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Deletion and alanine mutation analyses for the formation of active homo- or hetero-dimer complexes of mouse choline kinase-α and -β

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Abstract

Choline kinase (CK) is the first-step regulatory enzyme for the biosynthesis of phosphatidylcholine in all mammalian cells. It exists as at least three isoforms (α1, α2 and β) that are encoded by two separate genes termed *ck*-α and *ck*-β. The active enzyme has been proposed to consist of either their homo- or hetero-dimeric forms. Here, we report on the identification of several essential domains and amino acid residues involved in their active dimer formation. Full-length cDNAs or their truncated or alanine-mutated versions for mouse CK-α1 and CK-β tagged with either HA or Myc at their N-termini were expressed in COS-7 cells. Each dimer formation was analyzed by immuno-precipitation followed by Western blotting. Kinetic analysis for CK reaction was performed with different expression products. Both the N-terminal domain-1 and C-terminal portions (E424–K430 for CK-α1 and Q379–K385 for CK-β) were shown to be critical for the formation of active homo- or hetero-dimer complex. Interestingly, D320 in the CK-motif of CK-α1 was found to be essential for α1/α1 homo-dimerization but not for α1/β hetero-dimerization. A mutation of the corresponding D276 of CK-β to A276 did not show any effect on either its homo- or hetero-dimerization but it caused a strong inhibition of CK activity in either case.
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Keywords: Choline kinase; Phosphocholine; Isoform; Homo-dimer; Hetero-dimer

1. Introduction

Choline kinase (CK) catalyzes the first phosphorylation reaction in the CDP-choline (or Kennedy) pathway for the biosynthesis of phosphatidylcholine (PC), yielding phosphocholine (P-cho) from choline and ATP in the presence of Mg^{2+}. This enzyme exists in mammalian cells as at least three isoforms (CK-α1, -α2 and CK-β) that are encoded by two separate genes termed *ck*-α and *ck*-β (*chka* and *chkb* in the NCBI database, respectively) [1,2]. Each isoform is not active in its monomeric form. While several earlier studies with the purified enzyme from rat tissues [3–5] indicated that the active enzyme consists of either homo-dimer or homo-oligomer, our recent investigation using specific rabbit antibodies against mouse CK-α and CK-β suggested that a significant or even predominant part of CK activity in several intact mouse tissues may consist of α/β hetero-dimer [6]. In the paper, it was predicted that the actual combinations to form active dimer complexes may be further diversified because our CK-α isoform-specific antibody did not distinguish between CK-α1 and CK-α2. The activity of CK in a given cell type could be regulated not only by the level of each isoform in the cell but also by the combination of each isoform subunit (α1, α2, β) to generate active dimer complexes (α1/α1, α1/α2, α2/α2, α1/β, α2/β and β/β). Thus, it now appears that the molecular mechanism of regulation of CK activity in the cell is much more complicated than was expected. The kinetic characterization of each dimer complex, which might be prepared from each or combined over-expressed cell system, will be required for further evaluation of the physiological significance of the

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concurrent presence of several CK isoforms in mammalian cells.

For the CDP-choline pathway in mammals, the regulatory step as well as rate-limiting step for PC biosynthesis is generally considered to be at the P-cho cytidylyltransferase, the next-step enzyme to CK [7,8]. There have been several reports, however, which indicate that the CK step is also slow and can be regulatory for the net biosynthesis of PC [8,9].

Two interesting topics on CK and/or P-cho have been reported in recent years. Both seem to be not related directly to the net PC biosynthesis but predict other important roles of the CK and its reaction product, P-cho, in higher eukaryote. One is that P-cho can be a second messenger in cell growth signaling [10,11]. The activation of CK and the resulting increase in P-cho level are both proposed as necessary events for certain cells to proliferate under some growth-factor stimulations. From this concept, the development of novel CK inhibitors as the possible anti-cancer drugs has been actively investigated by several working groups [12,13]. How and where P-cho should be involved in the growth-signaling cascade, however, remain to be determined. The second topic is that CK gene induction seems to be associated with certain cell stresses such as the one caused by carbon tetrachloride intoxication on murine hepatocytes [6,14]. A similar rapid induction of CK gene expression has been demonstrated to occur also in plant *Arabidopsis* upon mechanical wounding [15] and salt stress [16]. These findings indicate that certain CK gene could be a member of the acute responsive gene family, although the exact role of CK induction as well as newly generated P-cho in various cell systems needs to be clarified.

Thus, we thought that it would be very important to know the nature of active CK dimer complexes and to obtain information for the critical domains as well as amino acid residues involved in each subunit association. The results presented in this report demonstrated clearly that the active CK exists as both homo- and hetero-dimer components in the cell and identified several critical peptide domains required for the formation of each dimer complex.

