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Cloning of a human choline kinase cDNA by complementation of the yeast *cki* mutation

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A human choline kinase cDNA was cloned by complementation of the yeast choline kinase mutation, *cki*, from a human glioblastoma cDNA expression library. The deduced sequence of the human enzyme comprised 456 amino acids with a calculated relative molecular mass of 52,065. The human enzyme resembled the rat liver enzyme over the entire sequence. It also resembled the yeast enzyme in the carboxy-terminal region, but not much in the amino-terminal region.

Human choline kinase cDNA; Functional cloning; Yeast mutant

1. INTRODUCTION

It is generally accepted that cholinephosphate cytidylyltransferase plays a major role in the regulation of phosphatidylcholine synthesis (for a review, see [1]). But recently a regulatory role of choline kinase in phosphatidylcholine synthesis has also been suggested (for a review, see [2]). To elucidate the regulatory mechanism of choline kinase, its cDNA and gene would be extremely useful. DNA encoding choline kinase has been isolated from two sources, yeast and rat liver, in our laboratory [3,4]. The yeast gene was obtained by complementation of the choline kinase mutation, *cki* [5,6], and rat liver choline kinase cDNA was recently cloned, using specific antibodies, from a λgt11 rat liver cDNA library. The open reading frames in the yeast and rat liver clones encoded 582 and 435 amino acid residues with relative molecular masses of 66,316 and 49,737, respectively.

Recently, several mammalian cDNAs were successfully cloned by complementation of the corresponding yeast mutations [7–12], but so far no successful cloning of cDNAs for phospholipid metabolism has been reported. Here we demonstrate the cloning of a mammalian cDNA encoding choline kinase by complementation of the yeast *cki* mutation from a human glioblastoma cDNA library constructed by Colicelli et al. [11]. The results raises the possibility that other mammalian cDNAs involved in phospholipid metabolism would also be available through yeast complementation (for yeast phospholipid metabolism mutants, see review [13]).

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2. MATERIALS AND METHODS

2.1. *Yeast strains and cDNA library*

Wild-type strain X2180-1A (*a SUC2 mal mel gal2 CUP1*) was provided by the Yeast Genetic Stock Center (University of California). Phosphatidylserine synthase mutant 667 (*a pss*) was isolated as an ethanolamine auxotroph from X2180-1A as described previously [14]. *cki* disruptant 2013 (αcki::HIS3 leu2 his3 trp1 can1) was constructed essentially as described previously [3], except that the gene was disrupted by inserting *HIS3* [15] between the *Bgl*II and *Bam*HI sites of the coding frame. Cloning strain D493-2 (*a pss cki::HIS3 leu2 his3 trp1 can1*) was constructed from 667 and 2013 by the standard genetic method. The composition of the inositol-free minimum medium (M-i medium) was as described previously [16]. The human glioblastoma cDNA library [11] was a gift from M. Wigler (Cold Spring Harbor Laboratory) to J.N.

2.2. *Materials*

Restriction endonucleases and other reagents were obtained from Takara Shuzo (Kyoto) and Nippon Gene (Toyama). [α-^{32}P]dCTP (409 Ci/mmol) and [*methyl*-^{14}C]choline chloride (52.0 Ci/mol) were purchased from Amersham Corp. and DuPont-New England Nuclear, respectively.

3. RESULTS AND DISCUSSION

3.1. *Cloning strategy*

Mutants defective only in the choline kinase gene (*CKI*) do not show any apparent growth phenotype [6], and are thus not suitable for complementation cloning. We introduced *cki* into the *pss* background to construct a *cki pss* double mutant. The *pss* mutation defective in phosphatidylserine synthase causes auxotrophic requirement for serine, ethanolamine, or choline [14].

