Cold-induced ependymin expression in zebrafish and carp brain: implications for cold acclimation

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Abstract Cold acclimation has been suggested to be mediated by alternations in the gene expression pattern in the cold-adapted fish. To investigate the mechanism of cold acclimation in fish brain at the molecular level, relevant subsets of differentially expressed genes of interest were identified and cloned by the PCR-based subtraction suppression hybridization. Characterization of the selected cold-induced cDNA clones revealed one encoding ependymin. This gene was shown to be brain-specific. The expression of ependymin was induced by a temperature shift from 25°C to 6°C in *Cyprinus carpio* or 12°C in *Danio rerio*. Activation of ependymin was detected 2 h after cold exposure and peaked at more than 10-fold at 12 h. This peak level remains unchanged until the temperature returns to 25°C. Although the amount of soluble ependymin protein in brain was not changed by cold treatment, its level in the fibrous insoluble polymers increased 2-fold after exposure to low temperature. These findings indicate that the increase in ependymin expression is an early event that may play an important role in the cold acclimation of fish.

Key words: Ependymin; Cold-induced gene; Cold acclimation; Brain-specific gene; Fibrous insoluble polymer; Fish

1. Introduction

Low temperature may threaten the survival of cells by lowering the membrane fluidity, disassembling the cytoskeleton, descending enzyme activities, inhibiting secretory processes, and affecting metabolic rates. Animals respond to this environmental stress through a number of physiological and developmental changes [1]. Moreover, a number of genes that protect cells from temperature change have been discovered in recent studies [2]. The expression of these genes enables animals to have cold acclimation by two strategies. Structural acclimation induces the expression of the desaturase gene for the desaturation of phospholipids leading to an increase in the membrane fluidity [3,4]. Functional acclimation involves in the expression of metabolic isozymes as well as protecting proteins, such as chaperones, to assist enzyme folding under the cold stress [5,6].

Fish may live in a wide range of temperatures. They should have evolved cellular responses to the damage and rate-depressing effect of cooling. The species living in cold climate have been demonstrated to have changes in enzyme activities, lipid composition, and behavior for cold acclimation [7–9]. Subsequently, the brain is the organ that senses temperature and makes instruction to cold acclimation. It is possible that cold-induced genes in the brain may exist and contribute to the control and regulation of the acclimation responses. However, information on this aspect is not available.

This study is aimed at elucidating the mechanisms of cold acclimation in fish brain; therefore, we set out to identify the cold-induced genes. A selected cold-induced cDNA clone, ependymin (epn), has been shown to be the brain-specific gene. In addition, ependymin glycoprotein (EPN) in goldfish enhances the rate of synthesis after learning [10]. Further studies demonstrated that the expression of epn mRNA is also learning-induced and its existence implies the formation of synaptic changes during the consolidation step of learning [11,12].

EPN is secreted by meningeal cells [13]. It has two glycoforms (37 kDa and 32 kDa) and is an important glycoprotein in the cerebrospinal fluid of teleost fish [14]. A bound form of EPN has been identified in the extracellular matrix of the cerebral blood vessels. This glycoprotein is probably associated with collagen fibrils [15,16]. Moreover, EPN has the capacity to bind calcium and results in a conformational transition [17,18]. In vitro and in vivo experiments have demonstrated that EPN may form fibrous insoluble polymers (FIP) [19,20]. In addition, this glycoprotein has been attributed to involve in optic nerve regeneration and neuroplasticity [21–24].

We have showed that the expression of epn mRNA and protein may be induced in the fish brain under cold stress. Taken together our finding suggests a possible role of EPN in the cold acclimation of fish.

2. Materials and methods

2.1. Experimental animals

Common carps (*Cyprinus carpio*) were purchased from a local supplier and the AB strains of zebrafish (*Danio rerio*) were obtained from the Institute of Neuroscience, University of Oregon. These fishes were maintained in 25-gal tanks with circulating aerated water at 25°C. The temperature of aerated water was changed at a rate of 20°C/h.

