Expression and characterization of the active molecular forms of choline/ethanolamine kinase-α and -β in mouse tissues, including carbon tetrachloride-induced liver

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Choline/ethanolamine kinase (ChoK/EtnK) exists as at least three isoforms (α1, α2 and β) in mammalian cells. The physiological significance for the existence of more than one form of the enzyme, however, remains to be determined. In the present study, we examined the expression and distribution of the isoforms in mouse tissues using isoform-specific cDNA probes and polyclonal antibodies raised against each N-terminal peptide sequence. Both Northern- and Western-blot analyses indicated that either the α (α1 plus α2) or the β isoform appeared to be the ubiquitously expressed enzyme. The mRNA abundance for the α isoform was highest in testis, whereas that for the β isoform was relatively high in heart and liver. While the native form of each isoform was reported to consist of either homodimers or homotetramers, our immunotitration studies clearly indicated that a considerable part of the active form of the enzyme consists of α/β hetero-oligomers, with relatively small parts of activity expressed by α/α and β/β homo-oligomers. This is the first experimental evidence for the presence of heteromeric ChoK/EtnK in any source. Thus our results strongly suggested that the activity of ChoK/EtnK in the cell is controlled not only by the level of each isoform but also by their combination to form the active oligomer complex. Carbon tetrachloride (CCl_4) was shown to induce ChoK activity 2–4-fold in murine liver. Our analysis for the mechanism involved in this induction revealed that the responsible isoform for CCl_4 was α, not β. The level of α mRNA was strongly induced in mouse liver, which resulted in a sustained increase in the amount of the α isoform. Consequently, the composition of α/α homo-oligomers came to represent up to 80 % of the total active molecular form of ChoK in CCl_4-induced liver, whereas it was less than 20 % in normal uninduced liver.

Key words: gene expression, isoforms, phosphatidylcholine biosynthesis, phosphocholine.

INTRODUCTION

Choline/ethanolamine kinase (ChoK/EtnK; EC 2.7.1.32 for ChoK and EC 2.7.1.82 for EtnK) catalyses the phosphorylation of choline/ethanolamine by ATP yielding phosphocholine/phosphoethanolamine [1]. This step commits choline/ethanolamine to the CDP-choline/CDP-ethanolamine pathway for the biosynthesis of phosphatidylcholine (PtdCho)/phosphatidylethanolamine (PtdEtn) in all animal cells [2,3]. We have demonstrated that there exist at least three isoforms of ChoK/EtnK, termed α1 (50 kDa), α2 (52 kDa) and β (45 kDa), in the mouse and rat, and probably also in the human [4]. The first two isoforms are derived from the same gene *chok/etnk-α* by alternative splicing, whereas the third isoform is a product of the separate gene, *chok/etnk-β*. Although all of these isoforms were shown to catalyse both ChoK and EtnK reactions *in vitro*, it has been reported from studies with the purified enzymes [5–7], as well as with the recombinant enzymes [8,9], that they had much higher specific activities with choline compared with ethanolamine. Most recently, an isoform of ethanolamine-specific kinase, termed EKI-1, has been cloned from a human cDNA library [10], which may suggest that ChoK and EtnK reactions can be catalysed by separate enzymes *in vivo*. The relatively ethanolamine-specific and highly ethanolamine-specific kinases have also been cloned from yeast [11] and *Drosophila* [12] respectively.

Earlier purification studies demonstrated that the homogeneous rat kidney ChoK/EtnK (ChoK/EtnK-β) had a molecular mass of 42 kDa when resolved by SDS/PAGE and its native form appeared to exist as a homodimer, because the activity of the purified preparation eluted at a molecular size of 90 kDa from the pre-equilibrated Sephadex G-150 gel-filtration column [5]. The highly purified ChoK/EtnK preparation from rat brain showed a single 44 kDa band when resolved by SDS/PAGE and its native form was also shown to be a homodimer by means of gel-permeation and ultra-centrifugation analyses [7]. The homogeneously purified enzyme from rat liver showed a single 47 kDa band when resolved by SDS/PAGE and its native form was reported to exist as a homotetramer, because an approximate molecular mass of 160 kDa was obtained by gel-filtration of the purified enzyme [6]. Existence of some microheterogeneity of the rat liver enzyme has also been suggested in the latter study [6]. The purified enzyme from rat liver probably corresponds to ChoK/EtnK-α (α1 or α2) from its subunit molecular mass. All of these studies indicated that no active isoform of ChoK/EtnK in rat tissues exists in a monomeric form, but exists in a homo-oligomeric (either homodimeric or homotetrameric) form.

One of the most intriguing characteristics of mammalian ChoK/EtnK is its inducibility in various experimental systems [13]. We previously reported that administration to rats of certain hepatotoxins, such as polycyclic aromatic hydrocarbon carcinogens [14] and carbon tetrachloride (CCl_4) [15], caused a 2–4-fold induction of hepatic ChoK/EtnK activity. This induction was found to be liver-specific and due most probably to the synthesis of new ChoK/EtnK protein. While most (but not all) ChoK activity in untreated rat liver could be immunoprecipitated

Abbreviations used: ChoK/EtnK choline/ethanolamine kinase; G3PDH, glycerol-3-phosphate dehydrogenase; GST, glutathione S-transferase; M-CPT I, muscle-type carnitine palmitoyltransferase I; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine.

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by rabbit antibody raised against the purified rat kidney ChoK/EtnK (ChoK/EtnK-β), the CCl_4-inducible activity could not [5], indicating that the newly-synthesized form of ChoK by CCl_4 administration was not a major form or forms of ChoK in normal rat liver. Although the exact physiological meaning of the inducibility has not been fully understood, the induction of ChoK/EtnK by several means [16] suggests that the enzyme plays an important role in the long-term regulation of the CDP-choline pathway and may be involved in the co-ordinate regulation of PtdEtn biosynthesis. On the other hand, the induction of ChoK/EtnK could be associated with the increased generation of phosphocholine and/or phosphoethanolamine in the cell, both of which have been proposed recently [17,18] as an essential event in the activation of a classical mitogen-activated protein kinase cascade during certain growth factor-induced mitogenic stimulation. The latter mechanism appears to be independent of the rate of net PtdCho/PtdEtn synthesis. Alternatively, an apparently liver-specific induction of ChoK/EtnK by several hepatotoxins may be involved in the hepatic detoxification and/or protection mechanisms against certain xenobiotics.

