Non-muscle myosin IIB-like immunoreactivity is present at the drebrin-binding cytoskeleton in neurons

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Abstract

Dendritic spines are extremely motile, providing a structural mechanism for synaptic plasticity. Actin-myosin interaction is thought to be responsible for the change in the shape of spine. We have already reported that drebrin, an actin-binding protein, inhibits actin-myosin interaction and is enriched in the dendritic spine of mature neurons. In this study, we prepared the actin cytoskeleton of dendritic spines as an immunoprecipitate with anti-drebrin antibody from adult guinea-pig brain, immunized mice with the cytoskeleton, and obtained a monoclonal antibody (MAb) called MAb G650. MAb G650 reacted with non-muscle myosin IIB, but it did not react with muscle myosin II or non-muscle myosin IIA. Immunoblotting with this antibody revealed that drebrin-binding cytoskeleton contains this myosin IIB-like immunoreactivity. Immunohistochemistry using MAb G650 demonstrated that this myosin IIB-like immunoreactivity can be detected in the neuronal cell bodies and their apical dendrites, where drebrin is hardly detected. These data demonstrate that a myosin subtype associated with drebrin-binding actin filaments in the dendritic spines is myosin IIB, although this myosin is widely distributed in somato-dendritic subdomains of neurons. Furthermore, it is indicated that the cytoskeletons in dendritic spine were uniquely characterized with actin-binding proteins such as drebrin, but not with myosins. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Actin–myosin interaction is thought to be involved in the regulation of spine shape (Morales and Fifkova, 1989; Hayashi et al., 1996), which changes during the induction of synaptic plasticity (Van Harreveld and Fifkova, 1975; Fifkova and Van Harreveld, 1977; Lee et al., 1980; Chang and Greenough, 1984; Desmond and Levy, 1986; Hosokawa et al., 1995). Although the unique character of actin filaments in the dendritic spines are reported (Hayashi et al., 1996), very little is known about myosins in the dendritic spines.

Three classes of the myosin superfamily are represented in the vertebrate brain: myosins I, II and V. Brain myosin II is classified as non-muscle myosin II. Non-muscle myosin heavy chains are similar in size to muscle myosin heavy chains, but they are encoded by different genes. At least two distinct genes have been isolated for non-muscle myosin heavy chains, namely IIA and IIB (Takahashi et al., 1992), and four splice-variants of mRNA have been reported for the IIB genes (Itoh and Adelstein, 1995). This means that there are at least five different messages for the expression of non-muscle myosin heavy chains. Multiple isoforms of the non-muscle myosin heavy chain have also been detected with protein analyses (Kimura et al., 1993; Murakami et al. 1993). The tissue distributions of these isoforms were analyzed using antibodies. Some of them are ubiquitous, others are brain-enriched, and some are brain-specific (such as myosin IIB1; Takahashi et al., 1992; Murakami et al., 1993). A more specific antibody is necessary to reveal the exact localization of each myosin isoform.

We previously reported that cytoskeletons in dendritic spines could be immunoprecipitated with anti-drebrin antibody (Shirao, 1995; Hayashi et al., 1996). The immunoprecipitate contains drebrin, actin, gelsolin and myosins. Using this immunoprecipitate as an anti-
gen, we obtained a monoclonal antibody (MAb) against myosin in the brain. This antibody reacted with myosin heavy chains in the cytoskeletons in the dendritic spines of brain. The biochemical and immunohistochemical characterization of this isoform was then analyzed.

2. Materials and methods

2.1. Immunoprecipitation with a monoclonal antibody

Immunoadfinity beads were prepared using the method described by Hayashi et al. (1996). A MAb M2F6 specific to drebrin (Shirao and Obata, 1986) was cross-linked to protein A-Sepharose 4FF (Pharmacia, Broma, Sweden) with dimethylpimelimidate (Harlow and Lane, 1988).

To prepare the immunogen used in this study, the cerebral cortices of adult guinea-pigs were homogenized in nine volumes of 5 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5 mM DTT, 1% NP-40 and protease inhibitors (1 μM leupeptin, 250 μM PMSF, 2 μM pepstatin) by ten strokes with a Teflon-glass homogenizer. The homogenate was centrifuged at 16,000 × g for 20 min. The immunoaffinity beads were incubated with the supernatant described above at 4°C for 2–3 h, recovered by centrifugation, and washed with the homogenization buffer. The extract from the beads with 0.25% SDS was used as the immunogen.

