CROP/Luc7A, a novel serine/arginine-rich nuclear protein, isolated from cisplatin-resistant cell line[1]

Yoichi Nishii[a], Masaki Morishima[a], Yoshiyuki Kakehi[b], Kouji Umehara[a], Noriyuki Kioka[a], Yoshitake Terano[c], Teruo Amachi[a], Kazumitsu Ueda[a,*]

[a] *Laboratory of Biochemistry, Division of Applied Life Sciences, Kyoto University Graduate School of Agriculture, Kyoto 606-8502, Japan*
[b] *Department of Urology, Kyoto University Graduate School of Medicine, Kyoto 606-8502, Japan*
[c] *Osaka City University Medical School, Osaka 545, Japan*

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Abstract A novel putative SR protein, designated cisplatin resistance-associated overexpressed protein (*CROP*), has been cloned from cisplatin-resistant cell lines by differential display. The N-half of the deduced amino acid sequence of 432 amino acids of CROP contains cysteine/histidine motifs and leucine zipper-like repeats. The C-half consists mostly of charged and polar amino acids: arginine (58 residues or 25%), glutamate (36 residues or 16%), serine (35 residues or 15%), lysine (30 residues, 13%), and aspartate (20 residues or 9%). The C-half is extremely hydrophilic and comprises domains rich in lysine and glutamate residues, rich in alternating arginine and glutamate residues, and rich in arginine and serine residues. The arginine/serine-rich domain is dominated by a series of 8 amino acid imperfect repetitive motif (consensus sequence, Ser-Arg-Ser-Arg-Asp/Glu-Arg-Arg-Arg), which has been found in RNA splicing factors. The RNase protection assay and Western blotting analysis indicate that the expression of CROP is about 2–3-fold higher in mRNA and protein levels in cisplatin-resistant ACHN/CDDP cells than in host ACHN cells. CROP is the human homologue of yeast Luc7p, which is supposed to be involved in 5′-splice site recognition and is essential for vegetative growth.

Key words: SR protein; RNA splicing; Cisplatin resistance; Nuclear protein; Differential display

1. Introduction

cis-Diamminedichloroplatinum (II) (cisplatin) has become one of the most widely used anticancer drugs targeting a variety of malignant tumors, including those of testes, head and neck, esophagus, lung, ovary and bladder [1]. However, their clinical effectiveness is frequently limited by the emergence of drug-resistant tumor cell populations that are often cross-resistant to structurally unrelated drugs [2,3]. Cisplatin reacts with DNA to produce 1,2-intrastrand cross-links between adjacent purines. The initial cytotoxic effects of the drug are believed to be due to an inhibition of DNA synthesis at the site of adduct formation [4,5]. It is also believed that cisplatin reacts with RNA to produce adducts [6–8]. They inhibit transcription by interfering with chromatin remodeling, and with transcription factor binding as well as with RNA synthesis itself [6]. Furthermore, RNA adducts of cisplatin affect RNA splicing [9].

Cisplatin resistance could be mediated through various mechanisms, including inactivation of cisplatin by thiol-containing molecules such as glutathione [10] and metallothionein [11], increased DNA repair [12], decreased drug accumulation [13], reduction in total cellular topoisomerase activity [14]. Increased expression of thioredoxin [15] and T-plastin [16] has also been reported. Because several of these mechanisms appear to operate in individual resistant cell lines, it is difficult to set the target for overcoming cisplatin resistance.

To explore a key molecule of cisplatin resistance, we tried to isolate genes that are commonly overexpressed in several cisplatin-resistant cell lines. Here, we report that a novel arginine/serine-rich nuclear protein, cisplatin resistance-associated overexpressed protein (CROP)/hLuc7A, is overexpressed in cisplatin-resistant cell lines.

2. Materials and methods

2.1. Materials

Cisplatin was obtained from Sigma. [α-^{32}P]CTP was obtained from Amersham.

