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Downregulation of myosin II-B by siRNA alters the subcellular localization of the amyloid precursor protein and increases amyloid-β deposition in N2a cells

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Abstract

The Alzheimer's disease (AD) brain pathology is characterized by extracellular deposits of amyloid-β (Aβ) peptides and intraneuronal fibrillar structures. These pathological features may be functionally linked, but the mechanism by which Aβ accumulation relates to neuronal degeneration is still poorly understood. Aβ peptides are fragments cleaved from the amyloid precursor protein (APP), a transmembrane protein ubiquitously expressed in the nervous system. Although the proteolytic processing of APP has been implicated in AD, the physiological function of APP and the subcellular site of APP cleavages remain unknown. The overall structure of the protein and its fast anterograde transport along the axon support the idea that APP functions as a vesicular receptor for cytoskeletal motor proteins.

In the current study, we test the hypothesis that myosin II, important contributor to the cytoskeleton of neuronal cells, may influence the trafficking and/or the processing of APP. Our results demonstrate that downregulation of myosin II-B, the major myosin isoform in neurons, is able to increase Aβ deposition, concomitantly altering the subcellular localization of APP. These new insights might be important for the understanding of the function of APP and provide a novel conceptual framework in which to analyze its pathological role.

Keywords: Alzheimer's disease; Amyloid precursor protein (APP); Amyloid-β; Myosin II

Alzheimer's disease (AD) is the most common neurodegenerative disorder of the elderly and is characterized clinically by a progressive memory loss, as well as other cognitive impairments. The neuropathological hallmarks of AD include abundant deposits of amyloid-β (Aβ) peptides organized in senile plaques, accumulation of abnormal tau protein in neurofibrillary tangles (NFTs), and extensive neuronal degeneration and loss. The pathogenic role of Aβ deposition is underscored by the evidence that each of the disease-causing mutations in familial AD results in enhanced production of amyloidogenic Aβ peptides. However, the numbers of NFTs are more closely correlated with neuronal loss and dementia than are the numbers of senile plaques, and the issue of whether Aβ deposits are neurotoxic, protective, or simply incidental markers of disease has remained controversial. The Aβ peptides found in senile plaques derive by the proteolytic cleavage of the β-amyloid precursor protein (APP), which is cleaved sequentially by enzymes termed β- and γ-secretase. The β-site APP cleavage enzyme (BACE) has been identified recently as a membrane-bound aspartyl-protease [1]. Cleavage of APP by BACE, at the N-terminus of the Aβ sequence, is the first prerequisite for generation of Aβ peptides. An additional cleavage in the transmembrane domain of APP by γ-secretase generates the C-terminal

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end of the peptide and the release of Aβ into the extracellular compartment. Recent studies indicate that γ-secretase is a multiprotein complex consisting of Presenilin, Nicastrin, Aph-1, and Pen-2 and that all four proteins are necessary for full proteolytic activity [2]. Alternative processing of APP by α-secretase precludes Aβ production, as this enzyme cleaves within the Aβ sequence. Although the proteolytic processing of APP has been implicated in the pathogenesis of AD, the normal function of APP, whether this function is related to the proteolytic processing, and where this processing takes place in neurons remain unknown. Production of Aβ has been localized to different compartments of the secretory pathway [3,4]. However, more recent studies ruled out production of Aβ in the endoplasmic reticulum and the Golgi [5].

The overall structure of APP and the observation of its fast anterograde transport along the axon, support the hypothesis that APP functions as a vesicular receptor for cytoskeletal motor proteins. Nonmuscle myosin II, represents one of the major contributors to the cytoskeleton of neuronal cells [6,7] and has been involved in several functional activities, including cell adhesion [8], migration [7], neuritic outgrowth [9], and possibly transport of cellular cargo along the actin cytoskeleton [10]. The class II nonmuscle myosin family consists of two widely expressed isoforms, myosin II-A (NMMHC-IIA) and myosin II-B (NMMHC-IIB), and a third, recently identified isoform, myosin II-C (NMMHC-IIC), expressed mainly in small intestine, colon, skeletal muscle, and lung [11]. Their structure consists of a globular head that can bind actin and has ATPase activity, and a tail that can either promote multimerization or bind different cellular cargo. Although each family member has a unique C-terminal 'cargo' domain, the identity of the specific players involved in neuronal responses remains obscure.

In the current study, we test the hypothesis that myosin II may influence the trafficking and/or the processing of APP. Our results demonstrate that, in neuronal cultured cells, downregulation of myosin II-B is able to increase Aβ secretion, concomitantly altering the subcellular localization of APP. These new insights might be important for the understanding of the function of APP and provide a novel potential link between APP and the neuronal cytoskeleton.

