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Identification and characterization of a novel Cut family cDNA that encodes human copper transporter protein CutC

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Abstract

Copper is an essential heavy metal trace element that plays important roles in cell physiology. The Cut family was associated with the copper homeostasis and involved in several important metabolisms, such as uptake, storage, delivery, and efflux of copper. In this study, a novel Cut family cDNA was isolated from the human fetal brain library, which encodes a 273 amino acid protein with a molecular mass of about 29.3 kDa and a calculated p*I* of 8.17. It was named *hCutC* (human copper transporter protein CutC). The ORF of *hCutC* gene was cloned into pQE30 vector and expressed in *Escherichia coli* M15. The secreted hCutC protein was purified to a homogenicity of 95% by using the Ni–NTA affinity chromatography. RT-PCR analysis showed that the *hCutC* gene expressed extensively in human tissues. Subcellular location analysis of hCutC-EGFP fusion protein revealed that hCutC was distributed to cytoplasm of COS-7 cells, and both cytoplasm and nucleus of AD293 cells. The results suggest that hCutC may be one shuttle protein and play important roles in intracellular copper trafficking.

Keywords: hCutC; Expression; Purification; Tissue distributions; Subcellular location

Copper (Cu) is an essential metal cofactor, which interacts with cuproenzymes that modify neuropeptides, generate energy, detoxify oxygen derived radicals, mobilize iron, coagulate blood, and cross-link connective tissue [1]. Copper homeostasis is regulated strictly in vivo, and the genetic diseases including Menkes disease and Wilson disease underscore critical roles for Cu uptake and distribution [2,3]. Copper was imported by the plasma membrane transport Ctr family proteins [1], which rapidly bind to intracellular copper chaperone proteins. The known copper chaperones are divided into three functional groups: the Atx1-like chaperones, the copper chaperones for superoxide dismutase, and the copper chaperones for cytochrome *c* oxidase [4].

Two types of gene families appear to be associated with the copper homeostasis and involved in the uptake, storage, delivery, and efflux of copper in bacteria [5]. One is the cop gene family including four genes, *cop*A, *cop*B, *cop*Y, and *cop*Z [6]. The cop family is a well-understood system of active transport efflux pumps and the cop proteins associate with efflux ATPase or as regulators of the *cop* operon [7,8]. The other is the cut gene family, which has at least 6 *cut* gene members (*cut*A, *cut*B, *cut*C, *cut*D, *cut*E, and *cutF*) [9]. A mutation in one or more of these genes results in an increased copper sensitivity [10,11]. The *cutA* and the *cut*E genes have been cloned and sequenced, respectively, in the *Escherichia coli* [12,13]. The *cut*A gene is universally distributed in a wide variety of bacteria. Containing two different operons, the *cut*A gene encodes a cytoplasmic protein (CutA1) and two inner membrane proteins (CutA2 and CutA3). The cutA2 plays roles in the biogenesis of *c* type cytochrome [8] and the functions of CutA1 and CutA3 are not clear [12]. The crystal structure of *Pyrococcus horikoshii* CutA revealed the structural implications for heavy metal-induced reversible

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assembly and aggregation of CutA protein [14]. CutE has been identical as an intracellular copper-binding protein [13] and functions as an apolipoprotein *N*-acyltransferase [15]. CutF is an outer membrane lipoprotein and involves in copper tolerance in *E. coli* [10,16]. Both copper-sensitive cutC and cutF mutants accumulate copper but have apparently normal kinetics of copper uptake [10] and appear to be required for the copper-sensitive phenotype in each mutant. The CutF protein was proposed to be responsible for protecting the cell from copper toxicity and for delivering copper to the sites of assembly of copper proteins.

Previous studies showed that CutC may play a role in intracellular trafficking of Cu(I) [17,18]. Recently, the crystal structure of *Shigella flexneri* CutC, (43% amino acid sequence with putative human CutC protein) has been resolved [19], but it is still unclear about the actual functions of CutC. The putative copper transporter protein CutC (GenBank: NP_057044) consisting of 273 amino acid residues was released by the "full-length long Japan" (FLJ) collection project at NCBI [20]. To further characterize the genetic determinants and disease relationships of copper metabolism in human, we isolated the human copper transporter CutC (*hCutC*) from human fetal brain library. The *hCutC* gene belongs to the CutC family (Pfam-PF03932) [21]. Here we report the isolation, expression, purification, and characterization of human copper transporter CutC.

