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Developmentally Regulated Expression of a Nonmuscle Myosin Heavy Chain IIB Inserted Isoform in Rat Brain

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The alternatively spliced isoform of the nonmuscle myosin II heavy chain B (MHC-B) with an insert of 21 amino acids at the 50- to 20-kDa junction of the globular region of myosin has been demonstrated to be expressed specifically in the central nervous system (CNS) in chicken. To explore the role of this B2 inserted isoform (MHC-B(B2)), immunoblot and immunoprecipitation analyses were performed using specific antibodies and extracts from rat tissues. MHC-B(B2) is present throughout the CNS, but is less abundant in the cerebrum and not expressed in the olfactory lobe at all. In the developing rat brain, MHC-B(B2) is expressed from postnatal day 10 (P10) in the cerebellum and increases markedly from P14. The appearance of MHC-B(B2) in the cerebrum (P28) is later than in the cerebellum. Additionally, we show that myosin IIB(B2) is homodimeric in its heavy chain subunit composition. These results suggest that myosin IIB(B2) might participate in cell motility in the neuronal cells of the mature CNS. © 1999 Academic Press

Myosin is a ubiquitous cytoskeletal protein present in all eukaryotic cells. It produces motor activity together with actin filaments generated by ATP hydrolysis. Recently a number of novel myosin isoforms have been identified by molecular cloning, constituting a superfamily of myosin molecules (for reviews, see Ref. 1, 2). Myosin II (conventional myosin) molecules are the best studied isoforms and are composed of a pair of heavy chains and two pairs of light chains. All vertebrate cells, including muscle cells, contain a form of myosin II referred to as nonmuscle myosin II. Nonmuscle myosin II plays a role in cell motility processes such as cytokinesis, migration, and shape change (for a review, see Ref. 3). To date, at least two different isoforms of the nonmuscle myosin II heavy chain have been identified in vertebrate cells (4, 5). They are referred to as MHC-A and MHC-B (5). The expression of the mRNAs encoding these two isoforms is tissue-dependent (5). The entire cDNA sequence encoding both isoforms has been determined from chicken (6, 7), human (8–11), *Xenopus* (12, 13) and that encoding MHC-A from rat (14).

Alternatively spliced isoforms of MHC-B encoding two cassettes of inserted amino acids at different locations in the MHC head region were found during the cloning of the cDNA encoding chicken brain MHC-B (7). These isoforms were expressed in a tissue dependent manner in that they were specific to the central nervous system (CNS). One insert of 10 amino acids (referred to as B1) was located at the 25–50-kDa domain junction close to the ATP binding region and another insert of 21 amino acids (referred to as B2) was located at the 50–20-kDa domain junction close to the actin binding region.[2] The mRNA's encoding these inserted isoforms are generated by the tissue specific alternative splicing of a single pre-mRNA for MHC-B. The mechanism regulating the neuron specific alternative splicing of the MHC-B(B1) was studied in cultured cells(15). It was reported previously that the mRNA encoding the B1 inserted isoform was expressed early in embryonic development, while expression of the mRNA encoding the B2 inserted isoform began just before birth in the developing chicken (16). It was also reported that the heavier isoform of MHC-B started to appear two weeks after birth in the mouse cerebellum (17). The knockout mice study demonstrated that the myosin IIB was required for normal development of a brain as well as a heart (18).

In this work, we report the MHC-B(B2) is expressed exclusively in the CNS of the rat. We also show the exact timing for the appearance of MHC-B(B2) during the postnatal development of rat brain. Finally, we demonstrate that myosin IIB(B2) exists as a homodimer, with no evidence for heterodimeric formation between the inserted and noninserted isoforms.

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The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank under Accession Number U73303.

[2] According to the proposal of Itoh and Adelstein (16), the names of insert B1 and B2 were exchanged from the original naming (7).

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MATERIALS AND METHODS

RT-PCR and subcloning of PCR products. Total RNA was prepared from adult rat brain by the acid guanidinium thiocyanate-phenol-chroloform extraction method (19). One microgram of total RNA was reverse transcribed to generate first strand cDNA using random hexamer and AMV Reverse Transcriptase (Promega) The resulting cDNA was amplified using *Taq* DNA polymerase (Perkin Elmer). The oligonucleotides used as primers were 5′-CAACGT-GGCTA-CACTCCTGCACCAG-3′ for 5′ sense primer and 5′-CCT-TCTTGGTCTTGTA-TGCAGAGCC-3′ for 3′ antisense primer. This primer set was designed from the cDNA sequence encoding chicken MHC-B to flank the B2 insert region. Thirty cycles of 93°C for 1 min, 58°C for 2 min, and 72°C for 1 min with a final extension of 3 min were performed. The fragments of expected size, 140 and 203 base, were eluted from the agarose gel, filled in by Klenow fragment and subcloned into the *Eco*RV site of pBluescriptIISK$^+$ (Stratagene). cDNA clones were sequenced with Sequenase enzyme kits (Amersham) using the dideoxy chain-termination method (20).