Materials and methods

Cell culture. The cells used in this study (mouse Neuro-2a (N2a), stably expressing wild type human APP695) were obtained from Peter Davies (Albert Einstein College of Medicine, Bronx, NY).

Immunoblot analysis. Immunoblots were done according to standard methods, using the following antibodies: monoclonal mouse anti-human APP (22C11, Chemicon), monoclonal mouse anti-human Aβ (4G8, Signet, and 6E10, Chemicon), monoclonal mouse anti-human β-actin (Sigma), monoclonal mouse anti-Pan-myosin (Covance Research Products), monoclonal mouse anti-transferrin receptor and polyclonal rabbit anti-human APP (Zymed), polyclonal rabbit anti-myosin II-A and polyclonal rabbit anti-myosin II-B (Abcam). The polyclonal rabbit anti-myosin II-C antibody was a generous gift from Robert S. Adelstein (National Institutes of Health, Bethesda, MD). Anti-mouse and anti-rabbit secondary antibodies were coupled to horseradish peroxidase (Amersham).

RNA interference. Myosin II-A, myosin II-B siRNA duplexes (directed at the mouse sequences, Accession Nos. NM_022410 and NM_175260, respectively) and control siRNA non-targeting duplex were purchased from Dharmacon, Inc. Myosin II-C siRNA duplex (directed at the mouse sequence, Accession No. NM_028021) was obtained from Qiagen. The control siRNA used in this study interacts with the RNA-induced silencing complex, but lacks sufficient homology with any known gene to effectively induce mRNA knockdown. Silencing efficiency, analyzed by immunoblotting, indicated that each silenced myosin was decreased at 48 h and that the lowest content was found at 72 h following transfection (data not shown). Transfections were performed using DharmaFect® (Dharmacon) at 4 μl/100 nM siRNA, according to the manufacturer's instructions.

BACE activity. The activity of BACE was determined using a commercially available secretase kit from R&D Systems, according to the manufacturer's protocol. The method is based on the secretase-dependent cleavage of a secretase-specific peptide conjugated to the fluorescent reporter molecules EDANS and DABCYL, which results in the release of a fluorescent signal that can be detected on a fluorescence microplate reader (excitation at 355 nm/emission at 510 nm). The level of secretase enzymatic activity is proportional to the fluorimetric reaction.

Immunofluorescence. N2a cells stably expressing human APP695 were grown overnight on culture slides and then transfected with siRNA duplexes for 72 h. Thereafter, the cells were incubated for 30 min with wheat germ agglutinin, Alexa Fluor® 594 conjugate (Invitrogen SRL). After fixing and permeabilization with ice-cold methanol, non-specific binding sites were blocked with 3% BSA. Cells were subsequently incubated with anti-APP monoclonal antibody (6E10, 1:200) together with anti-myosin II-B polyclonal antibody (1:100). Cells were then labeled with secondary antibodies: Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 680 goat anti-rabbit IgG (Invitrogen SRL) at a 1:500 dilution. Immunostained cells were observed with the appropriate filters on a Bio-Rad 1024 confocal three-channel microscope.

Cell surface biotinylation. N2a cells were surface biotinylated by incubating with Sulfo–NHS–SS–Biotin (Pierce) at 2 mg/ml in ice-cold PBS. After 30 min, cells were washed and quenched with PBS containing 100 mM glycine. Cells were then lysed in NP-40 1% buffer and incubated with NeutrAvidin™ Protein immobilized onto 6% crosslinked beaded agarose (Pierce). After washing in NP-40 buffer, the beads were boiled in sample buffer, fractionated in SDS–PAGE and immunoblotted with the indicated antibodies.

Results

Myosin II-B is the major myosin II isoform in N2a cells

In order to identify the myosin II isoforms expressed in neuronal N2a cells and compare the percent of each isoform at the protein level, we made use of specific antibodies and siRNAs. The immunoblot analyses shown in Fig. 1 indicate that N2a cells express myosin II-A, II-B, and II-C. We lowered the mRNA encoding each isoform using the appropriate siRNA (see Material and methods) and correlated the protein decrease obtained for each isoform with the change seen following detection with the pan-myosin antibody. As shown in Fig. 1, siRNA directed against myosin II-A efficiently reduced the II-A protein level, without altering the expression of the other two isoforms. Myosin II-A siRNA also significantly reduced the amount of total myosin II detected by the pan-myosin antibody. siRNA targeting myosin II-B had even a stronger impact on the total amount of myosin II (see Fig. 1, pan-myosin,