2.2. RNA preparation

After killing, brain and liver of the fishes were dissected and transferred to centrifugation tubes containing 7.5 ml of GTC buffer (4 M guanidium thiocyanate; 25 mM sodium citrate; 0.5% *N*-sodium lauroyl sacosine; 0.2 M 2-mercaptoethanol) with 1 g of glass beads. The

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Abbreviations: EPN, ependymin glycoprotein; epn, gene encoding EPN; SSH, subtraction suppression hybridization; FIP, the fibrous insoluble polymers

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tissues were then vigorously vortexed for 1 min and CsCl (3 g) was added. Lysates were collected by centrifugation at 500×*g* at 25°C for 30 min. The suspension was passed through an 18-gauge needle for 15 times to disrupt the chromosomal DNA. Total RNA was collected by centrifugation of the lysates in an ultracentrifuging tube containing 5 ml cushion (5.7 M CsCl, 0.01 M EDTA, pH 7.5) at 25 000×*g* and 20°C for 24 h [25]. The A_{260}/A_{280} ratio of the purified RNA exhibited ranged from 1.7–2.0 and its integrity and equalization were determined by 1.2% native agarose and stained with ethidium bromide.

2.3. Construction of subtractive cDNA libraries

The cold-induced cDNA in the carp brain was isolated by the polymerase chain reaction (PCR)-based suppression subtraction hybridization (SSH) according to the procedures provided by Clontech. Tester and driver cDNAs were synthesized from poly(A) RNA (1 μg) isolated from the carps cultured at 25°C (driver) and 6°C (tester) for 48 h, respectively. These cDNAs were then digested with *Rsa*I. The tester cDNA was divided into two portions and each was ligated with a different cDNA adaptor (adaptor 1 or adaptor 2) before two hybridizations were performed. In the first hybridization, an excess amount of drivers were added to each portion of tester (driver:tester = 30:1). The two samples from the first hybridization were mixed and fresh denatured driver cDNA were added. In the second hybridization, new hybrid molecules corresponding to differentially expressed cDNA with different adaptors on each end were formed. These hybrids were amplified exponentially by PCR. A secondary PCR amplification was then performed using nested primers to enrich for differentially expressed sequences. The PCR products were cloned into pCR2.1 (Invitrogen). The subtraction cDNA library was obtained after transformation into TOP10 *Escherichia coli* strain. Twenty randomly selected clones have sequenced and nucleic acid homology was determined using the BLAST program. Only one clone was found to be homologous with a known gene encoding EPN. The sequence data of epn in carp and zebrafish are available from GenBank under accession numbers U00432 and X52502, respectively.

2.4. Northern blot analysis

After denaturing, brain (5 μg) or liver (20 μg) RNA was treated with formamide/formaldehyde and applied to 1.2% agarose gel containing 18% v/v formaldehyde for electrophoresis. The RNA was then capillary transferred to a nitrocellulose membrane. DNA probes were radiolabeled by random priming to a specific activity of more than 1×10^9 cpm/μg DNA. Hybridization was then performed according to Church and Gilbert [26] and the radioactivity was detected by exposing the blot to a Fuji X-ray film. The RNA levels were evaluated by scanning the resulting autoradiography with a densitometer (Molecular Dynamics).

2.5. In vitro transcription and RNase protection analysis

Preparations (1 μg) of the plasmid DNA, pGEMT-ependymin and pGEMT-actin were linearized with *Rsa*I. In vitro transcription was allowed to proceed by adding SP6 RNA polymerase to a mixture containing a linearized DNA, nucleotides, RNasin, and [^{32}P]-UTP in the transcription buffer at 37°C for 60 min [27]. RNA (1 μg), for the analysis of epn expression, 5 μg, for the analysis of actin expression, was mixed with 5×10^5 cpm of the RNA probe in 30 μl of a hybridization buffer (40 mM PIPES, 1 mM EDTA, 0.4 M NaCl, 80% formamide, pH 6.4) [27]. RNase-resistant fragment was separated on 6% polyacrylamide/6 M urea gel and the radioactivity was detected by autoradiography. RNA expression was quantified by densitometric scanning after autoradiographic detection on a Fuji X-ray film at −70°C with an intensifying screen.

