Molecular Identification of a Calcium-Inhibited Catalytic Subunit of Casein Kinase Type 2 from Paramecium tetraurelia

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We have previously described the occurrence in Paramecium of a casein kinase (CK) activity (EC 2.7.1.37) with some unusual properties, including inhibition by Ca2+. We now have cloned four genes, PtCK2α1 to PtCK2α4, all of which encode the catalytic α subunit of type 2 CK (CK2) with calculated molecular masses ranging from 38.9 to 39.4 kDa and pl values ranging from 8.8 to 9.0. They can be classified into two groups, which differ from each other by 28% on the nucleotide level and by 18% on the derived amino acid level. One of them, PtCK2α3, has been expressed in Escherichia coli and characterized in vitro. As we also have observed with the isolated CK, the recombinant protein preferentially phosphorylates casein but also phosphorylates some Paramecium-specific substrates, including the exocytosis-sensitive phosphoprotein pp63/parafusin. Characteristically, Ca2+ inhibits the phosphorylation at elevated concentrations occurring during stimulation of a cell. Reconstitution with a recombinant form of the regulatory subunit from Xenopus laevis, XlCK2β, confirms Ca2+ sensitivity also under conditions of autophosphorylation. This is unusual for CK2 but correlates with the presence of two EF-hand calcium-binding motifs, one of which is located in the N-terminal segment essential for constitutive activity, as well as with an aberrant composition of normally basic domains recognizing acidic substrate domains. Immunogold localization reveals a considerable enrichment in the outermost cell cortex layers, excluding cilia. We discuss a potential role of this Ca2+-inhibited PtCK2α species in a late step of signal transduction.

The ciliated protozoan Paramecium tetraurelia is an established model system for the analysis of a variety of cell functions in which Ca2+ and protein phosphorylation play a role. This holds for surface pattern formation (24, 50, 57), cilary beat regulation (36, 55, 60), and dense core vesicle exocytosis (38). This and some other unusual features of this Paramecium kinase in comparison to the well-characterized types of mammalian CKs (EC 2.7.1.37), CK1 and CK2 (1, 9, 15), did not allow its unequivocal identification at that time. This is now possible after further analysis on the molecular level. We describe here the cloning of four Paramecium genes, PtCK2α1 to PtCK2α4, all encoding isoforms of the catalytic α subunit of CK2, which suggests the existence of a multigene family for this subunit. In other eukaryotes CK2 has been found as a tetramer consisting of two catalytic (α and α’) and two regulatory (β) subunits in various compositions (1, 9, 42). However, the gene encoding the regulatory subunit in Paramecium is still not known.

In order to express one of the PtCK2α genes in Escherichia coli, Paramecium glutamate codons had to be changed to glutamine codons of the universal code due to the deviant genetic code of Paramecium (5, 48). The active recombinant enzyme, PtCK2α3, has been used for antibody (Ab) production as well as for further biochemical analysis in vitro, including reconstitution analysis with a recombinant form of CK2β from Xenopus laevis (22). The latter has been used because the Paramecium ortholog of CK2β is not known. The unusual feature of Ca2+-sensitive phosphorylation is not restricted to casein or the autophosphorylation of the CK2 subunits but also occurs with the exocytosis-sensitive phosphoprotein, pp63/parafusin (pf), which we have previously cloned from Paramecium (16). In the presence of elevated Ca2+ concentrations pp63/pf is selectively dephosphorylated during exocytosis of the specialized dense-core vesicles (trichocysts) by a Ca2+-stimulated protein phosphatase (21, 26). Since pp63/pf isolated in vivo contains CK2 phosphorylation sites (34) and since CK2 has been found to be enriched also in domains where pp63/pf is located, this may explain the antagonistic effects of Ca2+ on the dephosphorylation-phosphorylation cycle of pp63/pf observed during exocytosis in Paramecium.

(Most of this work is part of a thesis by D. Vetter.)

MATERIALS AND METHODS

Materials. Aprotinin, N-p-tosyl-l-arginine methyl ester, phosphocellulose, DEAE-cellulose, and casein from bovine milk were obtained from Sigma (Deisenhofen, Germany). Pepstatin A was obtained from Serva (Heidelberg, Germany), leupeptin and Pefabloc SC were obtained from Biomol (Hamburg, Germany), [γ-32P]ATP was obtained from ICN (Eschwege, Germany), and sodium dodecyl sulfate (SDS) protein molecular weight markers were obtained from Pharmacia (Freiburg, Germany). Restriction enzymes were from New England Biolabs (Beverly, Mass.). All other reagents and all solvents used were of analytical grade.
Cell cultures and cell fractionation. *P. tetraurelia* wild-type cells (strain 7S) were grown at 25°C in a sterile synthetic medium and processed as previously described (26). Cell fractionation was done as described by Kissmehl et al. (29).

Isolation of CK. Cells were harvested from an 18-liter culture (2,000 cells/ml), concentrated, and washed on ice twice in 600 ml of 20 mM Tris-HCl (pH 7.0) and once in 300 ml of homogenization medium (HM) containing 20 mM triethanolamine-HCl, 10% glycerol, 1 mM dithioerythritol, 1 μM pepstatin A, 20 μM leupeptin, and 0.26 mM N-p-tosyl-L-arginine methyl ester (pH 7.5). The cells were homogenized in 40 ml of HM with an Ultraturrax type 18-10 instrument (Janke & Kunkel KG, Staufen, Germany) for 30 s at 20,000 rpm. The homogenate was centrifuged at 100,000 × g for 60 min at 4°C.

