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Localization of myosin IIB at the leading edge of growth cones from rat dorsal root ganglionic cells

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Primary cultures of rat dorsal root ganglionic (DRG) cells were stained with isoform-specific antibodies against non-muscle myosin II. Antibodies against the brain type myosin (MIIB) stained the peripheries of growth cones and non-neuronal cells. Double staining of the cells with the anti-myosin antibodies and rhodamine–phalloidin or anti-actin antibodies indicated that MIIB co-exists, with F-actin, at the leading edge. Antibodies against platelet myosin stained neither leading edges nor neurites, but stained the cell bodies of neurons and the stress fibers of non-neuronal cells. These results suggest that MIIB functions in the motility of the leading edge of DRG cells.

Myosin II; Heavy chain isoform; Growth cone

1. INTRODUCTION

Neuronal extension occurs primarily at the tip of growing neurites, but the mechanism for this process is not known. Involvement of an actin-based force generation system has long been suspected because actin is abundant in the leading edge of growth cones [1–3]. From previous studies it has been suggested that myosin II is not involved in the outgrowth of the growth cone, since myosin II has not been found there [4]. Recent studies on non-muscle myosins and their corresponding cDNAs indicate that distinctive myosin isoforms are expressed in non-muscle cells [5–9]. At least two heavy chain genes have been identified, MIIA (macrophage-type) [6] and MIIB (brain type) [8]. Based on their electrophoretic migration rates, these isoforms can be further subdivided [9,10]. In order to study the function(s) of non-muscle myosin in cell motility, it is essential to employ reagents that are specific to these myosin isoforms as molecular probes. In the studies reported here, we used anti-peptide antibodies specific for MIIB, and localized the MIIB isoform in the growth cones of cells cultured from rat dorsal root ganglion (DRG).

2. MATERIALS AND METHODS

The preparation and affinity purification of polyclonal antibodies against synthetic peptides having sequences corresponding to the C-terminal of human macrophage myosin (peptide IIA) and bovine brain myosin (peptide IIB) were described previously [9]. Antibodies against human platelet myosin heavy chains were purchased from Biomedical Tech. Inc. (Stoughton, MA). DRGs were homogenized with 10 mM Tris-HCl (pH 7.0), 5 mM EGTA, 5 mM EDTA, 5 mM 2-mercaptoethanol, 0.1 mM PMSF, 0.1 mM benzamidine-HCl, and 0.1 mg/ml of leupeptin, pepstatin A, and aprotinin. The homogenates (20 μg total protein for each lane) were applied for electrophoresis on 7% SDS-polyacrylamide gels followed by immunoblotting, as described [9,10]. Cultured cells grown on cover-glasses were permeated with cold methanol (−20°C), immediately fixed with 4% buffered paraformaldehyde and preincubated with 10% normal goat serum in phosphate-buffered saline. Polyclonal antibodies for myosin (1:500) and monoclonal antibodies for tubulin (Tago Inc., Burlingame, CA; 1:5,000) and actin (ICN, Costa Mesa, CA; 1:500) were used in combination with FITC-conjugated anti-rabbit IgG (Tago Inc.; 1:200) and rhodamine-conjugated anti-mouse IgG (Sigma, St. Louis, MO; 1:100). F-Actins were also stained by rhodamine–phalloidin (Molecular Probes Inc., Eugene, OR; 1:500). The cells were examined with a Zeiss 405 inverted light microscope and photomicrographs were taken with Kodak TMX-400 film.

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Abbreviations: PMSF, phenylmethylsulfonylfluoride; EGTA, ethylene bis(oxylenenitrilo)tetraacetic acid.

