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IMMUNOELECTRONIC LOCALIZATION OF MYOSIN-Va IN RAT CEREBELLUM

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ABSTRACT

Myosin V is an unconventional type of actin-binding myosin that participates in cytoplasmic organelle transport. Although this unconventional myosin has been extensively studied, its subcellular localization in the mammalian cerebellum has not been determined. In this work, we used an antibody against the tail domain of the myosin-Va heavy chain and a secondary antibody labeled with protein A-gold (15 nm) to study the subcellular distribution of this protein. Myosin-Va was found in the cytoplasm, where it was associated with a filament (probably actin). This protein was also detected in the plasma membrane of axons and dendrites in the molecular layer in rat cerebellum.

Key words: Cerebellum, electronic microscopy, myosin-Va, Purkinje cells

INTRODUCTION

Myosin-Va is an important member of a group of proteins collectively known as myosin-V. These proteins are involved in various cellular functions, including intracellular transport, signal transduction and the mechanoregulation of channels [28]. Myosin-V has been detected in cultured vertebrate [29] and invertebrate [26] neural cells, in proteins extracted from vertebrate brains [3,21], in the neural enteric system of rats [6], in guinea pig cochlea [4], and in the centrosome of the rat hippocampus [8]. Myosin-V has also been found in the dendritic and axonal terminations (growth cones) of chicken neurons [9,10,23,29], where it is responsible for the fusion of synaptic vesicles with the plasma membrane [21]. Other actin-binding proteins have also been found in neuronal cytoplasm of the adult rat forebrain [15,14,29].

Myosin-V belongs to a superfamily of myosins found in lower and higher eukaryotes [29] and was originally identified as an ATPase-dependent, calmodulin-binding protein [7,29]. Structurally, myosin-V contains two heads connected to a long chain with 12 light chains. As a motor protein, myosin-V associates with actin [17] and is involved in the movement of cellular organelles [4,24] and in the segregation and generation of subcellular compartments [29]. Myosin-V is also associated with the membranes of melanosomes, synaptic vesicles and the smooth endoplasmatic reticulum [19,29] in the growing cones of neurons [2,29] and in synaptosomes of the rat cerebral cortex [29]. In neurons, the motor activity that results from the association of myosin-V with actin contributes to the formation of dendritic spines and to the formation of new connections [10]. A lack of myosin-V results in neural and pigmentation deficiencies in mice and humans [3], with these deficiencies being characteristic of the rare Griscelli syndrome in humans [13].

The sublocalization of unconventional myosins, mainly myosin-V, may provide morphological evidence that would be helpful in understanding the movements or formation of dendritic spines in adult mammalian brains. In this work, we examined the sublocalization of myosin-V in adult rat cerebellum.

MATERIAL AND METHODS

The procedures involving animals were done within the guidelines of the Brazilian College for Animal Experimentation (COBEA) and were approved by the institutional ethics committee of the Federal University of Uberlândia. Four male Wistar rats (270 ± 32 g) that had been housed at 22 ± 0.4°C, on a 12 h light/dark cycle, with free access to water and food were anesthetized intramuscularly with a mixture of xylazine (40 mg/kg)

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and ketamine (100 mg/kg). The encephalon was removed from the cranium and dissected on ice to separate the cerebral cortex and the cerebellar cortex and medulla. All of the tissues were homogenized in 25 mM Tris-HCl, pH 8.0, containing 10 mM ATP, 10 mM EDTA, 0.3 mM PMSF, and 1 mM β-mercaptoethanol. The supernatant was obtained by centrifugation at 15,000 *g* for 15 min at 4ºC. Total proteins were measured by the Bradford assay [1] and aliquots were immediately diluted with sample buffer prior to electrophoresis. Aliquots containing 20 µg of protein were applied to 7-14% polyacrylamide gels containing sodium dodecylsulphate (SDS-PAGE) [18] and analyzed by western blotting [31]. A polyclonal antibody was generated in rabbits by inoculating a recombinant protein corresponding to the tail domain of chicken myosin-Va, as described elsewhere [7]. Monospecific antibodies were obtained by affinity purification of this serum over a column of immobilized antigen. The secondary antibody used was a rabbit anti-IgG antibody conjugated with biotin (Sigma Chemical Co., St. Louis, MO, USA). Immunoreactive bands in the blots were detected with avidin-peroxidase and diaminobenzidene (Sigma).