The supernatant was filtered through a layer of glass wool and applied to a 75-ml DEAE-cellulose column. The column was washed with 600 ml of HM and eluted with a linear gradient of 0 to 300 mM NaCl in HM. The total elution volume was 340 ml. Fractions of 6 to 7 ml were collected and assayed for protein kinase activity (see below). Kinase activity-containing fractions, eluting at 180 to 200 mM NaCl, were pooled (65 ml) and dialyzed against HM without glycerol and protease inhibitors. The sample was concentrated with Centriplus 10,000 (Amicon, Beverly, Mass.) and applied to a 4-ml phosphocellulose column. The column was washed with 16 ml of HM without glycerol and protease inhibitors and eluted with a linear salt gradient (22 ml) ranging from 0 to 500 mM NaCl, followed by a step to 1.2 M NaCl. Fractions of 0.5 ml were collected and tested for protein kinase activity.

Reconstitution of PtCK2α3 and XlCK2β. Reconstitution assays of the catalytic α subunit from *Paramecium* (PtCK2α3) and the regulatory β subunit from *X. laevis* (XlCK2β) were performed at 22°C for 10 min in a medium (10-μl final

![FIG. 1. Isolation of a CK which phosphorylates recombinant pp63/pf. (A) The fractions, after DEAE chromatography of a 100,000 × g supernatant, showing pp63/pf phosphorylation activity were further purified on a phosphocellulose column. (B and C) Kinase activity was eluted as a single sharp peak (B). The reaction products of the peak fraction (—3.75 ng of kinase with or without —6.50 μg of substrate) were subjected to electrophoresis, and the corresponding autoradiogram is shown (C). In the presence of Ca2+, phosphorylation of recombinant pp63/pf (filled arrowhead) and its 50-kDa polypeptide degradation product (open arrowhead) is inhibited by more than 30%.](image)
Fractons were collected and subjected to N-terminal sequencing analysis with a 492 protein sequencer (Applied Biosystems, Perkin-Elmer, Elangen, Germany) according to the instructions of the manufacturer.

**PCR strategy.** PCR was performed with $3 \times 10^7$ PFU of a P. tereatulae 51S cDNA library in zXAP Express (Strategene GmbH, Heidelberg, Germany) or $5 \times 10^7$ PFU of a XAPPII (Strategene) genomic library of EcoRI-digested macronuclear DNA from the same cells. Degenerative primers p1 and p2 derived from the sequences of the proteolytic peptides (p1, G152ATGTGTATGAAGG WATWAAG130; p2, A50CGTTGTAATCCCT RKCWWGGATGTTAATTTGC G135) were used in a PCR with the XAP Express cDNA library under following conditions: 5 min at 95°C, 15 cycles of 45 s at 95°C, 30 s at 43°C, and 1 min at 72°C; 15 cycles of 45 s at 95°C, 30 s at 46°C, and 1 min at 72°C; 5 cycles of 45 s at 95°C, 30 s at 49°C, and 1 min at 72°C; and a final 10-min extension at 72°C.

To identify PCK2a1 and PCK2a2, PCR were performed with primers p1 to p5 (p1 and p2 as above; p3, T52AAGGCTTACAACTTGATGAATTG TCA52; p4, A27GGATAATTTACAAATTTCTTTTGGATGAC52; p5, A50 CGTTGTAATCTTGCTCTGCG52) and primers derived from zXAPII or zXAP Express sequences. PCR with zXAP Express cDNA library was done as follows: 5 min at 95°C; 15 cycles of 45 s at 95°C, 2 min at 49°C, and 1 min at 72°C; 15 cycles of 45 s at 95°C, 2 min at 46°C, and 1 min at 72°C; 15 cycles of 45 s at 95°C, 2 min at 43°C, and 1 min at 72°C; and a final 10-min extension at 72°C. PCR with the XAPPII genomic library was done as follows: 5 min at 94°C; 1 min at 50°C; 7 min at 72°C; 3 cycles of 1 min at 94°C, 1 min at 55°C, and 7 min at 72°C; and a final 10-min extension at 72°C. PCR products were cloned into the EcoRV restriction site of pBlueScriptII SK(−), and several clones were sequenced.

**Screening of a ZAPX Express cDNA library.** A total of $3 \times 10^7$ plaques of a ZAPX Express cDNA library of P. tereatulae 51S from stationary phase were screened according to the instruction manual of Strategene’s ZAP Express cloning kit. A sample of 3.5 μg of the purified PCR fragment (see Fig. 3A) was labeled with 1.85 × 10^6 Bq of [γ-^32P]CTP by using the Ready To Go DNA labeling kit from Pharmacia and used as a probe. Because of the high AT content of Paramecium DNA, hybridization was carried out at 38°C in 50% formamide–0.1% (wt/vol) SDS–5× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate [pH 7.3]–5× Denhardt’s solution (52)–100 μg of denatured herring sperm DNA per ml. Washing was carried out in 0.1% (wt/vol) SDS–SSC twice for 10 min each at 38°C and twice for 30 min each at 54°C. Isolated positive plaques were screened in a second round as described above. Positive plaques were rescued by in vivo excision, cloned, and sequenced. To obtain the missing 5' ends, one of the clones was chosen for subsequent genomic library screens (see below). Together with the genomic 5' ends, the two cDNA clones were named PCK2a1 and PCK2a2.

**Screening of a genomic DNA library.** A total of $5 \times 10^5$ primary plaques of a ZAPII genomic library of EcoRIdigested macronuclear DNA from P. tereatulae 51S was screened as described above by using the insert from one of the cDNA clones as a probe after removal of poly(A) sequences by PCR. Two additional genomic clones were obtained and named PCK2a3 and PCK2a4.

**Removal of introns.** Removal of intron sequences was done with the ExSite PCR-based site-directed mutagenesis kit (Strategene). For primers used, see Results. PCR was carried out as follows: 5 min at 94°C, 2 min at 45°C (intron 2, 50°C), and 6 min at 72°C; 30 cycles of 1 min at 94°C, 2 min at 50°C (intron 2, 56°C), and 6 min at 72°C; and a final 10-min extension at 72°C.