When 20 μg/ml inositol was added to the culture, the requirement for ethanolamine and choline was more pronounced, and serine was no longer effective, probably due to a further decrease in phosphatidylserine synthesis caused by inhibition of the synthase by inositol [17] and by the lowered availability of CDPdiacylglycerol. When the *cki* mutation was added to this *pss* background, the resulting double mutant was unable to grow on choline because the *cki* mutation prevented choline utilization. Transformation of the double mutant with choline kinase cDNA should restore growth. Thus we thought that a choline kinase cDNA could be obtained by complementation of the *cki* mutation in the *cki pss* mutant.

3.2. *Cloning of a human cDNA capable of complementing the yeast* cki *mutation*

We cultured D493-2 (*a pss cki::HIS3 leu2 his3 trp1 can1*) in inositol-free minimum medium (M-i) supplemented with 20 μg/ml leucine, 20 μg/ml tryptophan and 200 μg/ml serine to the logarithmic phase at 23°C. Competent cells were prepared and transformed with a human glioblastoma cDNA library [11] by the method of Ito et al. [18]. The library had been constructed on yeast expression vector pADANS [11] containing the *ADH1* promoter and 14 amino acids derived from *ADH1*, and the *Not*I cloning site. Thus the cDNAs could be expressed under the control of the *ADH1* promoter in yeast cells. In this experiment, we used 10 μg of the library per 10^9 cells, and cultured the transformants for one week on one minimum plate supplemented with 20 μg/ml each of inositol, choline and tryptophan. From five such plates, we obtained 9 colonies. Seven were found to contain identical plasmids carrying a 2.4-kbp insert. We named this plasmid, pHGCKI-1. The other two colonies contained plasmids with smaller inserts, 1.8 and 1.6 kbp, and were not studied further. Upon retransformation with purified pHGCKI-1, D493-2 acquired the ability to grow on choline in the presence of 20 μg/ml inositol. The transformant did not grow without choline, indicating that the plasmid complemented the *cki* mutation, but not the *pss* mutation.

3.3. *pHGCKI-1 encodes choline kinase*

To confirm that pHGCKI-1 encodes choline kinase, we first examined whether or not the plasmid could restore the activity of the CDPcholine pathway in the parental strain by determining the incorporation of [*methyl*-^{14}C]choline into phosphatidylcholine. Fig. 1 shows that parental strain D493-2 incorporated the la-

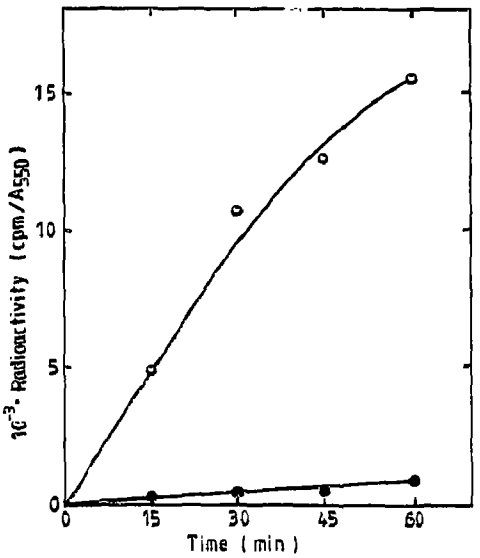

Fig. 1. Incorporation of [*methyl*-^{14}C]choline into phosphatidylcholine in mutant D493-2 (●) and its transformant carrying pHGCKI-1 (○). D493-2 and D493-2 [pHGCKI-1] were grown in M-i medium supplemented with 20 μg/ml leucine, 20 μg/ml tryptophan and 200 μg/ml serine to the logarithmic phase (A_{550}, 0.3–0.5). To 1 ml of each culture was added 0.1 μCi [*methyl*-^{14}C]choline (10 Ci/mol), and then the mixture were incubated at 30°C for the indicated times with shaking. Lipids were extracted as described previously [19] and separated on a Silica Gel 60 plate (Merck) with chloroform/methanol/acetic acid/88% formic acid/water (35:15:6:2:0.25, v/v) as the solvent system. The gel containing phosphatidylcholine was scraped off and counted in a toluene/Triton X-100 scintillant.