For histological analysis, the cerebellum was sectioned (2 mm) and immersed overnight in a modified Karnovsky solution containing 3.5% paraformaldehyde and 0.5% glutaraldehyde. The sections were then immersed in 0.1 M sodium cacodylate buffer containing 0.2% picric acid and washed overnight with 0.1 M sodium cacodylate buffer containing 10% sucrose, followed by post-fixation in 2% uranyl acetate in 15% acetone for 2 h at 4ºC.

The tissues were subsequently dehydrated in an acetone series (30, 50, 70 and 90%) at 4ºC for 30 min followed by a series of infiltrations: 1) overnight with a 2:1 mixture of 90% acetone and 4 M LRWhite at 20ºC, 2) 24 h with a 1:1 mixture of 90% acetone and LRWhite at 20ºC, 3) 48 h with a 1:2 mixture of 90% acetone and LRWhite at 20°C, and 4) three 24 h cycles with pure LRWhite, at 20°C, in the presence of ultraviolet light.

Sections were obtained using an ultramicrotome and were collected on slides in order to evaluate the synaptic regions of the cerebellar pyriform cells by light microscopy. The cerebellar region rich in Purkinje cell synapses was intimately associated with the molecular layer. After localization of the region to be sliced, ultrathin sections were obtained with a diamond knife and collected on nickel grids that were then processed for the immunocytochemical detection of myosin-V, as follows: 1) a 10 min incubation with saturated periodic acid, 2) a 20 min wash with ultrafiltrated Tris-buffered saline (TBS, pH 7.4), 3) a 15 min blockade with 0.02 M TBS containing 5% albumin, 0.025% Triton X-100 and 0.025% Tween-20 (TBS-ATT) in a humidified chamber, 4) a 20 h incubation with anti-myosin-Va antibody solution in TBS-ATT in a humidified chamber at 10ºC, 5) a 3 h wash with TBS-ATT, with the solution being changed every 20 min, 6) a 60 min incubation with secondary antibody conjugated to protein A and 15 nm gold particles (diluted 1:50 in TBS-ATT), 7) an overnight wash in 0.02 M TBS and 0.025% Triton X-100, and 8) four washes with deionized water. The sections were contrasted for 1 min with lead citrate and for 10 min with 5% uranyl acetate prior to examination with a Zeiss transmission electronic microscope.

RESULTS

Labeling with 15 nm gold particles was found inside the axonal and dendritic cytoplasm in rat cerebellar synapses in the molecular layer (Fig. 1), and was sometimes associated with actin (Fig. 2) and the plasma membrane (Fig. 1). Figure 1 shows various membrane units in an arrangement characteristic of the cerebellar molecular layer in which there are extensive ramifications of the dendritic expansions of pyriform cells. Axons and dendrites were indistinguishable, although both regions were stained.

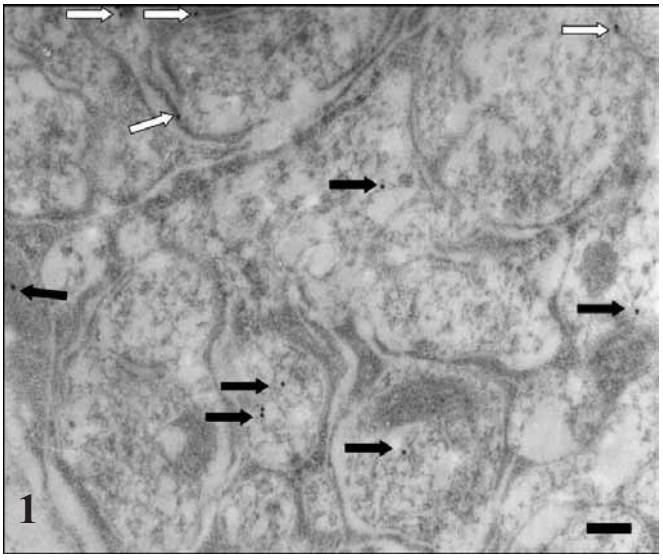
At high magnification, a line was seen between two colloidal gold particles (Fig. 2) and probably represented an actin molecule, based on its characteristic shape and the fact that actin is a myosin-binding protein [15]. This line was not a microtubule protein, which also binds myosin, because its diameter was smaller than the gold particle (15 nm).