In the present investigation, in order to assess the physiological meaning of the presence of more than one form of ChoK/EtnK in mammalian cells, we prepared both cDNA probes and rabbit antibodies specific for ChoK/EtnK isoforms α and β, and examined their relative expression and distribution in major mouse tissues, including CCl_4-induced liver. The results strongly indicated that the active form of ChoK in most mouse tissues exists predominantly as an α/β heterodimer (or hetero-oligomer) with a relatively smaller part of activity consisting of either α/α or β/β homodimers (or homo-oligomers). It was also clearly demonstrated that CCl_4 caused a strong induction of *chok/etnk-α* gene expression in liver and, as a result, the induced ChoK activity was mainly composed of homo-oligomeric forms of ChoK/EtnK-α. The expression of hepatic ChoK/EtnK-β was not significantly affected by CCl_4 administration.

EXPERIMENTAL

Animal treatment

Animal care was carried out in accordance with the guidelines of the Tokyo Medical and Dental University. Male ddY mice (6–7 weeks old) were used for all animal experiments. CCl_4 was dissolved in 2 vols. of olive oil and injected intraperitoneally at a dosage of 1 ml/kg of body weight. Control mice received equivalent volumes of olive oil. Three mice in each group were killed at 3, 6, 9, 12 and 24 h by cervical dislocation and the liver was quickly removed and subjected to total RNA extraction, as well as to subcellular fractionation. Animals had free access to water, but food was removed from 12 h before the start of the experiment until the end of the experiment.

Northern-blot analysis

Multiple mouse, rat and human tissue Northern blots were purchased from ClonTech Laboratories, and were hybridized and washed according to the manufacturer's instructions. In separate experiments, an aliquot (usually 20 μg) of freshly isolated total RNA (extracted with ISOGEN, a product from Nippon Gene, Tokyo, Japan) from several mouse tissues, as well as CCl_4-induced mouse liver, was run on a 1 % (w/v) agarose gel, then transferred on to a positively charged nylon membrane (Hybond N^+; Amersham Biosciences). The membrane was subsequently hybridized with a freshly radiolabelled (Rediprime DNA Labeling System; Amersham Biosciences) cDNA probe, then

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α1  MKTKFCTGGEAEPSPLGLLLSCGGNAAPTPGVGQQADAAGELESKQLGGRTQPLALPPPP  60

β                                           MAADGTGVVGGGAVGGGLPKD  21
                              d-1                         d-2
α1  PPPLPLPPPPSPPLADEQPEPR-TRRRAYLWCKEFLPGAWAGLREDQFHISVIRGGLSNM 119
           * * *    . . .* ..**** **.*.* ****  * ... .. ..*****.
β   GLQDAKCPEPIPNRAAASSLSRDAQRRAYQWCREYLGGAWARARPEELSVCPVSGGLSNL  81
                 d-3                              d-4
α1  LFQCSLPDSIASVGDEPRKVLLRLYGAILKMGAEAMVLESVMFAILAERSLGPKLYGIFP 179
    **.****. ..***.***.**********. *....*****************.***.**
β   LFRCSLPNHVPSVGGEPREVLLRLYGAILQ-GVDSLVLESVMFAILAERSLGPQLYGVFP 140
                                           d-5
α1  QGRLEQFIPSRRLDTEELRLPDISAEIAEKMATFHGMKMPFNKEPKWLFGTMEKYLNQVL 239
    .*****..***.*.*.*** * .*..**..** ****.***.***.*******.**.*.
β   EGRLEQYLPSRPLKTQELRDPVLSGAIATRMARFHGMEMPFTKEPRWLFGTMERYLKQIQ 200
                                               d-6
α1  RLKFSREARVQQLHKILSYNLPLELENLRSLLQYTRSPVVFCHNDCQEGNILLLEGQENS 299
     *   ......*.. .   *.*  *...**.**. *.********* ********   * .
β   DL---PSTSLPQMNLVEMYSLKDEMNSLRKLLDDTPSPVVFCHNDIQEGNILLL--SEPD 255
                d-7
α1  ERRKLMLIDFEYSSYNYRGFDIGNHFCEWMYDYTYEKYPFFRANIQKYPSRKQQLHFISS 359
    .  .***.*********************.*******..**..*.  .**.*.******.
β   SDDNLMLVDFEYSSYNYRGFDIGNHFCEWVYDYTYEEWPFYKARPTDYPTREQQLHFIRH 315
                                          d-8
α1  YLTTFQNDFESLSSEEQFATKEDMLLEVNRFALASHFLWGLWSIVQAKISSIEFGYMEYA 419
    **.. *.. * **.***   .*..***..*..*****.*****..**..*.*****.***
β   YLAEVQKG-EILSEEEQKKREEELLLEISRYSLASHFFWGLWSTLQASMSTIEFGYLEYA 374

α1  QARFEAYFDQKRKLGV                                             435
    *.**. **.** .*
β   QSRFQFYFQQKGQLTSSPSS                                         394


α2  RSCNKEGSEQAQNENEFQ
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Figure 1 Deduced amino acid sequences for mouse ChoK/EtnK-α and ChoK/EtnK-β from their isolated cDNAs

The arrow indicates the position (between Met^{150} and Gly^{151} of the α1 sequence) where an extra 18-amino-acid sequence (shown at the bottom) can be inserted to give the α2 isoform, created by alternative splicing. The highly conserved domains among mouse, rat and human ChoK/EtnK-α1 and -β isoforms are boxed and numbered d-1–d-8. The predicted critical amino acid residues that have been completely conserved in the deduced amino acid sequences from 17 eukaryotic ChoKs or EtnKs so far reported are shown by white letters on a black background. Both d-6 and d-7 are considered to be catalytic domains for all ChoK and EtnK activities as discussed elsewhere [4]. The N-terminal sequences upstream of d-1 were found to have no significant similarity between α and β isoforms, thus these sequences (indicated by black letters on a grey background; 2–84 for the α isoform and 2–46 for the β isoform) were used to obtain each GST fusion protein in *E. coli* to prepare the isoform-specific rabbit antibodies used in the present study. Highly conserved cysteine residues among all mammalian ChoK/EtnK isoform sequences [4] are indicated in bold.