Materials and methods

Cloning and bioinformatic analysis of hCutC. A high quality cDNA library was constructed by using the human fetal brain poly(A)$^+$ mRNA and a SMART PCR cDNA library construction kit (Clontech). After *Sfi*I digestion, cDNAs greater than 500 bp were ligated into the *Sfi*I A and *Sfi*I B sites of the modified pBluescript II SK (+) vector and then transformed into the *E. coli* DH5α using electroporation (*E. coli* pulser, Bio-Rad). Both 5′ and 3′ ESTs were generated with either dye primer or dye terminator chemistries on an ABI377 sequencer using M13 consensus primers. Primer walking was performed when necessary. Assembly program (Sanger Center) was used to assemble the full-length cDNA sequences. Through high-throughput cDNA sequencing, a human CutC cDNA clone was isolated.

DNA and the protein sequence comparisons were carried out using BLAST at NCBI (http://www.ncbi.nlm.nih.gov/blast). Multiple sequence alignment analysis was performed using Clustal W algorithms. Protein sorting analysis was done with PSORT II (http://psort.nibb.ac.jp:8800). Other sequence analyses were performed using Genedoc software.

Expression pattern of hCutC. To determine the expression profile of *hCutC* gene, one human Multiple Tissue cDNA (MTC) panel was used as PCR template according to the manufacturer's protocol (Clontech). The primer sequences of the *hCutC* were 5′-ACGAGCGCGGATACCGTCC-3′ (*hCutC*-f, corresponding to nucleotides 133–159) and 5′-CCTG GCATTACCACAATCCTG-3′ (*hCutC*-r, corresponding to nucleotides 684–704). Twenty-four cycles (for the control, GAPD) or 32 cycles (for *hCutC*) of amplifications (30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C) were performed using *Pfu* polymerase in a volume of 50 μl. The PCR product of *hCutC* was resolved on 1.5% metaphor agarose.

Expression and purification of hCutC. The open reading frame (ORF) of *hCutC* gene was cloned by PCR using the *Pfu* DNA polymerase. The sequences of forward and reverse synthetic primers were 5′-CGAG CTCAAAAGGCAGGGGGCCTCCTCTGA-3′ and 5′-GGGGTACCCT ACACCAGGATGTTCTTTGCGATA-3′, respectively. They were designed to anneal to upstream and downstream flanking sequences just outside the *hCutC* gene and to contain *Sac*I and *Kpn*I recognition sequences (underlined). The 820-bp PCR product was inserted into the *Sac*I and *Kpn*I sites of pQE30 vector (Qiagen). Purification of plasmids, agarose gel electrophoresis, and transformation of cells were performed according to the procedures previously described by Maniatis et al. [22]. The recombinant *hCutC* plasmid was transformed into *E. coli* M15. Then the transformed M15 cells were grown in Luria–Bertani (LB) medium (containing 100 mg/ml ampicillin and 50 mg/ml kanamycin) at 37 °C until the culture reached an OD_{600} of 0.6–0.8. After 6-h induction with 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) at 25 °C, cells were harvested by centrifugation and resuspended in cold sodium phosphate buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; and 10 mM imidazole), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) at 10 ml/g wet weight. The purification steps of hCutC fusion protein were performed according to the previous report with slight modification [19]. The purified protein was dialyzed against the stock buffer containing 5 mM Tris–HCl (pH 8.5) and 50% glycerol. The purified protein was stored at −70 °C until used. The fractions were analyzed by 12% SDS–PAGE and bands were visualized by Coomassie blue staining by previously described standard procedures. Protein concentrations in extracts and during purification evaluation were determined by the method of Bradford [23], using bovine serum albumin (BSA) as the protein standard.

Subcellular location analysis of hCutC. The ORF of *hCutC* was cloned into pEGFP-C1 expression vector (Clontech) allowing the expression of *hCutC* as a green fluorescent fusion protein (GFP). The COS-7 cells and AD293 cells were maintained in DMEM supplemented with 10% fetal calf serum and 100 U/ml ampicillin. Cells were grown at 37 °C and 5% CO_2. After the growth density of the mammal cells reached 70% completion, the generated fusion plasmid (2 μg) was transfected into COS-7 cells and AD293 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Results and discussion

Cloning and bioinformatic analysis of hCutC

The Cut genes play roles in copper homeostasis and the mutation in one or more of Cut genes results in an increased copper sensitivity. The *hCutC* gene was isolated from the human fetal brain cDNA library by the PCR method, which encodes a 273 amino acid protein with a molecular mass of about 29.3 kDa and a calculated p*I* of 8.17. The amino acid sequence of the human CutC is highly conserved and shows 90% identity to *Pan troglodytes* CutC, 89% identity with *Mus musculus* CutC, 85% identity to *Gallus gallus* CutC, 80% identity to *Xenopus tropicalis* CutC, and 44% *E. coli* CutC (Fig. 1). In bacterial, CutC contains the MPRMEKIM sequence in the N-terminal which was similar to a potential copper-binding motif (M-X-X-X-X-M-X-X-M) presented in the CopB ATPase from *Enterococcus hirae* [7,10]. While in human CutC, the corresponding amino acid sequence (127–134) is KELCMSLM which shares high homology with the other eukaryotes (Fig. 1). In Ctr family, Ctr1 has several potential copper binding motifs (M-X-X-M-X-M) in the N-terminal domain while Ctr3 lacks these motifs, but has an abundance of cysteine residues throughout the protein [24]. The deduced amino acid sequence of the CutC protein suggests that it is a cytoplasmic copper-binding protein.

