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Molecular Cloning of cDNA Encoding a 20S Proteasome α2 Subunit from Goldfish (*Carassius auratus*) and Its Expression Analysis

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ABSTRACT—Proteasomes are large, multisubunit particles that act as the proteolytic machinery for most regulated intracellular protein breakdown in eukaryotic cells. The core proteinase of this complex, the 20S proteasome, is comprised of four stacked rings with seven subunits each. The outer two rings are made up of seven, distinct α-type subunits, while the two inner rings are composed of seven, different β-type subunits. Here we present the cloning, sequencing and expression analysis of *Carassius auratus*, *α2_ca*, which encodes one of the proteasome α subunits from goldfish ovary. The cloned cDNA is 838 bp long and encodes 234 amino acids. The deduced amino acid sequence is highly homologous to α2 subunits from other vertebrates. The expression of mRNA for *α2_ca* occurs at very high levels in ovary and muscle and moderately high levels in testis, brain and gill. It was also shown that protein content was different from mRNA expression levels.

INTRODUCTION

The proteasome is an essential component of the ATP-dependent proteolytic pathway in eukaryotic cells. As such, it is responsible for the degradation of most cellular proteins (for review see Orlowsky 1990; Coux *et al*., 1996). Proteasomes are divided into two types, 20S and 26S. The 26S proteasome is involved in the ubiquitin-dependent protein degradation pathway. 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 (Glotzer *et al*., 1991; Nishizawa *et al*., 1993; Pagano *et al*., 1995; Tokumoto *et al*., 1997). Thus, the 26S proteasome is thought to be involved in the regulation of cell cycle events. The core particle of the eukaryotic 26S proteasome, the 20S proteasome, is highly conserved in its structure. Eukaryotic 20S proteasomes are cylindrical structures comprised of two pairs of stacked rings. Two inner rings are identical and comprised of seven different β-type subunits. In contrast, the two identical outer rings each contain seven distinct α-type subunits (Groll *et al*., 1997). Some of the β-type subunits which are situated within the central chamber contain the catalytically active sites for proteolysis. The α-type subunits play a role in the assembly of the 26S proteasome via interactions with components that cap the 20S proteasome at each end to yield the 26S complex.

Previously, we isolated the 20S proteasome from the cytosol of goldfish oocytes and made some monoclonal antibodies against it (Tokumoto *et al*., 1995a). We studied the changes in the activity and protein levels of subunits of the 20S proteasomes during oocyte maturation (Tokumoto *et al*., 1993). The 25 kDa subunit detected by the GC4/5 antibody was ubiquitous during oocyte maturation, suggesting that this subunit can be used to trace the 20S proteasome in further investigations.

To learn more about the biological functions of proteasomes in the regulation of meiotic maturation, a molecular study of proteasomes in goldfish has been undertaken. In this report, we describe the cDNA cloning for the 25 kDa subunit named *α2_ca*. We also report the expression pattern of the cloned gene at both the mRNA and protein levels.

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MATERIALS AND METHODS

Purification of 26S proteasome

The 26S proteasome was purified from immature ovaries of goldfish as previously described (Tokumoto *et al.*, 1995b).

Electrophoresis and immunoblotting

Electrophoresis was carried out according to the method of Laemmli (Laemmli, 1970). Electroblotting and immuno-detection were conducted as described previously (Tokumoto *et al.*, 1995a).

cDNA cloning by immuno-screening and sequencing

A cDNA library from goldfish ovary was constructed in λZAPII vector (STRATAGENE). Using a previously described anti-goldfish 20S proteasome monoclonal antibody (GC4/5; Tokumoto *et al.*, 1995a), immuno-screening was carried out on about 2×10^5 independent clones. Clones were plated with XLI-Blue MRF' *E. coli*, and incubated at 42°C for 3 to 3.5 hr. Hybond-C nitrocellulose membranes, immersed in 1 mM IPTG, were left on the plates and incubated at 37°C for 3.5 hr. After the incubation, membranes were washed in TBS (Tris Buffered Saline: 20 mM Tris-Cl/ 150 mM NaCl, pH 7.5) containing 0.1% Tween 20 (TTBS), blocked for 1 hr using Blocking Buffer (5% skim milk/ TTBS), incubated for 1 hr with GC4/5, washed in TTBS, incubated with alkaline-phosphatase conjugated goat anti-mouse IgG (ZYMED) for 1 hr and washed in TTBS. The membranes were then rinsed in coloring buffer (0.1 M Tris-Cl/ 1 mM EDTA/ 5 mM $MgCl_2$, pH 9.5) and coloring solution (4 μl of 75 mg/ml nitroblue tetrazolium chloride in 70% dimethylformamide/ 3 μl of 50 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt in dimethylformamide/ 1 ml coloring buffer) was added. After positive signals were detected, the membranes were washed with water. 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).

