostelium discoideum* (Dd GPK2) [4], with 80% homology. Therefore, the PK-2 cDNA fragment was employed as a hybridization probe to obtain the full-length cDNA clone from the *Euglena* cDNA library. Sequential plaque screening of the library detected 39 clones among 5×10^5 plaques. DNA sequencing revealed that these 39 clones contain the overlapping sequences but different length encoding the same open reading frame. The clone containing the longest insert was named *epk2* and used in the following analysis. The nucleotide and deduced amino acid sequences of *epk2* are depicted in Fig. 2. The nucleotides were numbered from the first ATG triplet encoding the putative initiating methionine, which has a purine at the -3 position and a purine at the +4 position, making it optimal for translation initiation [24]. The *epk2* has a poly T sequence at the 5′ terminus. We have three *Euglena* protein kinase genes in addition to *epk2*, which also have a poly T sequence similar to that of *epk2* (unpublished observation). Two of them contain an in-frame stop codon between the poly T sequence and the initiation codon. The evidence strongly supports that the assigned initiation codon shown in Fig. 2 is a relevant one. An in-frame stop codon TAA was found at nucleotides 1009–1011 downstream of the 132-amino acid sequence of PK-2. The nucleotide sequence of *epk2* predicted an open reading frame of 336 amino acids with a calculated molecular weight of 38 365, which encodes a protein kinase catalytic domain at residues 22–276, containing all of the hallmark motifs of 12 subdomains [6]. The catalytic domain of EPK2 shares high homology with the PKA catalytic domain from other species (Fig. 3C). The amino acid sequence of the catalytic domain of EPK2 shows 74.5% similarity to that of *Leishmania major* c-lpk2 protein kinase [25], 66.7% to *Dictyostelium discoideum* GPK2 [4], 62.0% to *Drosophila melanogaster* DC2 [26], 58.4% to *Homo sapiens* PKA-Cβ [27], and 56.8% to the *Saccharomyces cerevisiae* TPK1 [2]. A particular motif (FXXF) was found at the very end of the C-terminus of