By searching the human genome database, the *hCutC* gene was mapped to 10q24 in a contig NT_086775.1. The comparison result also showed that the *hCutC* gene has

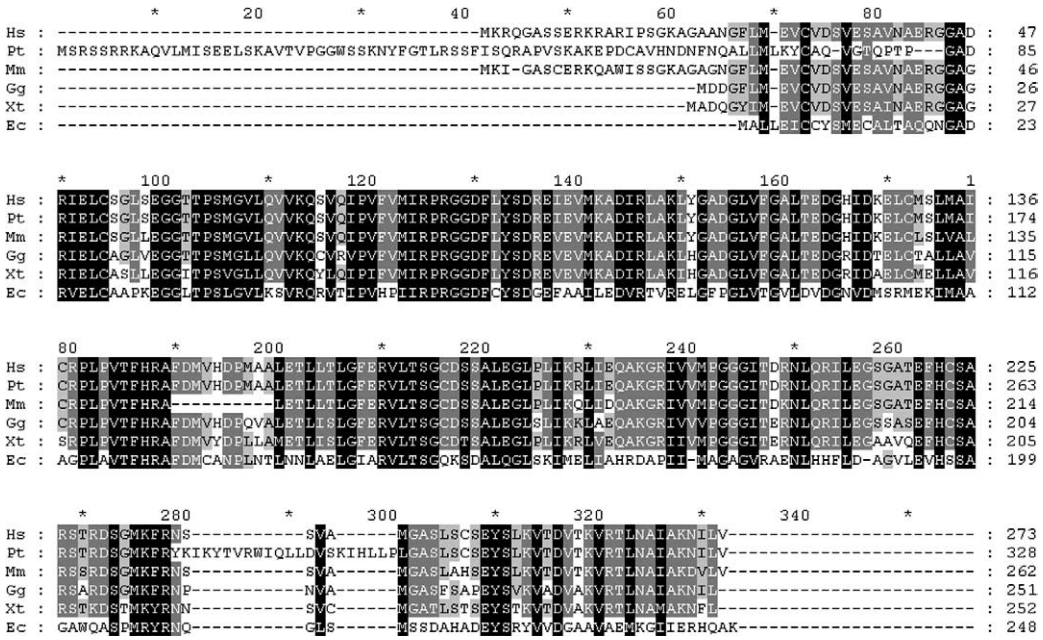

Fig. 1. Protein sequence alignment of human CutC (Hs, NP_057044), *P. troglodytes* CutC (Pt, XP_507975), *M. musculus* CutC (Mm, NP_079806), *G. gallus* CutC (Gg, NP_001006503), *X. tropicalis* CutC (Xt, NP_001011220), and *E. coli* CutC (Ec, NP_310611). Conserved amino acids are shaded.

nine exons and eight introns spanning 23.6 kb of human genomic DNA. All sequences at the exon–intron junction were consensus with the AG-GT consensus sequence [25].

Tissue distribution of hCutC

A MTC basic RT-PCR was employed to inspect the expression pattern of *hCutC*. The *hCutC* gene was expressed in all the detected tissues, with lower expression levels in skeletal muscle, pancreas, prostate, and small intestine (Fig. 2). The size of the product was the expected 570 bp. This result is consensus with the other copper transporters such as CutA, Ctr1, and Ctr3 [8,26,27], which may be associate with copper trafficking and tolerance.

Expression and purification of hCutC

The *hCutC* gene was cloned into pQE30 vector and then was expressed in *E. coli* M15 in soluble forms. The expected 32 kDa fraction was detected in SDS–PAGE, which included one 6× His tag in the recombinant protein (Fig. 3). The soluble recombinant protein was purified by Ni–NTA affinity chromatography to homogeneity. Approximately, 4 mg of the recombinant protein in 800 mg of crude protein was purified from 1 L LB culture medium.