Isolation of total RNA and proteins from goldfish tissues

Total RNA and proteins from various kinds of goldfish tissues were isolated using ISOGEN (WAKO) according to the manufacturer's instructions.

Northern blot analysis

Twenty μg of denatured total RNA from each tissue was electrophoresed using the formaldehyde-denatured gel method, then blotted onto Hybond N^+ nylon membrane (Amersham). A digoxigenin-labelled cRNA probe was synthesized using RNA Labeling Kit according to the manufacturer's instructions (Boehringer Mannheim Biochemica). The membrane was immersed in hybridization solution (50% formamide/ 10 mM Tris-Cl, pH 7.6/ 1 × Denhardt's/ 0.6 M NaCl/ 0.25% SDS/ 1 mM EDTA/ 100 μg/ml yeast tRNA) and pre-hybridized at 65°C for 3 hr. The cRNA probe was added and allowed to hybridize for 16 hr at 65°C. The membrane was then washed with SSC (20 × SSC: 3 M NaCl/ 0.3 M sodium citrate, pH 7.0). A final wash was performed with 0.2 × SSC/ 0.1% SDS at 65°C. Chemiluminescence detection was carried out using CDP-star substrate according to the manufacturer's methods (Boehringer Mannheim Biochemica). After detection of RNA, the membrane was soaked in 5% acetic acid and stained with 0.04% methylene blue/ 0.5 M sodium acetate (pH 5.2) to detect the total RNA blotted on the membrane.

Expression of *α2_ca* in bacteria

The full-length ORF of *α2_ca* was amplified by PCR with primers designed to produce *Nde*I and *Xho*I sites at the 5' and 3' ends, respectively. PCR fragments were inserted into the pET21b expression vector (STRATAGENE) between *Nde*I and *Xho*I site. The recombinant proteins were produced in BL21(LysE) *E. coli* using methods described previously (Hirai *et al.*, 1992).

RESULTS AND DISCUSSION

Isolation and characterization of *α2_ca* cDNA clones

A monoclonal antibody (GC4/5) against goldfish 20S proteasome specifically cross-reacted with a 25 kDa subunit of 26S proteasome (Fig. 1), which is also present in goldfish ovarian crude extracts (Tokumoto *et al.*, 1995a). Three positive clones were isolated from a goldfish ovarian cDNA library by immuno-screening with this antibody. The cDNA clones all contained an insert of 0.9 kb. Further analysis revealed that these three clones were identical. The cDNA was sequenced in its entirety (Fig. 2). Recombinant protein from the cDNA clearly cross-reacted with anti-GC4/5 (Fig. 3). Thus, we concluded that the cDNA isolated in this study encodes the 25 kDa subunit of goldfish 20S proteasome. The clone encodes a protein subunit of 234 amino acid residues with a predicted molecular mass of 25,877 dalton. Comparison of the amino acid sequence revealed that this molecule is highly homologous to the C3 subunits of rat (92%, Tanaka *et al.*, 1990), human (93%, Tamura *et al.*, 1991), *Xenopus* (95%, Fujii *et al.*, 1991) and mouse (92%, Seeling *et al.*, 1993) (Fig. 4). The sequence of *α2_ca* contains a conserved region which may be a nuclear location signal. Homology of this clone to the other six α-type subunits of human is under 34%. Similarly, homology to the seven β-type subunits is under 20%. These results further suggest that this goldfish cDNA encodes a proteasome subunit corresponding to the C3 subunit.

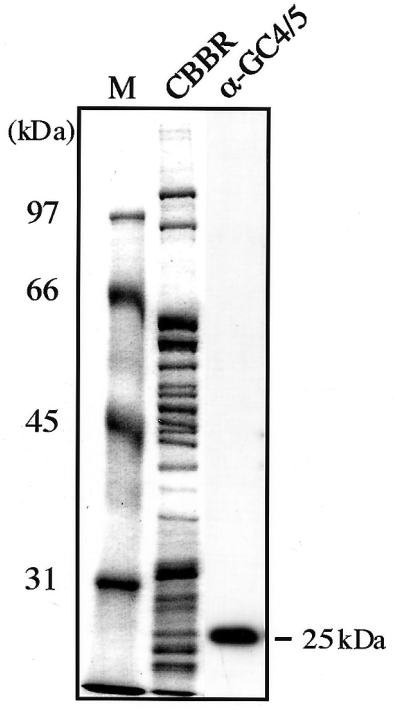

Fig. 1. Immunoblot of the purified 26S proteasome. 26S proteasome was electrophoresed under denaturing conditions (12.0% gel) and stained with Coomassie Brilliant Blue R-250 (CBBR), or immunostained with a monoclonal antibody (α-GC4/5) after electroblotting. Molecular masses of standard proteins are indicated at the left (M).