subjected to autoradiography. The fragments of cDNA used as probes were: mouse cDNA 708–1464 for ChoK/EtnK-α [19], rat cDNA 846–1432 for ChoK/EtnK-β [8] and mouse cDNA −1 to 466 for muscle-type carnitine palmitoyltransferase I (M-CPT I; kindly provided by Dr Naoshi Yamazaki, University of Tokushima, Japan). The cDNA fragment (450 bp) for mouse glycerol-3-phosphate dehydrogenase (G3PDH) was prepared by reverse-transcriptase PCR with freshly isolated mouse liver RNA as a template and the following primers: 5′-ACCACAGTCCA-TGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. The hybridization temperature of 65 °C was chosen where the cross-hybridization did not occur to a significant level between ChoK/EtnK-α and ChoK/EtnK-β transcripts.

Preparation of isoform-specific rabbit antibodies

The cDNA fragment corresponding to the N-terminal peptide sequence (2–84 for ChoK/EtnK-α and 2–46 for ChoK/EtnK-β; see Figure 1) of each isoform was ligated in-frame to pGEX-2T (Pharmacia Biotech) at the *Eco*RI/*Bam*HI sites and expressed in *Escherichia coli* (DH5α). The resulting glutathione S-transferase

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(GST) fusion proteins were purified through a GSH–Sepharose column. Each of the purified proteins (1 mg of each) was injected subcutaneously into female New Zealand White rabbits (2.3–2.5 kg) with Freund's complete adjuvant, then, booster injections (500 μg of each protein with Freund's incomplete adjuvant) were repeated every 3 weeks. Anti-GST antibody was also raised in a similar manner. For Western-blot analysis, each isoform-specific antiserum was purified through an affinity column that was prepared by conjugation of each antigen peptide with CNBr-activated Sepharose 4B (Pharmacia Biotech). The antibody raised against GST was removed first by passing through the column of GST-conjugated affinity beads, then, each isoform-specific antibody was purified through the respective antigen-conjugated column. The final IgG concentration of the purified antibody was 0.47 mg/ml for anti-ChoK/EtnK-α and 0.61 mg/ml for anti-ChoK/EtnK-β.

Western-blot analysis

Freshly isolated mouse tissues were homogenized in 3 vols. of 20 mM Tris/HCl (pH 7.5), 154 mM KCl and 2 mM 2-mercaptoethanol [including 1 tablet of protease inhibitor cocktail (Complete™; Roche, Mannheim, Germany)/50 ml] with a Teflon-pestle/glass homogenizer. An aliquot (20–40 μg of protein) of the 105000 ***g*** high-speed supernatant of tissue homogenates was resolved by SDS/PAGE (10 % gel), then transferred on to a PVDF membrane and incubated with the affinity-purified isoform-specific antibody (× 100 dilution for anti-ChoK/EtnK-α and × 300 dilution for antiChoK/EtnK-β). Horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences; catalogue no. NA9340) and an enhanced chemiluminescence detection kit (Renaissance Chemiluminescence Reagent Plus; NEN) were used for the subsequent signal detection procedure.

Immunoprecipitation and ChoK assay

Newly prepared 105000 ***g*** supernatant fractions from mouse tissue homogenates (280–580 μg of protein) were incubated with an antiserum raised against GST (control), GST–ChoK/EtnK-α and/or GST–ChoK/EtnK-β fusion proteins, either independently or in combination, overnight at 4 °C. PANSORBIN® [10 % (w/v); 50 μl; Calbiochem] suspension was added to each incubation to precipitate rabbit IgG, and then an aliquot of the resultant high-speed supernatant was used for the ChoK assay. In some experiments, an aliquot (approx. 30 μg of protein) of the post-immunoprecipitated fraction was resolved by SDS/PAGE for Western-blot analysis. The ChoK activity was determined essentially as described elsewhere [16]. Typical incubation mixtures contained 0.1 M Tris/HCl (pH 8.75), 10 mM ATP-2Na, 15 mM $MgCl_2$, 0.25 mM [*Me*-^{14}C]choline-chloride (10.5 μCi/ml) and enzyme preparation (approx. 50 μg of protein) in a final volume of 100 μl. The incubation was carried out at 37 °C for 20 min. The reaction product [^{14}C]phosphocholine was separated from [^{14}C]choline using a small AG 1-X8 (200–400 mesh, OH^- form) column (Bio-Rad). The activity was estimated by the amount of [^{14}C]phosphocholine formed/min per mg of protein.

Other methods

Protein concentration was estimated using a bicinchoninic acid ('BCA') protein assay kit (Pierce), with BSA as a standard. Statistical significance was determined by unpaired Student's *t* tests.

RESULTS

Comparison of the predicted amino acid sequence between ChoK/EtnK-α and ChoK/EtnK-β in mouse

Three isoforms of ChoK/EtnK have so far been identified from rat tissues [5–7] and their full-size cDNAs have been cloned [8,9,20]. The homologous isoforms were shown to exist in the mouse [19] and were predicted to also be present in the human [4]. Two of them, termed ChoK/EtnK-α1 and -α2, are splice variants from the same gene, *chok/etnk-α*, whereas another isoform, ChoK/EtnK-β, is a product of a separate gene, *chok/etnk-β*. Although both genes from the mouse are composed of 11 major exons (I–XI) and there is more than 50 % sequence similarity between each of their corresponding exons, the entire gene sizes are quite different, 40 kb for *chok/etnk-α* and 3.5 kb for *chok/etnk-β* [4]. In addition, the *chok/etnk-α* gene has an extra exon (II′) that can be spliced out during processing for the α1 isoform, but retained in the α2 isoform. The predicted amino acid sequences for the mouse α1 and β isoforms are aligned in Figure 1, together with the location (an arrow) and the predicted sequence (bottom) of the 18-amino-acid insertion for α2. The highly conserved domains between the two isoforms, not only in the mouse but also in the rat and human sequences, are boxed

