Hypothermia induces changes in the alternative splicing pattern of cold-inducible RNA-binding protein transcripts in a non-hibernator, the mouse

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ABSTRACT

Cold-inducible RNA-binding protein (CIRBP) plays important roles in protection against harmful effects of cold temperature. We previously found that several splicing variants of CIRBP mRNA are constitutively expressed in the heart of non-hibernating euthermic hamsters and that one of the variants is predominantly expressed with remarkable reduction in the expression of other variants in hibernating hypothermic hamsters. The aim of this study was to determine whether the regulation of alternative splicing is a common function in a non-hibernator, the mouse. The expression of CIRBP mRNA was assessed by RT-PCR. In euthermic control mice, several splicing variants of CIRBP mRNA were detected in various organs. When hypothermia was induced in mice by using isoflurane anesthesia, the short form variant, which encodes full-length functional CIRBP, was predominantly detected. Keeping body temperature of anesthetized mice at 37°C prevented changes in the splicing pattern. Exposure of mice to a low temperature did not change the splicing pattern, suggesting that endogenous neuronal and/or humoral pathways activated in response to cold stimuli applied to the body surface play minor roles. In agreement with this, the shift in alternative splicing was reproduced in isolated leukocytes in vitro when they were incubated at 28°C. Since application of a TRPM8 or TRPA1 agonist at 37°C failed to promote the shift in the splicing pattern, it seems likely that cold-sensitive channels are not involved in the splicing regulation. Therefore, it is probable that a substantial reduction of temperature is a major cause of the regulation of alternative splicing of CIRBP transcripts. The regulatory system of CIRBP expression at the level of alternative splicing, which was originally discovered in the hibernating hamster, commonly exists in non-hibernators such as mice.

Cold-inducible RNA-binding protein (CIRBP) is one of the cold-shock proteins, for which expression is increased in response to cold stress (15, 16, 29). The protein has an RNA-recognition motif in the N-terminal region and an arginine-glycine-rich motif in the C-terminal region (10, 11, 31). CIRBP has the capacity to bind RNAs and thereby regulate the expression of various proteins at the post-transcriptional level through modulating mRNA splicing, stability and transport (10, 11, 14, 25, 28, 30–32). In addition, there is several lines of evidence that cold-shock proteins in various types of cells confer protection against the harmful effects of a low tem-

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perature (6, 9, 10, 26, 31, 32).

Mammalian hibernators such as ground squirrels and hamsters experience drastic reduction in body temperature in a food-restricted cold environment (4, 19). Although the heart rate is greatly lowered, normal sinus rhythm is maintained under the condition of extreme hypothermia during hibernation (12, 13, 18). It is thus apparent that the hearts of hibernating animals possess a cold-resistant property. The fact that CIRBP exerts protective action in a low temperature range allowed us to speculate that the protein also plays an important role during hibernation. In our previous study, we found that several splicing variants of CIRBP mRNA are present in the heart of a non-hibernating euthermic hamster. Furthermore, we found that the splicing pattern observed in the heart of a hibernating hypothermic hamster is markedly different from that in euthermic animals. In hibernating hamsters, one of the variants is predominantly expressed with remarkable reduction of other variants (20). Considering that the variant predominantly expressed during hibernation encodes full-length functional CIRBP, the changes in the pattern of alternative splicing would be beneficial for protective effects of CIRBP to be exerted.

CIRBP is conserved in various mammals including humans and mice, which are non-hibernators (15, 16). However, it is unclear whether regulation of CIRBP expression at the level of alternative splicing is specific to hibernators or whether it commonly functions in non-hibernators. To address this issue, we used a non-hibernator, the mouse. Firstly, we examined whether several splicing variants are constitutively expressed under a euthermic condition. This would be important because the presence of multiple variants is the basis for the regulation at the level of alternative splicing. Then we examined whether a shift in the splicing pattern is induced in mice in response to reduction of body temperature. We also carried out in vitro experiments using isolated leukocytes to determine factors that contribute to the shift of the splicing pattern.

MATERIALS AND METHODS

Animals. Male ddY mice (38 ± 2.7 g in weight; Japan SLC, Inc., Shizuoka, Japan) were used in all of the experiments. The animals were maintained at an ambient temperature of 22°C with a 12:12-h light-dark cycle (lights on 07:00–19:00 h) and given free access to food and water before experiments. In experiments in which the effect of cold exposure was examined, mice were placed in a cold room kept at 4°C for 1 week. The Animal Care and Use Committee of Gifu University approved the animal experiments (permission numbers: 16030 and 17015). All efforts were made to minimize the number of animals used.