Figure 3A shows the SDS-PAGE profile of the proteins present in the cerebral cortex, cerebellar cortex and spinal medulla. Immunoblotting of these proteins with an antibody against the tail region of myosin-Va resulted in a single immunoreactive band (Fig. 3B) and indicated that the protein seen close to 205 kDa in SDS-PAGE in the cerebral cortex, cerebellar cortex and spinal medulla homogenates was myosin-Va.

DISCUSSION

Myosin-V have been described in cell cultures [25,29], vertebrate brain extracts [3,17], the neural enteric system of rats [6], guinea pig cochlea [4], the centrosome of the rat hippocampus [8], and in the dendritic and axonal terminations (growth cones) of chicken neurons [9,10,29]. However, only a few studies have examined the subcellular localization of myosin-V [11,20,25]. To our knowledge, the detection of myosin-V in cerebellum cells using an immunoelectronic method has not previously been described.

In this paper, we used *in situ* electronic microscopy with a post-embedding method to detect myosin-Va



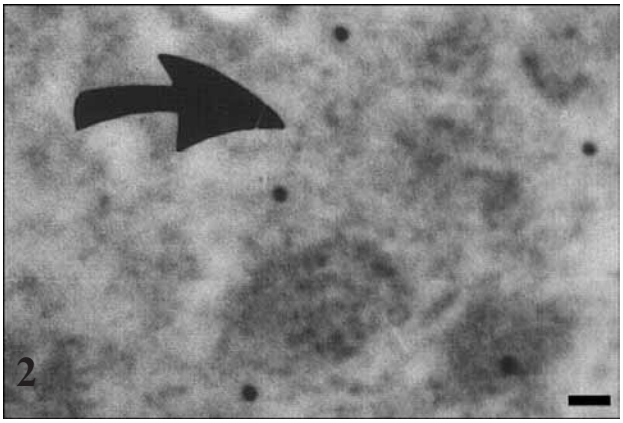


Figure 1. Myosin-V molecules labeled with 15 nm gold particles in the plasma membrane (white arrows) and cytoplasm (black arrows) of rat cerebellum. Bar = 167 nm.
Figure 2. A putative actin filament (black arrow) between two myosin V molecules. Bar = 50 nm.