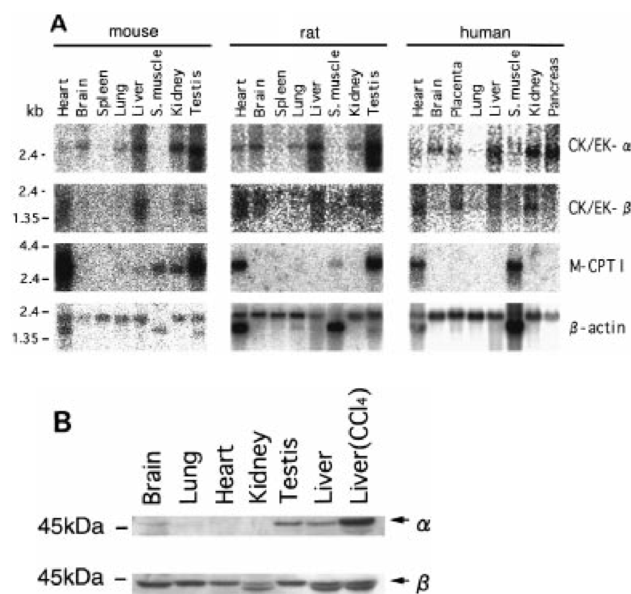

Figure 2 Northern- and Western-blot analyses for ChoK/EtnK-α and ChoK/EtnK-β in several mammalian tissues

(**A**) In Northern-blot analysis, commercially available multiple mRNA blot sheets (ClonTech Laboratories) were used. cDNA probes used were; mouse cDNA fragment 708–1464 [19] for ChoK/EtnK-α, rat cDNA fragment 846–1432 [8] for ChoK/EtnK-β and mouse cDNA fragment −1–466 for M-CPT I. The first exon of the M-CPT I gene has been shown to locate at only 300 bp (human) and 550 bp (mouse and rat) downstream of the *chok/etnk-β* gene. Other details are described in the text. CK/EK, choline/ethanolamine kinase. (**B**) In Western-blot analysis, the newly prepared 105000 ***g*** supernatant from each mouse tissue homogenate was resolved by SDS/PAGE (10% gel) (20 μg and 38 μg protein/lane for ChoK/EtnK-α and ChoK/EtnK-β respectively). Affinity-purified anti-ChoK/EtnK-α or anti-ChoK/EtnK-β was used for the first antibody and horseradish peroxidase-conjugated donkey anti-rabbit IgG F(ab′)$_2$ was used for the second antibody. Other details are described in the text. The band corresponding to 47 or 43 kDa could be detected differentially by the anti-ChoK/EtnK-α or the anti-ChoK/EtnK-β antibody respectively. The band just below the 43 kDa ChoK/EtnK-β band was always seen in both liver and kidney samples, but this band was found not to be specific, because pre-immunoprecipitation treatment of the applied tissue sample did not affect its appearance on the blot (see the legend for Figure 3).

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and numbered from d-1–d-8. There are several amino acid residues (white letters on a black background) that were found to be completely conserved among the reported or predicted 17 eukaryotic ChoK and EtnK sequences, all of which are thus considered to be more or less involved in the catalytic and/or regulatory functions [4]. The sequence FXHNDXXXXNhh--------hhDhEXXXXNXXXhDhXXHhXE (where single-letter amino-acid notation has been used, and where h stands for a highly hydrophobic amino acid: F, L, I, M, V, W or Y) has been postulated [4] as a ChoK/EtnK catalytic domain, because it contains a putative phosphotransferase (Brenner's) consensus sequence [21]. While there was high similarity (59.7 %) between the entire α1 and β sequences from the mouse, the N-terminal parts of the sequences ahead of d-1 (black letters on a grey background) were found to have no significant similarity. Thus these parts of the sequences (2–84 for α and 2–46 for β) were used to generate each GST fusion protein in *E. coli*, which were then used, after purification through a GSH–Sepharose column, for the preparation of the isoform-specific antibodies in the present investigation. Attempts to generate each isoform-specific and/or -nonspecific (reactive to both isoforms) antibodies against several synthetic peptides, including the C-terminal sequence, have so far been unsuccessful.

Tissue distribution of ChoK/EtnK-α and ChoK/EtnK-β

The relative abundance of ChoK/EtnK-α and ChoK/EtnK-β mRNAs and proteins in several mouse tissues was addressed by Northern- and Western-blot analyses respectively (Figures 2A and 2B). For mRNA analysis, rat and human tissues were also examined. From the results shown in Figure 2(A), both ChoK/EtnK-α and -β are suspected to be ubiquitously distributed proteins in the tissues of three mammalian species. On the other hand, M-CPT I showed relatively tissue-specific distribution, with high expression in both testis and heart (mouse and rat), as well as in skeletal muscle (human). As described elsewhere [22,23], the *m-cptI* gene was shown to locate at only 550 bp (mouse and rat) or 300 bp (human) downstream of the *chok/etnk-β* gene and a possible co-ordinate regulation of expression for both genes has recently been suggested [24,25]. From our Northern-blot results, both mRNAs were relatively high in heart and testis, whereas mRNA for ChoK/EtnK-β was detected in only a trace amount in skeletal muscle where M-CPT I expression was one of the highest among the tissues examined. This observation suggests that both ChoK/EtnK-β and M-CPT I gene expressions are regulated in a tissue-specific manner. The mRNA abundance for ChoK/EtnK-α was highest in testis, followed by liver and kidney in both mouse and rat, whereas an equally high distribution was seen in human pancreas, liver and kidney. On the other hand, the distribution for ChoK/EtnK-β mRNA was relatively high in both heart and liver with all mammalian tissues examined. Intense signals of 2.5 kb for ChoK/EtnK-α, 1.7 kb for ChoK/EtnK-β and 3.0 kb for M-CPT I roughly corresponded to their cDNA sizes. In some cases, i.e. ChoK/EtnK-α signals in testis from the mouse and rat and ChoK/EtnK-β signals in human tissues, intense signals of smaller or larger sizes respectively were detected, but it is not clear whether they were due to slow maturation, degradation, alternative splicing or alternative polyadenylation of the mRNAs. It should be noted that both of the ChoK/EtnK cDNA probes used did not cross-hybridize with the other isoform mRNA under our experimental conditions.

