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The actin cross-linking protein AFAP120 regulates axon elongation in a tyrosine phosphorylation-dependent manner

Jennifer Harder, Xiaohua Xu, Paul Letourneau, Lorene M. Lanier *

Department of Neuroscience, University of Minnesota, 6-145 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455, United States

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ABSTRACT

Growth cone guidance and axon elongation require the dynamic coordinated regulation of the actin cytoskeleton. As the growth cone moves, actin-dependent forces generate tension that enables protrusive activity in the periphery and drives growth cone translocation. This dynamic remodeling of the actin cytoskeleton in response to membrane tension requires activation of Src kinase. Although it has been proposed that these actin-dependent forces vary with the extent of actin cross-linking, the identity of the cross-linking protein(s) remains unknown. AFAP120 is a nervous system specific actin cross-linking protein that is regulated by Src kinase phosphorylation. Here, we report that AFAP120 is expressed and tyrosine phosphorylated in differentiating cerebellar granule cells, where it is enriched in the axon and growth cone. Over-expression of AFAP120 enhances neurite elongation in a tyrosine phosphorylation-dependent manner. These findings suggest that AFAP120 may coordinate Src signaling with the dynamic changes in the actin cytoskeleton that drive growth cone motility and axon elongation.

In developing neurons, the elongation of axons is guided by the motility of the growth cone, a highly dynamic structure at the distal end of the axon. Growth cones sense and translate extracellular signals into directed migration and axon extension. This motile morphogenic process is driven by dynamic reorganization of the actin and microtubule cytoskeletons [4]. Guidance signals, whether soluble or substrate bound, can induce receptor clustering which may in turn induce local changes in membrane tension and trigger intracellular signaling cascades and cytoskeletal reorganization. In growth cones, Src activation in response to ligand-induced tension is essential for cytoskeletal reorganization preceding growth cone turning [14,27,28]. Although the mechanism of Src activation and its downstream targets has not been determined, electron microscopy studies of actin organization in the growth cone suggest that at least one actin cross-linking protein is involved [19,20,26].

The Actin Filament Associated Proteins of 110/120 kDa (AFAP110/120) have the molecular binding properties required to coordinate Src signaling with actin remodeling. AFAP110/120 (AFAPs) are multi-domain actin cross-linking proteins that are capable of oligomerizing and binding to Src and protein kinase C (PKC, Fig. 1A; [2]). AFAP110 is ubiquitously expressed, while alternative splicing of the AFAP gene produces AFAP120, which is expressed specifically in the nervous system [6]. In non-neuronal cells AFAP110 plays an important role in formation of actin stress fibers, focal adhesions [5] and podosomes (adhesive actin-based structures found in Src transformed cells [9]) and is required for mechanical stretch-induced activation of Src [12,21,22]; these functions are blocked by a mutation in the AFAP110 SH2-binding domain that inhibits Src binding and AFAP110 tyrosine phosphorylation [1,12].

AFAPs contain a single actin-binding domain, so their ability to cross-link actin filaments depends on oligomerization [2]. Although phosphorylation is not required for AFAP binding to F-actin [25], AFAP oligomerization is regulated by Src-dependent tyrosine phosphorylation [24], so phosphorylation also regulates the ability of AFAPs to cross-link actin filaments.

Relatively little is known about the function of AFAPs in the nervous system. Staining of brain sections with an antiserum that recognizes both AFAPs indicates that AFAPs are widely expressed in the developing brain and cerebellum [3]. AFAP expression decreases in the adult brain, remaining high only in regions that undergo continuous adult neurogenesis [3]. These data suggest that AFAPs may play a role in differentiating neurons. In this report, we demonstrate for the first time that AFAPs are present in the growth cone and axon shaft of differentiating cerebellar granule neurons and that AFAP120 regulates axon extension in a tyrosine phosphorylation-dependent manner. Together, these findings suggest that AFAP120 may be one of the actin cross-linking proteins that regulate growth cone actin dynamics in response to Src activity.

