The phosphocreatine shuttle of sea urchin sperm: Flagellar creatine kinase resulted from a gene triplication

D. D. WOTHE*, H. CHARBONNEAU, AND B. M. SHAPIRO

Department of Biochemistry, SJ-70, University of Washington, Seattle, WA 98195

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ABSTRACT TCK, the creatine kinase (ATP:creatine N-phosphotransferase) from sperm flagella of the sea urchin Strongylocentrotus purpuratus, is a M, 145,000 aminolal protein that is employed in energy transport. Its amino acid sequence was obtained by analysis of fragments from cyanogen bromide digestion and by sequencing cDNA clones from two sea urchin testis libraries. TCK contains three complete but nonidentical creatine kinase segments joined by regions of sequence that are not creatine kinase-like and flanked by unique amino and carboxyl termini. Each creatine kinase segment is homologous to vertebrate creatines of both muscle and brain types, and all three repeats contain the essential active-site cysteine. The sequence differences among repeats suggest an ancient gene triplication, around the time of the chordate—echinoderm divergence. The echinoderm, with a unique creatine kinase in sperm, arginine kinase in eggs, and both phosphagen kinases in somatic cells, may represent a preserved branch point in evolution, and TCK may be a relic of this event.

Structurally similar creatine kinases (CKs; EC 2.7.3.2) are found in many invertebrates and vertebrates (1), existing as dimers or octamers of subunit M, 40,000 (2, 3). However, an echinoderm-specific CK is significantly larger: purified from the flagellum of Strongylocentrotus purpuratus sperm is monomeric, with an estimated M, of 145,000 (4). This CK participates in an energy shuttle that utilizes phosphocreatine to transfer the energy from ATP generated by the mitochondrion in the sperm head to dynein in the distal portions of the flagellum (5). A mitochondrial CK transphosphorylates creatine and ATP, maintaining the high levels of ADP that permit maximum respiration (5, 6). As phosphocreatine diffuses along the flagellum, the flagellar creatine kinase (TCK) regenerates ATP for use as a substrate by dynein. Specific inhibition of CKs by low levels of 1-fluoro-2,4-dinitrobenzene attenuates flagellar beating (6), indicating that the energy shuttle is essential for normal flagellar motion. TCK specifically associates with the axoneme and may bind directly to polymerized microtubules (7). To ascertain the primary structure of this unusually large CK, and to provide tools to examine its association with the axoneme, we have purified TCK, isolated and sequenced six CNBr peptides, and obtained the sequence of clones from two independent cDNA libraries.

MATERIALS AND METHODS

S. purpuratus were obtained at low tide along the northern shore of the Olympic Peninsula in Washington. Restriction endonucleases were purchased from United States Biochemical and Boehringer Mannheim. All other reagents were of the highest grade available.

Sperm were obtained and TCK was purified as described (4). TCK (46 mg) was dialyzed against 1.44 M Tris (pH 8.6) and S-carboxymethylated (8); the sample was then dialyzed against 70% formic acid and cleaved at methionine residues with CNBr (9). CNBr peptides were lyophilized, dissolved in 6 M guanidinium chloride buffered to pH 6.0 with 10 mM phosphate, and separated into size classes by size-exclusion HPLC, using two TSK-G3000SW columns (LKB) in series and a buffer containing 6 M guanidinium chloride in 10 mM phosphate (pH 6.0) at an elution rate of 0.5 ml/min. Individual peptide were separated from each size class by loading onto either a SynChropak RP-P C18 or a SynChropak RP-P C18 column (SynChron) and eluting with a 0–60% acetonitrile gradient (0.5%/min) in aqueous 0.085% trifluoroacetic acid at 1 ml/min.

Amino acid analysis of CNBr peptides was performed with a Waters system using the Pico-Tag derivatization protocol (10). Automated Edman degradations were performed in a Beckman 890C sequencer and derivatives were identified by HPLC (11).

A Agt11 cDNA library was generously provided by E. Davidson (California Institute of Technology, Pasadena, CA). This library was constructed by David Nishiyoda, by ligating inserts from a randomly primed Agt10 library made using S. purpuratus testis mRNA (12) into Agt11 vector arms. Plaques (620,000) were screened with antisense oligodeoxy-nucleotides (GTGGCAGAAACGCTTGAAGAC, GTTGATGGCTCTCTCCCTTCAT) designed using sequences from TCK CNBr peptides 1 and 6 (Fig. 1). The oligonucleotide sequence synthesized was based on codon-bias estimates for S. purpuratus from available cDNA sequences (13). Oligonucleotides were end-labeled with 32P by phage T4 polynucleotide kinase and used to screen filters representing ~25,000 Agt11 plaques each, according to standard techniques (14). A second, oligo(dT)-primed AZAP (Stratagene) cDNA library made using S. purpuratus testis mRNA by Dangott et al. (15) was generously provided by D. Garbers (Vanderbilt University Medical Center, Nashville, TN). This library was screened (164,000 plaques) with a TCK cDNA fragment from the Agt11 library labeled to a specific activity of 106 cpm/μg by the random priming method (16). cDNA clones that hybridized to both oligonucleotide probes were rescreened until only hybridizing phage were present, and phage DNA was isolated (14). Inserts were cut out with EcoRI and cloned into pBluescript KS(-) (Stratagene).