3. RESULTS AND DISCUSSION

Immunoblots of DRG homogenates (Fig. 1) indicated that anti-peptide IIB recognized one MIIB band. Comparison of the staining pattern with that seen in immunoblots of brain extracts revealed that the MIIB band in DRG cells is $MIIB_2$ (data not shown). This is consistent with the finding that $MIIB_2$ is the major cellular myosin isoform in nervous tissue of newborn animals (submitted for publication). Anti-peptide IIA antibody stained one band of MIIA weakly, which migrated at a different position from MIIB. Commercially available anti-platelet myosin, used here and by others [4,11], reacted with the MIIA band but did not react with the MIIB band found as the major isoform in newborn

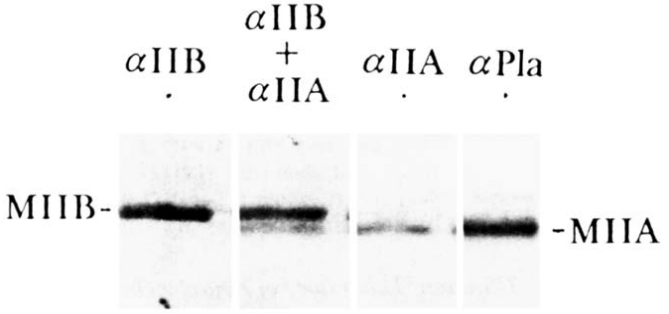

Fig. 1. Immunoblots of a DRG homogenate separated by SDS-7% polyacrylamide gel electrophoresis. α-IIB, α-IIA, α-pla represent anti-peptide IIB, anti-peptide IIA, and anti-human platelet myosin antibodies, respectively.

brain. In addition, anti-platelet myosin antibodies immunoprecipitated MIIA but failed to precipitate MIIB [10]. These data indicate that anti-platelet myosin antibodies do not react with the MIIB isoform both in native and denatured forms. Anti-peptide IIA stained the cells with a pattern similar to that given by anti-platelet myosin antibodies, but the staining was much weaker; the results of cell staining with anti-peptide IIA antibody are not shown.

Fig. 2a and c show the staining of a large, early-stage growth cone by anti-peptide IIB and rhodamine–phalloidin, respectively. The strong staining of the peripheral region of the growth cone by both reagents indicates that $MIIB_2$ and F-actin are co-localized in the motile leading edge of the growth cone. Both reagents