Ubiquitous expression of both ChoK/EtnK isoforms was confirmed further from the results of Western-blot data for several mouse tissue samples (Figure 2B). The bands with approx. molecular masses of 47 and 43 kDa were recognized by blotting with anti-ChoK/EtnK-α and anti-ChoK/EtnK-β antibodies respectively. Only the arrow-indicated bands were found to disappear when the post-immunoprecipitated tissue extracts by the respective antiserum were applied to SDS/PAGE, then blotted with each immunoaffinity-purified antibody. A relatively high amount of ChoK/EtnK-α protein was detected both in testis and liver, and especially in the CCl_4-induced liver (see below). The reason why there was no detectable 47 kDa band in kidney, where the ChoK/EtnK-α mRNA was shown to be abundantly expressed in our Northern-blot analysis, is not known at present. ChoK/EtnK-β protein, on the other hand, showed almost equal distribution in all mouse tissues examined, except that the signal was relatively low in kidney. The band located just below the 43 kDa ChoK/EtnK-β band, which was always seen in Western blots for liver and kidney samples, was revealed not to be specific, because this band could not be immunoprecipitated by preincubation with the anti-ChoK/EtnK-β serum (see below).

Analysis for active molecular forms of ChoK/EtnK in mouse tissues

Previous findings for the native molecular form of ChoK/EtnK suggested that the enzyme most likely exists as either a homodimer [5,7] or a homotetramer [6] in rat tissues. As it now became evident that both ChoK/EtnK-α and -β isoforms coexist in most mouse tissues, we next examined immunotitration analysis using the isoform-specific antibodies to assess what molecular form actually catalyses the phosphorylation reaction and how each isoform works differently in the given mouse tissues. The experiment was carried out with several mouse tissue extracts by incubating with either each isoform-specific antiserum or both anisera, and the ChoK activity remaining in the post-immunoprecipitated supernatant was determined as a function of added antiserum content. The results of these experiments are shown in Figures 3(A)–3(E). In all tissues examined, the addition of both isoform-specific antisera in combination was found to completely immunoprecipitate ChoK activity, indicating that there is no other ChoK isoform having epitopes totally distinct from either ChoK/EtnK-α or -β. The intriguing result was that, in each tissue examined, the sum of inhibition of ChoK activity by either one of the isoform-specific antiserum was found to far exceed 100 %. Since the antiserum raised against one ChoK/EtnK isoform did not appear to cross-react with the other ChoK/EtnK isoform in our experiment, this result strongly indicated the presence of α/β heterodimers (or hetero-oligomers) in all mouse tissues examined. Thus we calculated the possible content of active ChoK molecular forms by an assumption that the activity that was not immunoprecipitated by α-specific antiserum should result from β/β constructs, whereas the activity that was not precipitated by the addition of β-specific antiserum should result from α/α constructs. The remaining activity that was not derived from either α/α or β/β constructs should then be derived from α/β heteromeric constructs. For example, in the liver, the addition of α-specific antiserum caused 80 % inhibition of the total ChoK activity and the addition of β-specific antiserum also caused 80 % inhibition (Figure 3A), which indicated the presence of 20 % of each homodimer (or homo-oligomer) and 60 % heterodimer (or hetero-oligomer) in this tissue. In contrast, in the heart, 70 % of total ChoK activity did not respond to the α isoform-specific antiserum, whereas more than 95 % of ChoK activity was immunoprecipitated by the β isoform-specific antiserum (Figure 3B), indicating the presence of 70 % β/β and < 5 % α/α, and the remaining 25 % was estimated to be present as a heteromeric form. The results of these calculations for the relative

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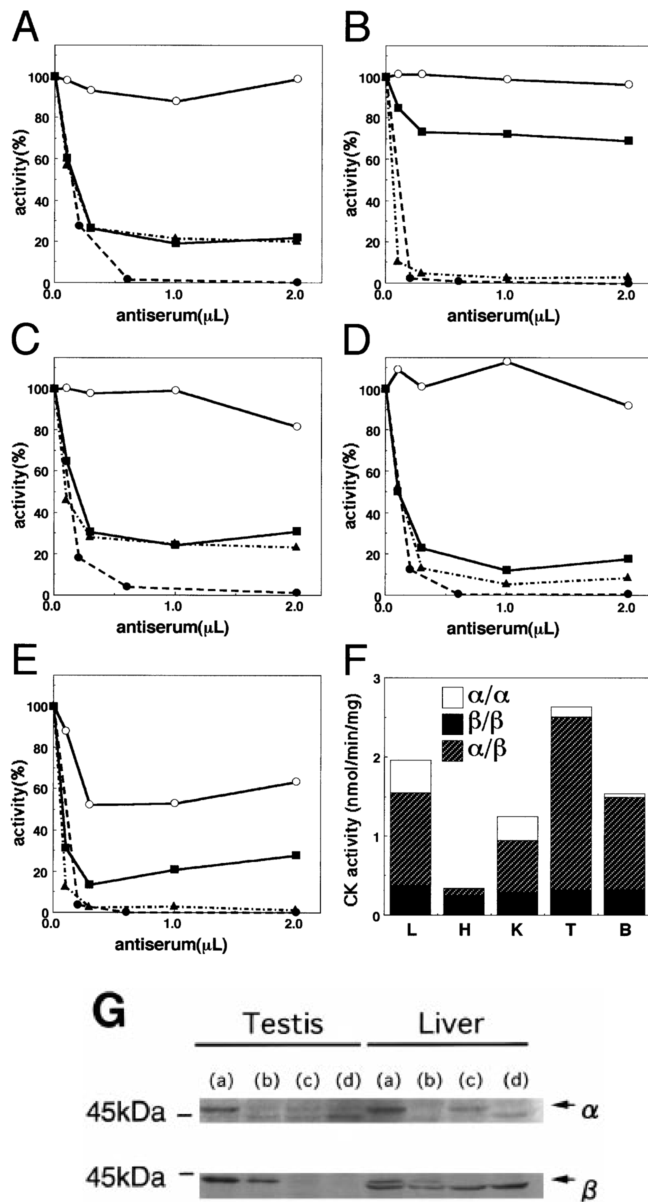