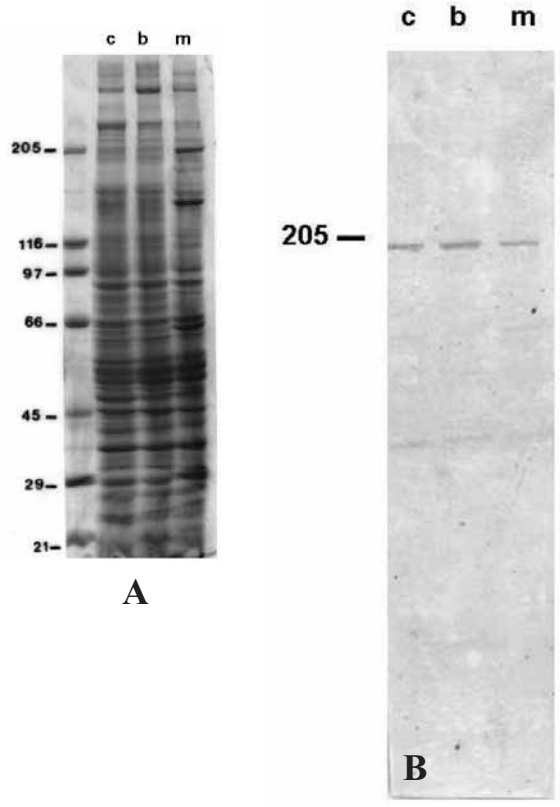


Figure 3. A) SDS-PAGE profile of proteins in rat cerebral cortex (**b**), cerebellum cortex (**c**) and spinal medulla (**m**). The left scale shows the relative molecular mass (Mr x 10^{-3}) of standard proteins. Myosin Va has an Mr of ~205 kDa. **B**) Immunoblotting of the proteins after electrotransfer to a nitrocellulose filter. Myosin Va was detected using an antibody against the tail region of this molecule and a biotin-conjugated secondary antibody followed by incubation with avidin-peroxidase-DAB in cerebral cortex (**b**), cerebellum cortex (**c**) and spinal medulla (**m**).

in the cerebellum. Since the tissue penetration by conjugated antibodies is limited in this post-embedding method, we used western blotting to confirm the presence of myosin-Va. In agreement with previous reports [7,10,15,16,25,27,30], a single band corresponding to myosin-Va was seen in the cerebral cortex, cerebellar cortex, and spinal medulla.

Brown and Bridgman [2] described the involvement of myosin-V in the migration of embryonic neurons. Since mature neurons can migrate, and since in adult brains new neurons can be formed from the subependimal layer [22], the localization of myosin-V in the synaptic region of the rat encephalon seen here was an important morphological and biochemical finding that could contribute to our understanding of the reorganization of the neural net after brain lesions [12].

The interaction of actin with myosin is involved in the movement of growing cones (filopodia and lamellipodia), as well as in the amoeboid movement of several cells and in organelle transport. According to DePina and Langford [5], myosin-V plays an important role in organelle transport in growing neurons [9,10]. The association of myosin-V with the plasma membrane agrees with such motor activity and with the presence of debrin in dendritic spines of the adult rat forebrain [15]. Since motor activity depends on protein fixation in the cells, the occurrence of actin-bound myosin and the association of myosin with the plasma membrane supports the involvement of myosin-Va in the intracellular movement of organelles in the cerebellum.

In conclusion, these findings demonstrate the presence of myosin-Va in the pre- and postsynaptic regions of rat cerebellum and indicate that this protein is probably associated with actin.