Induction of hypothermia with isoflurane inhalation. Since it is well known that reduction of body temperature is associated with general anesthesia (22, 24), hypothermia was experimentally induced by using anesthesia with isoflurane inhalation. After inhalation of isoflurane (concentration: 2%), each mouse was placed on a stainless plate warmed gently so that the body temperature of the mouse was kept constant at 28°C. To ascertain the effects of the anesthetic itself, each mouse after inhalation of isoflurane was placed on the warmed stainless plate to prevent reduction of body temperature. A thermistor probe was inserted into the rectum for monitoring rectal temperature.

Analysis of the expression of CIRBP in peripheral blood leukocytes. Whole peripheral blood was collected by cardiopuncture from euthermic or hypothermic mice under isoflurane anesthesia (concentration: 5%). Ten mL of hemolytic buffer (0.14 M NH₄Cl, 0.017 M Tris-HCl, pH 8.0) was added to 1 mL of the blood and incubated at 4°C for 10 min. The hemolysates were centrifuged at 1,300 × g for 10 min, and the precipitated leukocytes were used for reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Blood samples obtained from euthermic mice were used for the in vitro experiments. One mL of heparinized blood was incubated with a TRPM8 agonist, l-Menthol (KANTO CHEMICAL, Tokyo, Japan), or a TRPA1 agonist, allyl isothiocyanate (AITC) (FUJIFILM Wako, Osaka, Japan), at 37°C for 1 h. The agonists were dissolved in 100% dimethyl sulfoxide (DMSO; Nacalai Tesque, Inc., Kyoto, Japan) and diluted in saline (final concentrations of DMSO: 0.01% and 0.1% in TRPM8 and TRPA1 solutions, respectively). In another experiment, the blood samples were incubated at 37°C or at 28°C for 10, 30, and 60 min. After each treatment, we immediately added 10 mL of the hemolytic buffer and obtained leukocytes for reverse transcription-polymerase chain reaction (RT-PCR) analysis as described above.

RT-PCR. The expression of CIRBP mRNA was assessed by RT-PCR according to our previous study (20). In brief, frozen tissues or leukocyte samples were homogenized and total cellular RNA was ex-
Splicing pattern of mouse CIRBP

RESULTS

Expression of CIRBP mRNA in various organs of euthermic mice

We examined the expression of CIRBP mRNA variants in the brain, heart, lung, liver, kidney and testis of euthermic mice by using RT-PCR. Three splicing variants were identified in the brain, heart, lung and testis of euthermic mice. RT-PCR was performed using total RNAs from the organs. The PCR products were analyzed by gel electrophoresis. The photograph shows three representative results. Three DNA bands, approximately 500 bp, 700 bp and 800 bp in size, were identified with primer sets for CIRBP. β-actin mRNA was used as an internal control. Similar results were reproducibly obtained in five animals.

DNA sequencing. We excised PCR products from agarose gel and purified them with PCR clean-up gel extraction (MACHEREY-NAGEL, Düren, Germany). The purified cDNA was sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on an ABI Prism 3130 Genetic Analyzer (Thermo Fisher Scientific). Moreover, the cDNA including the entire open reading frame (ORF) of CIRBP was amplified by RT-PCR using RNA obtained from the heart of a euthermic mouse and was sequenced. The primer set used was as follows: sense 5’- TCA GGG ACC TGC CCG ACT CA -3’ and anti-sense 5’- AAC GTT CAG CGA AGC TCC C -3’. The sequence of the ORF was compared with the sequences of PCR products.

Data analysis. Data are presented as means ± standard deviation (SD). n indicates the number of different tissue samples from different animals. The significance of differences between mean values was determined by one-way analysis of variance followed by Scheffe’s F test for comparison of multiple groups. A P value less than 0.05 denotes the presence of a statistically significant difference. Statistical analysis was performed by using Statcel4 software (OMS, Saitama, Japan).
amplified depending on the cycle number, and 30 cycles were considered to be appropriate for RT-PCR of CIRBP. In addition, we carried out RT-PCR of β-actin mRNA as an internal standard to normalize the abundance of cDNA in each sample. Since all samples reached the same plateau level in RT-PCR with 30 cycles, 20 cycles were selected to confirm the abundance of cDNA (Fig. 2B).