**Site-directed mutagenesis.** Site-directed mutagenesis was carried out as described by Deng and Nickoloff (7) with the Transformer site-directed mutagenesis kit (Stratagene). For primers used, see Results. PCR with the mutant cDNA was cloned into the EcoRV restriction site of pBlueScript SK(−), and several clones were sequenced.

**Expression of CK2 subunits in E. coli.** After elimination of all introns and changing of all different Paramecium glutamine codons (TAA and TAG) into universal glutamine codons (CAA and CAG), the coding sequence of PCK2a2 was cloned into the XhoI restriction site of the pET16b expression vector of the pET system from Novagen (Madison, Wis.), which expresses a His6 tag for purification of the recombinant protein. The regulatory β subunit from X. laevis, XcKلا (cly), cloned into pT7H6 (6) was also expressed in E. coli strain B21(DE3). Purification of recombinant PtCK2α and XcKα was performed in a yield of 100 mg/L. Recombinant PCK2a2 and XcKα were purified by affinity chromatography on Ni^2+−nicotinylateagarose under native conditions, as recommended by the manufacturer (Qiagen, volume) containing 50 mM triethanolamine-HCl (pH 7.3), 1 mM dithioerythritol, and 30 mM NaCl.

**Assays for protein kinase activity.** Standard assays for protein kinases were performed in a volume of 30 μl containing 5 mM MgCl2, 120 to 180 μM ATP, 30 × 10^3 Bq of [γ-^32P]ATP, 19.8 μg of single-protein substrates or 30 μg of heat-inactivated Paramecium extracts (100,000 × g supernatant), and buffer (50 mM triethanolamine-HCl, 10 mM NaCl, 1 mM dithioerythritol [pH 7.3]). The MgCl2 concentration used was 1 or 7.5 mM. Ca2+ sensitivity was assayed in the presence of 1 mM EGTA or different concentrations of free Ca2+, as calculated by the computer program Webmaxclite, version 1.0 (3). Reactions were started by adding kinase samples (see figure legends) and terminated after 20 to 30 min at 22°C, and products were measured either by spotting 20-μl aliquots onto trichloroacetic acid-prepared Whatman 1 Chr 3 MM filter papers (59) or by addition of Laemmli sample buffer and subsequent SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and preparation for autoradiography (see below). Autoradiograms were evaluated densitometrically by relative peak area measurements.

**In situ gel kinase assay.** The modified method of Kissmehl et al. (28) was used to identify CK activity. Aliquots of kinase fractions from the phosphocellulose chromatography were analyzed by SDS–10% PAGE with gels mixed with 1 mg of casein per ml. After electrophoresis, SDS was removed by washing the gels with two changes of 20% 2-propanol in 50 mM Tris-HCl (pH 8.0), followed by two changes of 50 mM Tris-HCl–5 mM 2-mercaptoethanol (pH 8.0), each for 30 min. The proteins in the gel were denatured with 6 M guanidine-HCl in the same buffer for 1 h and then renatured with the above buffer containing 0.04% Tween 40 at 4°C for 16 h. Phosphorylation was performed by equilibrating the gels first in reaction buffer (20 mM triethanolamine-HCl, 10 mM MgCl2 [pH 7.5]) at 22°C for 2 h and then in the same buffer supplemented with 13 μM Bq of [γ-^32P]ATP. After 3 h the gels were washed extensively in 5% trichloroacetic acid containing 1% sodium pyrophosphate and prepared for autoradiography.

**Autoradiography.** Gels were dried onto cellophane sheets under vacuum and exposed to Amersham Hyperfilm-MP in Kodak X-Omatic cassettes with intensifier screens for up to 1 week at −70°C.

**Sequencing of proteolytic peptides.** About 8 μg of the 36/37-kDa protein was separated by SDS-PAGE and cleaved in the gel with the endoproteinase Lys C by the method of Eckerorsen and Lottspeich (10). Peptides were separated by reverse-phase high-pressure liquid chromatography on Supersphere 60 RP (Merck, Darmstadt, Germany). The solvent system was 0.1% (vol/vol) trifluoroacetic acid in water (solvent A) and 0.1% (vol/vol) trifluoroacetic acid in acetonitrile (solvent B). A gradient from 0 to 60% solvent B in solvent A was formed over 60 min at a flow rate of 300 μl/min. The detection wavelength was 206 nm.

FIG. 2. In situ gel kinase assay for CKs. The kinase peak fraction obtained during chromatography on phosphocellulose was subjected to electrophoresis on SDS–10% polyacrylamide gels, with or without addition of 1 mg of casein per ml to the matrix prior to polymerization. After SDS-PAGE, the gel with casein included was processed for in situ phosphorylation and subjected to autoradiography (lane 1). The gel without substrate was stained with silver (lane 2). Radioactive phosphate was incorporated into casein (lane 1), forming two strong bands of 48 and 46 kDa and a weaker band of 36/37 kDa, the latter corresponding to the kinase isolated by Kissmehl et al. (28). All phosphorylation activities could be assigned to single protein bands in the silver-stained gel (lane 2).
The recombinant CK2 subunits were eluted with a step gradient in seven steps from 100 to 800 mM imidazole in 50 mM sodium phosphate (pH 6.0)–300 mM NaCl–10% (vol/vol) glycerol. The fractions collected were analyzed on SDS-polyacrylamide gels, and the fractions containing the recombinant proteins were pooled and dialyzed with the buffer of interest for subsequent experiments.