Antibody production. A peptide of 12 amino acids was synthesized based on the derived amino acid sequence at the rat MHC-B(B2) insertion (ASFYDSVSGLHE) (see Fig. 1). This sequence is specific for MHC-B(B2) (the inserted sequence). Conjugation of the peptide to keyhole limpet hemocyanin and immunization of rabbits were performed according to the protocol (21). The titer was checked by ELISA using the specific peptide and by immunoblot analysis with rat brain extracts. Anti-rat MHC-B(B2) was purified on a peptide antigen column prepared by coupling 15 mg of the peptide with 25 ml of Affi-Gel 15 affinity support (Bio-Rad) in a total volume of 50 ml of 0.2 M MOPS (pH 7.2) according to the manufacturer's instructions. Antiserum and affinity purified antibodies specific for human MHC-A and MHC-B that recognize the carboxyl-terminal region of each isoform were generously donated by Dr. Robert S. Adelstein (NHLBI, NIH).

SDS-Polyacrylamide gel electrophoresis and immunoblotting. Rat tissues were homogenized in an extraction buffer (0.6 M NaCl, 5 mM EDTA, 5 mM EGTA, 14 mM 2-mercaptoethanol, 0.1 mM PMSF, and 40 mM MOPS, pH 7.6), and centrifuged at 15,000 g for 60 min at 4°C. The resulting supernatants were used as tissue extracts. The tissue extracts were separated on an SDS–5% polyacrylamide gel with 0.065% bisacrylamide, using the buffer system of Laemmli (22). The same amount of protein was loaded on each lane in each gel. The gel was electroblotted onto Immobilon-P (Millipore) and immunostained with antiserum. The antiserum was diluted as follows; anti-MHC-A 1 to 1000; anti-MHC-B 1 to 3000; anti-MHC-B(B2) 1 to 100. Following incubation with a second antibody coupled to HRP, the blots were developed using 4-chloro 1-naphtol as substrate.

Immunoprecipitation. Immunoprecipitation was performed according to a standard protocol (23). Cerebellum was dissected from adult rats, and homogenized in a buffer (150 mM NaCl, 1% IGEPAL CA-630 (Sigma), 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 5 μg/ml leupeptin, 20 mM Tris-HCl, pH 7.5). The extracts were made 5 mM with respect to ATP, and the insoluble material was sedimented at 265,000 g for 10 min at 4°C. Protein A–Sepharose beads (Pharmacia) were equilibrated with homogenization buffer containing 1 mg/ml BSA. The supernatants were pre-cleared using Protein A-Sepharose beads, and incubated with Protein A–Sepharose beads adsorbed with specific antibodies at 4°C for 2 hours. The immunocomplex was washed three times with homogenization buffer containing 1 mg/ml BSA followed by three times with PBS, eluted by boiling in sample buffer for SDS-PAGE, and subjected to immunoblot analysis and silver staining.

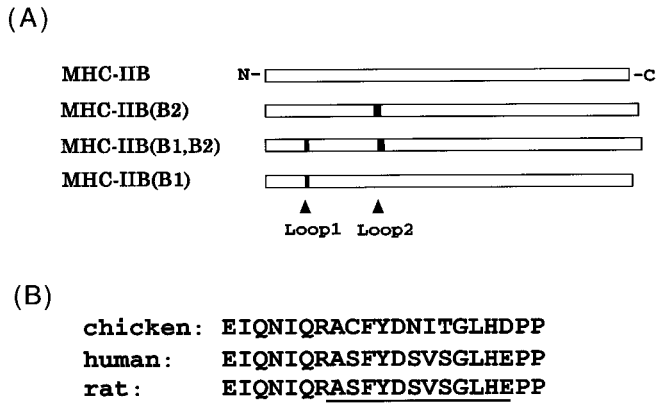

FIG. 1. Diagrams of the MHC-B alternatively spliced isoforms. (A) Four isoforms of MHC-B are expressed by alternative splicing in central nervous system tissues. The differences among the isoforms depend on whether B1 or B2 is inserted at the 25- to 50-kDa junction (loop 1) and the 50- to 25-kDa junction (loop 2), respectively. (B) The sequences of the B2 insert are aligned. The chicken and human sequences are from Takahashi *et al.* (7). The rat sequence was determined in this report. The underlined amino acids indicate the peptide used for generating antibodies to recognize the B2 insert specifically.