2.6. Isolation of soluble and insoluble protein fractions from brain

The soluble protein fraction was isolated by the method of Schmidt and Lapp [28] and the insoluble protein fraction by that of Shashoua [20]. To collect extracellular proteins, brains from the fishes were extracted in 1 ml (carp) or 0.5 ml (zebrafish) of an extraction buffer (2.5 mM calcium acetate and 0.14 M NaCl in 0.03 M Tris-HCl, pH 7.4) at 4°C for 2 h. The extracted brains were then homogenized in ice-cold extraction buffer using a Teflon glass homogenizer for 5 min. The homogenates were centrifugated at 10 000×*g* at 4°C for 30 min and the supernatant was designed as the intracellular fraction. The pellets were washed in triplicate with extraction buffer and were designed as the purified FIP fraction.

2.7. Western blot and dot blot analysis

The purified FIP fraction from one carp brain or three zebrafish brains was homogenized in extraction buffer using a glass homogenizer to give suspensions a milky appearance. Aliquots of FIP were then spotted onto a nitrocellulose paper (0.45 μm pore) by a vacuum suction to ensure a tight attachment of the FIP onto the paper. The total soluble protein and extracellular fractions (1 μg) or intracellular fractions (10 μg) were resolved by a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to a nitrocellulose paper. The membranes and dot blots were blocked in 5% non-fat milk for 45 min. Immunoblotting was performed using a rabbit polyclonal antibody generated by injecting purified recombinant thioredoxin-EPN fusion protein. A goat anti-rabbit antibody conjugated to horseradish peroxidase (1:5000) was used to detect protein-antibody complexes. After treating the membrane with ECL reagents (Amersham) for 1 min, the membrane was exposed to an X-ray film.

3. Results

3.1. Identification of ependymin as a cold induction gene

The PCR-based SSH was used to identify genes differentially induced by cold. RNAs were prepared from carp brain cultured at 25°C and 6°C. SSH was performed and a cDNA library containing genes with differential expression at low temperature was established. Twenty randomly selected clones were sequenced and sequence analysis revealed that one of these clones was the brain-specific epn.

A significant increase (approximately 9-fold) in the transcript level of epn at 6°C for 48 h was demonstrated by Northern blot analysis of RNA isolated from the brain of carps (Fig. 1). However, no significant expression or induction was detected in the liver, indicating that the cold-induced epn expression was a brain-specific phenomenon.

3.2. Time course of epn induction by low temperature

The time course of epn induction by low temperature was next examined using RNase protection analysis. There was a

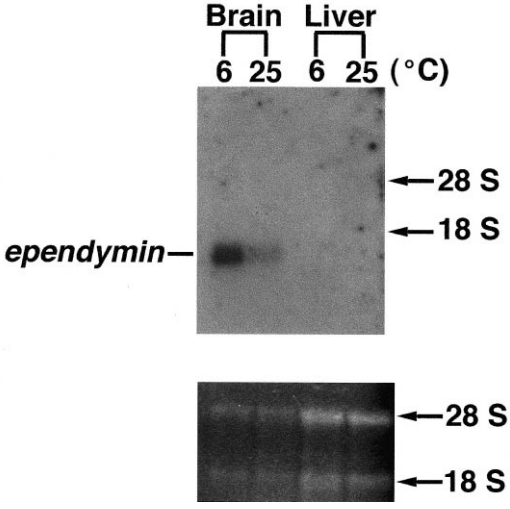

Fig. 1. Effect of low temperature on ependymin mRNA expression in carp brain and liver. Carps were exposed to 6°C for 48 h, or maintained at 25°C, before total RNA was extracted. Then brain RNA (5 μg) or liver RNA (20 μg) were separated by electrophoresis on a 1.2% agarose. The RNA samples were stained with ethidium bromide as shown in the lower diagram. The RNA was blotted onto a nitrocellulose paper and hybridized with the ^{32}P-labeled epn DNA probe. The radioactivity was visualized by autoradiography, the exposure time was 6 h. Arrows indicate the positions of 28S and 18S RNA.

