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Hibernation-specific alternative splicing of the mRNA encoding cold-inducible RNA-binding protein in the hearts of hamsters

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ABSTRACT

The hearts of hibernating animals are capable of maintaining constant beating despite a decrease in body temperature to less than 10 °C during hibernation, suggesting that the hearts of hibernators are highly tolerant to a cold temperature. In the present study, we examined the expression pattern of cold-inducible RNA-binding protein (CIRP) in the hearts of hibernating hamsters, since CIRP plays important roles in protection of various types of cells against harmful effects of cold temperature. RT-PCR analysis revealed that CIRP mRNA is constitutively expressed in the heart of a non-hibernating euthermic hamster with several different forms probably due to alternative splicing. The short product contained the complete open reading frame for full-length CIRP. On the other hand, the long product had inserted sequences containing a stop codon, suggesting production of a C-terminal deletion isoform of CIRP. In contrast to non-hibernating hamsters, only the short product was amplified in hibernating animals. Induction of artificial hypothermia in non-hibernating hamsters did not completely mimic the splicing patterns observed in hibernating animals, although a partial shift from long form mRNA to short form was observed. Our results indicate that CIRP expression in the hamster heart is regulated at the level of alternative splicing, which would permit a rapid increment of functional CIRP when entering hibernation.

1. Introduction

Homothermal animals are able to maintain a constant body temperature at approximately 37 °C even in a cold environment. The ability to maintain a warm body temperature is essential for survival of homeotherms since the heart cannot keep beating in deep hypothermic condition [1]. In fact, induction of deep hypothermia in rats causes ventricular dysfunction and arrhythmias such as atrioventricular block and ventricular fibrillation [2–4]. On the other hand, some rodents undergo hibernation to survive a severe environment during winter. During hibernation, the heart beats constantly despite the fact that body temperature is decreased to less than 10 °C. This suggests that the heart of hibernators is highly tolerant to cold temperature compared with that of non-hibernators. However, the maintenance of heartbeat during deep hypothermia seems unlikely to be totally associated with innate properties of the hibernator's heart, because the heart of a hamster suffers severe damage when deep hypothermia is forcibly induced by a combination of anesthetic injection and cooling [4]. It is therefore expected that some beneficial mechanisms operate during natural hibernation that protect the heart from harmful low temperatures.

It has been demonstrated that cold-inducible RNA-binding protein (CIRP) is induced by cold stress in cultured cells [5]. The CIRP protein regulates gene expression at the level of translation (i.e., mRNA splicing, stability and transport) and thus allows cells to respond rapidly to cold stress. Accumulating evidence indicates that CIRP plays important roles in protection of various types of cells against harmful effects of cold temperature [6,7]. It has been reported that CIRP is constitutively expressed in the testis of mice and humans, the temperature of which is maintained at 2–8 °C lower than core temperature, and an increase in the testis temperature induced by experimental cryptorchidism or immersion of the lower abdomen in warm water decreased the expression of CIRP [8]. This indicates that CIRP functions not only in cultured cells

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in vitro but also in tissues *in vivo* when temperature is lowered. Accordingly, the function of CIRP, in addition to its cold-inducible nature, prompted us to assume that the protein may participate in the maintenance of heartbeat of hibernating hamsters. In support of this assumption, it has been reported that CIRP expression in the Japanese treefrog is increased during the hibernation season [9]. Accordingly, the aim of the present study was to determine the expression pattern of CIRP in the heart of hibernating hamsters.

2. Methods

2.1. Animals and induction of hibernation

Male Syrian hamsters (Japan SLC, Inc., Shizuoka, Japan) weighing 130–160 g were used. Animals were maintained at an ambient temperature of 22 °C with a 12-h light/dark cycle (lights on 07:00–19:00 h) and given free access to food and water before induction of hibernation. Procedures for induction of hibernation were previously described [4,10]. In brief, animals were transferred to a constantly darkened cold (4 °C) room and acclimated to this condition for 2 months. During this acclimation period, 4 animals were housed together per cage. To induce hibernation, each acclimated hamster was housed in an individual cage with a reduced amount of food. This procedure allowed the hamster to go into hibernation within a few weeks. Hamsters were checked every morning and were judged to have entered deep hibernation by their immobility, curled posture, unresponsiveness to handling and profound reduction in body temperature. The experimental procedures were performed according to the guidelines for the care and use of laboratory animals approved by the Animal Care and Use Committee of Gifu University (permission numbers: 13078 and 14102).

