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Full length talin stimulates integrin activation and axon regeneration

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ABSTRACT

Integrin function is regulated by activation involving conformational changes that modulate ligand-binding affinity and downstream signaling. Activation is regulated through inside-out signaling which is controlled by many signaling pathways via a final common pathway through kindlin and talin, which bind to the intracellular tail of beta integrins. Previous studies have shown that the axon growth inhibitory molecules NogoA and chondroitin sulfate proteoglycans (CSPGs) inactivate integrins. Overexpressing kindlin-1 in dorsal root ganglion (DRG) neurons activates integrins, enabling their axons to overcome inhibitory molecules in the environment, and promoting regeneration in vivo following dorsal root crush. Other studies have indicated that expression of the talin head alone or with kindlin can enhance integrin activation. Here, using adult rat DRG neurons, we investigate the effects of overexpressing various forms of talin on axon growth and integrin signaling. We found that overexpression of the talin head activated axonal integrins but inhibited downstream signaling via FAK, and did not promote axon growth. Similarly, co-expression of the talin head and kindlin-1 prevented the growth-promoting effect of kindlin-1, suggesting that the talin head acts as a form of dominant negative for integrin function. Using full-length talin constructs in PC12 cells we observed that neurite growth was enhanced by the expression of wild-type talin and more so by two 'activated' forms of talin produced by point mutation (on laminin and aggrecan–laminin substrates). Nevertheless, co-expression of full-length talin with kindlin did not promote neurite growth more than either molecule alone. In vivo, we find that talin is present in PNS axons (sciatic nerve), and also in CNS axons of the corticospinal tract.

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1. Introduction

Integrins are αβ heterodimeric transmembrane molecules found on the surface of many different cell types that interact with extracellular matrix glycoproteins. In the nervous system, they are involved in cell migration, axon growth, synaptogenesis and axon regeneration (Eva et al., 2012a; Lemons and Condic, 2008; Winograd-Katz et al., 2014). Integrin function is regulated in various ways, including inside-out signaling, in which binding of molecules to the intracellular domain can switch the molecules from a low ligand-binding affinity state to a high affinity one (Hynes, 2002). Integrin–ligand binding depends on the affinity state, and subsequently allows the activation and propagation of intracellular outside-in signaling. Enhancing integrin activation promotes axon growth from cultured neurons (Ivins et al., 2000; Lein et al., 2000; Lemons and Condic, 2008; Tan et al., 2011), even in the presence of growth-inhibitory substrates such as chondroitin sulfate proteoglycans (CSPGs) and amino-Nogo (Hu and Strittmatter, 2008; Tan et al., 2011).

Integrin activation is affected by many signaling pathways, whose actions converge onto two families of proteins, talin and kindlins, which interact with the β-integrin cytoplasmic tail at two distinct sites. Talin is a large protein comprising a long C-terminal flexible rod domain (~220 kDa) that interacts with F-actin and vinculin while the N-terminal head (~50 kDa), contains an atypical four point one protein, ezrin, radixin and moesin (FERM) domain that binds to integrin cytoplasmic tails (Kim et al., 2011; Critchley, 2009; Ye et al., 2014; Calderwood et al., 2013). Binding of the talin head to integrin was identified as a final common step required for integrin activation (Goult et al., 2013; Tadokoro et al., 2003), and overexpression of the head domain is sufficient to induce integrin activation (Calderwood et al., 1999; Kim et al., 2003). Kindlins also associate with the cytoplasmic tail of beta integrins, promoting activation and clustering (Ye et al., 2013, 2014; Calderwood et al., 2013) and recent data suggest that kindlins promote integrin clustering thereby increasing the avidity of integrins for ligands (Ye et al., 2014). Our previous work has shown that expression of kindlin-1, which is not normally expressed in neurons, promotes integrin activation and axon regeneration in the spinal cord (Tan et al., 2012). Kindlin-1 influences Wnt and TGFbeta signaling in addition to its direct effects on integrins (Rognoni et al., 2014). Furthermore, co-expression of the talin head with kindlin-2 results in a synergistic enhancement of integrin activation, as observed in αIIbβ3-expressing CHO cells (Ma et al., 2008; Montanez et al., 2008). Coupled with our previous observation that overexpression of kindlin-1

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promotes axon regeneration over inhibitory substrates in vitro and in vivo (Tan et al., 2012), these findings make talin an attractive candidate for promoting axon regeneration.

Here we have investigated the effects of talin and the talin head domain on axon growth and the integrin signaling pathway, either singly or in combination with kindlin-1 or kindlin-2. In addition, we compared the in vivo distribution of talin molecules within the central and peripheral nervous systems.

2. Materials and methods

2.1. Dorsal root ganglion (DRG) neuron culture

DRGs were dissected from Sprague–Dawley rats (~3 months). The neurons were collected, dissociated with collagenase and trypsin, rinsed in calcium- and magnesium-free phosphate-buffered solution (PBS), transfected with expression constructs encoding GFP or talin head–GFP, and plated onto laminin (1 μg/ml) or aggrecan–laminin (25 μg/ml:1 μg/ml) in DMEM supplemented with insulin–transferrin–selenium (1×), penicillin–streptomycin–fungizone (1×) and 10 ng/ml nerve growth factor.