**Preparation of Abs, Western blotting, and immunolocalization.** Abs against recombinant PtCK2/H9251 were raised in rabbits. After several boosts, positive sera were taken at day 60 and purified by two subsequent chromatography steps: a first step on a His tag peptide column (24-amino-acid peptide, to remove His tag-specific Abs), followed by an affinity step on PtCK2/H9251. Western blotting was performed either with affinity-purified polyclonal Abs against recombinant PtCK2/H9251 or with a monoclonal penta-His Ab from Qiagen according to the QIAexpress protocol.

For quantitative immunogold localization on the electron microscopy level, we used methods for fixation (8% formaldehyde–0.1% glutaraldehyde, 12 h, 4°C), embedding (LR Gold, UV polymerization at −35°C), immunogold labeling, electron microscopy analysis, and quantification as outlined by Linder et al. (35). Briefly, ultrathin sections were incubated with primary Ab, followed by protein A (pA) coupled to 5-nm-gold particles (Au5). Controls using irrelevant Abs or omitting the primary Ab gave negative results. Au5 grains were referred to areas determined by a square hit point lattice (43) with a lattice constant depending on the size of structures to be analyzed. Gold-labeling density was referred to the structures defined in Results.

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**FIG. 3.** DNA and deduced amino acid sequences of PtCK2a1 and PtCK2a2. (A) The top two lines in each group show DNA sequences, and the bottom two lines show the amino acid sequences. Dots indicate identical nucleotides or amino acids in the two sequences. Nucleotides shown in lowercase are derived from the genomic sequences (g); those shown in uppercase are from cDNA (c). Boxes, amino acid sequences obtained by microsequencing of two proteolytic peptides of the isolated kinase; double underlining, positions of primers p1 and p2 to yield the underlined PCR fragment used to screen a zaphar3/ZAP Express cDNA library; asterisk, stop codon. (B) PCR strategy used to clone the two genes shown in panel A. Grey boxes, gene sequences in the DNA libraries; black boxes, positions of sequences coding for the microsequenced proteolytic peptides; filled line on the top, PCR fragment shown in panel A; filled lines on the bottom, rapid amplification of cDNA ends-PCR; arrows, positions of primers p1 to p5; x, primers derived from zaphar3 or zaphar3/ZAP Express sequences.
FIG. 4. DNA and deduced amino acid sequences of PtCK2α3 and PtCK2α4. The top two lines in each group show the DNA sequences derived from the genomic sequences, and the bottom two lines show the amino acid sequences. Dots indicate identical nucleotides or amino acids in the two sequences. Nucleotides shown in lowercase are intron sequences. Underlined sequences indicate primers used for removal of introns.
TABLE 1. Comparison of PtCK2α genes in P. tetraureliaa

| Gene       | No. of bp | Derived size (kDa) | Derived pl | Introns |
|------------|-----------|--------------------|------------|---------|
| PtCKα1     | 993       | 38.9               | 8.8        |         |
| PtCKα2     | 491       | 19.4               | 9.0        |         |
| PtCKα3     | 999       | 39.4               | 8.9        | 1       |
| PtCKα4     | 999       | 39.4               | 8.9        | 2       |

|            | Position (beginning) | Size (bp) |
|------------|----------------------|-----------|
| PtCKα1     | 102                  | 22        |
| PtCKα2     | 675                  | 32        |
| PtCKα3     | 824                  | 24        |
| PtCKα4     | 927                  | 22        |

a The percent identities of the derived amino acid sequences are as follows: for PtCKα1 and PtCKα2, 98%; for PtCKα3 and PtCKα4, 97%; and for all forms, ~84%.

RESULTS

We have isolated and purified CK2 from a 100,000 × g supernatant by use of DEAE-cellulose (Fig. 1A) and then, after dialysis, by phosphocellulose column chromatography (Fig. 1B). The resulting kinase activity, with a maximum in fraction 90, was verified not only with casein (data not shown) but also with recombinant pp63/pf (Fig. 1B). In the corresponding autoradiogram, the 63-kDa band of pp63/pf was labeled significantly more (>30%) in the absence than in the presence of Ca2+ (Fig. 1C). The same was true of a 50-kDa degradation product of pp63/pf, which has been identified by several criteria, including Ab binding. Therefore, fraction 90 from the phosphocellulose column was used for further analysis.

As shown in Fig. 2, we probed the capacity of the PtCK2 fraction from Fig. 1 to phosphorylate casein included in an SDS-polyacrylamide gel before polymerization. In the autoradiogram, several phosphorylation bands of 46 and 48 kDa and much weaker ones of 36/37 kDa are visible (Fig. 2, lane 1). These peaks correspond to distinct protein bands in lane 2. All bands were microsequenced. The 48-kDa protein contained the sequence 1YTNLNAQILK. From the 46-kDa band, the sequences NTHGLLK, DFAAHRK, SOFOOQVYDQGK, and MRELVPVK were obtained. However, no sequence homology to any known kinase could be established for these sequences, and the nature of these phosphorylation activities remains to be established. From the 36/37-kDa band we found two sequences, DVYEGIK and DFGLAEYYHPQODYNRVA SRYFK, the latter corresponding unequivocally to sequences common to CK2α isoforms. Therefore, the 36/37-kDa band may represent the Paramecium homologue of the catalytic α subunit of CK2 (PtCK2α), and this band was used to obtain primers for subsequent gene cloning.

Figure 3A shows the sequences of the PtCK2α1 and PtCK2α2 isoforms, which were obtained as follows. From microsequencing studies we derived oligonucleotide primers p1 and p2 by using a degenerate code and considering the codon usage in Paramecium as known until this work was started (17). Primer p1 represents the sequence 5'-D-K58 in PtCK2α1 and PtCK2α2, while primer p2 corresponds to 177A-V190 in PtCK2α1. By PCR we amplified a fragment which itself was used to create primers p3 to p5 (Fig. 3B). To obtain the complete gene sequence, these primers were combined with primers derived from λZAPII or λZAP Express in various PCRs. Only for PtCK2α1 could the whole cDNA sequence be established this way (Fig. 3).