REFERENCES

1. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248-254.
2. Brown ME, Bridgman PC (2004) Myosin function in nervous and sensory systems. *J. Neurobiol.* **58,** 118-130.
3. Catlett NL, Duex JE, Tang F, Weisman LS (2000) Two distinct regions in a yeast myosin-V tail domain are required for the movement of different cargoes. *J. Cell Biol.* **150,** 513-525.
4. Coling DE, Espreafico EM, Kachar B (1997) Cellular distribution of myosin-V in the guinea pig cochlea. *J. Neurocytol*. **26,** 113-120.
5. DePina AS, Langford GM (1999) Vesicle transport: the role of actin filaments and myosin motors. *Microsc. Res. Tech.* **47,** 93-106.
6. Drengk AC, Kajiwara JK, Garcia SB, Carmo VS, Larson RE, Zucoloto S, Espreafico EM (2000) Immunolocalization of myosin-V in the enteric nervous system of the rat. *J. Auton. Nerv. Syst.* **78,** 109-112.
7. Espindola FS, Suter DM, Partata LBE, Cao T, Wolenski JS, Cheney RE, King SM, Mooseker MS. (2000) The light chain composition of chicken brain myosin-Va: calmodulin, myosin-II essential light chains, and 8-kDa dynein light chain/PIN. *Cell Motil. Cytoskeleton* **47**, 269-281.
8. Espindola FS, Espreafico EM, Coelho MV, Martins AR, Costa FR, Mooseker MS, Larson RE (1992) Biochemical and immunological characterization of p190-calmodulin complex from vertebrate brain: a novel calmodulin-binding myosin. *J. Cell Biol.* **118**, 359-368.
9. Espreafico EM, Coling DE, Tsakraklides V, Krogh K, Wolenski JS, Kalinec G, Kachar B (1998) Localization of myosin-V in the centrosome. *Proc. Natl. Acad. Sci. USA* **95**, 8636-8641.
10. Espreafico EM, Cheney RE, Matteoli M, Nascimento AA, De Camilli PV, Larson RE, Mooseker MS (1992) Primary structure and cellular localization of chicken brain myosin-V (p190), an unconventional myosin with calmodulin light chains. *J. Cell Biol.* **119,** 1541-1557.
11. Evans LL, Lee AJ, Bridgman PC, Mooseker MS (1998) Vesicle-associated brain myosin-V can be activated to catalyze actin-based transport. *J. Cell Sci*. **111,** 2055-2066.
12. Gage FH (2003) Auto-recuperação cerebral. *Sci. Am.* **17,** 40-47.
13. Griscelli C, Durandy A, Guy-Grand D, Daguillard F, Herzog C, Prunieras M (1978) A syndrome associating partial albinism and immunodeficiency. *Am. J. Med.* **65,** 691-702.
14. Hannemann DE, Cao W, Olivares AO, Robblee JP, De La Cruz EM (2005) Magnesium, ADP, and actin binding linkage of myosin V: evidence for multiple myosin V-ADP and actomyosin V-ADP states. *Biochemistry* **44,** 8826-8840.
15. Hayashi K, Ishikawa R, Ye LH, He XL, Takata K, Kohama K, Shirao T (1996) Modulatory role of drebrin on the cytoskeleton within dendritic spines in the rat cerebral cortex. *J. Neurosci.* **16,** 7161-7170.
16. Homma K, Saito J, Ikebe R, Ikebe M (2000) Ca^{2+} dependent regulation of the motor activity of myosin V. *J. Biol. Chem.* **275,** 34766-34771.
17. Krementsov DN, Krementsova EB, Trybus KM (2004) Myosin V: regulation by calcium, calmodulin, and the tail domain. *J. Cell Biol.* **164,** 877-886.
18. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680-685.
19. Lambert J, Naeyaert JM, De Paepe A, Van Coster R, Ferster A, Song M, Messiaen L (2000) Arg-cys substitution at codon 1246 of the human myosin Va gene is not associated with Griscelli syndrome. *J. Invest. Dermatol.* **114,** 731-733.
20. Langford GM (1999) ER transport on actin filaments in squid giant axon: implications for signal transduction at synapse. *FASEB J.* ***1*3,** 248-250.
21. Larson RE (1996) Myosin-V: a class of unconventional molecular motors. *Braz. J. Med. Biol. Res.* **29,** 309-318.
22. Lim DA, Alvarez-Buylla A (1999) Interaction between astrocytes and adult subventricular zone precursors stimulates neurogenesis. *Proc. Natl. Acad. Sci. USA* **96,** 7526-7531.
23. Mani F, Espreafico EM, Larson RE (1994) Myosin-V is present in synaptosomes from rat cerebral cortex. *Braz. J. Med. Biol. Res.* **27,** 2639-2643.
24. Mermall V, Post PL, Mooseker MS (1998) Unconventional myosin in cell movement, membrane traffic, and signal transduction. *Science* **279,** 527-533.
25. Miller KE, Sheetz MP (2000) Characterization of myosin V binding to brain vesicles. *J. Biol. Chem.* **275,** 2598-2606.
26. Molyneaux BJ, Mulcahey MK, Stafford P, Langford GM (2000) Sequence and phylogenetic analysis of squid myosin-V: a vesicle motor in nerve cells. *Cell Motil. Cytoskeleton* **46,** 108-115.
27. Nguyen H, Higuchi H (2005) Motility of myosin V regulated by the dissociation of single calmodulin. *Nat. Struct. Mol. Biol*. **12,** 127-132.
28. Reck-Peterson SL, Provance Jr DW, Mooseker MS, Mercer J (2000) Class V myosins. *Biochim. Biophys. Acta* **1496**, 36-51.
29. Suter DM, Espindola FS, Lin CH, Forscher P, Mooseker MS (2000) Localization of unconventional myosins V and VI in neuronal growth cones. *J. Neurobiol.* **42,** 370-382.
30. Tilelli CQ, Martins AR, Larson RE, Garcia-Cairasco N (2003) Immunohistochemical localization of myosin Va in the adult rat brain. *Neuroscience* **121,** 573-586.
31. Towbin H, Staehelin J, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

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