2. Materials and methods

2.1. Materials

COS-7 cells were maintained under standard conditions of temperature (37 °C), humidity (95%), and carbon dioxide (5%). Cell culture reagents were from Invitrogen (Carlsbad, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA). COS-7 cells (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, as previously described [17,18]. Protein G-Sepharose™ 4 Fast Flow was obtained from Amersham Biosciences (Uppsala, Sweden). Anti-HA-probe (Y-11) and anti-c-Myc (9E10) antibodies (sc-805 and sc-40, respectively) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Oligonucleotide primers for every mutagenesis were from Qiagen K.K. (Tokyo, Japan). Restriction endonucleases unless otherwise indicated were from TaKaRa BIO INC. (Shiga, Japan). Protease inhibitor cocktail tablets (Complete™) were from Roche Molecular Biochemicals (Mannheim, Germany). Choline chloride was from Nacalai Tesque INC. (Kyoto, Japan). [*methyl*-^{14}C]Choline chloride was from Amersham Biosciences Corp. (Piscataway, NJ, USA). Other chemicals were purchased at their highest available purity from Sigma-Aldrich.

Escherichia coli (*E. coli*) XL1-blue was used for the routine subcloning. Plasmid pCMV-Script® vector (Stratagene, La Jolla, CA, USA) was used for the cloning of PCR fragments and their expression studies. *E. coli* cells were grown in either LB broth or LB agar medium supplemented with kanamycin (50 μg/ml).

2.2. Expression constructs

Complementary DNA fragments of 1.4-kb and 1.2-kb encoding full-length mouse CK-α1 and CK-β, respectively, [19] which had been tagged with either Myc or HA at each N-terminus were cloned into the donor plasmid pCMV using *Sal*I and *Bam*HI sites. These plasmids were then used as the templates for all deletion experiments or site-directed mutagenesis. The point mutation was introduced by an overlap extension PCR using Platinum® *Pfx* DNA polymerase draft from Invitrogen. The codon GCN was used for the amino acid mutation to Ala. The PCR products were treated with *Dpn*I restriction enzyme (New England Biolabs® Inc, Beverly, MA, USA) to digest the template, then transfected into *E. coli* XL1-Blue. The sequence of every construct was verified by sequencing with ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) in the Genome Laboratory of the Medical Research Institute, Tokyo Medical and Dental University.

2.3. Protein expression

For the immuno-precipitation study, COS-7 cells were seeded on 6-cm dishes at a density of 7×10^5 cells/dish on the day prior to transfection. Transfection of every cDNA construct (1 μg DNA/dish) using Lipofectamine™ 2000 (Invitrogen) was performed according to the manufacturer's instruction. Cells were usually cultivated for 24 h after the transfection.

For the enzyme assay, COS-7 cells were plated on 10-cm dishes at a density of 2×10^6 cells/dish on the day prior to transfection. Twenty-four hours after the transfection cells were collected in 0.3 ml homogenization buffer (20 mM Tris–HCl, pH 7.5, 2 mM 2-mercaptoethanol (2-ME), 154 mM KCl, and 1 tablet of Complete™ in each 50 ml buffer) and homogenized by brief sonication (twice with 15 s each on ice), the cell homogenate was then centrifuged at 105,000×*g* for 30 min. An aliquot (usually 20 μl) of the resulting supernatant was used for CK assay. The recoveries of total cytosolic protein from cells transfected with any deleted or alanine-mutated constructs were almost at the comparable levels as those transfected with wild-type constructs.

2.4. Immuno-precipitation

All manipulations were carried out at 4 °C. COS-7 cells were washed with PBS, then harvested and mixed in 0.5 ml lysis buffer (20 mM Tris–HCl, pH 7.5, 2 mM 2-ME, 0.5% NP-40, 154 mM KCl, and 1 tablet of Complete™ in each 50 ml buffer) for 15 min on ice, then centrifuged (19,000×*g*) for 15 min. An aliquot (usually 200 μl) of the resulting supernatant was used for immuno-precipitation. Protein concentration was measured with a Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Samples were pre-incubated with 20 μl of 50% (v/v) Protein G-Sepharose™ 4 Fast Flow for 1 h at 4 °C. After removing the beads by brief centrifugation, primary antibody was incubated with the supernatant for 1 h, usually with the dilution of 1:100 for either anti-HA-probe (Y-11) or anti-c-Myc (9E10) antibodies. Protein G-Sepharose™ beads (20 μl) were then added to the mixtures, and they were incubated for additional 12 h with constant mixing. Beads were collected by centrifugation and washed three times with the lysis buffer, bound proteins were then eluted by boiling for 5 min in ×2 sodium dodecylsulfate (SDS) sample buffer. An aliquot of the supernatant was analyzed by Western blotting [20].

2.5. Western blotting

Equivalent amounts of protein were resolved by SDS-13% (unless otherwise indicated) polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membrane (Hybond-P, Amersham Pharmacia Biotech, Uppsala, Sweden). Primary antibodies were incubated usually with the dilution of 1:200 for both c-Myc and HA-probe antibodies. Band detection was performed with either HRP-conjugated anti-rabbit IgG antibody (NA931V; Amersham Biosciences UK Ltd., Buckinghamshire, England) for anti-HA

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