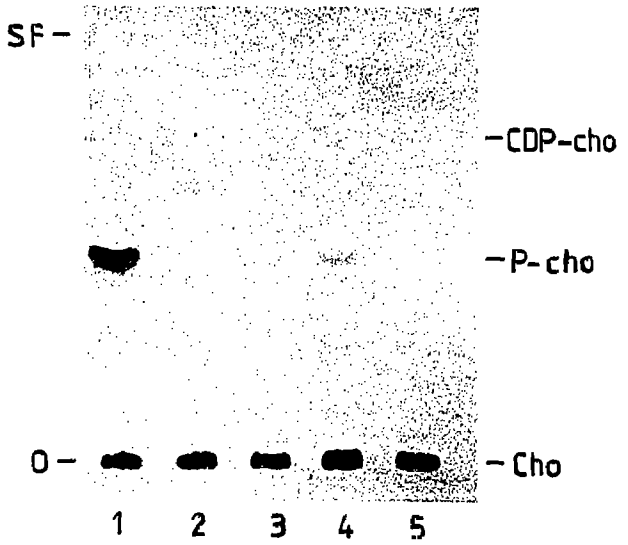

Fig. 2. Choline kinase activity of the D493-2 transformant harboring pHGCKI-1. D493-2 harboring pHGCKI-1 was cultured in 250 ml of M-i medium supplemented with 20 μg/ml tryptophan and 200 μg/ml serine, and harvested at the mid-logarithmic phase. The soluble fraction of the cells was prepared, and 6.8 μg was incubated for 10 min at 30°C in a 10 μl assay mixture containing the same ingredients as described previously [3] except that the concentration of [*methyl*-^{14}C]choline (52 Ci/mol) was reduced to 0.37 mM. A 9 μl portion of the reaction mixture was directly spotted onto a Silica Gel 60 plate (Merck), and then the plate was developed with 95% ethanol/2% ammonium hydroxide (1:1, v/v). Autoradiography was carried out for 2 days at room temperature with Fuji X-ray film. (Lane 1) Complete system; (lane 2) minus ATP; (lane 3) 10 mM EDTA instead of $MgSO_4$; (lane 4) the reaction mixture was boiled for 1 min at the end of the incubation and then treated with 10 units of calf intestine alkaline phosphatase for 10 min at 37°C; (lane 5) boiled enzyme. SF, solvent front; O, origin; CDP-Cho, CDPcholine; P-cho, phosphorylcholine; Cho, choline.

belled choline at a very low rate, but the transformant carrying pHGCKI-1 at a much faster rate. The rate was comparable to that in the case of wild type X2180-1A (data not shown). Thus pHGCKI-1 restored the CDP-choline pathway in the mutant. Next, we assayed the choline kinase activity. A 100,000×*g* supernatant was prepared, and incubated with [*methyl*-^{14}C]choline and ATP in the presence of Mg^{2+} for 10 min at 30°C. After incubation, the reaction mixture was directly applied onto a Silica Gel H plate. The plate was developed and then autoradiographed. As shown in Fig. 2, the 100,000×*g* supernatant of the pHGCKI-1 transformant produced phosphorylcholine (lane 1). The phosphorylcholine formation was dependent on ATP (lane 2) and Mg^{2+} (lane 3). Alkaline phosphatase treatment converted the reaction product to choline (lane 4), confirming the identity of the product. Heat treatment abolished the activity (lane 5). The present results show that pHGCKI-1 carries choline kinase cDNA. When choline kinase activity was assayed by the method described previously [20], the activities of the transformant and untransformed cells were 6 and 0.1 nmol·min^{-1}·mg^{-1} protein, respectively. Thus the choline kinase activity of the transformant was comparable to or even higher than that in rat tissues [20–22]. We concluded that the cloned cDNA contained a considerable length, or even the full-length of the choline kinase coding frame.

