Molecular cloning of cDNA encoding a cyclin-selective ubiquitin carrier protein (E2-C) from *Carassius auratus* (goldfish) and expression analysis of the cloned gene

Mika Tokumoto[a,b], Yoshitaka Nagahama[a], Toshinobu Tokumoto[c,*]

[a] *Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan*
[b] *JSPS Research Fellow, Faculty of Science, Shizuoka University, Shizuoka 422-8529, Japan*
[c] *Department of Biology and Geosciences, Faculty of Science, Shizuoka University, Shizuoka 422-8529, Japan*

Received 17 May 1999; received in revised form 22 July 1999

Abstract Destruction of cyclin B is required for exit from mitosis and meiosis. A cyclin-specific ubiquitinating system, including cyclin-selective ubiquitin carrier protein (E2-C), is thought to be responsible for cyclin B destruction. Here we present the cloning, sequencing and expression analysis of goldfish, *Carassius auratus*, E2-C which encodes the cyclin-selective ubiquitin carrier protein from goldfish ovary. The cloned cDNA is 677 bp long and encodes 172 amino acids. The deduced amino acid sequence is highly homologous to E2-C from other species. Recombinant goldfish E2-C possesses ubiquitinating activity against cyclin B. The expression of mRNA for E2-C was similar to that of mRNA for cyclin B, occurring at very high level in the ovary. The similarity of the expression pattern of E2-C and cyclin B suggests that E2-C mediates a cyclin-specific ubiquitination.

Key words: Ubiquitin carrier protein; E2-C; Cyclin B

1. Introduction

Proteins critical to the regulation of the cell cycle (e.g. cyclins, cdk inhibitors, or c-mos products) are known to be degraded by the ubiquitin pathway [1–4]. Degradation of cyclin B is essential for exit from M-phase [5]. The evidence that cyclin B degradation is mediated by ubiquitin-dependent proteolysis was provided by Glotzer et al. [1]. In the case of the degradation by the ubiquitin pathway, proteins are ligated to ubiquitin through their lysine amino acid groups, then degraded by the 26S proteasome [6]. A cell cycle-regulated ubiquitin ligase complex, APC/cyclosome, has been characterized biochemically in clam and *Xenopus* [7,8] and genetically in yeast [9,10]. These ubiquitin ligases catalyze polyubiquitination using a specialized ubiquitin carrier protein (E2). Among the multiple species of E2s, UBC9 protein is required for cell cycle progression in late G2 or early M-phase [11]. UBC4 protein can ubiquitinate cyclins in *Xenopus* egg extracts [8]. A novel cyclin-selective UBC family member, E2-C, was reported which can ubiquitinate cyclin B (13–91)/protein A fusion protein in a APC/cyclosome-dependent manner [12]. The counterparts of E2-C were identified in *Xenopus* [13] and human [14]. These findings suggest that the cell-cycle specific cyclin degradation occurs through a ubiquitin-dependent proteolytic system.

The 26S proteasome is an essential member of the ubiquitin-dependent proteolytic system in eukaryotic cells [15]. Physiological functions of the 26S proteasome include the regulation of the meiotic cell cycle [16]. Previously, we reported that purified 26S proteasome digested cyclin B and this digestion was an initial step of cyclin B degradation in fish [4]. The mechanism of cyclin B degradation after initial cleavage by the 26S proteasome remains to be determined.

To learn more about the molecular mechanism of cyclin B degradation, a molecular study of the ubiquitinating system in goldfish has been undertaken. In this report, we describe the cDNA cloning of the goldfish E2-C and the tissue specific expression pattern of the cloned gene.

2. Materials and methods

2.1. cDNA cloning of goldfish E2-C

A PCR fragment was amplified from goldfish ovary cDNA with primers E2-C #1 (GAYAAYYTBTTYAARTGGGT) and E2-C #4 (CCAYTTYTCYTTVARRATRTC)(Y = C or T; B = G,T or C; R = A or G; V = A,C or G). The amplified PCR fragment was subcloned into pBluescript II SK(−) vector and was sequenced. The PCR fragment was amplified using digoxigenin (DIG) DNA labeling mixture (Boehringer) to use as a probe. A λ ZAPII goldfish ovarian cDNA library was screened using the DIG-labeled probe. A total of 3×10^5 plaques were blotted onto the Hybond N^+ nylon membranes (Amersham) and hybridized at 60°C in hybridization solution (5×SSC, 0.5% blocking reagent (Boehringer), 0.1% *N*-lauroyl sarcosine, 0.02% SDS) with the DIG-labeled probe. Through two rounds of hybridization the positive plaques were isolated. Plasmid DNA was prepared by the in vivo excision protocol using the ExAssist/SOLR system (Stratagene). DNA sequencing was performed using a 377A DNA sequencer (Perkin Elmer ABI) with the Dye Terminator Cycle sequencing kit (Perkin Elmer ABI).