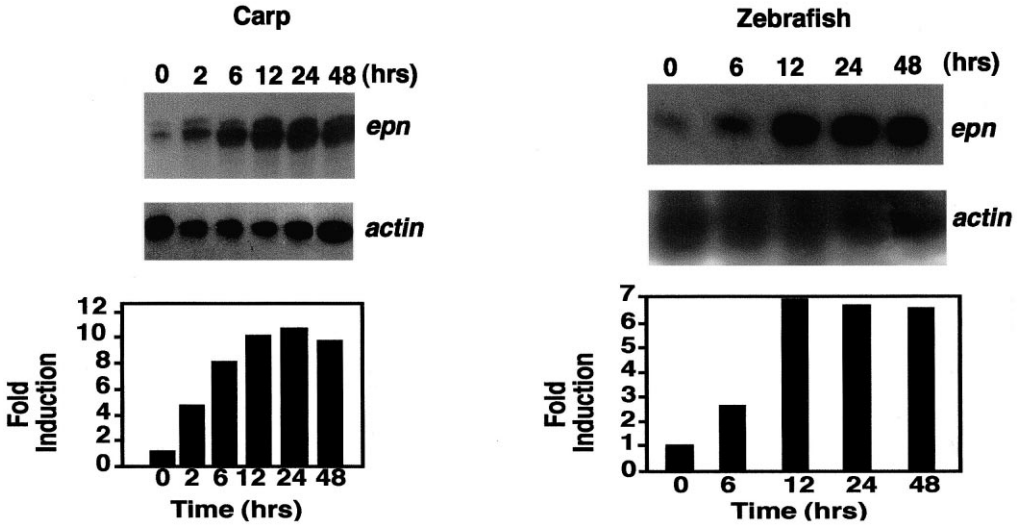

Fig. 2. Time course of ependymin mRNA induction by low temperature. Carp (left panel) and zebrafish (right panel) were incubated respectively at 6°C and 12°C for various intervals and total RNA of the brain was isolated. RNA was hybridized with antisense radiolabeled RNA probes for carp (left panel) and zebrafish (right panel) epn and β-actin. For epn and β-actin, the protected fragments were ~390 and ~230 nt, respectively. The radioactivity is visualized by autoradiography. The exposure times for ependymin and actin were 1 h and 6 h, respectively. The actin transcripts were employed as an internal control. The lower diagram shows the expression of ependymin mRNA relative to that of β-actin by quantitative densitometry. The fold induction of ependymin mRNA at 25°C was normalized to 1. Data from three separate experiments were averaged and the standard deviations were less than 15%.

significant increase in epn transcripts 2 h after lowering the temperature from 25°C to 6°C. At 12 h, the level of induction increased to approximately 10-fold and this high level remained unchanged at 6°C (Fig. 2, left panel). As shown in Fig. 2, right panel, the level of epn mRNA increased with time in zebrafish cultured at 12°C. The induction reached 7-fold at 12 h and unchanged under this temperature. Transcripts of β-*actin* (an internal control) did not show significant difference under the same condition. These results demonstrate the brain-specific induction of epn transcripts under cold stress in the *Cypriniformes* species.

3.3. Induction of ependymin protein by cold exposure

The expression of EPN in the brain of cold-treated zebrafish (Fig. 3) and carp (data not shown) was examined by Western blot analysis with an antibody that recognized both the 32- and 37-kDa forms of the protein. In the soluble fractions of total protein as well as extracellular and intracellular proteins, the level of expression did not show any change with temperature. However, analysis using dot blotting demonstrated that the expression of EPN in the FIP fractions increased by 2-fold 24 h after cold exposure (Fig. 4). These results suggest that only the insoluble bound form of EPN in the FIP increased at low temperatures.