2.2. Cell lines

Human renal cancer cells, ACHN and NC65, were propagated in RPMI 1640 medium with 10% fetal bovine serum under 5% CO_2 at 37°C. Cisplatin-resistant cell lines, ACHN/CDDP and NC65/CDDP, were established by propagation in the presence of 5 μg/ml cisplatin [17].

2.3. Differential display and cDNA cloning

Differential display was done as described [18] by using RNAmap kit (GenHunter, Brookline, MA, USA). Briefly, total RNA was reverse-transcribed with T_{12}MN oligonucleotides (M = G, C or A; N = G, A, T or C) as primer. PCR was done with synthesized cDNAs as templates and with the respective T_{12}MN oligonucleotide and specific arbitrary 10-mer according to the manufacturer's directions. PCR products were analyzed by electrophoresis on 7% sequencing gels and cloned into pT7Blue T-Vector (Novagen). ACHN/CDDP cDNA library was made with λ SHlox cloning kits (Novagen).

2.4. Reverse transcriptase (RT-) PCR analysis

Total RNA was converted to cDNA by reverse transcriptase and the cDNA was subjected to PCR (94°C for denaturation, 50°C for annealing, and 72°C for extension) with human G3PDH control primers (Clontech) and with CROP primers (5′-ACGTTCTGATCTTGGTCCG-3′ and 5′-TCGACGTTGTGGACCTTAGC-3′). Amplification was done for 25 cycles.

*Corresponding author. Fax: (81)-75-753 6104.
E-mail: uedak@kais.kyoto-u.ac.jp

[1] The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence database with accession number AB034205.

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2.5. Cloning of 5′-region of cDNA

The 5′-region of cDNA of the gene was isolated with 5′-Amplifinder RACE kit (Clontech). The first strand cDNA synthesis was done with total RNA from ACHN/CDDP and with an oligonucleotide 5′-CTGCTGCATCTTCTGTCTGATG-3′. After an Amplifinder anchor oligonucleotide was ligated to the 3′-end of cDNA, PCR was done with an Amplifinder anchor primer and an oligonucleotide 5′-GTGTCTACTTCGTGAACGACTTCTC-3′.

2.6. RNase protection assay

The 162 nucleotide fragment (from 64 to 235 from the translation start site) was synthesized from the long-type CROP cDNA by PCR with primers CCCAAGCTTAGCAACGTGCG (*Hin*dIII site is underlined) and CCTGGATCCACTTGTCATCTTCC (*Bam*HI site is underlined). The PCR products were inserted into pGEM vector. The plasmid DNA was linearized with *Hin*dIII and the antisense RNA probes were synthesized with T7 RNA polymerase. Total RNA (35 μg) was hybridized with ^{32}P-labeled antisense RNA probes. Products were analyzed by electrophoresis on a 7% sequencing gel.

2.7. Preparation of antibody against CROP

The amino-terminal half of CROP (amino acids 1–245) was fused with histidine tag in a pET28a vector (Novagen), and the fusion protein was expressed in *Escherichia coli* strain BL21. The fusion protein was purified with His-bind resin (Novagen), and a rabbit polyclonal antibody was raised against the purified protein.

3. Results

3.1. Isolation of cDNAs overexpressed in cisplatin-resistant cell lines

cDNA fragments, expressed higher in cisplatin-resistant ACHN/CDDP cells than its host ACHN cells, were isolated by differential display. Among them, 11 fragments were found to hybridize with mRNAs overexpressed in ACHN/CDDP by Northern analysis (data not shown). The isolated fragments being used as probes, 1.7 kb and 2.6 kb fragments, were isolated from a cDNA library prepared from ACHN/CDDP mRNAs. The sequence analysis revealed that the 1.7 kb cDNA encodes plastin. It was reported that the expression of T-plastin gene is higher in cisplatin-resistant human cancer cells [16]. This suggested that the differential display in this study worked.