Figure 3 Immunological analysis of the active molecular form of ChoK/EtnK in mouse tissues

(**A**–**E**) Newly prepared 105 000 ***g*** supernatant fractions (280–580 μg of protein) from mouse liver (**A**), heart (**B**), kidney (**C**), testis (**D**) and whole brain (**E**) were incubated with either an antiserum raised against GST (control; ○) or GST–ChoK/EtnK-α (■) and GST–ChoK/EtnK-β (▲) independently or in combination (●), overnight at 4 °C. PANSORBIN® [10% (w/v); 50 μl; Calbiochem] was added to each incubation to precipitate rabbit IgG and an aliquot (approx. 50 μg of protein) of the resulting high-speed supernatant was used for the determination of ChoK activity. The data are plotted as ChoK activities remaining in the post-immunoprecipitation supernatants as a function of the amount of added antiserum. (**F**) On the assumption that the activity not precipitated by α-specific antiserum was due to β/β homodimers (homo-oligomers), that the activity not precipitated by β-specific antiserum was due to α/α homodimers (homo-oligomers), and that the remaining activity should result from $\alpha\beta$ heterodimers (hetro-oligomers), the composition of active holoenzyme was estimated in the above mouse tissues. L, liver; H, heart; K, kidney; T, testis; and B, whole brain. The experiment was performed three times (from three mice) with similar results. The data from a single mouse are shown. CK, choline kinase. (**G**) An aliquot (30 μg of protein) of the post-immumoprecipitated supernatant from mouse testis and liver samples was resolved by SDS/PAGE (10% gel), and subjected to Western blotting after immunoprecipitation with: (a) 1 μl of anti-GST serum, (b) 1 μl of anti-ChoK/EtnK-α serum, (c) 1 μl of anti-ChoK/EtnK-β serum, and (d) 0.5 μl of anti-ChoK/EtnK-α serum and 0.5 μl of anti-ChoK/EtnK-β serum. Other details are described in the legend for Figure 2. The data clearly show that the signal for 47 kDa ChoK/EtnK-α (43 kDa ChoK/EtnK-β) was found to disappear completely after the pretreatment with the respective antiserum, but it was also significantly diminished after the treatment with the counterpart, anti-ChoK/EtnK-β (anti-ChoK/EtnK-α) serum. The data from liver samples indicate that the intensity of the band just below the 43 kDa ChoK/EtnK-β band did not change through the immunoprecipitation.

composition of active molecular forms of ChoK in several mouse tissues are shown in Figure 3(F). In these experiments, the control incubation was performed in the presence of corresponding amounts of anti-GST serum. The reason for the partial inhibition of brain ChoK activity observed in control incubations (Figure 3E) is not clear at present, but it was reproducible. The reliability of the above estimation for the considerable presence of heteromeric ChoK in mouse tissues was further confirmed by Western-blot analysis (Figure 3G). The data clearly show that either of the ChoK/EtnK isoform bands (47 kDa for the α isoform and 43 kDa for the β isoform) was decreased after immunoprecipitation with not only the respective antiserum but also the other isoform-specific antiserum in both testis and liver samples. Essentially identical results were observed with other mouse tissue preparations (results not shown).

Characterization of the CCl_4-inducible form of ChoK/EtnK in mouse liver

Our earlier study demonstrated that the administration of CCl_4 caused an acute, liver-specific induction of ChoK activity in the rat [15]. This induction appeared to be associated with an increased synthesis of a certain form of ChoK protein, because either actinomycin D or cycloheximide completely inhibited this induction [15]. In addition, the CCl_4-inducible form of ChoK was shown to be different in both its native molecular mass [15,26] and immunological nature [5,26] from a form or forms existing in normal untreated rat liver. To further assess the nature of ChoK induction by CCl_4, we examined time-course experiments for changes in ChoK activity and mRNA expression, as well as the protein level of each ChoK/EtnK isoform in mouse liver. As shown in Figure 4(A), ChoK activity in liver cytosol was significantly increased by a single dosage of CCl_4 in a time-dependent manner. The peak of activation (2.5-fold) was seen at 9 h after CCl_4 administration and the increased activity was sustained until 24 h. It was found that a significant ChoK activity was released into blood plasma after 12 h of CCl_4 treatment (results not shown) where no activity could be detected in the normal state. Thus the data do not show the exact degree of induction of ChoK activity in liver cells at the later experimental period. The mRNA for ChoK/EtnK-α, but not that for ChoK/EtnK-β, was remarkably increased by CCl_4 (Figure 4B). This increase was transient, seen only at 3 and 6 h after CCl_4 administration, then rapidly returned to the control level by 9 h. The rapid disappearance of the induced α mRNA suggested that this hepatotoxin might also cause the induction of a certain repressive component against *chok/etnk-α* gene expression in mouse liver. In contrast with the transient change in its mRNA level, ChoK/EtnK-α protein in the CCl_4-induced liver showed a sustained increase throughout the experimental period (Figure 4C). The increase in the α isoform became significant after 3 h of CCl_4 administration, which was shortly delayed from its mRNA induction. There was no significant change in the level of ChoK/EtnK-β protein during CCl_4-induction. The 47 kDa band recognized by the α-specific antibody was sometimes separated into two bands (Figure 4C), which probably indicated the presence of both $\alpha 1$ and $\alpha 2$ isoforms in the blot. Either band was shown to disappear when the applied samples had been pre-