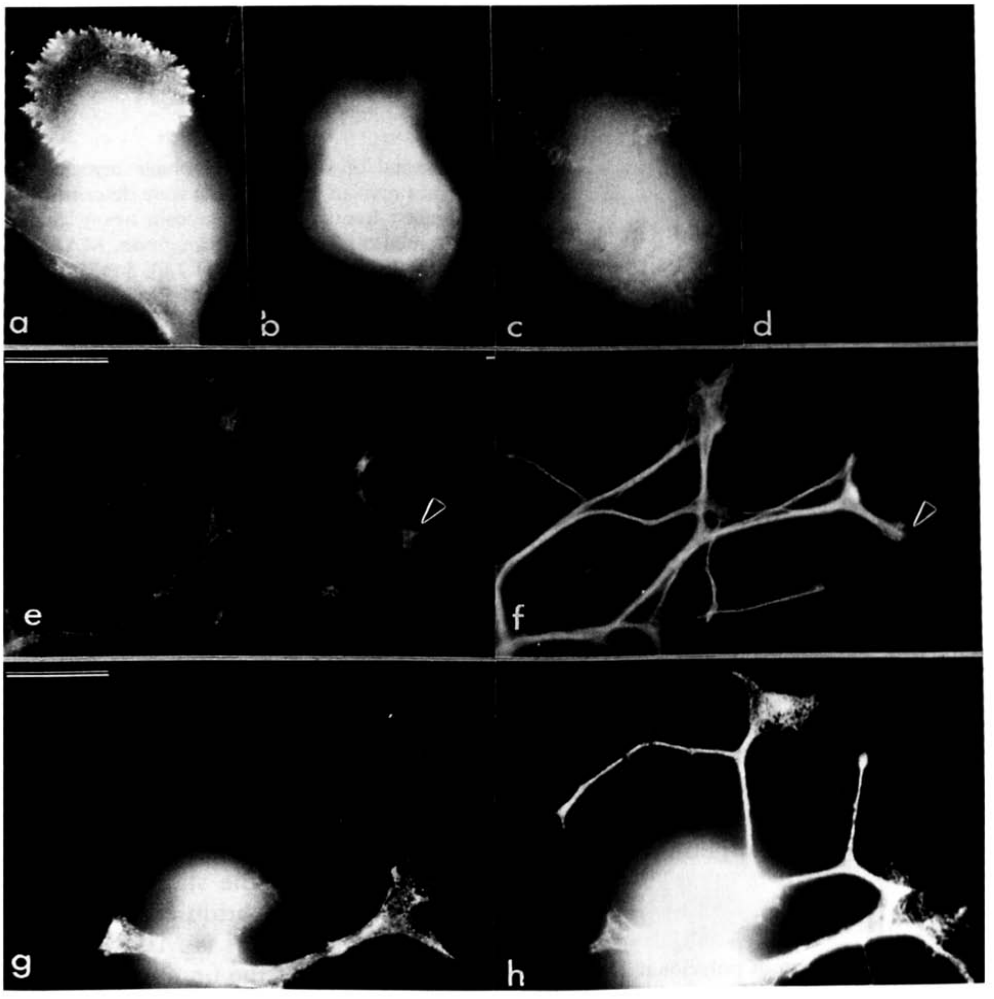

Fig. 2. Immunofluorescent staining of newborn rat DRG neurons. DRGs dissected from newborn rats were placed into Hank's balanced salt solution (HBSS) without calcium and magnesium (Sigma Chemicals, St. Louis, MO) and incubated with 3 U/ml of activated papain and 0.3 mg/ml of collagenase (Boehringer-Mannheim, Indianapolis, IN) at 37°C for 30–60 min followed by mechanical dispersal of the DRGs by using a pipette. The dispersed cells were rinsed with the HBSS with calcium and magnesium containing 10% fetal calf serum (Gibco Laboratories Inc., Grand Island, NY), resuspended with HBSS containing 10% fetal calf serum, 5% rat serum (Gibco), 0.3% glucose, 10 μg/ml of gentamycine, and 2 mM glutamine, and placed onto poly-L-lysine-coated glass coverslips in 35 mm dishes containing the same medium used for cell suspension. (a,b,e) Anti-peptide IIB; (c) rhodamine–phalloidin staining; (b) the cell seen in (a) and (c) is shown at a different focal plane; (d) anti-peptide IIB antibody preincubated with purified bovine brain myosin; (g) anti-human platelet myosin antibodies; (f,h), monoclonal anti-tubulin staining. In (a) and (c), a large new growth cone is shown. Arrowheads in (e) and (f) indicate the distal region of a growth cone. Bars = 20 μm.

also stained the cell body strongly and diffusely (Fig. 2a,c). In Fig. 2b, the same cell was photographed at a different focal plane to show that the intensely stained cell body was part of the cell from which the growth cone emanated. Preincubation of anti-peptide IIB with purified bovine brain myosin completely abolished the anti-peptide IIB staining (Fig. 2d), indicating that the staining with anti-peptide IIB (Fig. 2a and b) was specific to myosin IIB. In Fig. 2e and f, longer neurites with growth cones were double stained with anti-peptide IIB (Fig. 2e) and anti-tubulin (Fig. 2f) antibodies. Both antibodies stained neurites and growth cones, but not with exactly the same pattern, in that anti-peptide IIB stained filopodia-like structures at the distal regions of the growth cones beyond the area stained by anti-tubulin antibody (Fig. 2e and f, arrowheads). In contrast, anti-human platelet myosin antibodies did not clearly stain neurites or growth cones, but did stain neuronal cell bodies and non-neuronal cells (Fig. 2g); the neurites and growth cones, stained by the anti-tubulin antibodies (Fig. 2h), were refractory to the anti-platelet myosin antibodies (Fig. 2g). We conclude from these studies that MIIB is an integral constituent of the leading edges of nerve growth cones and of neurites, and also exists in cell bodies, while the myosin recognized by the anti-human platelet myosin antibodies exists, primarily or exclusively, in the neuronal cell bodies.

In non-neuronal cells in the same DRG cultures, the anti-peptide IIB antibodies stained the peripheral regions of the cells (Fig. 3a, arrowhead); these regions were visualized by anti-actin antibodies (Fig. 3b, arrowhead), indicating that MIIB and actin co-exist in this highly specialized region of these motile cells. However, the anti-platelet myosin antibodies stained the stress fibers intensely, and did not stain appreciably the leading edge (Fig. 3c and d, arrowheads). Thus cellular myosin isoform MIIB, but not MIIA, existed in the leading edges of both neuronal growth cones and non-neuronal cells in these cultures.