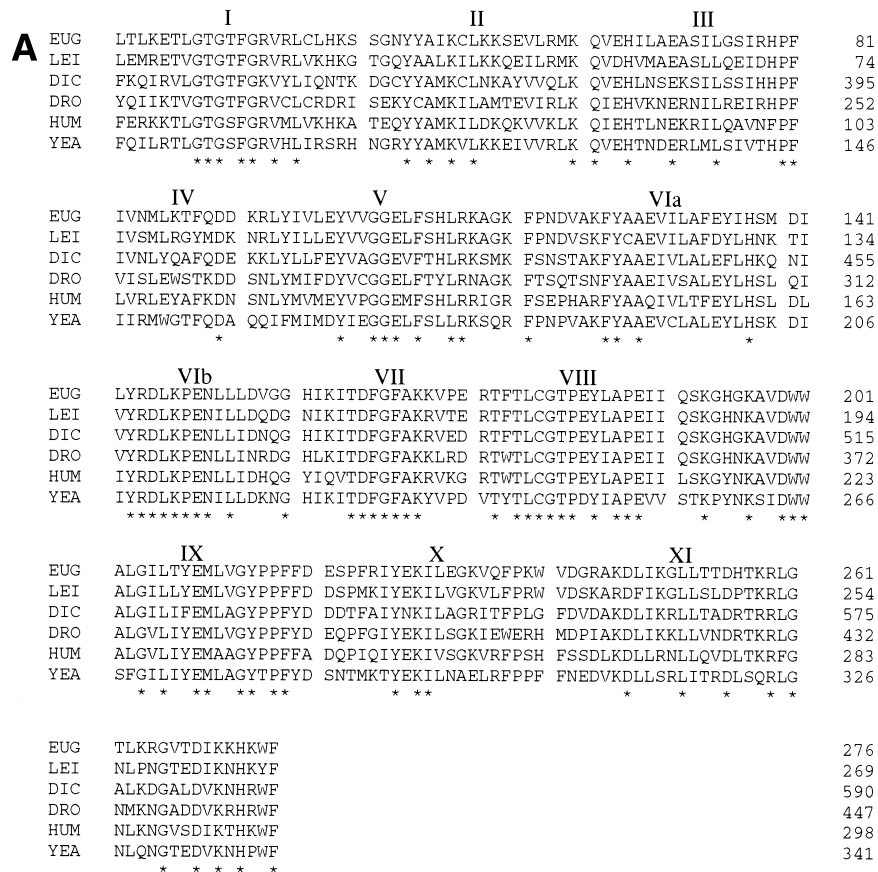

Fig. 3. Comparison of the catalytic domain of EPK2 with other protein kinases. A: Amino acid sequence alignment of PKA from different species. The roman figures indicate protein kinase subdomains. The asterisks indicate the amino acid residues conserved among the species. EUG, EPK2; LEI, *Leishmania major* (1438885); DIC, *Dictyostelium discoideum* (462434); DRO, *Drosophila melanogaster* (125318); HUM, *Homo sapiens* (125210); YEA, *Saccharomyces cerevisiae* (125208). B: Phylogenetic tree inferred from the amino acid alignments for the catalytic domain of PKA and PKG. PKA: PKA-Cα, *Homo sapiens* (125205); PKA-Cβ, *Homo sapiens* (125210); CePKA, *Caenorhabditis elegans* (1326365); DmPKA-C0, *Drosophila melanogaster* (125215); Aplc, *Aplysia californica* (102678); PKA-Cγ, *Homo sapiens* (125217); Sak, *Aplysia californica* (125219); DmPKA-C1, *Drosophila melanogaster* (125319); DdPKA, *Dictyostelium discoideum* (462434); DmPKA-C2, *Drosophila melanogaster* (125318); EPK2, *Euglena gracilis*; c-lpk2, *Leishmania major* (1438885); ScPKA-TPK1, *Saccharomyces cerevisiae* (125208); ScPKA-TPK3, *Saccharomyces cerevisiae* (547757); ScPKA-TPK2, *Saccharomyces cerevisiae* (1708610). PKG: PKG-I, *Homo sapiens* (125379); DmPKG-G2, *Drosophila melanogaster* (157212); DmPKG-G1, *Drosophila melanogaster* (157202). The numbers in parentheses indicate the accession numbers in the GenBank data base. The scale bar indicates the branch length in Kimura distance units. Bootstrap values (100 replicates) for the various nodes are shown on the tree.

EPK2, supporting that EPK2 is a catalytic subunit of PKA [28]. Furthermore, cluster analysis on NJ software indicated that EPK2 is located in the pedigree of the PKA catalytic subunits (Fig. 3B) [2,4,26,27,29–36]. A single band of 1.4 kb was detected by Northern blot analysis using the *epk2* fragment as a probe, confirming that the *epk2* gene is transcribed per se in *Euglena* cells (Fig. 4).

3.2. Association of EPK-2 to the regulatory subunit of PKA

The catalytic and regulatory subunits of PKA are associ-

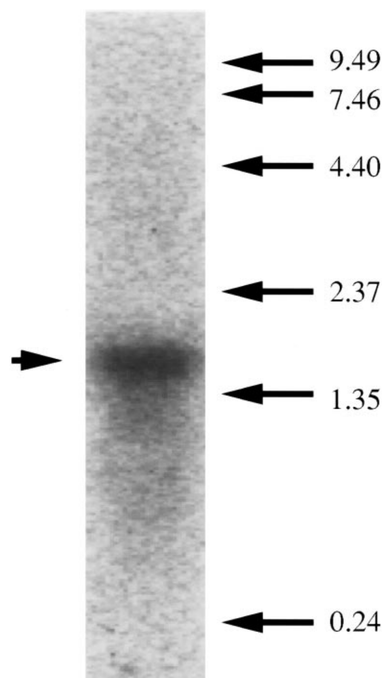

Fig. 4. Northern blot analysis of *epk2*. Poly(A)$^+$ RNA isolated from *E. gracilis* Z was employed. The positions of size markers are indicated in kb. The position of the *epk2* transcript is shown by an arrowhead.

ated in the resting cells, and cAMP generated upon stimulation binds to the regulatory subunit to release the catalytic subunit from the complex [1]. We have not yet obtained the clones encoding the regulatory subunit from *Euglena*. Therefore, the interaction of EPK2 protein with the regulatory subunit of rat PKA (RIα) was examined in the transfected COS-7 cells as shown in Fig. 5. FLAG-RIα was immunoprecipitated by the anti-HA epitope tag antibody from the cell lysate coexpressing FLAG-RIα and HA-EPK2. When the cell lysate was incubated with cAMP, FLAG-RIα was not detected in the immunoprecipitate. FLAG-RIα was not immunoprecipitated by the anti-HA epitope tag antibody from the cell lysate expressing FLAG-RIα alone. On the other hand, the association of HA-EPK2 with FLAG-RIα was also observed in the immunoprecipitate with the anti-FLAG epitope tag antibody in a cAMP-sensitive manner. These results indicate that EPK2 associates with RIα in the expressed cells, and cAMP dissociates the interaction of these two proteins.

3.3. Protein kinase activity of EPK2

The epitope-tagged EPK2 expressed in COS-7 cells was immunoprecipitated, and the protein kinase activity of the recombinant EPK2 protein was measured by monitoring the incorporation of the radioactivity from [γ-^{32}P]ATP to Kemptide, a synthetic peptide substrate of PKA [22]. As shown in Fig. 6, the recombinant protein phosphorylated Kemptide, and the activity was inhibited by PKI, a specific inhibitor of PKA [23]. cAMP stimulated the protein kinase activity of the immunoprecipitated enzyme slightly but reproducibly. It is probable that a part of HA-EPK2 is associated with the regulatory subunit of PKA endogenously expressed in the host cells. PKI inhibited the protein kinase activity to the basal level even in the presence of cAMP. A control test for comparison, using the catalytic subunit of rat PKA, resulted in almost the same inhibition to that of EPK2 (data not shown).