Two more PtCK2 genes, i.e., isoforms PtCKα3 and PtCKα4, have been identified and cloned, as follows. The PCR fragment underlined in Fig. 3A was used to screen a λZAP express cDNA library, resulting in several cDNA clones, one of which was used to screen a genomic library obtained from J. E. Schultz (Tübingen, Germany). We obtained two additional genomic clones, PtCKα3 and PtCKα4, which have also been established on the cDNA level (Fig. 4). Therefore, all isoforms may be expressed. Since the identity of the derived amino acid sequences for these two forms is also very high (~97%) (Fig. 4 and Table 1), their clone similarity would not allow for Northern hybridizations. Both genes, PtCKα3 and PtCKα4, contain four introns, each located at the same positions within the genes. As expected for Paramecium, the four introns are short, varying between 22 and 32 nucleotides in length (Table 1).

Table 1 shows a comparison of all of the isoforms found in Paramecium, and Fig. 5 shows a comparison with sequences from different organisms. All of the isoforms are very similar, suggesting the occurrence of a multigene family in Paramecium.

One of the isoforms, PtCKα3, was expressed in E. coli as a His10-tagged form. After its isolation, the recombinant protein was analyzed by SDS-PAGE and by Western blotting, where it showed a band of ~37 kDa (Fig. 6). The same isoform also was used to characterize the enzymatic activity of PtCK2α (Fig. 7), either in the absence or in the presence of the regulatory β subunit from X. laevis (XICK2β) used in recombinant form. The X. laevis subunit was used because the endogenous gene(s) and protein(s) for the regulatory β subunit are still not known. Whereas XICK2β had no known activity by itself (Fig. 7, lane 2), PtCKα3 was constitutively active (lane 1), and its autophosphorylation could be stimulated by XICK2β (lane 3). Depending on the molar ratio and on the assay conditions, stimulation of the catalytic activity was in the range of 3- to 15-fold and the β subunit was autophosphorylated. The autophosphorylation of both subunits was clearly inhibited by Ca2+.

The same is also true of the catalytic activity towards casein, independent of whether it was phosphorylated only by the catalytic α subunit (lanes 5 and 6) or by the reconstituted α and β subunits (lanes 7 and 8). Ca2+ sensitivity was also observed with several Paramecium substrates, including the exocytosis-sensitive phosphoprotein pp63/pf (Table 2). In all of these cases, the Ca2+ concentrations used were adjusted and were within the range (~10 μM) occurring in a Paramecium cell during stimulus-secretion coupling (46). Activity is not influenced at the low Ca2+ concentrations (~50 nM) occurring in the cytosol of nonactivated cells (data not shown). These ex-


Experiments clearly reveal some of the characteristics of the CK type IIA described by Kissmehl et al. (28) and used as the starting material for gene cloning.

We raised polyclonal Abs against recombinant PtCK2α/H9251, however, due to the considerable similarity of all isoforms, the Abs would not discriminate between the various isoforms, all of which seem to be expressed. Western blot analysis of subcellular fractions from Paramecium revealed PtCK2α to be distributed predominantly in the cytosolic compartment but the signal was considerably enriched in the cortical fraction (not shown). Quantitative immunogold section labeling revealed localization of PtCK2α outside nuclei and organelles, such as in mitochondria and dense-core secretory vesicle (trichocyst) contents, as well as absence from cilia (Fig. 8, Table 3). Values refer to overall cytoplasmic labeling because the antigen is cytosolic. Any deviation from the reference value (1.0 for cytosolic labeling density) directly indicates relative enrichment in specific cytosolic areas, such as the cortical regions specified in Table 3. This holds, with increasing density, for the following structures: trichocyst docking sites, the complex formed by the outer membrane of alveolar sacs (cortical Ca²⁺-stores) and the cell membrane (subplasmalemmal

\[ \text{FIG. 5. Alignment of } \alpha \text{ subunits of different CK2 sequences. Note large deviations in the N-terminal region.} \]
space), and, finally, the complex formed by the inner region of alveolar sacs plus adjacent epipilum. Since gold labeling in cortical regions is considerably greater than general cytoplasmic labeling, retention of PtCK2α in the subplasmalemmal space may explain signal enrichment in Western blots of isolated cell cortex fragments, with cell membranes and attached alveolar sacs but excluding cilia.

**DISCUSSION**

Our aim was to identify unequivocally the *Paramecium* CK, which combines several properties of both types of mammalian CKs but which also differs because of its inhibition by Ca\(^{2+}\) (28). This has now been achieved by analysis on the molecular level, where peptides of the enriched catalytic subunit were analyzed and used to identify the corresponding gene. Since the four isoforms that we found are very similar, only one of them, *PtCK2α*, was selected for heterologous expression and further analysis. Our finding is that the previously described CK in *Paramecium* corresponds to the catalytic α subunit of CK2.

**Aspects pertinent to molecular biology and biochemistry.**

The occurrence of two or more genes and expression of several isoforms of a certain protein frequently occurs in *Paramecium* (16, 18, 25, 30), as we show for the catalytic subunit of CK2 (this study). The open reading frames are interrupted by short AT-rich introns (51, 58), which vary between 22 and 32 bp (Table 1). As expected, in *Paramecium* (58) there is only restricted consensus with sequences usually found in promoter regions of higher eukaryotes, such as TATA or CAT boxes.