The PCR analysis suggested that the sequence from the 2.6 kb fragment is overexpressed in cisplatin-resistant cell lines derived from two different cell lines, ACHN and NC65 (Fig. 1). Because sequence analysis has suggested that the 2.6 kb cDNA fragment does not contain the 5′-region of the cDNA, we did 5′-RACE to isolate the 5′-region. We analyzed several clones and found that cDNA fragments isolated with 5′-RACE could be divided into two groups. One is about 1240 bp, and the other about 1050 bp. The short-type cDNAs (about 1050 bp) do not contain a region of 193 bp, which exists in the long-type cDNAs at 214–406 bases from A in the first ATG (Fig. 2A). The 193 bp region contains several

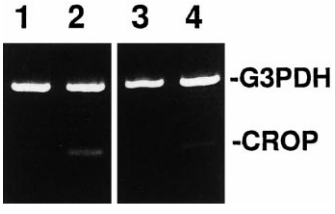

Fig. 1. RT-PCR analysis. 1, ACHN; 2, cisplatin-resistant ACHN/CDDP; 3, NC65; 4, cisplatin-resistant NC65/CDDP.

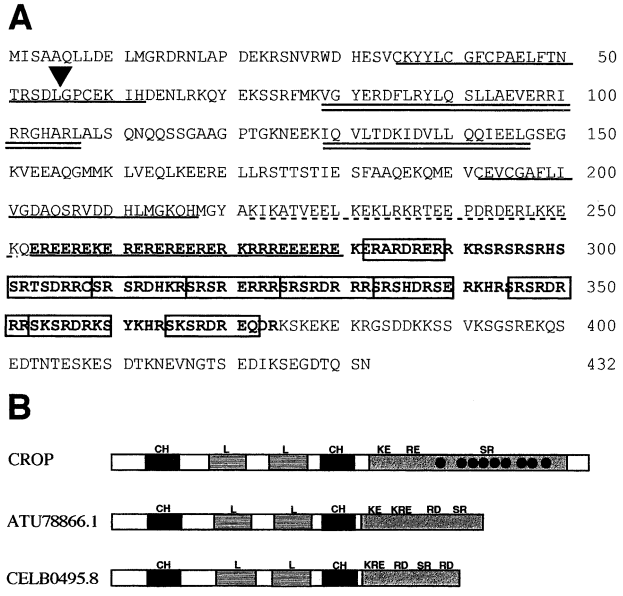

Fig. 2. Predicted amino acid sequence of CROP (A), and schematic domain organizations of CROP-related genes (B). A: The cysteine/histidine motifs are underlined once. The leucine zipper-like repeats are underlined twice. The lysine/glutamate-rich region is underlined with a dashed line. The arginine/glutamate-rich region is in bold and underlined letters. The arginine/serine-rich region is in bold letters. 8 Amino acid imperfect repetitive motifs in serine/arginine-rich domain are in boxes. The arrow head indicates the site in which the 193 base exon is inserted. B: *A. thaliana* putative arginine-aspartate-rich RNA binding protein (U78866.1) and *C. elegans* B0495.8. CH, cysteine/histidine motifs; L, leucine zipper-like repeats; KE, lysine/glutamate-rich region; KRE, lysine/arginine/glutamate-rich region; RD, arginine/serine-rich region; RS, arginine/serine-rich region; ●, 8 amino acid imperfect repetitive motif.

nonsense codons so that the long-type cDNA cannot encode any long open reading frame. In contrast, the short-type 1.05 kb cDNA, together with the 2.6 kb cDNA fragment, encodes a long open reading frame of 432 amino acids (Fig. 2A), which we named CROP.

To examine whether the translatable short-type mRNA of the *CROP* gene is expressed in the cisplatin-resistant ACHN/CDDP cells, RNase protection assay was done with a 230 base RNA probe (Fig. 3). This probe contains the 5′-terminal 63 base sequence of the 193 base exon, the 99 base cDNA sequence adjacent to the 5′-end of the 193 base exon, and a 68 base sequence of vector. Both cisplatin-sensitive ACHN cells and cisplatin-resistant ACHN/CDDP cells produced mainly two protected RNA bands of 99 base and 162 base. The amounts of 99 base and 162 base bands represent the amounts of the short-type mRNA and the long-type mRNA, respectively. This indicated that the amount of the short-type translatable mRNA is about three times more in cisplatin-resistant ACHN/CDDP cells, and that more than 90% of the *CROP* mRNA is the short-type mRNA in cisplatin-resistant ACHN/CDDP cells.