To characterize the antigen of a MAb, an extract of adult rat cerebral cortices was incubated with immunoaffinity beads prepared with the MAb. In some cases, immunoprecipitates were washed with 1 mM ATP to examine whether the antigen was an ATP-dependent actin-binding protein. Immunoaffinity beads prepared with anti-synaptophysin antibody, MAb 171B5 (Obata et al., 1986), were used as a control.

2.2. Production of monoclonal antibody

The immunoprecipitates of actin filaments with the anti-drebrin antibody from 10-week-old guinea-pig brains were injected into BALB/c mice. Spleen cells were fused with myeloma P3-X63Ag-U1 (P3U1), using standard selection and single cell cloning techniques (Harlow and Lane, 1988). Antibody-producing clones were screened by immunoblotting analysis of the detergent (1% NP-40)-insoluble fraction obtained from rat brains.

2.3. Electrophoresis and immunoblotting

The proteins that bound to the immunoaffinity beads or rat tissues were homogenized with eight volumes of Laemmli’s SDS-sample buffer, and boiled for 3 min (Laemmli, 1970). Samples (equivalent to 0.2 mg wet weight of tissue) were subjected to SDS-polyacrylamide gel electrophoresis as described previously (Shirao and Obata, 1985). To separate the isoforms of non-muscle myosin heavy chain, crude myosin extracts from rat brain prepared with 10 mM imidazole–HCl (pH 7.5) containing 0.5 M NaCl, 10 mM ATP, 10 mM MgCl₂, 2 mM EGTA, 5 mM 2-mercaptoethanol and 0.01% NaN₃ were electrophoresed in SDS-5% polyacrylamide gels with a ratio of acrylamide:bisacrylamide of 30:0.25 as described previously (Murakami et al., 1991). To distinguish myosin heavy and light chains, samples were electrophoresed in SDS-12.5% polyacrylamide gels with a ratio of acrylamide:bisacrylamide of 30:0.25 (Ishikawa et al. 1989). In some cases, the acrylamide gels were silver-stained with 2D-Silver Stain II (Daiichi Pure Chemicals, Tokyo, Japan).

The separated proteins were transferred to Immobilon transfer membranes (Millipore, MA). The membranes were incubated in skimmed milk for more than 4 h and then with the first antibody for 1 h at room temperature. The anti-myosin-IIA antibody was a polyclonal antibody against human platelet myosin (Biomedical Technologies, Stoughton, MA). The anti-drebrin antibody was a supernatant of hybridoma cells producing MAb M2F6 (Shirao and Obata, 1986). Anti-β-actin MAb (Sigma, St Louis, MO) was used to detect actin. After washing in phosphate-buffered saline (PBS) for 30 min, the membranes were incubated with the second antibody (peroxidase-conjugated goat IgG against rabbit or mouse IgG; Cappel, Durham, NC) for 1 h, washed again, and incubated with 3,3’-diaminobenzidine (DAB).

2.4. Immunohistochemistry

Ten-week-old Wistar rats were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and the brains were removed and immersed in the same fixative for 2 h. Cryosections, 10 μm thick, were treated with 0.1% Triton X-100 in PBS for 30 min and incubated with 3% bovine serum albumin (BSA) in PBS for more than 1 h. They were then incubated with the first antibody for 1 h. Immunoreactivity was visualized by the avidin–biotin–peroxidase complex method (Vectorstain ABC kit, Vector Labs). For double-immunostaining of G650 antigen and drebrin, permeabilized and BSA-treated sections were incubated with a mixture of rabbit antiserum against drebrin and MAb G650. The second antibody was a mixture of rhodamine-conjugated antibody against rabbit IgG and FITC-conjugated antibody against mouse IgG (Cappel, Durham, NC). Specimens were observed with a confocal laser microscope (MRC 1024; Bio-Rad, Richmond, CA). All the procedures took place at room temperature.
drebrin–actin complex in the spine, we immunoprecipitated actin cytoskeletons with anti-drebrin antibody from a detergent extract of guinea-pig brains. As with the rat, the major proteins in this precipitate were myosin, drebrin, gelsolin and actin (data not shown; Hayashi et al., 1996). We immunized mice with this precipitate and fused their spleen cells with myeloma cells to produce monoclonal antibodies. One MAb, named MAb G650, reacted with a 220-kDa band in the rat brain homogenate (Fig. 1A, left lane). The 220-kDa band was detected in the anti-drebrin immunoprecipitate (middle lane), but was not detected in the antisynaptophysin immunoprecipitate (right lane for negative control). When the anti-drebrin precipitate was washed with 1 mM ATP, the 220-kDa protein was eluted (Fig. 1B), raising the possibility that the 220-kDa protein may be a heavy chain of myosin II. Drebrin was not eluted with ATP, but was eluted with acidic glycine buffer (Fig. 1B).

