Characterization of goldfish heat shock protein–30 induced upon severe heat shock in cultured cells

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Abstract Temperature-dependent changes of growth rate and protein components were investigated for primary cultured cells derived from goldfish caudal fin. When the culture temperature was shifted from 20°C to 35°C and 40°C, the growth rate was increased at 35°C as compared with that at 20°C, but no cell growth was observed at 40°C. The differential scanning calorimetry demonstrated the onset of the endothermic reaction for goldfish cellular components at 40°C. Therefore, the temperature shift to 40°C was found to be of severe heat shock for goldfish cultured cells. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis revealed that, although expression of 70-kDa components was slightly induced at 35°C, the temperature shift to 40°C markedly induced the expression of the 30-kDa component in addition to that of 70-kDa component. The N-terminal amino acid sequencing identified the 30- and 70-kDa components to be heat shock protein (Hsp)–30 and Hsp70, respectively. Northern blot analysis revealed that the enhanced Hsp30 messenger ribonucleic acid (mRNA) levels were only observed at 40°C, whereas Hsp70 mRNA was slightly accumulated at 35°C. These results indicated that Hsp30 might have important functions under severe heat stress condition.

INTRODUCTION

Cultured cells respond to thermal stress by changing their physiological states. It is known that a moderate heat stress terminates or delays mammalian cell growth, whereas a severe heat stress is lethal (Kühl and Rensing 2000). In a similar manner, cells from poikilothermic teleosts are not able to grow at their sublethal temperatures (Hightower and Renfro 1988; Bols et al 1992). For example, the rainbow trout, *Oncorhynchus mykiss*, cell line RTG-2 cannot grow at 26–28°C and cannot survive at 30°C (Mosser et al 1986). It has been also reported that goldfish, *Carassius auratus*, cells easily change their growth rate in association with temperature shifts (Sato et al 1990): they maintain their proliferation even at 37°C (Shima et al 1980), terminate their growth at 40°C, and hardly survive at 42°C (Mitani et al 1989). Our previous study indicated that the population doubling times of goldfish cells at 30°C and 35°C were apparently shorter than those at 20°C and 25°C (Kondo and Watabe 2004).

The synthesis of heat shock proteins (Hsps) upon exposure to an acute temperature rise is one of the major biochemical responses of cells, regardless of poikilotherms and homeotherms (Sonnen et al 2002). The induction of major Hsps, whose molecular masses were 30, 42, 70, and 90 kDa, was evidenced by autoradiography when goldfish cells cultured at 27°C were exposed to 37°C (Sato et al 1990). Furthermore, goldfish cells exposed to 40°C significantly expressed 2 proteins of 30 and 70 kDa (Sato et al 1990).

The transcription of Hsps is induced by binding of heat shock transcription factors (Hsfs) to promoter regions of the Hsp genes (Morimoto 1998). Furthermore, Hsfs from certain fish species have been identified and characterized (Rabergh et al 2000; Buckley and Hofmann 2002; Ojima and Yamashita 2004). In response to severe stress conditions, Hsfs form a homotrimeric structure with deoxyribonucleic acid (DNA)–binding activity and enter into the nucleus (Morimoto 1993), thus enhancing the transcription of Hsps (Morimoto 1993). Hsp70 and Hsp90 bind to Hsfs under normal conditions, both negatively regulating the transcriptional activity of Hsfs (Mosser et

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al 1993; Ali et al 1998). It has been claimed that Hsps are dissociated from Hsf, which subsequently forms trimer to activate Hsp gene expression when Hsps alternatively bind to unfolded proteins as molecular chaperones under stress conditions (Morimoto 1998). In this regard, Lepock et al (1988) demonstrated using differential scanning calorimetry (DSC) that the denaturation of cellular proteins was related to Hsp induction and cell death during heat shock.