* Corresponding author. Tel.: +1 612 626 2399; fax: +1 612 624 7910.
E-mail address: lanie002@umn.edu (L.M. Lanier).
URL: http://www.neurosci.umn.edu/faculty/lanier.html (L.M. Lanier).

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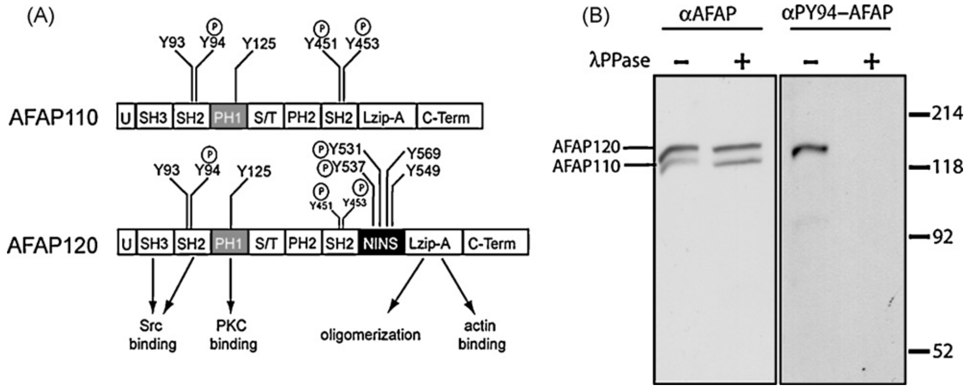

Fig. 1. AFAP120 expression and tyrosine phosphorylation in developing neurons. (A) Domain structure of AFAP110 and AFAP120. AFAP110 and AFAP120 contain identical SH3- and SH2-binding domains, two pleckstrin homology domains (PH1 and PH2), a serine/threonine rich region (S/T) and a leucine zipper motif within which lies an actin-binding domain (Lzip-A). The SH2- and SH3-binding domains mediate AFAP interaction with Src, while the PH1 domains regulates AFAP association with PKC. AFAP120 contains a neuronal insert (NINS) that is generated by alternative splicing of an exon between the SH2 and Lzip domains. Positions of the potential Src kinase phosphorylated tyrosines (*Y*) are shown; major sites of Src-dependent phosphorylation are indicated by an encircled 'P'. (B) Western blot analysis of AFAP expression in cerebellar granule cell cultures. Granule cell lysates were treated with (+) or without (−) λ-phosphatase, then immunoblotted and sequentially probed with PY94-AFAP antiserum that recognizes phosphorylated tyrosine 94 in both AFAP110 and AFAP120 and anti-AFAP antiserum that recognizes both AFAP110 and AFAP120. Positions of AFAP110 and AFAP110 are indicated on the left and molecular weight markers (kDa) are indicated on the right.

Replication-deficient recombinant adenoviruses expressing AFAP120-WT or phosphomutant AFAP120-9F (all the tyrosine phosphorylation sites mutated to phenylalanine) were produced using the pAdTrack-CMV shuttle vector and the AdEasy system [13]. The pAdTrack-CMV shuttle vector expresses the gene of interest (e.g. AFAP120) under a CMV promoter with EGFP expressed under a second CMV promoter. Viruses were purified on a CsCl gradient and tittered on 293HEK cells as previously described [26].

Cultured cerebellar granule cells (see below) were lysed in 50 mM Tris, 1.0% NP-40 and 150 mM NaCl containing protease and phosphatase inhibitors. Protein concentration was determined using the BCA assay (Pierce) and 10 μg/lane of cerebellar neuronal lysate were separated on an 8% SDS-PAGE gel. For phosphatase assays, phosphatase inhibitors were omitted from the lysis buffer and 40 μg of lysate were mixed with 4 μg of λ-phosphatase (Upstate) and 5 mM DTT in phosphatase reaction buffer, then incubated at 37 °C for 10 min. The reaction was stopped by addition of sample buffer and boiling for 5 min. Antibodies included anti-phosphotyrosine 4G10 (Upstate), anti-AFAP (F1), anti-phospho-AFAP (PY94-AFAP) and Horse radish peroxidase labeled secondary antisera (Sigma). Signal was developed with ECL reagent (Amersham).