**Effects of hypothermia on the splicing pattern of CIRBP transcripts**

We examined whether hypothermia induces changes in the splicing pattern of CIRBP transcripts. The rectal temperature of anesthetized mice was lowered to 28°C, and hypothermia was maintained for 1 h. Then the splicing pattern of CIRBP transcripts was analyzed by RT-PCR with focus on the brain and heart. Although both the long form and short form were detected in the brain and heart of euthermic
To assess the net effect of low temperature, we incubated peripheral blood at 28°C or 37°C. The long form and short form of CIRBP were identified at 10 min after starting incubation at 28°C. Shifts in alternative splicing of CIRBP became apparent at 30 and 60 min (Fig. 4C). On the other hand, the splicing pattern was unchanged in leukocytes incubated at 37°C for 60 min (Fig. 4C).

DISCUSSION

We previously reported that the shift in the alternative splicing pattern of CIRBP transcripts from a non-hibernating euthermic pattern to a hibernating hypothermic pattern would serve to express functional CIRBP in the heart of the hamster (20). The aim of this study was to determine whether the unique post-transcriptional regulation commonly operates in a non-hibernator, the mouse. We found that several splicing variants of CIRBP mRNA are constitutively present in various organs of euthermic mice. We also found that the short form variant, which encodes full-length functional CIRBP, is dominantly expressed after induction of hypothermia. These findings demonstrate that the regulation of alternative splicing of CIRBP transcripts is not specific to hibernators, but also occurs in a non-hibernator, the mouse, when body temperature is decreased.

The results of in vitro experiments using isolated leukocytes indicated that cold-sensitive channels are not involved in the splicing regulation and that a substantial reduction of temperature is a major cause of the regulation of alternative splicing of CIRBP transcripts.

We also examined the effects of the anesthetic used for induction of hypothermia (i.e., isoflurane) on the splicing pattern of CIRBP transcripts. Mice anesthetized with isoflurane were placed on a heating pad to prevent reduction of body temperature. Even after inhalation of the anesthetic for 1 h, similar splicing variants to those observed in control animals (see Fig. 1) were detected (Fig. 3B). There was no significant difference in the ratio of long form to short form between the anesthetized mice maintained at 37°C and control animals (Fig. 3D).

Effects of cold exposure on the splicing pattern of CIRBP transcripts

We then investigated whether cold exposure influences the splicing pattern of CIRBP transcripts without inducing hypothermia. Even after keeping mice at 4°C for 1 week, there was no change in the splicing pattern either in the brain or the heart (Fig. 3C). There was no significant difference in the ratio of long form to short form between the animals kept in a cold room and control animals kept at 22°C (Fig. 3D).

Effects of low temperature on the splicing pattern of CIRBP transcripts in peripheral blood leukocytes

We found that the shift in the splicing pattern was evident also in circulating peripheral blood leukocytes; that is, both the long form and short form of CIRBP were detected in leukocytes of euthermic mice anesthetized and maintained at 37°C, while the short form was dominantly expressed in leukocytes of hypothermic mice (Fig. 4A). Hence, we carried out in vitro experiments using isolated leukocytes to determine factors contributing to the shift in the splicing pattern.

To investigate whether cold-sensitive transient receptor potential (TRP) channels are involved in alternative splicing of CIRBP mRNA, we treated blood samples with a TRPM8 agonist, l-Menthol, or a TRPA1 agonist, AITC, at 37°C for 1 h. There was no change in the splicing pattern in the presence of either the TRPM8 agonist or the TRPA1 agonist (Fig. 4B).

To assess the net effect of low temperature, we incubated peripheral blood at 28°C or 37°C. The long form and short form of CIRBP were identified at 10 min after starting incubation at 28°C. Shifts in alternative splicing of CIRBP became apparent at 30 and 60 min (Fig. 4C). On the other hand, the splicing pattern was unchanged in leukocytes incubated at 37°C for 60 min (Fig. 4C).