2.2. Expression of goldfish E2-C in bacteria

The full-length ORF of goldfish E2-C was amplified by PCR with primers designed to produce a *Nde*I and *Xho*I site at the 5′ and 3′ end, respectively. PCR fragments were inserted into the pET21b expression vector (Stratagene) between the *Nde*I and *Xho*I site. The recombinant proteins were produced in *Escherichia coli* BL21(LysE) using methods described previously [17] and purified by affinity chromatography on a Ni-NTA Agarose (Qiagen) column according to the manufacturer's instructions.

2.3. Expression of cyclin B

A PCR fragment of N-terminal deletion mutant cyclin B (cyclin B Δ57), which lacks the N-terminal 57 amino acids as is cut by 26S proteasome, was amplified by PCR with primers designed to produce a *Nde*I and *Xho*I site at the 5′ and 3′ end, respectively. PCR fragments were inserted into the pET21b expression vector (Stratagene) between the *Nde*I and *Xho*I site. ^{35}S-labeled cyclin B Δ57 was produced in rabbit reticulocyte lysates and purified using methods described previously [4].

*Corresponding author. Fax: (81) (54) 238-4778.
E-mail: sbttoku@ipc.shizuoka.ac.jp

0014-5793/99/$20.00
PII: S0014-5793(99)01189-8

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ACAGCCGATCTTACGCCATGGCCTCTCAGAATATGGACCCCGCAGCAGCATCGTCCACAG  60
                              M  D  P  A  A  A  S  S  T  A
CAGCACTGAAGGGCAGCGAAAACAGCGTCAGCGTGTCGAAAGGATCCGTCACTAAAAGGC 120
  A  L  K  G  S  E  N  S  V  S  V  S  K  G  S  V  T  K  R  L
TTCAGCAAGAGCTGATGACGCTAATGATGTCAGGAGACAAGGGCATTTCAGCATTTCCAG 180
  Q  Q  E  L  M  T  L  M  M  S  G  D  K  G  I  S  A  F  P  E
AGTCTGACAATCTCTTCAAATGGATTGGCACAATAGATGGAGCTCAAGGAACGGTTTATG 240
  S  D  N  L  F  K  W  I  G  T  I  D  G  A  Q  G  T  V  Y  E
AAGGTTTGAGGTACAAACTTTCCCTGGAGTTCCTAGCGGTTACCCCTACAATGCCCCTC 300
  G  L  R  Y  K  L  S  L  E  F  P  S  G  Y  P  Y  N  A  P  R
GGGTGAAATTCGTCACTACTTGTTTCCACCCAAATGTTGATGAGAATGGCTTCATCTGCC 360
  V  K  F  V  T  T  C  F  H  P  N  V  D  E  N  G  F  I  C  L
TAGACATTTTAAAGGACAAGTGGTCTGCACTATATGATGTCCGCTCCATTCTATTGTCCA 420
  D  I  L  K  D  K  W  S  A  L  Y  D  V  R  S  I  L  L  S  I
TTCAGAGTTTATTAGGAGAACCCAACAATGATAGTCCAATGAACTCTGCAGCTGCTGAAC 480
  Q  S  L  L  G  E  P  N  N  D  S  P  M  N  S  A  A  A  E  L
TATGGGATGACCAGGAAGCATTCAAAGCCCATTTGCACGCAACCTACAAGAACTGAGCAT 540
  W  D  D  Q  E  A  F  K  A  H  L  H  A  T  Y  K  N  *
GAACATTAACATCTGCCTCTTGTTTTATCTGTGTCTGTTTTCTTGTTTGTAATGTAAATT 600
TTCTGTATTGGGTTTCTGTGTATATTTTTCAATGTTGTCCTAAATAAAATGCTGATTGAA 660
TTGAAAAAAAAAAAAAA
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Fig. 1. Nucleotide and amino acid sequences of goldfish E2-C. The upper letters correspond to the nucleotide sequence. The lower letters represent the amino acid sequence. The numbers refer to the nucleotide positions at the end of each line. The GenBank accession number for this sequence is AB022024.

2.4. Electrophoresis and immunoblotting

Electrophoresis was carried out according to the method of Laemmli [18]. Electroblotting and immunodetection were conducted as described previously [4].