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GCAAAAGACAAAAATGGCAGACCGAGGATACAGTTTTTCTCTCACAACATTTAGTCCCTC   60
              M  A  D  R  G  Y  S  F  S  L  T  T  F  S  P  S
GGGCAAACTGGTGCAGATTGAATATGCTCTGGCAGCTGTAGCAGCCGGTGCTCCATCGGT  120
  G  K  L  V  Q  I  E  Y  A  L  A  A  V  A  A  G  A  P  S  V
AGGAATTAAAGCATCAAATGGAGTTGTGCTGGCAACTGAGAAGAAACAGAAGTCCATACT  180
  G  I  K  A  S  N  G  V  V  L  A  T  E  K  K  Q  K  S  I  L
GTATGATGAACAGAGCGTGCACAAAATCGAGCCCATAACAAAACACATTGGCATGGTGTA  240
  Y  D  E  Q  S  V  H  K  I  E  P  I  T  K  H  I  G  M  V  Y
CAGTGGAATGGGTCCGGACTACAGGGTTCTGGTCAGAAGAGCAAGAAAGCTGGCGCAGCA  300
  S  G  M  G  P  D  Y  R  V  L  V  R  R  A  R  K  L  A  Q  Q
GTATTTCCTGGTATACCAAGAGCCAATCCCCACAGGCCAGCTGGTACAGAGAGTGGCTTC  360
  Y  F  L  V  Y  Q  E  P  I  P  T  G  Q  L  V  Q  R  V  A  S
TGTTATGCAAGAATACACACAGTCAGGAGGTGTGCGGCCATTTGGGGTCTCTCTTCTCAT  420
  V  M  Q  E  Y  T  Q  S  G  G  V  R  P  F  G  V  S  L  L  I
TGCCGGTTGGGATGAAGACAGACCATACTTATTTCAGTCAGACCCATCGGGAGCATACTT  480
  A  G  W  D  E  D  R  P  Y  L  F  Q  S  D  P  S  G  A  Y  F
TGCCTGGAAAGCCACTGCAATGGGAAAGAGCTATGTCAATGGAAAAACATTTCTTGAAAA  540
  A  W  K  A  T  A  M  G  K  S  Y  V  N  G  K  T  F  L  E  K
AAGATATAATGAAGATCTGGAACTTGAAGATGCTATACACACTGCCATCTTAACTTTGAA  600
  R  Y  N  E  D  L  E  L  E  D  A  I  H  T  A  I  L  T  L  K
GGAAAGTTTTGAAGGTCAGATGACAGAGGAAAACATTGAGGTGGGCATCTGTAATGAAGC  660
  E  S  F  E  G  Q  M  T  E  E  N  I  E  V  G  I  C  N  E  A
AGGATTTCGCAGACTGTCACCTGCCGAGGTCAAGGATTACTTGGCAGCAATTGCGTAAAC  720
  G  F  R  R  L  S  P  A  E  V  K  D  Y  L  A  A  I  A  *
CACAGCACTGCAACTGCTTACACTGTTTCCTGTTTTGGTTTGGTCATTCATTTGAATGTT  780
GCAGTTTTATTACGTGTACATCTGTGCAACTGATTTCTTTATAAAAGGATACATTATT    838
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Fig. 2. Nucleotide and amino acid sequences of *α2_ca*. The upper letters correspond to the nucleotide sequence. The lower letters represent amino acid sequence. The numbers refer to the nucleotide positions at the end of each line. The possible nuclear localization signal is underlined. The GenBank accession number for this sequence is AB013342.

Recently, a new systematic name has been suggested to clarify the relationship among the 20S proteasome subunits from different organisms (Groll *et al.*, 1997). Under this system, which is based on the solved crystal structure of the yeast proteasome, the different α-type and β-type subunits are numbered sequentially according to the direction of rotation within each ring. Adjacent α and β subunits have the same numbers. To prevent confusion due to difference in naming strategy, we named the clone *α2_ca* (α2 subunit of *Carassius auratus*) according to a systematic nomenclature.