RESULTS

Tissue specific expression of MHC-B(B2). The amino acids sequence of the rat B2 insert derived from the RT-PCR product was identical to that of the human B2 insert (Fig. 1). In order to investigate the expression of MHC-B(B2) in rat tissues, antibodies to MHC-B(B2) were raised against a peptide synthesized on the basis of the derived amino acid sequence of the rat MHC-B(B2) inserted residues. A second source of anti-MHC-B antibodies which cross-react with MHC-B(B2) as well as noninserted MHC-B (Mr = 200,000) was also used. This is expected because these isoforms share the same carboxyl-terminal residues. The anti-MHC-B(B2) antibodies cross-react only with the slower migrating of the two bands recognized by the anti-MHC-B antibodies. Figure 2 shows the immunoblots of rat tissue extracts using three different antibodies. We analyzed extracts from six different parts of the brain and compared the results to extracts from five non-neuronal tissues that were expected to show high expression of the MHC-B isoform. Figure 2 demonstrates that MHC-B(B2) is expressed in a tissue dependent manner, that is, it is present in CNS tissues especially the cerebellum, brain stem, spinal cord, and di- and mesencephalon. It is significantly less abundant in the cerebrum and not expressed in the olfactory lobe at all. The distribution of MHC-B(B2) protein in rat brain is similar to chicken brain (7). The slower migrating band recognized with anti-MHC-B antibodies showed the same tissue distribution as the band recognized by anti-MHC-B(B2) antibodies. Figure 2 demonstrates that anti-MHC-A antibodies cross-react with polypeptides

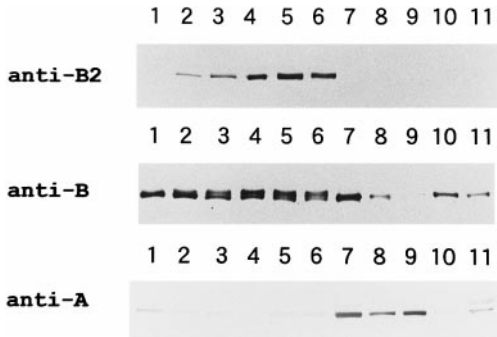

FIG. 2. Immunoblot analysis of extracts from rat tissues probed with specific antibodies. The extracts (75 μg/lane for anti-B2, 10 μg/lane for anti-B, and 50 μg/lane for anti-A) were subjected to SDS–5% polyacrylamide gel electrophoresis followed by immunoblotting. *Lanes 1–8* are as follows: *1*, olfactory lobe; *2*, cerebrum; *3*, di- and mesencephalon; *4*, cerebellum; *5*, brain stem; *6*, spinal cord; *7*, lung; *8*, kidney; *9*, adrenal; *10*, testis; *11*, posterior epididymis.

in extracts from lung, kidney, and adrenal. The anti-MHC-A antibodies detected a slower migrating band in addition to MHC-A in posterior epididymis.

Change in expression of MHC-B(B2) during rat brain development. To characterize whether expression of MHC-B(B2) protein in rat brain was developmentally regulated, selected rat brain tissues were individually dissected from newborn rats at various days after birth and were analyzed by immunoblotting. Figure 3 shows immunoblots of extracts from cerebrum, di- and mesencephalon, cerebellum, and brain stem. The figure demonstrates that expression of MHC-B(B2) protein is developmentally regulated in brain with different time profiles. MHC-B(B2) was not expressed in newborn rat brain. The presence of MHC-B(B2) was first observed in the cerebellum at postnatal day 10 and increased markedly from postnatal day 14 (P14). The appearance of MHC-B(B2) was slightly later in the brain stem (P14), obviously later in di- and mesencephalon (P18), and significantly later in cerebrum (P28) than in cerebellum. The figure also indicates that the total amount of MHC-B isoforms is almost the same after birth, that is, the amount of the non-inserted MHC-B isoform is decreased with the increase of MHC-B(B2).