Within minutes after heat shock, the translation of most preexisting messenger ribonucleic acids (mRNAs) excepting for those of Hsps is shut off, whereas Hsp mRNAs expressed newly are translated very efficiently (Panniers 1994; Sierra and Zapata 1994). Such responses are modulated by 2 initiation factors, eIF-2 and eIF-4 (Scorsone et al 1987; Thach 1992), the activities of which are inhibited during heat shock, terminating the translation of most preexisting mRNAs except for Hsps (Panniers 1994). It has been demonstrated that the long 5' untranslated sequences existing in Hsp mRNAs enable Hsps to be translated during heat shock, whereas eIF-2 and eIF-4 activities are inhibited (Bienz 1985; Pelham 1985). Thus, various lines of evidence have been accumulated about the heat-shock response. However, very little corresponding information is available on fish despite the fact that they are typical poikilotherms, and cellular organization is affected markedly by environmental temperatures (Somero 2003).

In this report, the effect of high temperatures on goldfish cells was investigated. Subsequently, DSC was used to investigate the endothermic reactions of goldfish cells. The 30- and 70-kDa components induced upon heat shock at 40°C were identified as Hsp30 and Hsp70, respectively, and their expression profiles were investigated.

MATERIALS AND METHODS

Cell culture

L-15 medium was purchased from Invitrogen Corporation (Carlsbad, CA, USA). Fetal bovine serum (FBS; Lot 20K2397) was obtained from Sigma (St Louis, MO, USA). Carp, *Cyprinus carpio*, blood was collected from the caudal vasculature of fish (25–40 cm in total length) reared at 25°C. Blood clots were formed by incubation at 25°C for 1 hour, and serum was separated by centrifugation. Carp serum (CS) was sterilized by filtration through a 0.22- μ m membrane and stored at –20°C until use. Primary culture cells were prepared from goldfish fin according to the method reported by Kondo and Watabe (2004) and had been cultured for more than 1 month at 35°C before heat treatment. The culture temperature of 35°C was selected because this condition facilitates much faster growing of

goldfish culture cells than 20°C (Kondo and Watabe 2004), thus shortening the period required to obtain enough quantities of cells for subsequent experiments. Then, cells grown at 35°C were placed into 24-well plates at 5.0×10^3 in the abovementioned L-15 medium containing 5% FBS and 5% CS and subjected to 1-day culture at 35°C to obtain appropriate cell concentrations. To attain the following heat shock responses, the culture temperature was decreased to 20°C and cultured at this temperature for another day. Subsequently, the culture temperature was raised to 35°C and 40°C to compare cellular heat shock responses between these 2 temperatures. During the treatments, cell numbers were counted on days indicated in the Results and subjected to various analytical procedures.

DSC analysis

Cells were suspended in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4) containing 137 mM NaCl and 5.4 mM KCl, and their concentration was adjusted to 1.0×10^7 cells/mL. Cells suspension, thus prepared, was subjected to DSC at a scan rate of 1°C/min in a temperature range of 5°C to 110°C using a differential scanning microcalorimeter VP-DSC (MicroCal Inc., Northampton, MA, USA). DSC data were analyzed using a software package, Origin, developed by MicroCal, and the molar excess heat capacity (ΔC_p) was obtained. To compare the scan for psychrophilic fish, the RTG-2 cell line derived from rainbow trout gonad (American Type Culture Collection, Rockville, MD, USA) was cultured and applied to DSC analysis.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and N-terminal amino acid sequencing

Cells cultured for days indicated after the temperature shift were harvested and washed with ice-cold phosphate-buffered saline. After centrifugation at $1000 \times g$ for 5 minutes, the pellet was dissolved in 50 mM Tris–HCl (pH 8.0) containing 0.15 M NaCl and 0.5% Nonidet-P40 and stored at –20°C until use. Thirty micrograms of proteins were analyzed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970) using 7.5–20% polyacrylamide gels. Standard molecular weight markers (Sigma) in SDS-PAGE were myosin heavy chain from rabbit muscle (205 000), β -galactosidase from *Escherichia coli* (116 000), phosphorylase b from rabbit muscle (97 400), bovine serum albumin (66 000), ovalbumin from chicken egg (45 000), and carbonic anhydrase from bovine erythrocytes (29 000). The gels after SDS-PAGE were stained with a solution containing 0.05% Coomassie Brilliant Blue (CBB) R-250, 25% methanol, and 7% acetic acid and destained

with a solution containing 25% methanol and 7% acetic acid. The band densities were quantified using the NIH Image Program (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