2.2. Induction of artificial hypothermia

Artificial hypothermia was induced forcibly in hamsters without adapting to a cold environment by the combination of isoflurane inhalation and cooling. After induction of isoflurane anesthesia at room temperature, the animals were placed in an ambient temperature of 4 °C. At this time, cable lead electrodes (ECG safety cable lead sets; Philips Medical Systems, USA) for recording ECG were placed at the forelimbs with a ground electrode placed at one hindlimb. A thermistor probe (ML312, ADInsturments) was also inserted into the rectum for monitoring body temperature. The concentration of inhaled isoflurane was decreased in a stepwise fashion depending on the heart rate (>300 beats/min (bpm), 2%; 200-300 bpm, 1.5%; 150-200 bpm, 1%; 100-150 bpm, 0.5%; <100 bpm, 0%). By this procedure, body temperature was decreased to less than 10 °C without resulting in cardiac arrest, though a skipped QRS complex was occasionally observed.

2.3. Tissue sampling and RT-PCR

The expression of CIRP mRNA was assessed by RT-PCR. Animals were anesthetized with isoflurane and were exsanguinated via the carotid arteries. Heart samples were dissected out totally and homogenized. Total cellular RNA was extracted from the homogenates using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). First-strand cDNA was synthesized from 3 μg of total RNA by using Superscript III RNase H^- reverse transcriptase and Random primers (Life Technologies). The absence of PCR-amplified DNA fragments in the samples without reverse transcription indicated the isolation of RNA free from genomic DNA contamination. The PCR was performed with platinum Taq DNA polymerase (Life Technologies). The primer set was as follows: CIRP sense 5′- GCC ATG GCA TCA GAT GAA GGC A -3′ and anti-sense 5′- GCT TTT ACT CGT TGT GTG TA -3′. All primers were purchased from Life Technologies. Amplifications were performed by 30 cycles. The reaction products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide (0.4 μg/mL). The gels were exposed to UV light with a UV transiluminator (UVP Laboratory Products, Upland, CA, USA) and photographed. Densitometry analyses of the results were performed with Image J software. Relative values of long form mRNA to short form mRNA were calculated by taking the value of the respective short form as unity (1.0).

2.4. DNA sequencing

PCR products were purified by PCR clean-up gel extraction (MACHEREY-NAGEL, Düren, Germany) and reacted using a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Reacted samples were sequenced on an ABI Prism 3130 Genetic Analyzer (Life Technologies).

2.5. Statistical analysis

Values are presented as means ± SD. Statistical analysis was performed by analysis of variance with post hoc testing using Duncan's multiple range test.

3. Results

3.1. Identification of CIRP expression in the hamster heart

To determine whether CIRP mRNA is expressed during a non-hibernating euthermic condition in the hamster, RT-PCR was performed using total RNAs from the hearts of normal-conditioned hamsters. As shown in Fig. 1, three DNA bands that differ in size were identified. The approximately 600-bp, 750-bp and 900-bp products were tentatively termed short, middle and long forms, respectively. We focused on short and long forms because they were relatively abundant. The results of sequence analysis of the short form indicated that the product contains the complete open reading frame (ORF) for full-length CIRP. The coding region sequence of hamster CIRP mRNA, which was submitted to GenBank (LC047854) in April 2015, was found to share 94.4%, 94.3% and 89.0% homology with the mouse, rat and human, respectively (Supplemental Fig. 1). The ORF of the cDNA encoded a 172-amino-acid polypeptide. At the amino acid level, the deduced protein shared high identity with that of the mouse (99.4%), rat (98.8%) and human (95.9%) (Supplemental Fig. 2).

The exon structure of the hamster CIRP deduced from that of human CIRP (GenBank NM_001280) is shown in Fig. 2. Sequence analysis revealed that 209-bp nucleic acid sequences were inserted into the 3′ region of exon 7 in the long form product. The inserted sequences contain a stop codon, suggesting production of a C-terminal deletion isoform of CIRP.