4. Discussion

In this report, five distinct PCR fragments were obtained by using the PCR primers based on the conserved sequences of the AGC family protein kinases. These fragments encoded amino acid sequences homologous to the protein kinases. A full-length clone named *epk2* was isolated using one of the PCR fragments as a probe, which has a high homology to the catalytic subunit of PKA isolated from other species. EPK2 was revealed to associate with the regulatory subunit of the mammalian PKA, indicating that this molecular property of the catalytic subunit of PKA is highly conserved during molecular evolution of the *Euglena* EPK2. In addition, EPK2 had a protein kinase activity toward the substrate peptide preferential to PKA, and the activity was inhibited by PKI, a specific inhibitor of PKA. These results indicate that EPK2 is a homologue of the catalytic subunit of PKA in *Euglena*. The PKA activity has been detected in an extract of *Euglena* [11], but the molecular basis has not been clarified. The present study provides the first molecular biological evidence of the catalytic subunit of PKA in the photosynthetic organisms. The PKA activity detected in the extract of *Euglena* [11] could be encoded, at least in part, by *epk2*. It has been indicated that the intracellular level of cAMP in *Euglena*

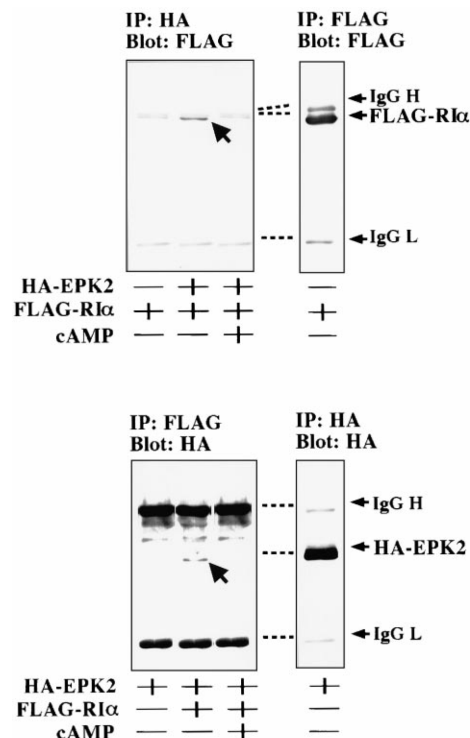

Fig. 5. Interaction of EPK2 with the regulatory subunit of rat PKA. COS-7 cells were transfected with the expression plasmids of HA-*epk2* and FLAG-RIα as indicated at the bottom of the panels. Where indicated, cAMP was added to the cell lysates at 10 μM. Immunoprecipitation (IP) and immunoblot (Blot) were carried out using the antibodies as indicated at the top of the panels. The positions of HA-EPK2, FLAG-RIα, and IgG (heavy (H) and light (L) chains) are indicated by arrows on the right. The positions of coimmunoprecipitated HA-EPK2 and FLAG-RIα are indicated by arrows in the panels.

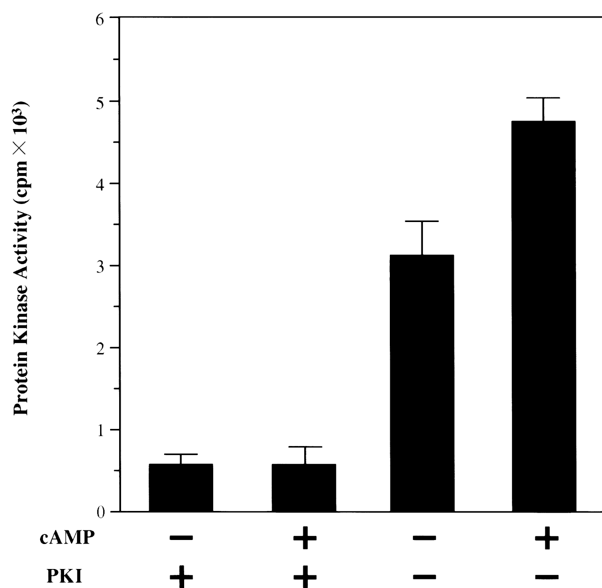

Fig. 6. Protein kinase activity of EPK2. HA-EPK2 was expressed in COS-7 cells and immunoprecipitated. Protein kinase activity was measured using Kemptide as a substrate in the presence or absence of cAMP and PKI. Bars = ±S.D. ($n = 3$).

shows a circadian rhythm, and that PKA is a candidate for an oscillator for circadian clock output which seems to mediate the phasing of the cell cycle [10]. The regulatory subunit of PKA has not yet been identified in *Euglena*, however, the protein kinase activity of *Euglena* PKA would be controlled by the interaction with the regulatory subunit in this organism. Further studies on *Euglena* PKA should give a clue to the roles of cAMP in the phasing of the cell division cycle and the circadian clock. In addition, such studies will contribute to further understanding of the function of PKA not only in *Euglena* but also in other photosynthetic organisms.

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