Determination of myosin heavy chain dimer composition. We performed immunoprecipitations from extracts of cerebellum to analyze the association of the two MHC-B isoforms. Immunoprecipitates were separated by SDS-PAGE, and visualized by silver staining or immunoblotting with either anti-MHC-B(B2) antibodies or anti-MHC-B antibodies (Fig. 4). The figure shows that only MHC-B(B2) heavy chains were immunoprecipitated with anti-MHC-B(B2) antibodies, while both heavy chains were immunoprecipitated with anti-MHC-B antibodies. If a heterodimer consisting of one MHC-B heavy chain and one MHC-B(B2) heavy chain exists in the extracts, both polypeptides should be visualized by silver staining or immunoblotting with anti-MHC-B antibodies. These results confirm that myosins IIB(B2) and IIB are homodimeric with respect to heavy chain subunit composition in the cerebellum.

DISCUSSION

We present data showing that the nonmuscle MHC-B(B2) isoform is expressed at the protein level in a tissue dependent and developmentally regulated manner in the rat CNS. We also present data showing that myosin IIB(B2) exists as homodimer in brain. The B2

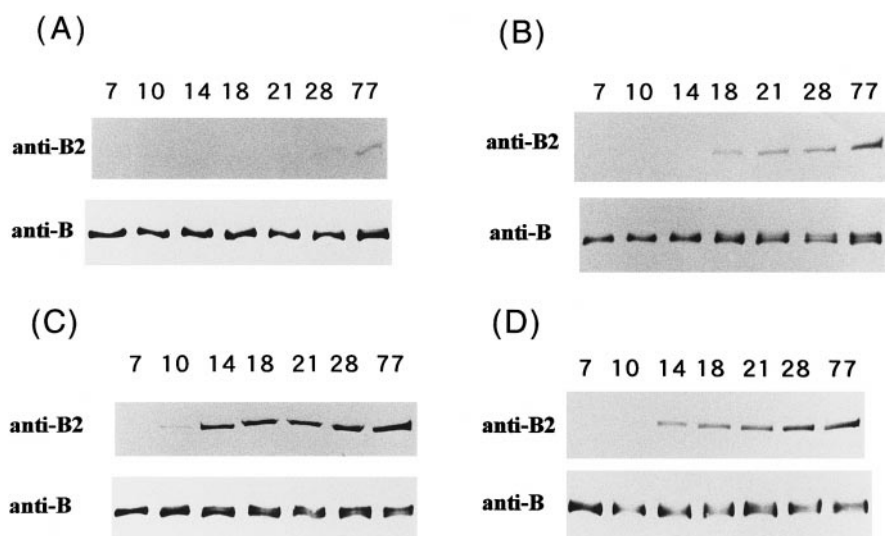

FIG. 3. Immunoblot analysis of extracts from postnatal developing rat cerebrum (A), di- and mesencephalon (B), cerebellum (C), brain stem (D) with specific antibodies. The extracts (75 μg/lane for anti-B2, 10 μg/lane for anti-B) were subjected to SDS–5% polyacrylamide gel electrophoresis followed by immunoblotting. The numbers above each set of immunoblots represent postnatal days.

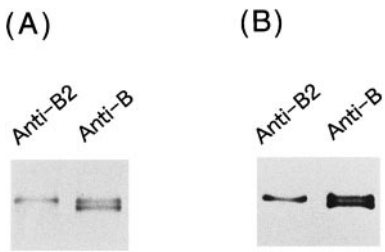

FIG. 4. Immunoprecipitation of myosin IIB isoforms from rat cerebellum extracts with specific antibodies. (A) Silver stained SDS–5% polyacrylamide gel of the immunoprecipitated products. (B) Immunoblot of the immunoprecipitated products with anti-B serum. Anti-B2 and Anti-B indicate the antibodies used for immunoprecipitation.

insert was first identified in chicken during the cloning of the cDNA encoding brain MHC-B (7). In the CNS tissues of adult chicken, the expression of MHC-B(B2) in the cerebrum was obviously lower than in cerebellum, brain stem, di- and mesencephalon, and the spinal cord (7). In adult human and bovine brains, however, MHC-B(B2) was expressed to comparable levels in the cerebrum and cerebellum (16). A similar distribution of MHC-B(B2) (referred to there as NM1) was also demonstrated in adult human brain by Kimura *et al.* using a monoclonal antibody specific for brain myosin (24). Our results on the distribution of MHC-B(B2) in rat brain were similar to that found for chicken brain. Murakami *et al.* (25) reported that the heavier isoform of MHC-B (referred to as $MIIB_1$) is expressed more abundantly in cerebellum than in cerebrum in rat brain. It is very possible that $MIIB_1$ is identical to MHC-B(B2). As Itoh and Adelstein (16) suggested, the distribution of MHC-B(B2) in brain differs markedly among species. However, the results demonstrated here point out that the differences are not caused by the differences between avian and mammalian species. Interestingly, MHC-B(B2) is not expressed in *Xenopus* tissues, whereas MHC-B(B1) is constitutively expressed in all *Xenopus* tissues (13). This suggests that MHC-B(B2) might have started to be expressed in di- and mesencephalon, cerebellum, brain stem, and spinal cord after the amphibian period. Thereafter the expression of this isoform may have extended to the cerebrum after the appearance of mammals. It is interesting that MHC-B(B2) expression is decreased in the part of the rat brain that originates from the forebrain.

