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Paxillin phosphorylation counteracts proteoglycan-mediated inhibition of axon regeneration

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ABSTRACT

In the adult central nervous system, the tips of axons severed by injury are commonly transformed into dystrophic endballs and cease migration upon encountering a rising concentration gradient of inhibitory proteoglycans. However, intracellular signaling networks mediating endball migration failure remain largely unknown. Here we show that manipulation of protein kinase A (PKA) or its downstream adhesion component paxillin can reactivate the locomotive machinery of endballs in vitro and facilitate axon growth after injury in vivo. In dissociated cultures of adult rat dorsal root ganglion neurons, PKA is activated in endballs formed on gradients of the inhibitory proteoglycan aggrecan, and pharmacological inhibition of PKA promotes axon growth on aggrecan gradients most likely through phosphorylation of paxillin at serine 301. Remarkably, pre-formed endballs on aggrecan gradients resume forward migration in response to PKA inhibition. This resumption of endball migration is associated with increased turnover of adhesive point contacts dependent upon paxillin phosphorylation. Furthermore, expression of phosphomimetic paxillin overcomes aggrecan-mediated growth arrest of endballs, and facilitates axon growth after optic nerve crush in vivo. These results point to the importance of adhesion dynamics in restoring endball migration and suggest a potential therapeutic target for axon tract repair.

Introduction

After neuronal networks have developed, injury to the adult central nervous system (CNS) induces the formation of glial scars and myelin debris that release or expose many inhibitors of axon regeneration (Yiu and He, 2006). Glial scars formed in the vicinity of injured regions consist predominantly of reactive astrocytes together with other cell types that secrete inhibitory proteoglycans such as chondroitin sulfate proteoglycans (CSPG) and keratan sulfate proteoglycans (KSPG). These proteoglycans pose a major impediment to axon regeneration (Bradbury et al., 2002; Imagama et al., 2011; Jones and Tuszynski, 2002; McKeon et al., 1991; Silver and Miller, 2004). CSPG receptors include two Type IIa receptor protein tyrosine phosphatases (RPTPs) as well as Nogo receptors (Dickendesher et al., 2012; Fisher et al., 2011; Fry et al., 2010; Shen et al., 2009). Because Type IIa RPTPs also interact with heparan sulfate proteoglycans (Aricescu et al., 2002; Johnson et al., 2006) that facilitate axon growth (Bandtlow and Zimmermann, 2000; Van Vactor et al., 2006), targeting receptor-downstream events specific to inhibitory proteoglycans is a reasonable strategy for axon tract repair after CNS injury. However, the intracellular signal transduction networks mediating CSPG-based inhibition of axon regeneration are largely unknown.

After CNS injury, the inhibitory proteoglycans CSPG and KSPG are organized as a gradient with higher concentration in the lesion core and lower in the penumbra (Davies et al., 1999; Ito et al., 2010; Jones and Tuszynski, 2002; Silver and Miller, 2004). As the tips of regenerating axons approach the injury site, they migrate into the penumbra but eventually stop advancing and become swollen into "dystrophic endballs" in the rising gradient of inhibitory proteoglycans. Ramón y Cajal first described this dystrophic structure and believed that it was a final resting state of the immobilized axon tip (Ramón y Cajal, 1928). However, in vitro reproduction of dystrophic endballs on a gradient of aggrecan, an inhibitory proteoglycan that contains chondroitin sulfate and keratan sulfate chains, revealed that endballs are highly motile for some time but incapable of forward translocation in this hostile environment (Tom et al., 2004). This finding

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suggests that axon growth on proteoglycan gradients in vitro and possibly across glial scars in vivo is achievable if we can properly manipulate intracellular molecular machinery for endball migration. Although in vitro searches so far have failed to identify compounds that restore the capability of endballs to grow robustly on aggrecan gradients (Steinmetz et al., 2005), we report here that endballs can resume forward migration in this hostile environment after pharmacological or genetic perturbation that induces serine phosphorylation on paxillin, an intracellular component of the adhesion machinery. We also show that chemical induction or genetic mimicking of paxillin phosphorylation is sufficient to overcome aggrecan-mediated growth arrest of endballs in vitro and to facilitate axon growth in the injured adult CNS in vivo, possibly through activation of adhesion turnover in endballs.

Materials and methods

cDNA constructs

Rat paxillin cDNA (clone ID: 7128801) was purchased from Open Biosystems. Rat p21-activated kinase 1 (PAK1) cDNA was obtained through RT-PCR of total brain RNA derived from embryonic day 18 Wistar rat (SLC) using the following primer pair: 5′-ACGCGTCGACG CCACCATGTCAAATAACGGCTTAGACG-3′ and 5′-ATGTTTAGCGGCCGCC AGGAAATGGGAGAAGCAAG-3′. These cDNAs were subcloned into pCA GGS, a mammalian expression vector under the control of the CAG promoter (provided by J. Miyazaki, Osaka University, Suita, Osaka, Japan) (Niwa et al., 1991). cDNAs encoding for the following fluorescent proteins were inserted into the vectors adjacent to the 3′ end of paxillin cDNA or adjacent to the 5′ end of PAK1 cDNA: Venus (provided by A. Miyawaki, RIKEN Brain Science Institute, Wako, Saitama, Japan) (Nagai et al., 2002) and TagRFP-T (Shaner et al., 2008), a modified version of TagRFP (Evrogen). Single amino acid mutations were introduced into paxillin and PAK1 sequences using QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's protocol.