3. Results

3.1. MAb G650

In order to identify the myosin subtype that bound to the 220-kDa protein, we immunoprecipitated myosin with MAb G650. In addition to IgG, the immunoprecipitate from the cerebral cortices of adult rats contained the 220-kDa protein and a 43-kDa protein. When the immunoprecipitate was washed extensively with 1 mM ATP, the 43-kDa protein was eluted from MAb-G650-affinity beads.
Fig. 3. Identification of the G650 antigen as a non-muscle type of myosin IIB. (A) A brain homogenate was electrophoresed in a wide lane of a less cross-linked polyacrylamide gel, and was cut into two strips. One strip was immunostained with MAb G650 (as indicated) and the other was stained with anti-platelet-myosin (indicated by IIA). The a G650 antigen migrated a little more slowly than the band recognized by the anti-platelet myosin heavy chain. (B) Extracts of human platelet (lanes 1 and 3) and COS cells (lanes 2 and 4) were electrophoresed and immunoblotted with MAb G650 (lanes 1 and 2) and anti-platelet-myosin antibody. Note that myosin IIA in human platelets was not detected with MAb G650.

The mobility of the G650 antigen was then examined in a less cross-linked polyacrylamide gel. The mobility was a little slower than that of the band stained with anti-platelet-myosin antibody that is supposed to recognize type IIA (Fig. 3A). Human platelets only contain myosin IIA (Maupin et al., 1994), and COS cells only contain myosin IIB (Tullio et al., 1997). MAb G650 recognized a 220-kDa band in COS cells by immunoblotting, but not in human platelets (Fig. 3B, left lanes); while anti-myosin-IIA antibody recognized a 220-kDa band in human platelets, but not in COS cells (Fig. 3B, right lanes). These data suggest that G650 antigen is a myosin IIB isoform.

3.3. Tissue distribution of G650 antigen

SDS extracts from the thymus, kidney, lung, liver, spleen, small intestine, stomach, heart, skeletal muscle, and brain were subjected to immunoblotting and the tissue distribution of G650 antigen was examined. With the anti-platelet myosin antibody, 220-kDa bands were clearly detected in most of the tissues except the heart and skeletal muscles (Fig. 4A). In contrast, MAb G650 recognized intense 220-kDa bands in the brain and lung, and a weak band in the thymus, and kidney, but not in other tissues (Fig. 4B). Fig. 4C shows the profile and the amount of protein loaded on the gel. When regional differences within the brain were examined, no clear difference was found in the amount of G650 antigen in different regions of the brain (Fig. 5A), whereas the amount and isoform composition of drebrin was region-dependent (Fig. 5B), as described previously (Kojima et al., 1993; Hayashi et al., 1996).

beads (Fig. 2A, lane 2). This 43-kDa band was immunostained with anti-ß-actin antibody (Fig. 2B, lane 3). Furthermore, silver staining (Fig. 2C) showed that the immunoprecipitate with MAb G650 contained two additional bands, of 20 and 14 kDa (Fig. 2C, lane 2). The apparent molecular sizes of the additional bands are similar to those of the regulatory light chains of smooth muscle myosin purified according to the method previously described by Ebashi (1979). Finally, the antiserum raised against the non-muscle myosin IIB (kindly gifted by Dr R S Adelstein) (Takahashi et al., 1992) immunostained the 220-kDa protein in the immunoprecipitate, although the antiserum against non-muscle myosin IIA (kindly gifted by Dr R S Adelstein) did not. These results indicate that MAb G650 recognizes a heavy chain of myosin II.

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3.4. Localization of G650 antigen in the brain

Immunocytochemical localization of G650 antigen in the rat brain sections was compared with the localization of drebrin. In the cerebral cortex, there is a similar distribution of G650 as compared with drebrin. They are heavily distributed in the neuropil region of the cortex, although G650 antigen was also weakly observed in the axonal tract (Fig. 6A and D). Higher magnification of light microscopy revealed that cell bodies and apical dendrites in addition to scattered dots were intensely immunostained with MAb G650 (Fig. 6B and C). By contrast, it may be noted that drebrin could be hardly observed in the cell bodies (Fig. 6E). Blood vessels were not stained in any of the regions examined with MAb G650. In contrast, the anti-myosin II A antibody stained capillaries and the endothelium of blood vessels (data not shown) as reported by Murakami and Elzinga (1992).