We have demonstrated that MHC-B(B2) is not present in the newborn rat brain and only becomes apparent with different timing in distinct regions during postnatal development of the rat brain. Murakami *et al.* showed that $MIIB_1$ started to appear two weeks after birth in the mouse cerebellum (17). Our observation on the timing of the appearance of MHC-B(B2) in the rat cerebellum was in good agreement with theirs. The emergence of the B2 inserted isoform in the cerebellum is similar in timing with the morphological development of the rat cerebellum with respect to the migration of the neuronal cells and the formation of the dendritic trees of Purkinje cells (26). Based on this, the role of MHC-B(B2) might be related to maintaining cell localization and morphology particular in a mature brain. It should be noted that the appearance of MHC-B(B2) in the cerebrum is remarkably later than in cerebellum, brain stem, and di- and mesencephalon. Morphological development such as the migration and differentiation of the neuroblasts in the cerebrum is completed much earlier than in cerebellum (27). Thus the appearance of MHC-B(B2) would be too late to be associated with the completion of morphological changes during development. There must be some unknown event of cell motility relating to the function of MHC-B(B2) in the postnatal developing cerebrum.

The locations of the inserted amino acids in the MHC-B head region are important for the function of the myosin molecule. The B1 insert is located at the 25–50-kDa junction close to the ATP-binding region, whereas the B2 insert is located at the 50–20-kDa junction close to the actin binding region. These sites are the locations of loop 1 and loop 2, respectively, which are the least conserved regions in the myosin molecule according to Spudich (28). He suggested that these regions would play an important role in the function of myosin ATPase activity during the interaction with actin. For example, the 50–20-kDa junction region (loop 2) has been shown to be important for determining the enzymatic activity of myosin by using a chimaeric myosin expressed in *Dictyostelium* (29). The 50–20-kDa junction of vertebrate smooth muscle myosin has been shown to be necessary for proper regulation mediated by the phosphorylation of the regulatory light chain of the myosin molecule (30, 31).

The above results suggest that the presence of the B2 insertion might alter the enzymatic or motor activity of myosin IIB by changing the interaction with actin in a CNS tissue. It has been demonstrated that myosin purified from brain showed different properties compared with those purified from other nonmuscle tissues. For example, brain myosin filaments were fairly stable, even in a low salt solution, in the presence of ATP in contrast to myosin isolated from other nonmuscle and smooth muscle (32, 33). The brain myosin filaments tended to form ladder-like and net-like aggregated structures mediated by head to head association of myosin molecules (32, 34). Since brain tissue is enriched for myosin IIB, these properties might be related to myosin IIB. Another possibility is that the B2 insertion provides brain myosin with a unique property. To prove this, however, it may be necessary to separate pure myosin IIB(B2) from the mixture of myosin IIB isoforms and then compare the biochemical characters of each isoform. We demonstrated that my-

osin IIB(B2) is homodimeric with respect to its heavy chain subunit composition. This result may help us to separate the isoforms from a brain.

The distribution of myosin IIB isoforms in brain tissue sections has been reported using immunohistochemical studies (35, 36). The expression of myosin IIB was observed in most neuronal cells. In rat cerebellum sections, the cell bodies of Purkinje cells and their dendrites in the molecular layer were stained dramatically. The myosin IIB(B2) isoform would most likely colocalize somewhere in this region. Although the role of myosin IIB itself in the neuronal cells is still unclear, Smith has proposed that an actin based motor could play an important role in the motility of the growth cones (37). It has been reported that myosin IIB was localized at the leading edge of the growth cones and might be involved in extension (36, 38) or retraction (39) of growth cone. On the other hand, it has been proposed that myosin II could be involved in neurotransmitter release (40). We speculate that myosin IIB(B2) might participate in cell motility with reference to the neuronal cells in mature CNS tissue, to modify the function of myosin IIB. It is still unclear whether myosin IIB(B2) and myosin IIB are expressed in the same cell or the former is expressed only in a particular set of cells in CNS tissues. An answer to this question is essential to understanding the function of myosin IIB(B2).

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