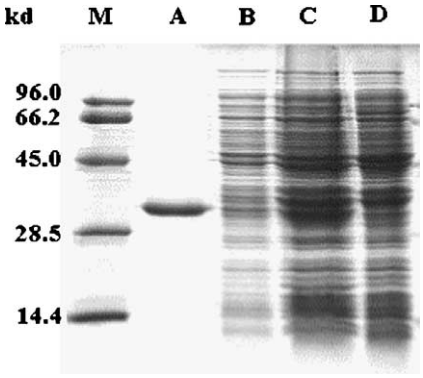

Fig. 3. SDS–PAGE showing expression in *E. coli* and purification of the recombinant hCutC protein. Lane M, protein molecular mass standards; (A) peak fraction from Ni–NTA chromatography; (B) the supernatant of bacteria after sonication (induced with IPTG); (C) crude extraction from the *E. coli* cells induced with IPTG; and (D) crude extraction from the *E. coli* cells without the IPTG induction.

Subcellular location of hCutC

The hCutC-pEGFP fusion plasmid was transfected into COS-7 cells and AD293 cells. After 24 h of expression, the hCutC-pEGFP product was detected with the fluorescence microscope. The results showed that the

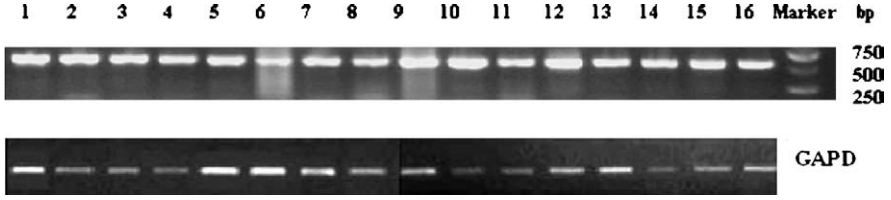

Fig. 2. Reverse transcription PCR analysis of *hCutC* with different human tissues. GAPD was used as a control. 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, thymus; 11, prostate; 12, testis; 13, ovary; 14, small intestine; 15, colon; 16, blood leukocyte.

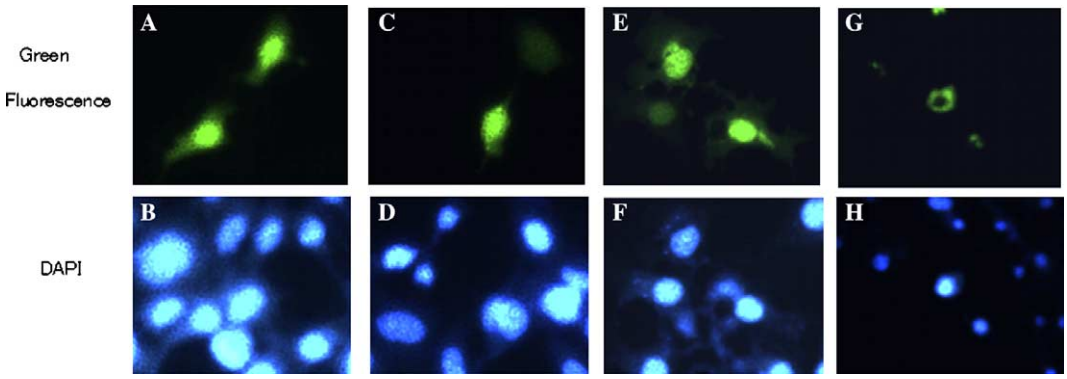

Fig. 4. Subcellular location of hCutC-pEGFP fusion protein in transfected COS-7 and AD293 cells. (A) pEGFP protein in AD293 cells; (C) hCutC-pEGFP fusion protein in AD293 cells; (E) pEGFP protein in COS-7 cells; (G) hCutC-pEGFP fusion protein in AD293 cells; (B,D,F,H) the proteins colored with DAPI in (A,C,E,G), respectively. The hCutC-pEGFP fusion product is distributed to cytoplasm of COS-7 cells and throughout the whole cell in AD293 cells. The pEGFP control is distributed throughout the whole cell in COS-7 and AD293 cells as expected.

hCutC-pEGFP fusion product was distributed to cytoplasm of COS-7 cells, and both cytoplasm and nucleus of AD293 cells. The pEGFP control was distributed throughout the whole cell in COS-7 cells and AD293 cells as expected (Fig. 4). It was reported that many members of Cut family are cytoplasmic copper-binding proteins [10,13]. For example, the bacterial CutC and CutE have been identified as intracellular copper-binding proteins and CutF is an outer membrane lipoprotein [10,13,16]. The result that CutC was distributed to cytoplasm is consensus with the previously reported one in *E. coli* CutC [10], which suggests that human CutC may be a cytoplasmic copper-binding protein and play roles in intracellular copper trafficking. Moreover, the hCutC was distributed to nucleus of AD293 cells, which suggests that hCutC may be one shuttle protein for copper homeostasis.

Acknowledgments

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