Cell culture substrates

Glass-based dishes were coated with 0.1% poly-D-lysine (PDL, Sigma) overnight at 37 °C. A uniform substrate of laminin or aggrecan was prepared by incubating PDL-coated glass coverslips with 10 μg/ml laminin (Invitrogen) or 10 μg/ml laminin plus 200 μg/ml aggrecan (Sigma), respectively, in calcium and magnesium-free Hank's balanced salt solution (Invitrogen) for 3 h at 37 °C. A gradient substrate of aggrecan was prepared as described previously (Tom et al., 2004) with minor modifications. Briefly, PDL-coated coverslips were spotted with 2 μl of a solution of 1.6 mg/ml aggrecan and 10 μg/ml laminin. This procedure caused a circular area of approximately 2.2 mm in diameter to be coated with aggrecan, in which the rim of the circle contained increasingly higher concentrations of aggrecan than the center. After the spots were air dried, the coverslips were completely covered with 10 μg/ml laminin.

Neuronal culture

Dorsal root ganglions (DRGs) of adult Sprague–Dawley rat (200–240 g, female, SLC) were dissociated and cultured as described previously (Tom et al., 2004). Briefly, DRGs were dissociated with 2.5 units/ml dispase II (Roche) and 200 units/ml collagenase type 2 (Worthington) for 70 min at 35 °C. Neuronal cultures were maintained in Neurobasal-A medium (Invitrogen) containing 2% B-27 (Invitrogen), 1% Glutamax (Invitrogen) and 1% Antibiotic–Antimycotic liquid (Invitrogen) in a humidified atmosphere of 5% CO_2 at 37 °C. During live cell imaging, neuronal cultures were maintained in Leibovitz's L-15 medium (Invitrogen) supplemented with 2% B-27 in a humidified atmosphere of 100% air at 37 °C. For gene transfection, dissociated DRG neurons were nucleofected using Nucleofector II (Lonza) according to the manufacturer's protocol G-013.

Quantification of axon growth in vitro

Axon crossing of aggrecan gradients was evaluated by measuring the total length of axons in the aggrecan rim. Four hours after plating DRG neurons on spot preparations, the cells were treated with the following compounds: 20 μM forskolin (adenylate cyclase activator, Sigma), 20 μM Sp-cAMPS (cAMP analog, Calbiochem), 10 μM 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8-CPT-2′-O-Me-cAMP, Epac activator, Biolog), 10 μM N^6-benzoyladenosine-3′,5′-cyclic monophosphate [6-Bnz-cAMP, protein kinase A (PKA) activator, Biolog], 1 μM KT5720 (PKA inhibitor, Calbiochem), 100 μM myristoylated protein kinase inhibitor-(14-22)-amide (mPKI, PKA inhibitor, BioMol), 20 μM 8-bromo-cGMP (8-Br-cGMP, cGMP analog, Calbiochem), 1 μM KT5823 (protein kinase G inhibitor, Calbiochem), 50 μM 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (NOC18, nitric oxide donor, Dojindo), 50 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO, nitric oxide scavenger, Calbiochem), 100 nM AG1478 (epidermal growth factor receptor inhibitor, Sigma), 100 nM PD168393 (epidermal growth factor receptor inhibitor, Calbiochem), 1 μM KN93 (calcium/calmodulin-dependent protein kinase inhibitor, Sigma), 100 nM phorbol 12-myristate 13-acetate (protein kinase C activator, Wako), 1 μM Gö6976 (protein kinase C inhibitor, LC laboratories), 100 nM wortmannin (phosphoinositide 3-kinase inhibitor, Calbiochem), 20 μM PD98059 (mitogen-activated protein kinase kinase inhibitor, Santa Cruz), 100 μM $NiCl_2$ (voltage-gated calcium channel blocker, Wako), 10 μM tetrodotoxin (sodium channel blocker, Alomone Labs), 10 nM paclitaxel (microtubule stabilizer, Calbiochem), 0.69 U/ml chondroitinase ABC, (Seikagaku Corporation).

Two days after gene transfection and/or pharmacological treatment, the cells were fixed and immunostained with rabbit anti-β tubulin III IgG (1:1000 dilution, Sigma, catalog No. T2200) and Alexa Fluor 594-conjugated anti-rabbit IgG (1:400 dilution, Invitrogen). Transfected cells were identified by Venus or TagRFP-T fluorescence. Aggrecan gradients were visualized with mouse anti-chondroitin sulfate IgM (clone CS-56, 1:500 dilution, Sigma) and Alexa Fluor 350-conjugated anti-mouse IgM (1:400 dilution, Invitrogen). The cells were observed with a 10× NA 0.40 objective lens (UPlanSApo, Olympus) on an inverted microscope (IX81, Olympus). Fluorescence images were acquired with a cooled charge coupled device (CCD) camera (ORCA-AG, Hamamatsu Photonics, binning set at 2 × 2) under the control of SlideBook software (Roper). The outer boundary of aggrecan rims was identified on the CS-56 fluorescence image, and an inner rim boundary was set at 120 μm distant from the outer boundary such that aggrecan rims on all images have equal width. Then, the total length of axons in the aggrecan rim was defined as the sum of lengths of all axonal segments located between the outer and inner boundaries. The measurements were performed using ImageJ software (National Institutes of Health).

Axon growth on uniform substrates was assessed by measuring the length of the longest axon in each neuron. Four hours after plating, DRG neurons were treated with 1 μM KT5720 or vehicle only. After an additional 20-h incubation, the cells were immunolabeled for β tubulin III and subjected to axon length measurements as described previously (Kamiguchi and Yoshihara, 2001).

Quantification of endball migration in vitro

Endball migration was quantified two days after plating on aggrecan spot preparations. DIC images of dystrophic endballs were acquired every 1 min with a 100× NA 1.40 oil objective lens (UPlanSApo, Olympus) and a CCD camera (ORCA-AG, binning set at 2x2) on an inverted microscope (IX81). Migration speed was