2.2. PC12 cell culture

PC12 cells were plated on collagen IV-coated T75 tissue culture flasks in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal calf serum, L-glutamine (25 mM) and penicillin–streptomycin–fungizone (1×). Neuronally-differentiated PC12 cells were prepared by adding NGF (100 μg/ml) to the culture medium, and incubated for another 10–14 days. Differentiated neurons were transfected with GFP, talin head, talin-1 (full-length), talin-1 (with T1767E mutation) and talin-1 (with E1770A mutation) DNA constructs using the Neon Transfection System (Invitrogen), and plated onto coverslips pre-coated with laminin (1 μg/ml) or aggrecan–laminin (25 μg/ml:1 μg/ml) in the aforementioned culture medium (with NGF).

2.3. Transfection with DNA constructs

Transfection was performed using a Neon Transfection Kit (Invitrogen), following the manufacturer's instructions. The transfection parameters used were: DRG neurons, 1200 V, 2 pulses and 20 ms pulse duration and PC12 cells, 1410 V, 1 pulse, and 30 ms pulse duration. The constructs used were kindlin-1 and kindlin-2 linked to cherry donated by Reinhard Fassler (Ussar et al., 2008; Montanez et al., 2008), full length and constitutively active full length talin from David Critchley (Goult et al., 2009) and talin head from Mark Ginsberg (Calderwood et al., 1999). Co-transfection rates using these constructs and methods are around 80% in our laboratory.

2.4. Post-fixation immunocytochemistry

Cell cultures on coverslips were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100, blocked with goat or donkey serum and incubated with primary antibodies at 4 °C overnight. They were then washed and incubated with secondary antibodies for 1 h at room temperature before being mounted on slides. Primary antibodies were used against beta III tubulin (Sigma, 1:400), pY397 FAK (BD Biosource, 1:100), talin (clone 8D4, Abcam, 1:20), GFP (Abcam, 1:500), and mCherry (Clontech, 1:200).

2.5. Live immunocytochemistry

The 9EG7 antibody was added to the cultures for 15 min at 37 °C. After washing with culture medium, the cultures were fixed with 4% PFA. They were then incubated in the FITC-conjugated goat anti-rat antibody for 1 h, before being mounted on slides.

2.6. Post-fixation immunohistochemistry of cryostat sections

Animals were first sacrificed with an overdose of sodium pentobarbital, and perfused transcardially with PBS followed by 4% PFA, before dissecting out the spinal cord and sciatic nerve. The tissue was cryoprotected in 30% sucrose solution at 4 °C for 3 days, embedded in OCT (RALamb UK) and cut longitudinally using a cryostat into 14 μm sections. Sections were washed in PBS (15 min, room temperature), permeabilized with 0.1% Triton X-100 (15 min, room temperature), blocked with 10% goat serum (1 h, room temperature) and incubated in primary antibodies (diluted in blocking solution) at 4 °C overnight. The following day, sections were washed with PBS, incubated with secondary antibodies (1 h, room temperature) and then mounted in Fluorosave. Primary antibodies were used against: talin (clone 8D4, Abcam, 1:20) and beta III tubulin (Sigma, 1:400).

2.7. Axon growth assay

For DRG neurons, two parameters were quantified as a measure of axon growth, i.e. (a) percentage of neurons with axons longer than the cell body diameter and (b) average of the longest axons extended by each neuron. For PC12 cells, only the axon length was quantified.

2.8. Quantitative immunofluorescence (QIF)

At least 20 axons per coverslip were first selected at random and imaged. An area of axons (>30 μm long) was then traced, and the fluorescence intensity of immunostaining analyzed using the Leica Application Suite (Leica Microsystems).

3. Results

3.1. Talin head overexpression does not affect axon growth

Talin and the kindlins are the main mediators of inside-out integrin signaling (Karakose et al., 2010; Goult et al., 2009; Kim et al., 2011; Calderwood et al., 2013; Ye et al., 2014). Overexpression of the head domain alone is sufficient to induce integrin activation (Calderwood et al., 1999; Kim et al., 2003). Activation of integrins by manganese, antibodies and kindlin-1 promotes axon growth (Hu and Strittmatter, 2008; Ivins et al., 2000; Lein et al., 2000; Tan et al., 2011). We therefore tested the effect of talin head domain overexpression on axon growth from cultured neurons growing on permissive and inhibitory substrates.

Dissociated adult DRG neurons were transfected with DNA constructs encoding the talin head or control GFP and cultured on growth-promoting (laminin) or growth-inhibitory (aggrecan–laminin) substrates for two days. Surprisingly, overexpression of the talin head did not affect axon growth on either substrate (Fig. 1A–C). We therefore asked if it had had any effect on the activation, expression level or signaling from integrins in the axons. While the total amount of surface β1 integrin was unchanged ($p = 0.42$, t-test) (Fig. 1D), the level of activated β1 integrin was significantly increased, as measured by 9EG7 immunostaining (+30.0%, $p < 0.05$, t-test) (Fig. 1E). However, this was not accompanied by an increase in the level of ‘outside-in’ integrin signaling, as measured by pY397 FAK intensity. In fact, the pY397 FAK level decreased following talin head overexpression in neurons cultured on laminin (−48.8%, $p < 0.01$, t-test) (Fig. 1F). We further determined that overexpression of the head domain of talin did not interfere with the production of endogenous full-length talin by the neurons, as determined using 8D4, an antibody that specifically recognizes the tail domain of talin (Praekelt et al., 2012) ($p = 0.81$, t-test) (Fig. 1G).

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