3.2. Amino acid sequence and domain organizations of CROP

The N-half of the deduced amino acid sequence of CROP contains cysteine/histidine motifs and leucine zipper-like repeats. The C-half of CROP consists mostly of charged and polar amino acids: arginine (58 residues or 25%), glutamate (36 residues or 16%), serine (35 residues or 15%), lysine

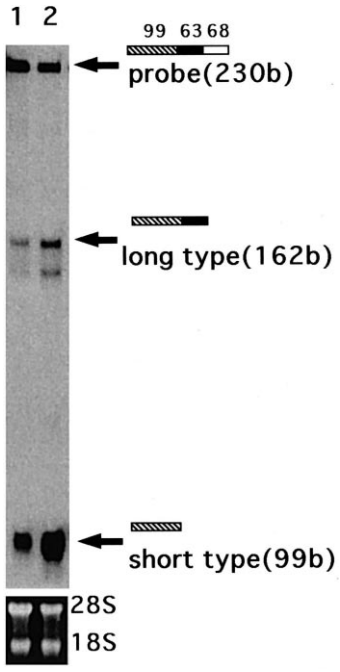

Fig. 3. RNase protection assay. The 230 base RNA probe is diagramed at the right. The 5′-terminal 63 base sequence of the 193 base exon is indicated by a black box. The 99 base cDNA sequence adjacent to the 5′-end of the 193 base exon is indicated by a hatched box. The 68 base sequence of vector is indicated by an open box. Lane 1, ACHN; lane 2, ACHN/CDDP. Lower panel, ethidium bromide staining of 5 μg total RNA used for the assay.

(30 residues or 13%), and aspartate (20 residues or 9%). It is extremely hydrophilic and comprises domains rich in lysine and glutamate residues, rich in alternating arginine and glutamate residues, and rich in arginine and serine residues. The arginine/serine-rich domain (RS domain) is dominated by a series of 8 amino acid imperfect repetitive motif (consensus sequence, Ser-Arg-Ser-Arg-Asp/Glu-Arg-Arg-Arg). This motif has been found in HEL117, an RNA helicase [19], and the 70K component of the U1 snRNP particles [20]. Interestingly, *Arabidopsis thaliana* putative arginine-aspartate-rich RNA binding protein and *Caenorhabditis elegans* B0495.8 also contain a region rich in lysine and glutamate residues, rich in

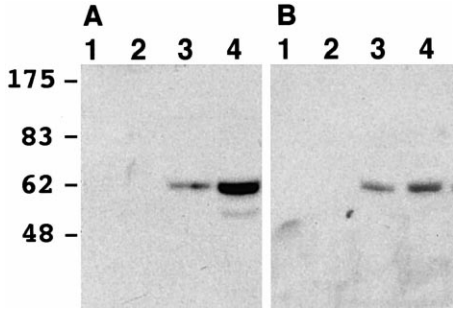

Fig. 4. Western blotting with the anti-CROP antibody. A: Cytosolic fraction (lanes 1 and 2) and nuclear extract (lanes 3 and 4) of Cos-7 cells (lanes 1 and 3) and Cos-7 cells transiently expressing CROP (lanes 2 and 4). B: Cytosolic fraction (lanes 1 and 2) and nuclear extract (lanes 3 and 4) of ACHN cells (lanes 1 and 3) and ACHN/CDDP (lanes 2 and 4). Proteins (50 μg) were separated by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel.