DISCUSSION

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We showed in the present study that several splicing variants of CIRBP mRNA are constitutively expressed in various organs of mice in a euthermic condition. This is unusual because the expression levels of cold shock proteins are generally low under a condition in which euthermic levels are maintained (15, 16). However, the constitutive expression may be important since the presence of multiple variants in a basal state is essential to regulate the expression of CIRBP by shifting the pattern of splicing. The functional role of the CIRBP isoform derived from the long form variant is not known. Sequence analysis predicts 5 amino acids in the C-terminal arginine-glycine-rich motif are replaced with 9 different amino acids. Considering that the C-terminal motif is related to CIRBP functions (1, 5), it can be postulated that the isoform encoded by the long form variant is insufficient to exert function as a cold shock protein. On the other hand, the iso-
Fig. 3 Effects of hypothermia on the expression pattern of CIRBP mRNA in the brain and heart of mice. (A) The photograph shows the splicing variants of CIRBP mRNA in the brain and the heart of three hypothermic mice with a representative example in a euthermic condition for comparison. The alternative splicing pattern was changed in hypothermic mice, in which the short form was dominantly detected. (B) Samples were obtained from mice for which body temperature was maintained at 37°C under isoflurane anesthesia. The photograph shows three representative examples with a representative example in an euthermic condition without anesthesia and that in a hypothermic condition for comparison. (C) Samples were obtained after keeping mice at 4°C for 1 week. The photograph shows three representative examples with a representative example in an euthermic condition and that in a hypothermic condition for comparison. (D) Summary graphs of ratios of the long form (800 bp) to the short form (500 bp) in an euthermic condition (n = 5), in a hypothermic condition (n = 4), in an anesthetic and maintained euthermic condition (n = 4) and in a cold exposure condition (n = 5) are shown. The values are normalized as ratios in the euthermic condition. Each bar represents the mean of data ± SD. *P < 0.05, **P < 0.01.
Splicing pattern of mouse CIRBP

prior to hypothermia as a preparatory response. Based on this idea, we consider that cold stimuli applied to the body surface of an animal would trigger endogenous neuronal and/or humoral pathways, which cause the shift in the splicing. However, keeping the mice in a cold environment for up to 1 week failed to promote a shift in the splicing. It can be expected that the applied cold stimuli were sufficient since exposure to cold for 1 week causes adaptive changes of brown adipose tissue (8, 23). Accordingly, it is reasonable to conclude that a substantial reduction of temperature is a major cause of the regulation of alternative splicing of CIRBP transcripts. In agreement with this, the testis, the temperature of which is lower than the core temperature (21), showed the splicing pattern observed in hypothermic animals even in an euthermic state.

The precise mechanism by which the alternative splicing is regulated remains unknown. Since cold-shock proteins exert protective effects to tolerate cold stress (6, 9, 10, 26, 31, 32), it is possible that the expression of functional CIRBP is brought about prior to hypothermia as a preparatory response. Based on this idea, we consider that cold stimuli applied to the body surface of an animal would trigger endogenous neuronal and/or humoral pathways, which cause the shift in the splicing. However, keeping the mice in a cold environment for up to 1 week failed to promote a shift in the splicing. It can be expected that the applied cold stimuli were sufficient since exposure to cold for 1 week in mice causes adaptive changes of brown adipose tissue (8, 23). Accordingly, it is reasonable to conclude that a substantial reduction of temperature is a major cause of the regulation of alternative splicing of CIRBP transcripts. In agreement with this, the testis, the temperature of which is lower than the core temperature (21), showed the splicing pattern observed in hypothermic animals even in an euthermic state.

Temperature-dependent change in alternative splicing has been reported as a regulatory mecha-
nism of β-globin expression in mammalian cells (7). In our study, the splicing pattern of CIRBP mRNA was changed in isolated leukocytes incubated at a low temperature in vitro. Also, similar temperature-dependent alteration of splicing has been reported in a flowering-related gene in plants (17, 27). The results of the present study together with results of reported studies indicate that change in alternative splicing in response to a low temperature is a universal and fundamental regulatory mechanism retained by a wide variety of cell types.

It is well known that TRP channels play roles in thermo-sensation. TRPM8 and TRPA1 channels can be activated within ranges of temperatures less than 37°C (2, 3). Therefore, it was of interest to assess the involvement of these cold-sensitive channels in the shift in the splicing pattern. Although the concentrations of agonists of TRPM8 and TRPA1 used in present study were sufficient to activate these channels (2, 3), these reagents caused no change in the splicing pattern in isolated leukocytes. On the other hand, a shift in the splicing pattern was promoted when the leukocytes were incubated at 28°C. Collectively, our results suggest that the shift of alternative splicing is promoted by a low temperature independently of the activation of TRP channels.

In conclusion, we revealed that the regulatory system of CIRBP expression at the level of alternative splicing, which was originally discovered in the hibernating hamster, commonly exists in non-hibernators such as mice. Enhancement of functional CIRBP expression might be applicable to advance therapeutic hypothermia and low temperature preservation of organs for transplantation.

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CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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