3.4. *Nucleotide sequence of a human choline kinase cDNA*

The 2.4-kbp insert was excised from pHGCKI-1 with *Not*I, and then the 2,408-bp sequence was determined by the dideoxy chain termination method [23] after subcloning into M13 mp10 and mp11 [24]. As shown in Fig. 3, there was a large open reading frame capable of encoding 465 amino acid residues. As expected, the reading frame was in-frame with the *ADH1* leader peptide in expression vector pADANS [11]. Since it was not known whether translation started at the first methionine or a further upstream one, we compared the deduced amino acid sequence with the recently determined rat liver choline kinase sequence [4].

3.5. *Sequence comparison with choline kinase from other sources*

In Fig. 4, the deduced amino acid sequence, starting from methionine-1 as the first amino acid, is aligned with that of rat liver choline kinase. The first 23 amino acids of both sequences perfectly matched each other, strongly suggesting that methionine-1 is the translational start site of human glioblastoma choline kinase. Furthermore, with some deletions or insertions, the rat liver and human glioblastoma enzymes showed a high degree of sequence similarity over their entire lengths; 84.9% amino acids were identical and 9% conservative.

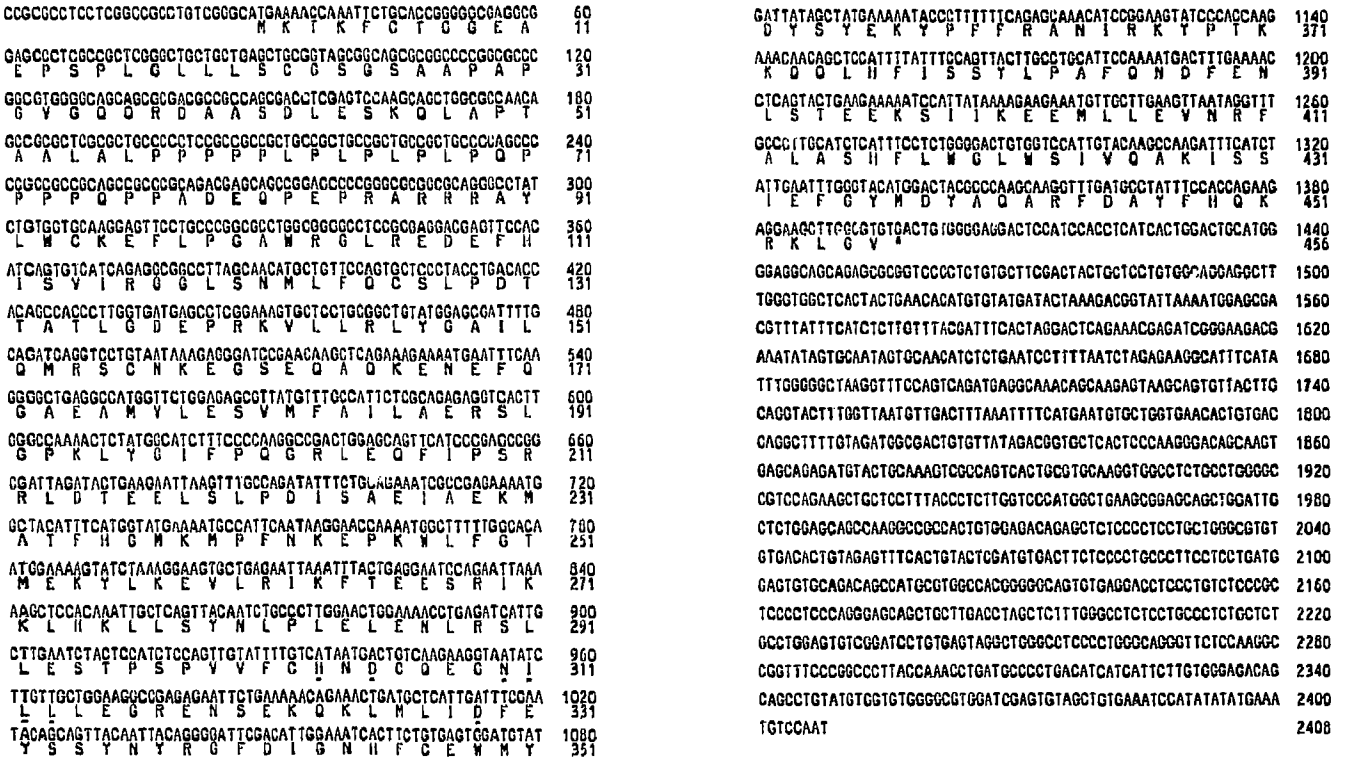

Fig. 3. Nucleotide sequence and deduced amino acid sequence of human glioblastoma choline kinase cDNA. The sequence was excised from pHGCKI-1 using *Not*I and determined by the dideoxy chain termination method [23]. The *Not*I site is not shown in the figure. The numbers at the right denote the positions of the last nucleotide and amino acid of each line. Amino acids are numbered with the first methionine taken as position 1. The stop codon is indicated by an asterisk. Brenner's phosphotransferase consensus [25] is indicated by underlinings.