The bands containing the proteins concerned were cut and immersed into 50 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl and 1% SDS overnight. The extracted proteins were precipitated with 5-fold volume of ice-cold acetone. The precipitates were dissolved in 20 μ L of 50 mM phosphate buffer (pH 7.4) containing 0.1% SDS and 0.4 μ g of V8 protease (Sigma). After 2 hours of digestion at 37°C, peptide fragments were applied to SDS-PAGE using 15% polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After the membranes were stained with CBB R-250, the bands appearing were cut, put onto a Blot cartridge block, and analyzed for N-terminal amino acid sequences with an ABI 467A protein sequencer (Applied Biosystems, Foster City, CA, USA).

Polymerase chain reaction amplification

To amplify a 3'-end region of complementary DNA (cDNA) encoding a component of interest, 3' rapid amplification of cDNA ends (RACE) was performed. Cells were lysed in Isogen (Nippon Gene, Toyama, Japan), and total RNA was extracted according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μ g of total RNA. The *NotI* oligodT primer was used at 50 μ g/mL to initiate first-strand cDNA synthesis with 0.5 U SuperScript II reverse transcriptase (Invitrogen) in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 500 mM [deoxy]nucleotide 5'-triphosphate (dNTP), and 10 mM dithiothreitol. Polymerase chain reaction (PCR) was carried out in a 10- μ L reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% Triton X-100, 0.2 mM dNTP, 0.05 unit/ μ L ExTaq DNA polymerase (Takara, Tokyo, Japan), 2 pmol gene-specific primer, 2 pmol *NotI* oligodT primer, and template cDNA. The PCR condition was at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, and this cycle was repeated 30 times. A 5'-end region of cDNA encoding a component of interest was amplified using 5' RACE kit (Invitrogen). The cDNA fragments amplified were subcloned into pGEMT easy (Promega Corporation, Madison, WI, USA), and their sequences determined with an ABI 373A DNA sequencer (Applied Biosystems).

Northern blot analysis

Fifteen micrograms of total RNA were denatured at 70°C for 15 minutes in 50% formamide, subjected to electro-

Fig 1. Growth curve of goldfish culture cells at 20°C (circle), 35°C (square), and 40°C (triangle). Cells cultured at 35°C were placed in 24-well plates at a density of 1×10^4 cells/well. After 1 day culture at 35°C, the culture temperature was shifted from 35°C to 20°C and cells were maintained at 20°C for another 24 hours. Then, the culture temperature was shifted to 35°C and 40°C. Three independent plates were counted for cell numbers at each culture temperature. Data are given as means $\pm$ SE, where the vertical bars of standard error are within the size of marks.

phoresis on a 0.9% agarose gel containing 0.2 M 3-(*N*-morpholino)-propanesulfonic acid (pH 7.0) and 18% formamide, and then transferred to Hybond N⁺ nylon membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were dried and baked at 80°C for 15 minutes. After prehybridization in a solution containing 0.5 M Na₂HPO₄ (pH 7.4), 1 mM ethylenediamine-tetraacetic acid, and 7% SDS at 65°C for 30 minutes, hybridization was performed at 65°C for 18 hours in the same solution containing a randomly labeled [³²P] DNA probe. The membranes were washed sequentially with 2 $\times$ standard saline citrate (SSC) containing 0.1% SDS at room temperature for 5 minutes, 2 $\times$ SSC containing 0.1% SDS at 65°C for 20 minutes, and 1 $\times$ SSC containing 0.1% SDS at 65°C for 20 minutes. The hybridized membranes were scanned with a Fujix Bas 1000 Bioimaging Analyzer (Fuji Film, Tokyo, Japan).