Confocal laser microscopic observation of double immunostaining with an anti-drebrin antibody and MAb G650 revealed that G650 antigen and drebrin colocalized on many scattered dots, although some G650-positive dots were not immunostained with anti-drebrin antibody and vice versa (Fig. 7). The cell bodies were only stained with MAb G650, but not with an anti-drebrin antibody.

Fig. 8 shows the sagital sections of cerebellar cortex stained with MAb G650. Immunostaining was observed in the molecular layer, Pukinje cell layer and granular layer, but was very faintly observed in the white matter. Closer examination revealed that the Purkinje cell bodies and their apical dendrites were strongly stained (Fig. 8B), which was similar to the staining pattern observed in the cerebral cortex.
4. Discussion

In this study, we obtained a MAb that recognizes a non-muscle myosin IIB. Immunoblotting using this antibody revealed that a myosin associated with the drebrin-binding cytoskeletons in the dendritic spine is non-muscle myosin IIB. Further we suggested that possible unique character of actin–myosin interaction in the dendritic spine depend on an actin-binding proteins but does not depend on a myosin subtype.

4.1. The antigen recognized by MAb G650

Our antibody, MAb G650, recognizes a 220-kDa protein in the drebrin-binding cytoskeletons of dendritic spines in the adult rat brain. This 220-kDa protein had non-muscle myosin IIB-like immunoreactivity. In addition to the 220-kDa protein, a 43-kDa protein, which was identified as actin by immunoblotting, and two smaller polypeptides were co-immunoprecipitated by immunoprecipitation with MAb G650. The apparent molecular weights of the two smaller polypeptides were similar to those of two light chains of smooth muscle myosin. Non-muscle myosin II consists of 220-kDa MHC and two smaller light chains, and binds to actin filament in an ATP-dependent manner. Taken together, it is thought that MAb G650 recognizes the MHC of non-muscle myosin II.

Many reports have classified MHC isoforms in the brain into types IIA and IIB (Murakami and Elzinga, 1992; Takahashi et al., 1992) which are distinguished by their electrophoretic mobility in less cross-linked polyacrylamide gels (Murakami et al., 1991). The MHC isoform that is recognized with MAb G650 migrated more slowly than myosin IIA in the less cross-linked polyacrylamide gel. Furthermore, immunoblotting analysis demonstrated that MAb G650 did not react with the MHC of myosin IIA in human platelets, but reacted with the MHC of myosin IIB in COS cells (monkey kidney epithelial cell line). Immunohistochemistry demonstrated that the cell bodies and apical dendrites of Purkinje neurons in the cerebellum and of pyramidal cells in the cerebral cortex were heavily stained with MAb G650. These staining patterns are very similar to patterns observed by others using anti-IIB antibodies (Murakami and Elzinga, 1992; Shimada et al., 1995). These data indicate that MAb G650 recognizes a myosin IIB type isoform, although it has not been known whether MAb G650 discriminate subtypes of MHC IIB, such as MHC IIB (B1) and (B2).

4.2. Myosin in the drebrin-binding cytoskeleton

In our previous study, we reported that the cytoskeleton of dendritic spine that was immunoprecipitated with anti-drebrin MAb contains myosin II (Hayashi et al., 1996). Immunohistochemical study using antiserum against myosin II also demonstrated that myosin II was found in dendritic spines (Miller et al., 1992). However it has not been elucidated which isoform of myosin II is in dendritic spines. In the present immunohistochemical study using MAb G650, the fine dot-like staining scattered throughout the grey matter in the cerebrum were much stronger than those reported previously by the others. Double labeling of myosin IIB and drebrin revealed partial colocalization of myosin IIB and drebrin, which is localized in the dendritic spine. Further myosin IIB isoform was enriched in the anti-drebrin immunoprecipitate. Taken together it is indicated that myosin IIB isoform is involved in the drebrin-binding cytoskeletons in the spine.

However, the subcellular distribution of myosin IIB is not restricted to the dendritic spine but more ubiqui-
tous compared with the restricted localization of drebrin in dendritic spines. Immunoblotting technique demonstrated that regional differences in myosin IIB expression within the brain have no relation with those of drebrin. Furthermore, drebrin was not enriched in the immunoprecipitate using MAb G650 (data not shown). These data suggest that myosin IIB is not unique in the dendritic spine, but is widely distributed within the neurons. By contrast, drebrin is uniquely localized in dendritic spines, and characterizes the actin filaments in the dendritic spine (Shirao et al. 1987; Hayashi et al., 1996). Taken all together, it is indicated that possible uniqueness of actin–myosin interactions in the dendritic spine, which may play roles in the synaptic plasticity, is not depend on the myosins but do on the actin-binding proteins such as drebrin.

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