2.5. Preparation of ubiquitinating enzymes from cytosol of goldfish ovulated oocytes

Ovulated oocytes were homogenized with four volumes of 20 mM HEPES-NaOH buffer, pH 7.5, containing 100 mM β-glycerophosphate, 15 mM MgCl2, 5 mM EGTA and 1 mM DTT and the homogenate was centrifuged at $100\,000\times g$ for 1 h. The supernatant was applied to a DEAE-cellulose column (4.5×6.3 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM DTT (TD buffer). The column was washed with 10 volumes of the same buffer and bound materials were eluted with TD buffer containing 0.5 M KCl and 20 ml fractions were collected. The flow-through fraction was added to saturated ammonium sulfate to 45% saturation. Precipitates were collected by centrifugation at $10\,000\times g$ for 10 min, then dialyzed against TD buffer. This fraction possessed ubiquitin ligating activity against cyclin B as described in clam [7] and was used as the APC/cyclosome fraction. Fractions eluted from the DEAE-cellulose column were pooled, diluted 10-fold with TD buffer and applied to a Q-Sepharose column (1.6×10.0 cm) equilibrated with TD buffer. Absorbed materials were eluted with a linear gradient of KCl from 0 to 1 M and fractions of 10 ml were collected. Ubiquitin-activating enzyme (E1) was assessed by the activity of ubiquitin thiolester complex formation with recombinant E2-C. Active fractions were pooled and used as a partially purified E1 fraction from goldfish oocytes.

2.6. Isolation of total RNA and Northern blot analysis

Total RNA from various kinds of goldfish tissues was isolated using ISOGEN (WAKO) according to the manufacturer's instructions. Northern blot analysis of mRNA for E2-C and cyclin B was carried out as described previously [19].

3. Results and discussion

3.1. Isolation and characterization of goldfish E2-C cDNA clones

Three positive clones were isolated from 3×10^5 plaques of the goldfish ovarian cDNA library by screening with PCR-probe. The cDNA clones all contained an insert of 0.7 kb long. Further analysis revealed that these three clones were identical. The cDNA was sequenced in its entirety (Fig. 1). The clone encodes a protein of 172 amino acid residues with a predicted molecular mass of 18 877 Da. Comparison of the amino acid sequence revealed that this molecule is highly homologous to the E2-C of clam (60%), frog (80%) and human (76%) [12–14] (Fig. 2). The sequence of goldfish E2-C contains a cysteine residue which may be used for thiolester formation with ubiquitin. The homology of this clone to the other ubiquitin carrier proteins is under 30%, which further suggests this goldfish cDNA encodes a E2-C. Recombinant protein from the cDNA formed a thiolester complex with ubiquitin in an E1-dependent manner (Fig. 3A). Furthermore, the recombinant E2-C can ubiquitinate cyclin B in a APC/cyclosome-dependent manner (Fig. 3B). Thus, we concluded that the cDNA isolated in this study encodes the goldfish E2-C.

3.2. Gene expression of goldfish E2-C in various tissues

Using the goldfish E2-C cRNA probe, Northern blot analysis of various tissues revealed a major band of ∼0.7 kb (Fig. 4A). The intensities of the signals differed significantly depending on the organs. Ovary showed relatively strong signal. Testis showed moderate signal. In other organs, signals were quite low under the assay conditions. We also analyzed mRNA levels of cyclin B (Fig. 4B). The expression patterns showed a clear relationship between E2-C and cyclin B. The similarity of the expression pattern of mRNAs supports the notion that E2-C mediates a cyclin B-specific ubiquitination.

Previously, we hypothesized that restricted digestion of cyclin B by 26S proteasome is an initial step in cyclin B degradation in a study using purified 26S proteasome and cyclin B [4]. In the present study, we identified a cyclin-selective ubiq-

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Goldfish    MASQNMDPAAASSTAALKGSENSVSVSKGSVTKRLQQELMTLMMSGDKGISAFPESDNLF  60
Xenopus     MASQNVDPAAASSVASRKGQESGTSAARGSVGKRLQQELMTLMMSGDKGISAFPESDNLF  60
Human       MASQNRDPAATSVAAARKGAEPSGGAARGPVGKRLQQELMTLMMSGDKGISAFPESDNLF  60
Clam        MSGQNIDPAANQVRQKERPRDMTTSKERHSVSKRLQQELRTLLMSGDPGITAFPDGDNLF  60

                                                    *
Goldfish    KWIGTIDGAQGTVYEGLRYKLSLEFPSGYPYNAPRVKFVTTCFHPNVDENGFICLDILKD 120
Xenopus     KWIGTIDGAVGTVYEDLRYKLSLEFPSGYPYNAPTVKFVTPCFHPNVDSHGNICLDILKD 120
Human       KWVGTIHGAAGTVYEDLRYKLSLEFPSGYPYNAPTVKFLTPCYHPNVDTQGNICLDILKE 120
Clam        KWVATLDGPKDTVYESLKYKLTLEFPSDYPYKPPVVKFTTPCWHPNVDQSGNICLDILKE 120

Goldfish    KWSALYDVRSILLSIQSLLGEPNNDSPMNSAAAELWDDQEAFKAHLHATY-KN------  172
Xenopus     KWSALYDVRTILLSLQSLLGEPNNESPLNPYAAELWQNQTAYKKHLHEQYQKQVREKEI  179
Human       KWSALYDVRTILLSIQSLLGEPNIDSPLNTHAAELWKNPTAFKKYLQETYSKQVTSQEP  179
Clam        NWTASYDVRTILLSLQSLLGEPNNASPLNAQAADMWSNQTEYKKVLHEKY-KTAQSDK-  177
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Fig. 2. Comparison of the amino acid sequences for the goldfish, clam, frog and human E2-C. Conserved residues are boxed. The cysteine residue responsible for ubiquitin thiolester complex formation is indicated with an asterisk. The numbers refer to the amino acid position at the end of each line.