**FIG. 6.** Expression of His\(_{10}\)-tagged PtCK2α3 in *E. coli*. Lysis of cells was performed with 6 M guanidinium hydrochloride, and the His\(_{10}\)-tagged protein with an apparent molecular mass of 37 kDa (arrowhead) was purified on an Ni-nitrilotriacetate-agarose column. (A) Silver-stained fraction of recombinant PtCK2α3 (500 ng) (B) Western blot of His\(_{10}\)-tagged PtCK2α3 (100 ng/lane) processed with either anti-His tag Abs (lane 2), affinity-purified anti-PtCK2α3 Abs (lane 3), or affinity-purified anti-PtCK2α3 Abs preadsorbed with PtCK2α3 (lane 4).

**FIG. 7.** Ca\(^{2+}\) inhibits the catalytic activity of PtCK2α3 before and after reconstitution with heterologous CK2β from *X. laevis*. Reconstitution and phosphorylation assays were performed as described in Materials and Methods. Note that PtCK2α (166 ng) is constitutively active (lane 1) and can be stimulated by a factor of ~10 when it is assayed in reconstituted form with 94 ng of XlCK2β (lane 3). CK2β has no activity by itself (lane 2) but is autophosphorylated in the presence of PtCK2α (lane 3). Autophosphorylation of both subunits is strongly inhibited by 10 μM CaCl\(_2\) (lane 4) when compared with assays containing 1 mM EGTA (lane 3). Ca\(^{2+}\) sensitivity is also observed with casein (4.5 μg) as a substrate (lanes 5 to 8), independent of whether only PtCK2α (lane 6) or reconstituted PtCK2α/XlCK2β (lane 8) is used.

Also, the sequence \(^{−3}\)AXYATGG\(^{+4}\), which is characteristic of the environment of the start codon in genes of higher eukaryotes (33), is not present, whereas the noncoding 3′ region displays the usual poly(A)\(^{+}\) tail when identified from cDNA libraries, as shown for the *PtCK2α1* isoform (Fig. 3).

There are also some deviations at the N terminus of PtCK2α. However, this is rather extensive in another protozoan, *Theileria parva*, and is less pronounced in the corn plant, *Zea mays* (Fig. 5). The difference from *Theileria* is noteworthy insofar as it belongs to the Apicomplexa, which, with the cili-

**TABLE 2.** Phosphorylation by reconstituted CK2 is inhibited by Ca\(^{2+}\)

| Phosphorylated band | Densitometric evaluation of reconstituted CK activity from autoradiograms with: EGTA (1 mM) | CaCl\(_2\) (10 μM) |
|---------------------|-----------------------------------------------|-------------------|
|                     | Arbitrary U                                   | Relative activity (%) |
| PtCK2α              | 162.0                                         | 35.5              | 21.9 |
| XICK2β              | 251.4                                         | 113.5             | 45.1 |
| Casein              | 456.2                                         | 219.6             | 48.1 |
| pp73                | 80.3                                          | 52.6              | 65.5 |
| pp63/pf             | 15.3                                          | 4.8               | 31.4 |
| pp47                | 9.0                                           | 3.2               | 35.6 |

\(a\) pp73, pp63/pf, and pp47 occur in the 100,000 x g supematant of *Paramecium* extracts.
FIG. 8. Immunogold (pA-Au₅⁺) localization of PtCK2α on a “grazing” section through the surface region of a *Paramecium* cell whose position is shown in a diagram (A) corresponding to the micrograph (B). Note that cells have an egg case-like surface relief, with cross-sectioned cilia (ci) emanating from the center of a surface impression. The section enters an increasingly deeper cytoplasmic region at the right side. Note the absence of label from plastic alone (left) and a cilium and scarce label in the cytosolic compartment (small arrowheads), except for larger groups of gold grains (large arrowheads) in some regions of the surface complex, i.e., at the interaction of cell membrane (cm) and outer region of alveolar sacs (as) and at the complex formed by the inner region of an alveolar sac and the epiplasm (ep). Two gold grains occur close to the docking site of a trichocyst (tt) (medium arrowhead).
ates, belong to the phylum of Alveolata. *Paramecium* isoforms display the highest resemblance to the enzymes of yeasts. Concomitantly, the evolutionary tree (Fig. 9) clearly shows that the CK2\(\alpha\) forms of yeast and *Paramecium* are positioned most closely to each other, in agreement with some functional aspects discussed below.

Several subdomains of the CK2\(\alpha\) subunit, as classified by Hanks and Hunter (15) (Fig. 10), contain characteristic substrate recognition motifs (42), which in part deviate in *Paramecium*. General CK2 motifs are 74KKK77, 79KREIK83, and 191RVASRYFK198. We find 74KKQK77, 79KRETK83, and 191RVASRYFK198 in PtCK2\(1/2\), as well as 74KKRR77, 79KRETK83, and 191RVASRYFK198 in PtCK2\(3/4\). Thus, only the last motif is fully conserved in *Paramecium*. Another feature critical for substrate binding is located in the 148Ht to H166 region of CK2. Generally, this conserved region consists of H148X5H154X5H160X5H166 (1). In the \(\alpha\) subunits of *Saccharomyces cerevisiae* (41) and of all PtCK2 isoforms (this paper), this sequence is changed to H148X5H154X5Q160X5P166. The changes observed may explain some of the deviating biochemical features previously reported (28), including Ca\(^2+\) sensitivity.