For dissociated cerebellar cultures, cerebella from postnatal day 3–7 mice were dissected, the meningies removed and the tissue chopped into small pieces. After digestion in phosphate-buffered saline solution containing 0.125% (w/v) trypsin (Invitrogen) for 20 min at 37 °C, the tissues were mechanically triturated by repeated passages through a polished Pasteur pipette in phosphate-buffered saline solution containing 0.05% (w/v) DNAse (Invitrogen). Dissociated neurons were resuspended in the neurobasal medium with B-27 supplement (Invitrogen) and plated poly-D-lysine coated coverslips.

For aggregated granule cell cultures, dissociated cerebellar granule cells were isolated and purified from P5-6 mice as essentially as described [8,16]. Briefly, isolated cerebella were incubated in 0.15% trypsin type XII-S (Sigma) and Ca^{2+}–Mg^{2+}-free PBS for 20 min, resuspended in media containing 0.25% DNAse I and triturated with a fire-polished glass pipette. Cells were kept in BME media (Gibco) supplemented with 10% FBS, 10% horse serum, penicillin/streptomycin, 0.2 mM L- glutamine and 6 mM glucose. Granule cells were then isolated from the interface of a 35–60% Percoll gradient and subsequently purified by sequential pre-plating on Petri dishes coated with 0.1 mg/ml poly-D-lysine for 1 h in media. 3×10^5 cells were then seeded in wells of 16-well Lab-Tek chamber slides (Nunc) and incubated in a humidified chamber at 37 °C, 5% CO_2. After 1–3 h, cells were infected with a multiplicity of infection (m.o.i.) of 20–40. Under these conditions, at least 50% of the cells were infected and no cyto-pathological effects due to virus infection were detected. 24 h following infection, cell aggregates were collected by brief low-speed centrifugation and resuspended in the above media supplemented with B-27 (Invitrogen), 10 mM D-serine (Sigma), and 25 mM KCl. Aggregates were then transferred to glass coverslips coated with 50 mg/ml PDL (Sigma) and 25 μg/ml laminin (Invitrogen) and incubated for an additional 24 h before processing for immunofluorescence.

Cells were fixed for 30 min at 37 °C in 4% paraformaldehyde in PHEM buffer (60 mM PIPES pH 7.0, 25 mM HEPES pH 7.0, 10 mM EGTA, 2 mM $MgCl_2$) with 0.12 M sucrose. After rinsing in PBS, coverslips were incubated in 10% fatty acid free bovine serum albumin (BSA) in PBS for 30 min, permeablized for 10 min in 0.2% triton/PBS, rinsed, and re-blocked in 10% BSA/PBS for 30 min. Alexa-labeled Phalloidin (molecular probes) was used to label F-actin and DAPI staining was used to visualize nuclei. Primary antibodies used included anti-AFAP [7], anti-βIII-tubulin (Promega) and anti-tyrosinated tubulin (Chemicon). Secondary antibodies were purchased from Jackson labs.

For granule cell aggregate axon imaging and analysis, aggregates were chosen if they were relatively symmetrical with aggregate diameters between 80 and 130 μm and their axons had minimal contact with axons from other aggregates. The centroid of each aggregate was determined in the DAPI channel image using the Openlab thresholding tool. Only regions of aggregates where all axons could be visualized in entirety were chosen for measurement. EGFP expressing cells were measured from the centroid to the distal tip of the axon and normalized to the mean length of control axons in the same experiment. Experiments were done in duplicate.

To determine which AFAP isoforms are expressed in the developing cerebellum, lysates from cultured cerebellar granule cells were probed with an antiserum that recognizes both AFAPs. This analysis revealed that while both AFAP110 and AFAP120 were expressed, AFAP120 was relatively more abundant (Fig. 1B, left panel).

In non-neuronal cells, AFAP110 function is regulated by Src-dependent tyrosine phosphorylation [1,10]. In the presence of