RESULTS

Temperature-dependent changes of cell growth rate

The changes of growth rate after temperature shifts from 20°C to 35°C and 40°C were investigated (Fig 1). The growth of goldfish culture cells was enhanced when the culture temperature was shifted to 35°C. However, the cell growth was terminated when the culture temperature was shifted to 40°C. Cells died within a few hours when the culture temperature was shifted to 45°C (data not shown).

Fig 2. Differential scanning calorimetry (DSC) scan of goldfish culture cells in comparison with that of rainbow trout RTG-2. Cells of 1×10^7 were subjected to DSC analysis. The black line indicates the scan of goldfish culture cells, whereas the gray line indicates that of RTG-2. Arrows represent the temperatures at which goldfish cells were cultured. All scans are arbitrarily offset for clarity.

DSC analysis of goldfish cells

DSC analysis was used to assess the thermal stability of goldfish cellular components in comparison with that of rainbow trout, which inhabit at temperatures different from goldfish. Cells suspended in 20 mM HEPES buffer (pH 7.4) containing 137 mM NaCl were subjected to DSC analysis, and the results in a temperature range of 10°C to 50°C are shown in Figure 2. Goldfish cells started the first endothermic reaction at about 40°C whereas RTG-2 did at about 30°C (Fig 2).

Time-dependent changes of cellular protein composition after temperature shift

SDS-PAGE showed that 30- and 70-kDa components were the major proteins induced at 40°C (Fig 3). Although the band density of the region where the 30-kDa component appeared at 40°C was not changed when cells were shifted to 35°C (Fig 4A), that of the 70-kDa component was slightly increased (Fig 4C). The band density of the 30-kDa component was maximal in cells at 40°C in 8 hours after the temperature shift (Fig 4B). The density at 24

Fig 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) patterns of goldfish cell extracts after culture temperature shifts. The culture temperature was shifted from 35°C to 20°C, and cells were maintained at 20°C for another 24 hours. Subsequently, the culture temperature was shifted to 35°C and 40°C, and then harvested after 1, 2, 4, 8, and 24 hours. Cell extracts were prepared, and 30 µg of extracted proteins was applied to SDS-PAGE using 7.5–20% linear-gradient gels. The open and filled arrowheads indicate 70- and 30-kDa components, respectively. The 44-kDa component supposed to correspond to actin was used as control of the changes for expressed proteins as shown in Figure 4.

Fig 4. Changes of goldfish 30- and 70-kDa components after the rise in culture temperature. Panels A and B represent changes in the relative 30-kDa-component levels upon temperature shift from 20°C to 35°C and 40°C, respectively. Panels C and D represent changes in the relative 70-kDa-component levels upon temperature shift from 20°C to 35°C and 40°C, respectively. The 44-kDa component of Figure 2 was used as the control, and relative levels in the ordinate represent those compared with the values of 30- and 70-kDa components before the temperature shift. Data are given as means $\pm$ SE from 3 culture plates.

hours was about 1.5 times higher than that before the temperature shift. However, the maximum band density of the 70-kDa component at 40°C was observed at 4 hours after the temperature shift, and the density after 24 hours was still about 3 times higher than that before the temperature shift (Fig 4D).