The observed Ca\(^2+\) sensitivity is based on several lines of evidence. (i) Inhibition of enzymatic activity by Ca\(^2+\) occurs not only with the catalytic \(\alpha\) subunit but also with the holoenzyme after reconstitution with the \(\beta\) subunit from *X. laevis*. Although such experiments still have to be repeated with the endogenous regulatory subunit once it is identified, a similar effect can be predicted. (ii) Ca\(^2+\) sensitivity is not restricted to the phosphorylation of a specific substrate but can also be observed with the CK2 subunits themselves under conditions of autophosphorylation. (iii) The Ca\(^2+\) concentration used to

### TABLE 3. Relative density of labeling by anti-PtCK2\(\alpha\) Abs, followed by pA-Au

| Structure                                                                 | Relative labeling density\(^a\) |
|---------------------------------------------------------------------------|---------------------------------|
| Cytosol (cytoplasm outside organelles).............................................| 1.00                           |
| Nuclei.............................................................| 0.07                           |
| Trichocyst\(^b\) contents ..................................................................| 0.00                           |
| Mitochondria ..................................................................................| 0.03                           |
| Cilia ............................................................................................| 0.04                           |
| Trichocyst tips + attached collar\(^c\) materials)................................| 3.12                           |
| Cortex                                                                    |
| Cell membrane + outer region of alveolar sac\(^d\) membrane ..................| 4.83                           |
| Inner region of alveolar sac membrane with attached epilasm\(^e\) ..........| 10.95                          |
| Extracellular (embedding material only)............................................| 0.02                           |

\(^a\) Data are pooled from eight cell sections and normalized to 1.00 for cytosolic labeling (absolute value, 4.22 pA-Au, grains/\(\mu\)m\(^2\); off-cell background, 0.01 pA-Au, grains/\(\mu\)m\(^2\)). For details, see Materials and Methods.

\(^b\) Dense-core secretory vesicles.

\(^c\) Cytosolic proteins attached to the trichocyst membrane in its upper region close to the docking site at the cell membrane.

\(^d\) Flat cortical Ca\(^2+\) stores underlying the cell membrane, with free sites for trichocyst insertion at the cell membrane and emergence of cilia.

\(^e\) Proteins underlying alveolar sacs.

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FIG. 9. Phylogenetic tree of CK2\(\alpha\) subunits, including the PtCK2\(\alpha\) isoforms. Multiple positions are sequences from different EMBL data bank accession numbers, aligned from top to bottom in the following sequence: X54962, M98431, M55265, J02853, M93665, X70251, U51866, M9456, X63775, M16534, D90994, D17712, M55268, M59457, AJ290815, AJ290814, AJ290813, L29508, X74275, Z30597, M33759, M22473, D10246, D10247, X61387, Y11526, L05535, and M92084. Protein sequences were analyzed with the program Megalign according to the Clustal method with the identity weight table (rating only identical amino acids). The length of a branch symbolizes the number of amino acid exchanges.
obtain half-maximal inhibition is within the physiological range (≤10 μM) occurring during stimulus-secretion coupling (46).

(iv) Motif searching (2) in the primary sequences of PtCK2α revealed the presence of two EF-hand Ca$^{2+}$-binding domains, which show up to 76 to 88% similarity with the EF-hand consensus pattern (23) (Table 4). Whereas one of them is located near the catalytic loop (Fig. 10), the position of the second motif differs among the four PtCK2α isoforms. In PtCK2α3

![Diagram](image)

**FIG. 10.** Domain structure of PtCK2 and amino acid motifs relevant for substrate recognition by CK2, indicated according to references 15 and 42, respectively.

**TABLE 4.** EF-hand calcium-binding motifs in the primary sequences of the catalytic subunits of *Paramecium* CK2 and other *Paramecium* Ca$^{2+}$-binding proteins

| Protein and motif | Amino acid at position: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|------------------|------------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **EF-hand consensus**<sup>a</sup> | | D | x | DNS | Not ILVFW | DENSTG | DNOGRHK | Not GP | LIVMC | DENQSTAGC | x | x | DE | LIVMFYW |
| CaM<sup>b</sup> | | D K D D G D G T I T K E L | | | | | | | | | | | | |
| EF121–33 | | D K D D G D G T I T K E L | | | | | | | | | | | | |
| EF257–69 | | D A D G D G T I D F P E F | | | | | | | | | | | | |
| EF394–106 | | D R D G N G L L S A A E L | | | | | | | | | | | | |
| EF4130–142 | | D T D G D G H I N Y E E F | | | | | | | | | | | | |
| **Centrin**<sup>c</sup> | | D T D G T O S T D PK E L | | | | | | | | | | | | |
| EF150–62 | | D T D G T O S T D PK E L | | | | | | | | | | | | |
| EF286–98 | | D T D G S G O T D F A E F | | | | | | | | | | | | |
| EF3123–135 | | D S E R A G V V T L K D L | | | | | | | | | | | | |
| EF4159–171 | | D S D G A O V V T F E D F | | | | | | | | | | | | |
| **CaN-B**<sup>d</sup> | | D K D D G S G O L E P S E L | | | | | | | | | | | | |
| EF130–42 | | D K D D G S G O L E P S E L | | | | | | | | | | | | |
| EF262–74 | | D T D G N D G K K S F A E F | | | | | | | | | | | | |
| EF398–110 | | D T D O D G F I T N G E L | | | | | | | | | | | | |
| EF4139–151 | | D E D F D D S K I S F A E F | | | | | | | | | | | | |
| **PtCK2α1** | | D P S S S K T P C I F D Y | | | | | | | | | | | | |
| EF1103–115 | | D Y C H S K G I M H R D V | | | | | | | | | | | | |
| EF2145–157 | | D Y C H S K G I M H R D V | | | | | | | | | | | | |
| **PtCK2α2** | | D P S S S K T P C I F D Y | | | | | | | | | | | | |
| EF1103–115 | | D Y C H S K G I M H R D V | | | | | | | | | | | | |
| EF2145–157 | | D Y C H S K G I M H R D V | | | | | | | | | | | | |
| **PtCK2α3** | | D Y N K N O P K E Y W D Y | | | | | | | | | | | | |
| EF114–26 | | D Y N K N O P K E Y W D Y | | | | | | | | | | | | |
| EF2145–157 | | D Y N K N O P K E Y W D Y | | | | | | | | | | | | |
| **PtCK2α4** | | D F N K N O P K E Y W D Y | | | | | | | | | | | | |
| EF114–26 | | D F N K N O P K E Y W D Y | | | | | | | | | | | | |
| EF2145–157 | | D F N K N O P K E Y W D Y | | | | | | | | | | | | |

<sup>a</sup> Motif searching was done by using Proscan (2) with a threshold of 70%.