Single- and double-stranded DNA sequencing was performed using a Sequenase 2.0 kit (United States Biochemical), which uses the dyeoxynucleotide chain-termination procedure of Sanger et al. (17). The amino acid sequence deduced from the cDNA sequence was compared to the Protein Identification Resource protein sequence data bank (release 21) and the GenBank data base (release 62.0).

Abbreviations: CK, creatine kinase; TCK, flagellar CK.

To whom reprint requests should be addressed.

The sequence reported in this paper has been deposited in the GenBank data base (accession no. M33763).
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CMCK 246-271  MKEVFRRFCVGLKKIEEIHKKAGHPF
Peptide 1  MKVFRKRECNLKVENALKAGYE
Peptide 2  MRAVEKRFEDQGFE
Peptide 3  MKVREFFCDQKLKVEISDKSKY

CMCK 74-90  CVAGDEESEYEFDKLFDF
Peptide 4  MVAGDEESEYDFAFADFD

CMCK 207-220  MARDWPDAARGIWHN
Peptide 5  MARDWPQQRGRIWHN

Peptide 6  MKGEDINSLLPEKLR

FIG. 1. Amino-terminal sequences (including the inferred methionines) for CNBr peptides from TCK aligned with regions of the chicken muscle CK (CMCK) sequence obtained from the cDNA sequence. The position of the CMCK sequence is indicated before each fragment. Peptides 1–5 are all similar to CMCK sequences. The underlined portions of peptides 1–3 are identical to each other. Each of the residues common to all three repeats is identical to equivalent positions in the CMCK sequence.

RESULTS AND DISCUSSION

Protein Sequencing. CNBr peptides of TCK were separated into nine size classes and each pooled size population was purified further by reverse-phase HPLC (data not shown). Amino acid sequence was obtained from six of the purified peptides and compared to sequences deduced from other CK cDNAs. Fig. 1 shows the peptide sequences aligned with the indicated portion of the protein sequence from chicken muscle CK cDNAs.

TCK peptides 1–5 were similar to regions of vertebrate CKs. In addition, peptides 1–3 were similar to one another, which indicated either a triplication of CK units or polymorphism of TCK. Peptide 6 had no significant sequence similarity to CKs.

cDNA Sequence. Two partial TCK cDNA clones were identified in the &kappa;11 library by screening with the two synthetic oligonucleotides, and several 4.0-kilobase (kb) clones were identified in the AZAP library by using the two partial cDNA clones. Restriction maps for the TCK cDNA, a 4.0-kb clone, and the two partial clones are presented in Fig. 2a. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 3. The longest clone identified was 3985 base pairs (bp), excluding the poly(A) tail, and contained a 3522-bp open reading frame beginning at position 95 with a consensus translation initiation sequence (18, 19). It is preceded by a potential translation initiation sequence beginning at position 70 that is almost certainly not recognized by 40S ribosomal subunits (18, 19). The single long open reading frame is followed by a 366-bp 3' untranslated sequence that includes an imperfect polyadenylation consensus sequence (AATACA) at position 3961 followed in 20 bases by poly(A) sequence.

Examination of the translation of the 3522-bp open reading frame reveals three nonidentical CK repeats flanked by unique regions (Fig. 2b; regions A and D). Each repeat is joined to the next by unique sequence (regions B and C). The protein sequence predicted by translation of the cDNA sequence contains all six peptide sequences in frame (Figs. 2b and 3). The protein is 1174 amino acids long with a predicted molecular weight of 130,877 and a pI of 5.85. All six peptide sequences are identical to the translations at amino acids 287–312, 488–504, 995–1008, 1034–1057, and 1156–1170. However, the translation of clone WB2.1 of Fig. 2 had an amino acid different from that of peptide 6 at position 1166, raising the possibility that this position may be polymorphic.

The triplication of CK units explains the large size of this monomeric CK. To our knowledge, this is the first example of an endotriplication of potentially functional enzyme units. Characterization of other proteins has demonstrated that gene duplication to form homologous domains is a common occurrence in evolution. For example, human CD45 has two phosphatase domains formed by a repeat of exons 17–25 (20). Both repeats are part of the cytoplasmic domain and may be involved in signaling in leukocytes. Precursor proteins such as prolifinigrin, which consists of a 250-amino acid filaggrin unit repeated 12 times in the primary transcript, are partially cleaved to form intermediates that, after use, are further