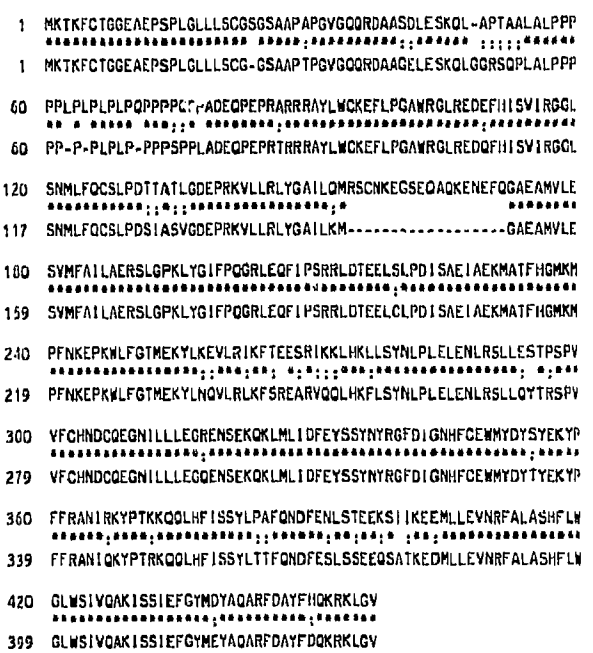

Fig. 4. Sequence alignment of human glioblastoma choline kinase and rat liver choline kinase. The sequences were compared using the GENETYX program (SDC Corp. Inc.) and aligned so that maximum matching was obtained. (Upper line) Human glioblastoma choline kinase; (lower line) rat liver choline kinase. The asterisks indicate identical amino acids, and the colons conservatively substituted amino acids. The figures at the left denote the positions of the left-end amino acids.

At the nucleotide level, the open reading frames resembled each other, 68% identity. Compared with yeast choline kinase [3], significant sequence identity (28.7%) was observed in the carboxy-terminal regions (human, amino acids 292–427; yeast, 357–487), but not much in the amino-terminal regions. The carboxy-terminal region contained Brenner's phosphotransferase consensus (HXDhXXXNhhh.....D, where h stands for large hydrophobic amino acids, FLIMVWY) [25] at positions 303–329 (Fig. 3). We previously showed that a truncated yeast choline kinase constructed by removing the amino-terminal 151 amino acids still exhibited slight but significant choline kinase activity [3]. Thus it is tempting to speculate that the carboxy-terminal region is involved in the catalytic function of choline kinase from yeast, rat liver and human glioblastoma.

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