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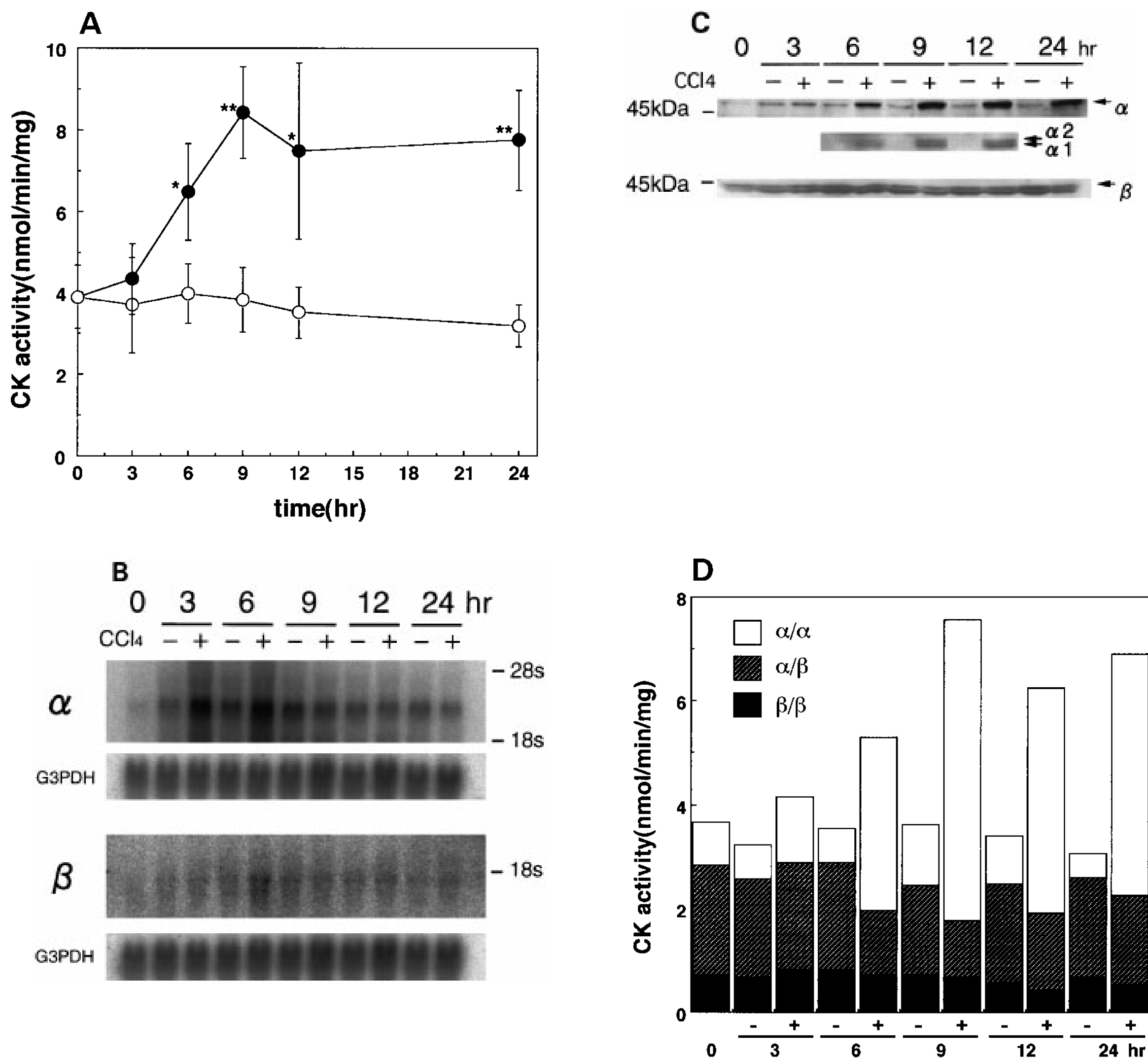

Figure 4 Characterization of the CCl_4-inducible form of ChoK/EtnK in mouse liver

CCl_4 was dissolved in 2 vols. of olive oil and injected intraperitoneally (1 ml/kg) into 6-week-old male ddY mice. Control mice received equivalent volumes of olive oil. Three mice from each group were killed at 3, 6, 9, 12 and 24 h, and the liver was quickly removed and subjected to total RNA extraction, as well as to subcellular fractionation. (**A**) ChoK (CK) activity was determined with a 105 000 ***g*** supernatant fraction of liver homogenates. (○) Values from control livers; (●) values from CCl_4-induced livers. Each bar represents the mean ± S.E.M. for three mice. Significant difference from the respective control value: $^{*}P < 0.05$; $^{**}P < 0.01$. (**B**) An aliquot (20 μg/lane) of the total RNA from either control livers or CCl_4-induced livers at the indicated time after CCl_4 injection was subjected to Northern-blot analysis for ChoK/EtnK-α and ChoK/EtnK-β. The locations of 28 S and 18 S ribosomal RNAs are indicated. Other details are described in the text. (**C**) Western-blot data for ChoK/EtnK-α and ChoK/EtnK-β. An aliquot (38 μg/lane) of the 105 000 ***g*** supernatant from either control livers or CCl_4-induced livers at the indicated time was resolved by SDS/PAGE (10% gel), then transferred on to a PVDF membrane and blotted with an affinity-purified isoform-specific antibody. In some experiments, the intense 47 kDa ChoK/EtnK-α band from the CCl_4-induced liver was found to give two separate bands as shown in the middle panel (tentatively indicated as α1 and α2), both of which were recognized specifically by an anti-ChoK/EtnK-α antibody. Other details are described in the text. (**D**) The change in the composition of the active (native) molecular form of ChoK (CK) in the CCl_4-induced liver was determined by the immunotitration assay according to the method described in the legend for Figure 3. The experiment was performed three times (from three mice in each group) with similar results. The data from a single mouse in each group are shown.

immunoprecipitated by anti-ChoK/EtnK-α serum. The possibility that CCl_4 could induce both α1 and α2 isoforms almost equally was suggested also by sequencing several cDNA clones for the α isoform isolated from a cDNA library that had been constructed from the CCl_4-treated rat liver (results not shown).