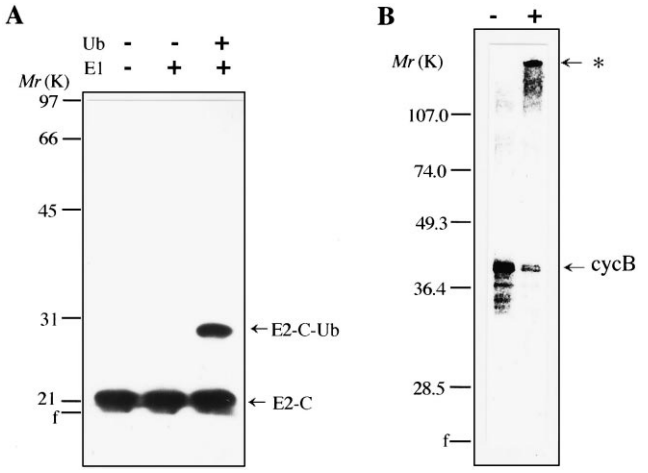

Fig. 3. Activities of *E. coli*-produced goldfish E2-C. A: Thiolester complex formation of recombinant goldfish E2-C with ubiquitin. Purified recombinant goldfish E2-C (0.1 mg/ml) was incubated with ubiquitin-activating enzyme (E1) fraction (10 μg/ml) and/or ubiquitin (1 mg/ml) as indicated. Samples were exposed to Laemmli's SDS sample buffer after incubation for 60 min at 30°C and analyzed by Western blotting. Blots were immunostained by an anti-Penta·Histidine monoclonal antibody (QIAGEN). The position to which the complex of ubiquitin and goldfish E2-C migrated is indicated as E2-C-Ub. B: Ubiquitination of cyclin B. ^{35}S-labeled goldfish cyclin B Δ57 was produced in reticulocyte lysates and purified as described [4]. Purified recombinant cyclin B Δ57 was incubated in a mixture of E1 and APC/cyclosome fractions in the presence (+) or absence (−) of recombinant E2-C. Samples were exposed to Laemmli's SDS sample buffer after incubation for 90 min at 30°C. Ubiquitinated cyclin B was detected by autoradiography on Imaging plates (Fuji Film). The position to which the major part of ubiquitinated cyclin B migrated is indicated with an asterisk. Molecular masses of standard proteins are indicated at the left.

uitin carrier protein and demonstrated in vitro the presence of a ubiquitinating system in goldfish oocytes. The reconstruction system for these ubiquitinating enzymes, therefore, makes it feasible to elucidate the molecular mechanism of cyclin B degradation.

Acknowledgements: This work was supported by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan (09740625 and 10160210 to T.T.). This study was performed as part of the National Institute for Basic Biology Cooperative Research Program (99-121 to T.T). M.T. is a Research Fellow of the Japan Society for Promotion of Science.

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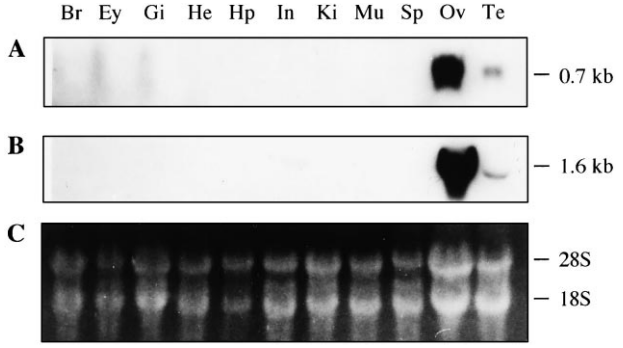

Fig. 4. Tissue distribution of mRNA for goldfish E2-C. Northern blot analysis of E2-C (A) and cyclin B (B). Twenty microgram of total RNAs from various organs (Br, brain; Ey, eye; Gi, gill; He, heart; Hp, hepatopancreous; In, intestine; Ki, kidney; Mu, muscle; Sp, spleen; Ov, ovary; Te, testis) were used. Ribosomal RNAs were detected by staining with ethidium bromide (C).

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