4. Discussion

The homeoviscous adaptation of membrane to changes in temperature not only has been widely observed in animals but also in plants and microorganisms as both phenotypic and genotypic responses [3]. By this cold acclimation, the proportion of unsaturated fatty acids in the membrane phospholipids increases, thereby promotes membrane fluidity [3,30,31]. In addition to cold-induced alternations in the composition of the phospholipids [4,7], the release of neurotransmitters and hormones in the brain has also been reported [29]. In accord

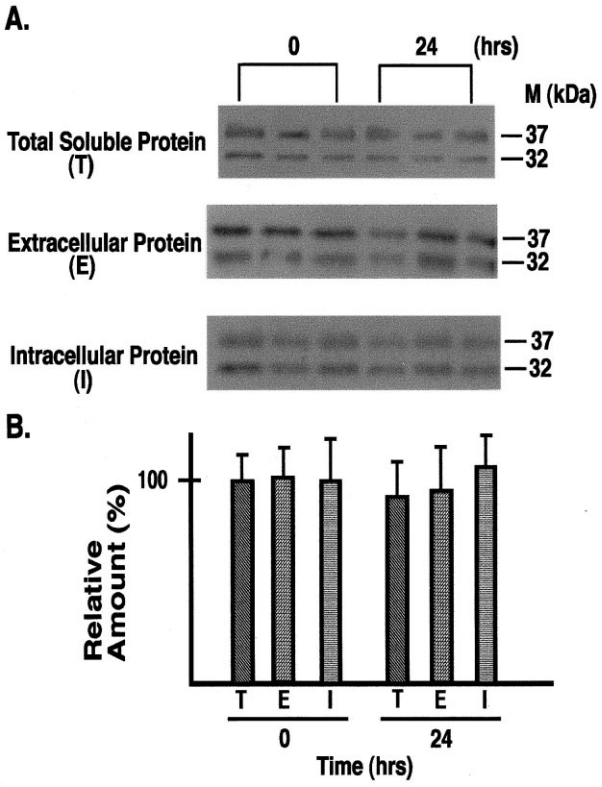

Fig. 3. Western blot analysis of ependymin protein expression in zebrafish brain. Zebrafish were incubated at 25°C as control or 12°C for 24 h. Proteins were prepared as described in Section 2. Total protein (T, 1 μg), extracellular protein (E, 1 μg) and intracellular protein (I, 10 μg) were separated by SDS-PAGE (12%) and analyzed with ependymin-specific antisera. The lower diagram shows the variations in ependymin protein by a quantitative densitometry. The level of ependymin protein at time 0 was normalized to 100. The data were the averages obtained from three separate experiments.

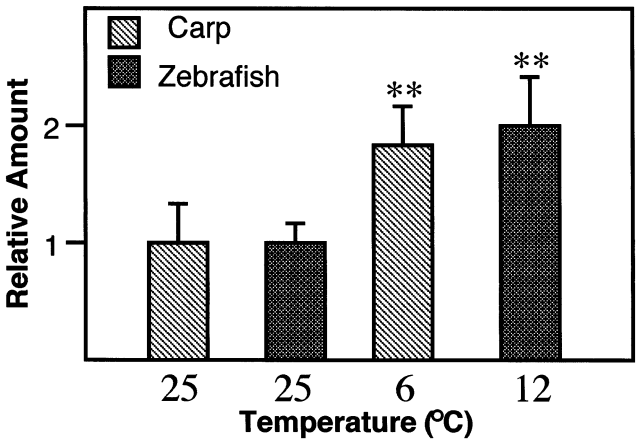

Fig. 4. Quantitation of ependymin protein contained in FIP after exposure to low temperature. Carp and zebrafish were incubated at 25°C and 6°C or 12°C for 24 h. Brain FIP was extracted and analyzed by dot blotting with ependymin-specific antisera as described in Section 2. The relative amount is defined as the ratio of the amount of EPN at 25°C to that at 6°C for carp or 12°C for zebrafish, respectively. Data were represented as means ± S.D. from nine fishes. Significant differences (versus the control) are shown by asterisks (Student's *t*-test, $^{**}P < 0.001$).

with this, it was demonstrated that the increase of transcript levels of desaturase enhances membrane fluidity by increasing unsaturated phospholipid in the carp liver. Up to now, analysis on the changes in gene expression pattern in the brain under low temperature has not yet been reported.