Gene expression of *α2_ca* in various tissues

Using the *α2_ca* cRNA probe, Northern blot analysis of various tissues revealed a major band of ~1.0 kb (Fig. 5A). The intensities of the signals were significantly different depending on the organs. Ovary, muscles and blood showed relatively strong signals. Testis, gill and brain, however, showed moderate signals. We also analyzed protein levels of α2 subunit by Western blot (Fig. 5B). Surprisingly, the expression patterns of mRNAs and proteins show no clear relationship. In contrast to mRNA expression levels, immunoblots of testis, kidney, hepatopancreas and gill showed strong signals. Only faint signals were detected on the immunoblots of blood, muscle and ovary; however, Nothern blots of these tissues showed strong signals. It has been reported that mRNAs of proteasome subunits are up-regulated in the proliferating period in cell cultures, while total amount of the proteasome protein showed only a small increase (Shimbara *et al.*, 1992). Similar observations were reported in *Xenopus laevis* α2 subunit in the liver (Fujii *et al.*, 1993). Our results also indicate that expression of mRNA for proteasome subunits does not directly correspond to the amount of protein.

The strategy used to isolate the *α2_ca* gene should be useful for the isolation of other proteasome subunit genes for which antibodies are already available. Recently, we found that at least two subunits in the 26S proteasome change during oocyte maturation. This finding suggests that some mechanism regulates the 26S proteasome. The molecular charac-

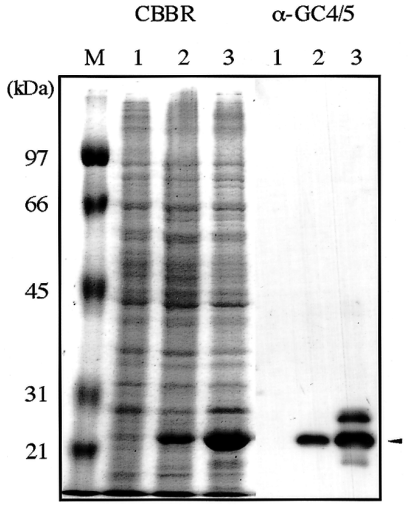

Fig. 3. Expression of *α2_ca* in bacteria. *E. coli* cell lysates were subjected to SDS-PAGE (12.0% gel) and stained with CBBR-250 or immunoblotted with α-GC4/5. Each lane corresponds to lysates from *E. coli* transformed with vector (pET21b) alone (lane1) or *α2_ca* construct before (lane2) and after (lane3) IPTG induction. Arrowhead indicates a recombinant *α2_ca* protein. Relatively high amounts of *α2_ca* protein were expressed without IPTG induction. Molecular masses of standard proteins are indicated at the left.

terization of these genes will, therefore, make it feasible to analyze proteasome function *in vivo*, thus allowing the importance of proteasome-mediated protein degradation in meiotic maturation to be addressed.

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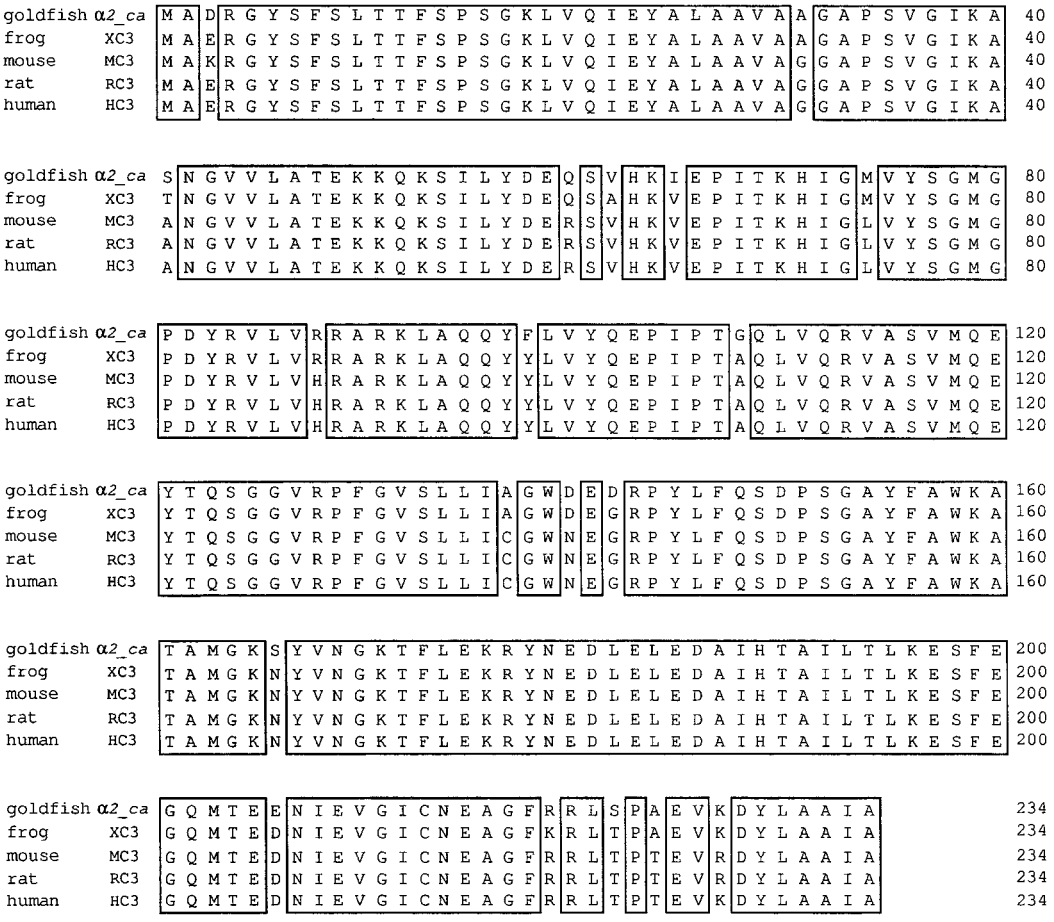