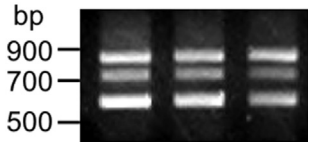

Fig. 1. Expression of CIRP mRNA in the hearts of non-hibernating euthermic hamsters. RT-PCR was performed using total RNAs from the hearts of non-hibernating, euthermic hamsters. The PCR products were analyzed by gel electrophoresis. A photograph shows three representative examples. Three DNA bands, approximately 600 bp, 750 bp and 900 bp in size, were identified. Similar results were reproductively obtained in five animals.

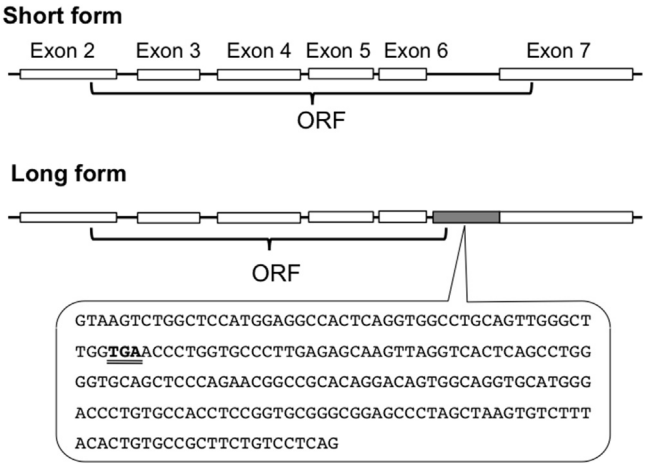

Fig. 2. Schematic representation of hamster CIRP gene constructs. The intron/exon structure hamster CIRP gene was deduced from that of human CIRP. In the long form product, 209-bp nucleic acid sequences were inserted into the 3′ region of exon 7. The inserted sequences contain a stop codon (double underline), suggesting production of a C-terminal deletion isoform of CIRP.

3.2. Expression of CIRP mRNA in the heart of a hibernating hamster

When an RNA sample isolated from the heart of a hibernating hamster was applied to RT-PCR, a single product of 600 bp in size was amplified without accompanying 750-bp and 900-bp products (Fig. 3). The nucleic acid sequences of the 600-bp amplicon were completely identical to those of the short form observed in the heart of a euthermic hamster. The single band was observed in all samples obtained from hibernating animals (n = 7), although sampling time varied from a few hours to 24 h after entering deep hibernation.

3.3. Expression of CIRP mRNA in the heart during artificial hypothermia

We then addressed the question of whether the differential splicing patterns in the heart of a hibernating hamster depend on reduction of tissue temperature or whether they are achieved by some specific mechanisms that operate when entering hibernation. To reduce body temperature forcibly, hamsters were anesthetized with isoflurane and cooled in a refrigerator (4 °C). Heart samples were obtained within 1 h or 3–6 h after body temperature had decreased to less than 10 °C and were applied to RT-PCR analysis. Induction of artificial hypothermia did not completely mimic the splicing patterns observed in hibernating hamsters; that is, 750-bp and 900-bp products as well as a 600-bp product were amplified (Fig. 4). However, densitometric analysis showed that the ratio of short and long forms was shifted from approximately 1:1 under a euthermic condition to 2:1 after induction of artificial hypothermia (relative values of long form to short form: 0.89 ± 0.10 in a euthermic condition vs 0.53 ± 0.15 in artificial hypothermia, $p < 0.05$, n = 4). Keeping the hypothermic condition for up to 6 h did not cause a further shift to the short form (0.56 ± 0.07, n = 4).

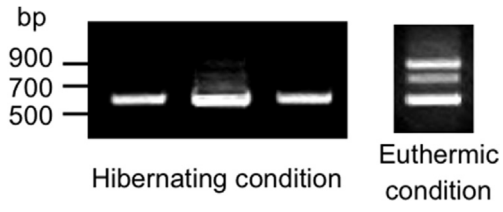

Fig. 3. Expression of CIRP mRNA in the hearts of hibernating hamsters. RT-PCR was performed using total RNAs from the hearts of hibernating hamsters. The PCR products were analyzed by gel electrophoresis. The photograph on the left shows three representative examples in a hibernating condition and the photograph on the right shows a representative example in a euthermic condition. A single product of 600 bp in size was amplified without accompanying 750-bp and 900-bp products. Similar results were reproductively obtained in seven animals.