Identification of the 30- and 70-kDa components

The bands of the 30- and 70-kDa components in SDS-PAGE gels were excised, and the proteins were extracted and digested. The digested proteins were again resolved by SDS-PAGE, and the bands appearing were subjected to N-terminal amino acid sequencing. As a result of homology search, the sequence determined for the fragment derived from 30-kDa component fragment was homologous to that of Hsp30s from desert fish, *Poeciliopsis lucida* (Norris et al 1997) (the DNA Data Bank of Japan [DDBJ]/European Molecular Biology Laboratory [EMBL]/GenBank databases with accession number U85502), and chinook salmon, *Oncorhynchus tshawytscha* (U19370) (Fig 5A). The sequences of fragments from the 70-kDa component were identical to those of goldfish Hsp70 (AB092839) (Fig 5B). It was noted that the amino acid sequence of goldfish Hsp70 was distinct from that of goldfish Hsc70 (AB092840), a Hsp70 family protein existing in cells constitutively (Fig 5B). The sequences that might imply the

Fig 5. N-terminal amino acid sequences of proteolytic peptides from goldfish 30- and 70-kDa components. The sequences of the 30- and 70-kDa components are shown in panels A and B, respectively. Residue X indicates the amino acid residue that could not be determined. The sequences cited from the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank databases were heat shock protein (Hsp)-70 (AB092839) and Hsc70 (AB092840) of goldfish (gf) and Hsp30 of chinook salmon (cs) (U19370) and desert fish (df) (U85502).

Table 1 Nucleotide sequences of primers used for PCR amplification of cDNA fragments encoding goldfish Hsp30^a

Primer name	Sequence
Hsp30-f	5'-TCAGC GGGAA GACAG AGAAG-3'
Hsp30-r1	5'-TCCTT GAGGA AGATC AAA-3'
Hsp30-r2	5'-TACGA GCCCT TCCCA TCCT-3'

The sequence of primer Hsp30-f was designed referring to the nucleotide sequences encoding Hsp30s from chinook salmon, *Oncorhynchus tshawytscha* (the DDBJ/EMBL/GenBank databases with accession number U19370), and desert fish, *Poeciliopsis lucida* (U85502). The sequences of primers Hsp30-r1 and Hsp30-r2 were designed from the nucleotide sequence determined by 3'-RACE in the present study using primer Hsp30-f.

^a PCR, polymerase chain reaction; cDNA, complementary deoxyribonucleic acid; Hsp, heat shock protein; DDBJ, DNA Data Bank of Japan; EMBL, European Molecular Biology Laboratory; RACE, rapid amplification of cDNA ends.

presence of other protein components were not detected in the present procedures.

cDNA cloning of goldfish Hsp30

Although the nucleotide sequence of goldfish Hsp70 has been reported, no corresponding information is available on goldfish Hsp30. To clone the cDNA encoding Hsp30, a primer to amplify cDNA fragment encoding a 3'-end region was designed, referring to the cDNA sequences of desert fish and chinook salmon Hsp30 (Table 1). The nucleotide sequence obtained by PCR was also used to design the reverse primers for 5' RACE (Table 1). The full-length cDNA thus obtained was ascertained by a single PCR with primers constructed from the 5'- and 3'-non-coding regions and registered into the DDBJ/EMBL/

Table 2 Amino acid identities between goldfish Hsp30 and other small Hsps in the full length and the region of alpha crystallin domain (%)^a

	Full length	Alpha crystallin domain
Chinook salmon Hsp30	49.7	75.6
Desert fish Hsp30	45.2	68.6
African clawed frog Hsp30C	25.3	43.0
Desert fish Hsp27	14.7	31.4
Human Hsp27	13.1	27.9
Human alphaA-crystallin	17.1	31.4
Human alphaB-crystallin	18.1	37.2

Amino acid sequences were cited from the DDBJ/EMBL/GenBank databases: chinook salmon, *Oncorhynchus tshawytscha*, Hsp30, U19370; desert fish, *Poeciliopsis lucida*, Hsp30, U85502; African clawed frog, *Xenopus laevis*, Hsp30C, X57962; desert fish Hsp27, U85501; human Hsp27, P04792; human alphaA-crystallin, P02489; human alphaB-crystallin, P02511. The sequences were aligned using clustalW program and alpha crystallin domains were determined referring to Kim et al (1998).

^a HSP, heat shock protein; DDBJ, DNA Data Bank of Japan; EMBL, European Molecular Biology Laboratory.