Our results strongly suggest that MIIB is an integral component of the force generation system responsible for extension of the leading edge of motile cells in the DRG cultures. However, the involvement of other molecules capable of generating force, such as myosin I [12], in neuronal growth cone motility cannot be excluded [11]. Bridgman and Daily [4] have reported that rat superior cervical ganglion neurons can be stained by anti-platelet myosin antibodies. They observed that the strongest staining was at the border between the central and peripheral regions of the growth cone, with little staining of the peripheral (leading edge) regions. We have found that the antibody they used is refractory to the MIIB isoform, under wide ranges of antibody concentrations [10]. Our immunocytochemical results agreed with those reported by others [11,13], using polyclonal antibodies made against purified myosins from mouse neuroblastoma cells and chicken brains, although the isoform-specific (MIIA vs. MIIB) distribution cannot be addressed by using these polyclonal antibodies. The results presented here strongly suggest that myosin isoforms are distributed in an isoform-specific pattern, and that isoform-specific probes are required in order to study the myosin function(s) within the cell.

There are at least two possible mechanisms for MIIB-mediated leading edge extension. One, analogous to muscle contraction, involves the interaction of MIIB filaments with anti-parallel F-actin filaments. Actin filaments at the edges of motile cells are known to be oriented with their pointed ends directed inward; this has been observed in growth cones [1–3], CHO cells [14], and at the leading lamella of fibroblasts [15]. However, if a small fraction of the actin filaments point outward and are not anchored, while a large fraction of the actin filaments point inward and are anchored at foci, bipolar

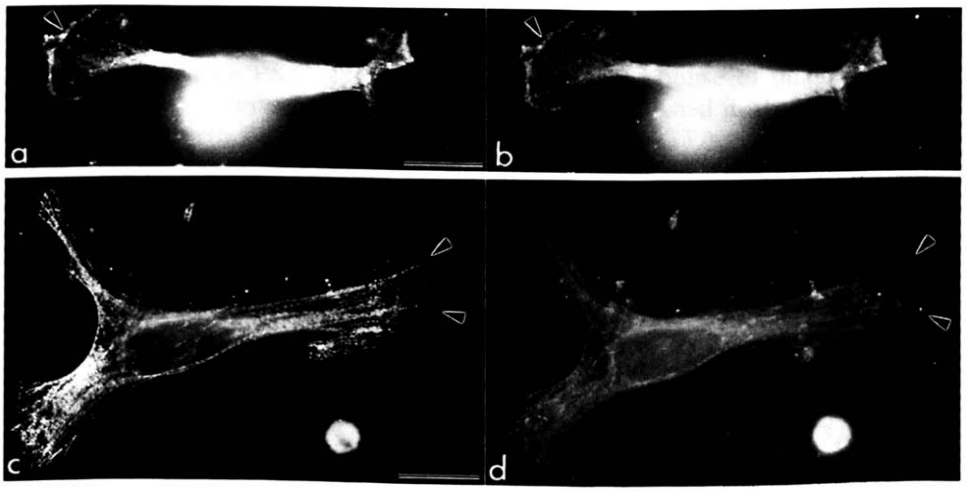

Fig. 3. Immunofluorescent micrographs of non-neuronal cells in the same DRG cultures. (a) Anti-peptide IIB; (b,d) monoclonal anti-actin antibody; (c) anti-human platelet myosin antibodies. Arrowheads indicate the leading edges of cells identified by anti-actin staining. Bars = 20 μm.

myosin filaments could generate shearing forces between the two actin filaments; the one that is anchored would remain fixed, while the other could be propelled outwards. Another possibility is that MIIB filaments interact with both cytoplasmic F-actin and membrane(s). The interaction of myosin with cytoplasmic membranes could be either direct, through binding to phospholipids [16], or indirect, through membrane bound F-actin [17,18]. The myosin heads interacting with the inward directed actin filaments could generate outward-directed force that would then tend to force the membrane in the direction of cell movement or growth.

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