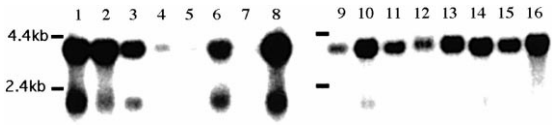

Fig. 5. Expression of CROP mRNA in human tissues. Human multiple tissue Northern blot I, II (Clontech), containing approximately 2 μg of poly(A) RNA per lane from the indicated human tissues, were analyzed by Northern blot hybridization with a CROP-specific probe. Lane 1, heart; 2, brain; 3, placenta; 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, peripheral blood leukocyte.

alternating arginine and glutamate residues, and rich in arginine and serine residues (Fig. 2B). Domain organizations of CROP, ATU78866.1 and CELB0495.8 are quite similar.

3.3. Higher expression of CROP in the nucleus of cisplatin-resistant cells

A 58 kDa protein was detected by the anti-CROP antibody in the nuclear extract from ACHN host cells and cisplatin-resistant ACHN/CDDP cells, but not in their cytosolic fraction (Fig. 4B). The amount of 58 kDa protein is about 2-fold more in ACHN/CDDP cells than ACHN host cells. To confirm that the 58 kDa protein is CROP, the CROP expression plasmid was transiently transfected into Cos-7 cells. The anti-CROP antibody recognized an endogenous 58 kDa protein in the nuclear extract from Cos-7 cells, and the overexpressed 58 kDa protein in the nuclear extract from the transfected cells (Fig. 4A). These results indicate that the 58 kDa nuclear protein is CROP, and that the expression of CROP is higher in ACHN/CDDP cells than ACHN host cells.

3.4. Expression of CROP mRNA in human tissues

Northern blot analysis indicated that the *CROP* gene is expressed in almost all tissues tested as mRNA of about 3.5 kb (Fig. 5). Importantly, the expression of the *CROP* gene showed the clear tissue-specificity: it is expressed strongly in heart, brain, pancreas, thymus, ovary, small intestine and peripheral blood leukocyte, and weakly in lung, liver and kidney.

4. Discussion

We cloned a cDNA for a nuclear protein of 432 amino acids from cisplatin-resistant ACHN/CDDP cells, and named this protein CROP. The expression of CROP in mRNA and protein levels is about 2–3-fold higher in the cisplatin-resistant cells than the host cisplatin-sensitive cells.

The N-half of CROP contained cysteine/histidine motifs and leucine zipper-like repeats. The cysteine/histidine motif in CROP is highly homologous to those in *A. thaliana* putative arginine-aspartate-rich RNA binding protein [21] and other three proteins from *C. elegans* and *Saccharomyces cerevisiae*. This cysteine/histidine motif (C-X_2-C-X_{13-15}-H-X_5-H) is also found in the human snRNP polypeptide C and the yeast PRP6, PRP9 and PRP11, involved in the nuclear splicing pathway [22]. The C-half of CROP comprises domains rich in lysine and glutamate residues, rich in alternating arginine and glutamate residues, and rich in arginine and serine residues (RS domain). The RS domain is dominated by a series of 8 amino acid imperfect repetitive motif (consensus

sequence, Ser-Arg-Ser-Arg-Asp/Glu-Arg-Arg-Arg), which has been found in RNA splicing factors.

The SR proteins, characterized by a C-terminal RS domain [23–26], are involved in the formation of spliceosome, localize in a nucleus, and show speckled intranuclear distribution, in which snRNP particles and SR proteins are suggested to be organized [27]. Some RS domains are predicted to mediate protein-protein interactions, to work as a nuclear localization signal, and formation of spliceosome assembly.

Recently, it was reported that yLuc7p, a novel yeast U1 snRNP protein, is essential for vegetative growth, and that mutation of yLuc7 causes a defect in pre-mRNA splicing [28]. yLuc7p is supposed to be involved in the interaction between U1 snRNP and the nuclear cap binding complex and in 5′-splice site recognition. The homologues of yLuc7 have been found from the EST data base of higher eukaryotes. Interestingly, all metazoan LUC7 family members contain a carboxyl-terminal extension with multiple arginine-serine (RS) or arginine-glutamate (RE) repeats [28]. The CROP protein was turned out to be identical to hLuc7A. Higher expression of CROP could influence 5′-splice site recognition and alternative splicing, which may be directly or indirectly involved in cisplatin resistance.

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