GenBank databases with accession number AB177389. The deduced amino acid sequence of goldfish Hsp30 contained the sequence of a proteolytic fragment determined by N-terminal amino acid sequencing. Goldfish Hsp30 showed higher homology to Hsp30 from other animals than to Hsp27 and alpha-crystallin (Table 2). Small Hsps contain the sequences known as alpha-crystallin domain that is important for their function as a molecular chaperone (Kim et al 1998; MacRae 2000). This region was also conserved in goldfish Hsp30 (Table 2).

Time-dependent changes of Hsp30 and Hsp70 mRNA levels after temperature shift

The time-dependent changes of Hsp30 and Hsp70 mRNA levels were investigated by Northern blot analysis (Fig 6). The goldfish Hsp30 cDNA fragment of 392 to 865 nucleotide (nt) and the goldfish Hsp70 cDNA fragment of 1795 to 1958 nt were used as probes. The latter sequence was cited from the DDBJ/EMBL/GenBank databases with accession number AB092839. Hsp30 transcripts were not observed when the culture temperature was shifted from 20°C to 35°C, whereas they were significantly induced when the temperature was shifted from 20°C to 40°C (Fig 6A). The maximum level of Hsp30 mRNA was observed in cells at 4 hours after the temperature shift to 40°C. Hsp70 mRNA expression was observed when temperature was shifted from 20°C to 35°C (Fig 6A,C). However, the Hsp70 mRNA level at 1 hour after the temperature shift to 40°C was about 8 times higher than that at 1 hour after the temperature shift to 35°C. The level at 2 hour after temperature shift to 40°C was highest.

DISCUSSION

Goldfish is a eurythermal temperate fish and survives at higher temperatures than those at which psychrophilic rainbow trout does. The lethal temperature of cultured cells derived from rainbow trout is about 30°C (Mosser et al 1986), which is lower than that of goldfish cells. Although the mechanisms involved in heat-induced cell death is still unclear, thermal denaturation of cellular components such as those contained in nuclear matrix is thought to be primarily attributable to such cell death (Roti et al 1997; Roti et al 1998). Ritchie et al (1995) also analyzed the endothermic reactions of the liver, muscle, and lens tissues homogenates derived from rat, rabbit, and rainbow trout and claimed that the temperature where the endothermic reaction first appears is correlated with their body temperatures. In this study, we also confirmed that the goldfish cellular components had higher thermostability than those of rainbow trout cultured cells did (Fig 2). Therefore, it is likely that the higher thermal stability of cellular components of goldfish cells determine the upper lethal temperatures of this eurythermal fish.

Although Hsps in RTG-2 were expressed when culture temperature was shifted from 22°C to 26°C–30°C, this cell line terminated the growth above 26°C (Mosser et al 1986). On the other hand, 4 major Hsps, Hsp90, Hsp70, Hsp42, and Hsp30, were induced when goldfish cells cultured at 27°C were exposed to 37°C (Sato et al 1990). The cell growth at 37°C was faster than that at 27°C. In this study, the 70-kDa band slightly induced at 35°C in goldfish culture cells was determined to be Hsp70 by N-terminal amino acid sequencing (see Fig 5). The 30-kDa band observed only when cells were exposed to 40°C was identified as that of Hsp30. The present sequencing analysis implied that the 70- and 30-kDa bands predominantly consisted of Hsp70 and Hsp30, respectively. However, there is a high possibility that the bands observed in SDS-PAGE analysis contained other proteins. Therefore, it is necessary to adopt a more precise method such as Western blot analysis using specific antibodies for quantitation of Hsp70 and Hsp30. Such a situation is also applicable to the present Northern blot analysis to follow changes in the accumulated mRNAs encoding Hsp70 and Hsp30 (see Fig 6) because these proteins formed large multigene families. It is urgent to develop probes specific to Hsp70 and Hsp30 mRNAs for a more precise interpretation.