In this study, epn was identified to be a cold-induced gene in the brain by the PCR-based SSH and the differential activation of this gene was confirmed by analyzing the RNA expression. In addition to demonstrate the cold-induced stimulation of epn gene expression, we also observed that the expression of this gene is reversible after increasing the temperature back to 25°C (data not shown). Upon exposure to cold stress, there were no detectable changes of the soluble form EPN, although there was a 10-fold increase in its mRNA. This phenomenon may in part be masked by the abundant constitutive expression of EPN in the brain. However, we could not rule out the possibility that localized changes in the soluble form of EPN may exist within the brain. Moreover, significant increase in the insoluble bound form of EPN in the FIP was observed. These findings suggest that EPN is a cold-induced gene in the *Cypriniformes* family. However, whether this finding is a general phenomenon in fish brain requires further investigations.

In mice, regulation of cold-induced HSP70 expression in brown adipose tissue appears to be mediated by norepinephrine. The expression of HSP70 increases and reaches a peak 4–6 h after treating with the hormone. However, the level of expression then returns to the baseline within 2 h [5]. Moreover, administration of noradrenaline may lead to acute cold-induced suppression of leptin gene expression in white adipose tissue [32]. In this study, we found that the cold induction of epn mRNA in the brain of carp and zebrafish is rapid and may be detected at 2 h after exposure to low temperature. This response is relatively quick when compared with that of the desaturase activation in liver of carp that has a peak at 2–3 days under cold stress [4,30]. These findings signify that increase in EPN expression is an early event in the cold acclimation of fish. Although it is unclear whether the induction of epn gene is through the transcriptional regulation, our preliminary studies on the epn promoter using gel mobility shift assay [33] did not reveal significant differences in the DNA-protein binding pattern between fish exposed to 25°C and low temperature (unpublished).

Consistent with the findings in goldfish brain [14], EPN is also expressed abundantly in the brain of carp and zebrafish. It has been reported that about 50% of the EPN are in the extracellular fluid of the brain [34]. Although soluble EPN is the predominant constituent of the cerebrospinal fluid, a bound form of EPN has also been found to be associated with the extracellular matrix. This bound form has been considered to be the one with physiological functions [15,16]. In fact, EPN is the predominant constituent of cerebrospinal fluid of teleost fish [13,34] and regeneration or plasticity of nerve system requires the presence of EPN [22]. In goldfish, a significant increase in the synthesis of EPN has been demonstration in the regeneration of retina. The sharpening of the regenerating retinotecal projection may be blocked by the antibodies against this protein [23,24]. In cold acclimation, induction of EPN may enhance the regeneration and plasticity of the nerve system.

The acclimation to cold has been reported to be associated with the increase of the rate of Ca^{2+} uptake in trout cardiac sarcoplasmic reticulum and reduction in that of the sarcoplasmic Ca^{2+} uptake rate in the heart of carp [35]. It has been shown that two-third of Ca^{2+} in the CSF of the rainbow trout is protein bound [14]. Since EPN may bind to Ca^{2+} and results in a conformational transition [17,18], this glycoprotein has been suggested to play an important role in maintaining Ca^{2+} homeostasis of the brain [34]. Since we have demonstrated that there is a high level of bound EPN in the brain of carp and zebrafish under low temperature treatment, it is possible that the cold-induced EPN may involve in the binding of Ca^{2+} that regulates the calcium homeostasis in the brain. However, this suggestion awaits to be clarified.

The presence of FIP in the synaptosomal fraction of the brain has been observed in fish [20]. It has been suggested that FIP may be a polymeric aggregate of a mixture of cytoskeletal and extracellular matrix proteins, including insoluble form of EPN [15,16]. Extracellular matrix proteins, such as fibronectin, and collagen, may be involved in cell adhesion and migration. These proteins may also serve as a reservoir for the hormones controlling cell growth and differentiation. Moreover, pre- and post-synaptic densities may contain FIP involved in the extracellular architecture and plasticity of nerve system [34,15]. The increase of expression of EPN in FIP under cold stress not only prevents the nerve system from damage but also promotes its regeneration.

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