Fig. 4. Comparison of the amino acid sequence for the α2 subunits from goldfish, frog, mouse, rat and human. Conserved residues are boxed. The numbers refer to the amino acid position at the end of each line.

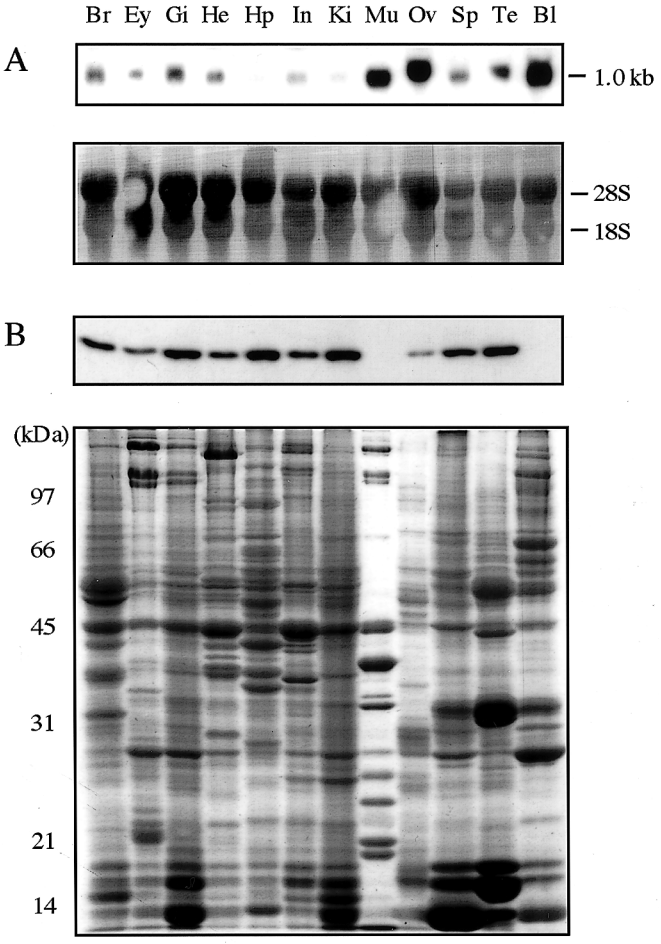

Fig. 5. Tissue distribution of mRNA and protein for goldfish proteasome α2 subunit. (**A**) Northern blot analysis (upper panel). Twenty μg of total RNAs from various organs were used. Ribosomal RNAs were detected by staining with methylene blue (lower panel). (**B**) Western blot analysis (upper panel). Proteins from 0.26 mg of each tissue were subjected to SDS-PAGE. Br, brain; Ey, eye; Gi, gill; He, heart; Hp, hepatopancreas; In, intestine; Ki, kidney; Mu, muscle; Ov, ovary; Sp, spleen; Te, testis; Bl, blood. After electroblotting, the membrane was immunostained with α-GC4/5. Total proteins were detected by CBBR-250 staining (lower panel).

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