a

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b

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Fig. 2. (a) Restriction endonuclease map for the TCK cDNA. Heavy line indicates the region of the cDNA that is translated. Restriction endonuclease sites are shown below the line: E, EcoRI; B, BamHI; S, SstI; T, SstII; X, XhoI. Identical restriction sites were found in the three cDNAs presented below the TCK cDNA. Clones WD2.1 and WC2.1 were selected from the &kappa;11 cDNA library. Clone WD2.2 was selected from the AZAP cDNA library and contains the entire coding sequence. (b) Line diagram of the TCK protein sequence with the three repeats boxed. The non-CK amino terminus is 60 amino acids long (A). Each repeat is 354 amino acids long. The first and second repeats are separated by a 19-amino acid linking region (B). The second and third are separated by a 20-amino acid linking region (C). The last repeat is followed by a 13-amino acid stretch of non-CK-like sequence (D). The positions of the six CNBr peptides that were sequenced are indicated by the numbered bars.
cleaved to form monomers (21). The endotriplated TCK is probably an example of the first sort, creating a protein with three active sites. There is no evidence that the three subunits are cleaved into individual CK units.

Triplated domains have been seen in two other cases. Ferredoxin, a non-heme iron protein, has three related domains resulting from an ancient gene tripliation (22). A rare variant of the hemoglobin-binding protein haptoglobin (Johnson type) contains a partial tripliation that may have resulted from the unequal crossing-over of an already duplicated allele (23). A similar mechanism might explain the tripliation of this echinoderm CK.

**Echinoderm CKs.** The only phosphen kinase of echinoderm sperm is the high molecular weight CK and a mitochondrial CK (4), whereas eggs have only arginine kinase (24, 25). This situation may have arisen from an early duplication of a primordial phosphen kinase, which then diverged into creatine and arginine kinases. A later duplication of the CK, followed by unequal crossing-over, could have resulted in TCK. These several phosphen kinases might have further evolved for specific functions in each gamete—for example, in producing the phosphocreatine shuttle that facilitates energy transport in sperm.

The high molecular weight form of CK is found in sperm from all echinoderms (24, 25) as well as in some somatic echinoderm tissues. The distribution in somatic tissues varies from one species to another. Thus the echinoderm may represent a preserved branch point in evolution in which the primary phosphen energy source is in transition from arginine kinase to CK.

**Comparisons with Other CKs.** The high degree of sequence similarity between TCK and other CKs confirms TCK as a member of the same family. The percent of identical residues when each domain is aligned with the other two domains is presented in Table 1. The percent identity between each repeated domain of TCK and representative muscle (26–28), brain (29), mitochondrial (30), piscine (31), mammalian (26), and avian (27, 28) CKs is also shown (Table 1). For compar-
Table 1. Percent sequence identity among various CKs

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<td>66</td>
<td>83</td>
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The percent sequence identity is shown for each repeat of TCK in comparison with the other two and with 10 vertebrate CK sequences when both are aligned. The alignment was done with the Intelligenetics Suite program of programs and is for the 354 residues comprising each repeat. Similar data are presented for the 381 residues of the human muscle and brain isozymes of CK and for the human mitochondrial CK.

ison, the percent identity to the human isozymes of CK is also presented (32–34). It is noteworthy that while even the human brain and muscle isoforms are 80% identical, no two domains of TCK share more than 72% identity. In fact, the differences between domains of TCK are as great as the differences between TCK and these other CKs. This finding suggests that the gene duplication events took place long ago, around the time of the chordate radiation.

Table 2. A TCK-specific pentapeptide sequence and the reactive cysteine sequence

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*See Table 1 for abbreviations.
†A conserved 5-amino acid portion of each TCK repeat differs from vertebrate CKs in the same well-conserved region. The three repeats of TCK were aligned with portions of other CKs. The changed region is underlined.
‡The protein sequences surrounding the putative reactive cysteines (*) of all three repeats are aligned with the same region of vertebrate CKs.

Although the endotripping was a distant event, features common to CKs are found in each of the three repeats of TCK. Four tryptophan residues exist in conserved positions as determined from previous CK sequences (2, 30) and one of these tryptophan residues is thought to bind the adenine of ADP at the active site (35). Hosse et al. (30) have described a “CK framework” consisting of regions of CK sequence that are conserved among all known sequences. Most regions making up this CK framework are intact in all three TCK repeats, but one region that is characteristic of the vertebrate enzyme differs in TCK and is identical in all three repeated domains (Table 2, upper half). Although no direct evidence exists as to whether each domain of TCK is catalytically active, residues that are part of the CK active site are conserved in each, including the reactive cysteine (31, 36) and surrounding peptide (Table 2, lower half). If each domain were catalytically active, we would expect the TCK to have a specific activity similar to that of the mammalian enzyme, as well as multiple fluorodinitrobenzene binding sites (37) per molecule of enzyme. Indeed, the specific activity of purified TCK is 235 units/mg, which is comparable to values obtained for mammalian CKs (4). By transfecting cells with appropriately modified cDNA clones, we should be able to directly examine the capacity of each domain to effect catalysis, as well as the sites in the TCK sequence (if any) responsible for association with microtubules (5) of the axoneme.

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