The transcriptions of Hsps are induced by the activation of Hsfs, which form trimers under stress conditions. Hsp70 binds to Hsf and Hsp90 maintains Hsf as an inactivated monomer, both negatively regulating the DNA binding and transcriptional activity of Hsf (Mosser et al 1993; Ali et al 1998). However, Hsfs are activated by the

Fig 6. Changes of heat shock protein (Hsp)-30 and Hsp70 messenger ribonucleic acid (mRNA) levels in goldfish culture cells after the rise in culture temperature. (A) Northern blot analysis using 15 μ g of total RNA. (B) Changes of relative Hsp30 mRNA levels after the raise of culture temperature. β -Actin mRNA levels were used as the control, and relative levels in the ordinate represent those compared with Hsp30 mRNA levels at 1 hour after the raise of culture temperature from 20°C to 40°C. (C) Changes of relative Hsp70 mRNA levels after the raise of culture temperature. β -Actin mRNA levels were used as the control, and relative levels in the ordinate represent those compared with Hsp70 mRNA levels at 1 hour after the raise of culture temperature from 20°C to 35°C. Data are given as means $\pm$ SE from 3 culture plates.

production of unfolded proteins, inducing Hsp70 and Hsp90 as molecular chaperones (Morimoto 1998). It has been claimed that Hsps are dissociated from Hsf, which subsequently forms trimer to activate Hsp gene expression when Hsps alternatively bind to unfolded proteins as molecular chaperones under stress conditions (Ananthan et al 1986; Baler et al 1996; Morimoto 1998). Therefore, goldfish Hsp30 may be induced only when unfolded proteins are accumulated at a concentration of much more than the levels that Hsp70 could deal with.

Although Hsp30 decreased almost to the original level after treatment at 40°C for 24 hours, the accumulated Hsp70 levels remained high even at this time (see Fig 4). The decreased mRNA levels of Hsp30 and Hsp70 at 40°C at 8 hours after the temperature shift suggest that these proteins are not much newly translated in this period. Taken together, Hsp70 seems more stable than Hsp30. It has been demonstrated that Hsp70 exerts its refolding activity for denatured as well as nascent proteins in an adenosine triphosphate (ATP)-dependent manner (Kiang

and Tsokos 1998). On the other hand, Hsp30 forms oligomers, binds to unfolded proteins, and prevents their aggregation independently of ATP (MacRae 2000). Because Hsp30 and Hsp70 have distinct properties to repair unfolded proteins, the degradation of these Hsps may occur at the different time lapses after temperature shift.

An Hsp27 homologue was induced as well as Hsp30 by heat treatment of the PLHC-1 cell line, which was derived from a hepatocellular carcinoma of desert fish (Norris et al 1997). Hsp27 is the major small Hsp in mammals and phosphorylated in a heat shock-dependent manner (Lavoie et al 1995). Hsp27 markedly prevents heat shock-induced cell death as Hsp70 does (Gabai and Sherman 2002). However, no apparent change in the expression of components having the molecular mass of around 27 kDa was detected in goldfish cells by SDS-PAGE (see Fig 3). Goldfish Hsp30 showed lower amino acid identity to Hsp27s than to Hsp30s (see Table 2). It is therefore uncertain whether goldfish Hsp30 can prevent heat shock-induced cell death as Hsp27 does. Because goldfish Hsp30 was significantly induced at 40°C, it is important to reveal the function of goldfish Hsp30 to protect cells against heat shock.

In conclusion, we showed that goldfish cell growth was terminated at 40°C, where the onset of the endothermic reaction for goldfish cellular components was observed. Furthermore, the temperature shift to 40°C markedly enhanced the expression of Hsp30, although Hsp70, but not Hsp30, was expressed even at 35°C. Further information on the mechanisms involved in the signaling pathways to induce the expressions of 2 major Hsps, Hsp30 and Hsp70, upon heat shock will give new insights into better understanding of why goldfish have high upper-lethal temperatures.

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