<sup>b</sup> Amino acids matching the consensus pattern are in boldface.

<sup>c</sup> Prosite accession number PS00018.

<sup>d</sup> EMBL accession number U35344.
and PtCK2α4, the EF-hand motif is located in the N-terminal segment at position 14 to 26, a region which is essential for the constitutive activity of mammalian CK2α (53, 54). Therefore, binding of Ca$^{2+}$ in this region might be critical for the interaction of the N-terminal segment and the activation segment, which is necessary to provide a fully active conformation state of the catalytic subunit (54). Interestingly, Ca$^{2+}$ sensitivity is also observed in the reconstituted holoenzyme, suggesting that in the presence of Ca$^{2+}$ the N-terminal region is still unable to keep the activation loop in an open conformation due to conformational changes within the N-terminal region. This aspect implies that Ca$^{2+}$ might play an important role in the regulation of the catalytic activity of this kinase.

**Aspects pertinent to cell biology.** In contrast to Ser/Thr phosphorylation-dephosphorylation, for ciliates only little is known about reversible Tyr phosphorylation, although at least one Tyr kinase seems to be present among the kinases detected in the 1 to 2% genome sequencing (58). This excludes dual-specificity CKs (20, 49). In *Paramecium*, neither protein kinase C nor Ca$^{2+}$/calmodulin (CaM)-dependent protein kinase has been identified unequivocally on the molecular level so far, although more than 119 eukaryotic protein kinase domains have been recently identified (8, 58). However, different types of Ca$^{2+}$-activated kinases (14, 56), including a family of Ca$^{2+}$-dependent kinases with integrated CaM motifs (25), have been described, as well as a Ca$^{2+}$-inhibited CK (28), which we now can assign as the *Paramecium* homologue of CK2. While we find that PtCK2α is absent from cilia (Table 3), there also exist soluble and axonema-bound CKs of the type 1 family (62), as well as Ca$^{2+}$-dependent protein kinases, CaPK1 and CaPK2, which have all been localized to cilia of *Paramecium* (14, 56).

Therefore, PtCK2α may operate in nonciliary cortical domains.

The model presented in Fig. 11 is based on the following observations. Induction of synchronous trichocyst exocytosis is accompanied by a rapid (30-ms) transient increase in the free Ca$^{2+}$ concentration, [Ca$^{2+}$]$_{i}$ in the cell cortex (11, 31, 32). In fluorescent analyses, [Ca$^{2+}$]$_{i}$ transients culminate at ~1 s, and they are downregulated within ~20 s (32). Induction of synchronous trichocyst exocytosis is also accompanied by a rapid dephosphorylation of the exocytosis-sensitive phosphoprotein pp63/pf (21, 26), which is one of the substrates used in this study (Fig. 1 and Table 2). The corresponding enzyme has been identified as calcineurin (CaN) (27, 38), a Ca$^{2+}$/CaM-dependent phosphatase also occurs in *Paramecium* (27) with enrichment in the cell cortex (39), where we already found pp63/pf (29) and where we now find enrichment of PtCK2α (this paper). Whereas pp63/pf dephosphorylation is accomplished within ~80 ms (21), rephosphorylation occurs within up to 20 s (26, 63). This temporal coincidence and the inverse Ca$^{2+}$ sensitivity of the various enzymes may imply that the cortical [Ca$^{2+}$] signals generated during trichocyst exocytosis could affect the activities not only of protein phosphatases but also of protein kinases with different Ca$^{2+}$ sensitivities. According to matrix-assisted laser desorption ionization analysis, three of the six Ser and Thr sites phosphorylated in vivo can be attributed to CK2 activities (34), with all sites located at the surface of the pp63/pf molecule (40). Ca$^{2+}$-stimulated (4) and Ca$^{2+}$-inhibited kinases may be active at different times, as the Ca$^{2+}$ transient develops and decreases during and after exocytosis performance, respectively. The feedback may include regulation of [Ca$^{2+}$]$_{i}$, since pp63/pf has been identified as...
phosphoglucomutase (16), an enzyme strongly involved in the regulation of [Ca^{2+}]], homeostasis in yeast (12). Considering the phosphoglucomutase activity of pp63/pf, we also have implied a role in establishing ATP and Ca^{2+} homeostasis in the subplasmalemmal domain (40), where CK2 is also localized. In addition, the Ca^{2+}-sensitive dephosphorylation-rephosphorylation cycle of pp63/pf upon exocytosis stimulation may affect microdomain localization (binding) and/or enzymatic activity of the molecule.

Although the identity of some other phosphorylated protein substrates still has to be established (Table 2), phosphorylation of other cortical components is known to regulate self-assembly processes during surface pattern formation (24, 57). Since this is accompanied by [Ca^{2+}] oscillations (47), some Ca^{2+}-sensitive protein phosphorylation steps may be included, although the type of Ser/Thr kinase involved in Partamentum is not known. In S. cerevisiae the homologue of PtCK2α has been discussed in the context of the establishment of cell polarity (13), and in fact these homologues share important features of substrate recognition domains (see preceding section). Ca^{2+}-sensitive CKs, including the one described here, may now be tested more stringently in different cell types for analysis of the complicated interplay between [Ca^{2+}], signals and any other second messengers.

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