4. Discussion

It has been demonstrated that CIRP plays important roles in protection of various types of cells against harmful effects of cold temperature [11]. Since CIRP expression is up-regulated in response to cold temperature, we expected that expression of CIRP mRNA would be enhanced during hibernation. In accordance with this, it has been reported that RNA-binding protein motif 3 (RBM3), one of the cold shock proteins having a function similar to that of CIRP, is up-regulated in hibernating mammals [12–14] and plays an important role in neuroprotection [15,16]. However, the most remarkable finding of the present study was that hibernation induces changes in alternative splicing of CIRP mRNA in the heart, leading to an increase in the functional form of CIRP. The CIRP protein enhances translational efficiency of target mRNAs by modulating mRNA splicing, stability and transport and thus allows cells to respond rapidly to cold stress [6,7]. Regulation at the level of alternative splicing, rather than at the level of transcription, would be beneficial especially in hibernating animals because it may enable rapid expression of a subset of proteins required for cold tolerance despite extensive reduction of enzymatic reactions due to a cold temperature.

Our results suggest that the short form mRNA encodes the full-length hamster homolog of CIPR. On the other hand, a C-terminally truncated CIRP would be produced from the long form mRNA in the heart of non-hibernating euthermic hamsters. The amino acid sequence, Met (1)-Lys (84), in the N-terminal region of human CIRP, which has been identified as an RNA-binding domain [17], was conserved in the C-terminally truncated isoform. This fact indicates that the isoform possesses RNA binding activity equally to full-length CIRP. However, the isoform lacked critical phosphorylation and methylation sites located at the C-terminal region, the phosphorylation and/or methylation of which are related to activation of CIRP [11,18]. It is thus probable that the C-terminally truncated isoform plays a dominant-negative role over the full-length CIRP. Considering that the enhanced expression of target proteins of CIRP is a response to cold stress, there is no need for CIRP to be expressed constitutively during a euthermic state. We therefore speculate that

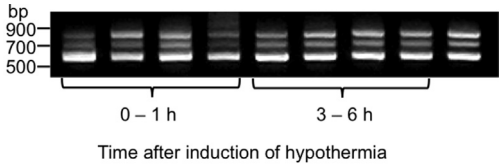

Fig. 4. Expression of CIRP mRNA in the hearts of artificial hypothermic hamsters. Deep hypothermia was induced forcibly by cooling hamsters anesthetized with isoflurane. Heart samples were obtained within 1 h or 3–6 h after body temperature had decreased to less than 10 °C and were applied to RT-PCR analysis. The PCR products were analyzed by gel electrophoresis. A photograph shows four examples in each hypothermic condition and a representative example in a euthermic condition for comparison. Products of 750 bp and 900 bp in size as well as a 600-bp product were amplified. Densitometric analysis showed that the ratio of short and long forms was shifted from approximately 1:1 under a euthermic condition to 2:1 after induction of artificial hypothermia. Keeping the hypothermic condition for up to 6 h did not cause a further shift to the short form. Similar results were reproductively obtained in five animals in each experimental condition.

the dominant-negative regulation is important to mask the function of CIRP under a non-hibernating condition. The apparently non-meaningful regulation may be beneficial when the hibernation mechanism is triggered. The dominant-negative regulation combined with constitutively active transcription may permit rapid expression of CIRP function by switching the splicing pattern. The mode of regulation is interesting as hibernation-specific gene expression.

Factors inducing the shift in alternative splicing of CIRP are unclear at present. Hypothermia is the most probable candidate due to its cold-inducible nature. In the present study, however, we showed that induction of artificial hypothermia was insufficient to reproduce the shift in alternative splicing during hibernation. Considering that a partial shift from long form to short form occurred in the artificial hypothermic hamsters, factors related to preparatory changes during adaptation to a cold and short photoperiod environment would be needed in addition to hypothermic stimulus.

In summary, we demonstrated that CIRP mRNA was constitutively expressed in the heart of a non-hibernating euthermic hamster with several different forms probably due to alternative splicing and that mRNA encoding C-terminally truncated CIRP was shifted to that encoding full-length CIRP during hibernation. Regulation at the level of alternative splicing would permit a rapid increment of functional CIRP and thereby be beneficial in hibernating animals.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.135.

Transparency document

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