Finally, we examined the immunoprecipitation assay to characterize the active molecular form of CCl_4-induced ChoK/EtnK by the method described above and the results are shown in Figure 4(D). As was expected from both Northern- and Western-blot analyses, the major inducible ChoK activity was shown to be derived from α/α homodimers (or homo-oligomers). More than 70% of total ChoK activity at 9, 12 and 24 h after CCl_4 administration was estimated to exist in this particular form, whereas it was not more than 20% in normal untreated mouse liver.

DISCUSSION

The present study provided several lines of new experimental evidence for the molecular characteristics of ChoK/EtnK in the mouse. First, both ChoK/EtnK-α and ChoK/EtnK-β are ubiquitously expressed isoforms and coexist in all mouse tissues examined. Secondly, there exist several different native molecular forms of ChoK/EtnK expressing ChoK activity, with the predominant form in most mouse tissues being α/β heterodimers (or hetero-oligomers). Since it now appears that the term α isoform

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includes the two splice variants, $\alpha 1$ and $\alpha 2$ [4,20], actual subunit combinations to form active dimer (or oligomer) complexes could be further diversified. Even though the present investigation did not differentiate the actual oligomeric structure of the native form of ChoK/EtnK (dimer or oligomer), this is the first report demonstrating the presence of a heteromeric structure of ChoK/EtnK in any source. Thus the activity of ChoK/EtnK in a given cell type must be regulated not merely by the level of each isoform in the cell but also by the combination of each isoform subunit ($\alpha 1$, $\alpha 2$ and β) to generate active dimer (or oligomer) complexes ($\alpha 1/\alpha 1$, $\alpha 1/\alpha 2$, $\alpha 2/\alpha 2$, $\alpha 1/\beta$, $\alpha 2/\beta$ and β/β). This finding strongly suggests that the molecular mechanism of regulation of ChoK activity, and probably also of EtnK activity, in the cell is much more complicated than was expected. The kinetic characterization of each dimer (oligomer) complex, which might be prepared from each or combined overexpressed cell system, will be required in future studies. It has not been established which domain(s) of each ChoK/EtnK isoform can contribute to formation of the holoenzyme. There exist several cysteine residues conserved in both ChoK/EtnK-α and ChoK/EtnK-β among the predicted mouse, rat and human sequences, i.e. one each in d-1, d-2, d-6 and d-7 (Figure 1). As discussed elsewhere [4], both d-6 and d-7 have been considered to be involved in the catalytic function. Thus either or both of the other two well-conserved cysteine residues located in d-1 and d-2 is/are probably involved in the formation of dimer (or oligomer) complexes. Our previous finding [8], that a truncated GST–ChoK/EtnK-β fusion protein lacking both d-1 and d-2 domains did not show any ChoK activity at all, may suggest the possible involvement of these domains for the formation of active β/β homodimer complexes. There are several other well-conserved, α- or β-isoform-specific cysteine residues located in the N-terminal part of each sequence (Figure 1), some of which may also be involved in holoenzyme formation.

Thirdly, it was clearly shown that the CCl_4-inducible form of ChoK/EtnK in murine liver is ChoK/EtnK-α and not ChoK/EtnK-β. This induction was most likely due to the enhanced *chok/etnk*-α gene expression through a mechanism still unresolved. Consequently, the composition of the α/α homo-oligomeric form of ChoK/EtnK becomes most predominant in the CCl_4-induced liver, whereas this form makes a relatively minor contribution to total ChoK activity expressed in the normal untreated liver. Our previous finding [15,26] that the CCl_4-inducible form of ChoK in rat liver had a significantly higher intact molecular mass than the normal form probably indicated the presence of α/α homo-oligomers rather than homodimers in the CCl_4-induced liver. It was reported that the possible native form of ChoK/EtnK-α ($\alpha 1$ or $\alpha 2$) in rat liver was homotetrameric [6,27]. If this were also the case in mouse liver, then the CCl_4-inducible native form could be more than tetrameric. At present, however, we have no direct evidence for the difference in native ChoK/EtnK-α molecular conformation between the normal and CCl_4-induced mouse liver.

A mechanism of the possible up-regulation of the *chok/etnk*-α gene by CCl_4 may be direct or indirect. CCl_4 itself or its metabolite(s) can be a ligand for a certain nuclear receptor, which, although such receptor molecule has not yet been identified, upon ligand biding would cause a stimulation of promoter activity to induce *chok/etnk*-α gene expression. However, the presence as well as the location of the CCl_4-responsive element in the 5′ upstream promoter region of the *chok/etnk*-α gene has not yet been identified. This will be one of our most important future targets. Alternatively, CCl_4 metabolism is an established model of liver necrosis and fibrosis, and the liver damage created by this metabolism is free radical-dependent. CCl_4 is rapidly oxidized by cytochrome P450 to the trichloromethyl radical, which generates oxygen radicals [28,29] and lipid peroxides [30,31] in abundance. These radicals and peroxides would cause a variety of acute phase responses of hepatic cells, such as activation of cellular synthesis of inflammatory mediators [32,33]. Thus it would be possible to speculate that *chok/etnk*-α gene expression is up-regulated by one of the metabolic products generated during the early process of liver injury by CCl_4.

Another important finding of the present investigation is the possible existence of a repressor component in the CCl_4-induction of ChoK/EtnK-α. The level of the ChoK/EtnK-α transcript was significantly induced at 3 and 6 h after CCl_4 administration, then rapidly returned to the control level by 9 h and thereafter. This finding indicated that the half-life of the induced message for ChoK/EtnK-α must have been quite short and also strongly suggested that CCl_4 induced certain suppressor component(s) with respect to *chok/etnk*-α gene expression. The presence of a relatively long 3′-untranslated region in ChoK/EtnK-α mRNA [19] may be partly related to the observed fast decay of the inducible transcript. Finally, identification of the repressive component would provide further evidence for the physiological meaning of the liver-specific ChoK/EtnK-α induction by CCl_4.

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