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The Effects of Tag-1 on the Maturation of Mouse Cerebellar Granule Neurons

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Abstract

The cell adhesion molecule Tag-1 is highly expressed in immature cerebellar granule neurons (CGNs) during axonogenesis and is down-regulated prior to onset of radial migration. However, its precise role(s) during development of mammalian CGNs has been unclear. Here we studied the effects of anti-Tag-1 function blocking antibodies on the development of mouse CGNs in primary cell culture and in situ. Interfering antibodies inhibited axon formation by mouse CGNs in both cell cultures and in cerebellar slices. Effects on axon extension in cell cultures were observed under conditions of homotypic cell–cell contact, consistent with inhibition of cell adhesion activity. Further, when used as a substratum Tag-1 protein strongly stimulated neurite outgrowth by CGNs. Antagonism of Tag-1 also enhanced CGN migration in modified Boyden chamber assays. Radial migration was inhibited by Tag-1 antibodies in cerebellar slices, possibly reflecting a block in early CGN maturation in situ. These findings are consistent with a regulatory role for Tag-1 in axon emergence as well as migratory behavior by developing mouse CGNs.

Keywords

Cerebellar granule neuron; Neuronal differentiation; Axonogenesis; Cell adhesion

Introduction

Development of mammalian cerebellar granule neurons (CGNs) occurs largely during the early postnatal period (reviewed in Goldowitz and Hamre 1998). Immature CGNs initiate differentiation by extending bipolar axons within the mid-region of the external germinal layer (EGL). In the deeper EGL (pre-migratory zone (PMZ)), axon extension continues and CGNs initiate radial migration through the molecular layer (ML) until reaching the internal granule cell layer (IGL). Upon onset of radial migration, CGN axons form fascicles of parallel fibers within the PMZ/ML.

Transient axonal glycoprotein 1 (Tag-1)/contactin-2 is a cell adhesion molecule possessing diverse roles in different neuronal populations, including the regulation of migration (Denaxa et al. 2001, 2005), neurite outgrowth (Furley et al. 1990; Stoeckli et al. 1991), and axon guidance (Stoeckli and Landmesser 1995). In the developing mouse cerebellum, Tag-1 is transiently up-regulated in CGNs within the PMZ during the first postnatal week, and it is present on both cell bodies and elongating parallel fibers just prior to onset of radial migration (Pickford et al. 1989; Yamamoto et al. 1990). Tag-1 is also associated with extending CGN growth cones (Buttiglione et al. 1998), suggesting an important role in parallel fiber extension and fasciculation. Further, soma expression suggested Tag-1 involvement in radial migration onset (Stottmann and Rivas 1998) and/or in tangential migration (Bailly et al. 1996).

While studies in chick cerebellum have implicated Tag-1/axonin-1 in parallel fiber alignment (Baeriswyl and Stoeckli 2008), there has been little evidence of a role for Tag-1 in parallel fiber formation by mammalian CGNs. For example, *Tag-1* null mice showed no obvious cerebellar phenotype at P2, apparently reflecting compensatory mechanisms (Fukamauchi et al. 2001). Here, we used acute function blocking studies to investigate the actions of Tag-1 during development of mouse CGNs.

Methods

Cell and Tissue Culture

Dissociated and re-aggregated CGNs and cerebellar slices were prepared from P5-P6 CD1 mice and cultured in Neurobasal/B27 medium as described previously (Wang et al. 2007). Recombinant VSVG-pseudotyped retroviruses expressing EGFP were used to infect cerebellar slice cultures (4×10^7 infectious units per well) (Wang et al. 2007). >90% of viral-transduced cells within the cerebellar slice cortical layers are CGN progenitors and their postmitotic progeny (Wang et al. 2007). All protocols employed for mouse studies were in full compliance with the National Institutes of Health Guide and Use of Laboratory Animals.

Immunofluorescence

Cerebellar granule neurons (CGN) cultures and cerebellar slices were fixed with 4% paraformaldehyde and analyzed by immunofluorescence as previously described (Wang et al. 2007). Samples were blocked with 10% normal goat serum and then incubated with primary and Cy3-conjugated secondary antibodies. The following primary antibodies were used: anti-Tag-1 (clone 4D7 1:100), anti-GFP monoclonal antibody (1:1000, Millipore, Temecula, CA), and pan-axonal neurofilament monoclonal antibody (pNFL) (SMI-312, 1:1000). Nuclei were stained with 1 μ g/ml bisbenzimidazole (Sigma) following treatment with secondary antibodies.

Axon Outgrowth Assays

96-well culture plates were coated with human Tag-1 protein (R&D System, Minneapolis, MN) or bovine serum albumin (BSA) (Fraction V; Sigma) for 2 h at 37°C. Dissociated CGNs were cultured for 24 h and neurites were visualized using Calcein AM (Invitrogen, Grand Island, NY). For Tag-1 antibody blocking studies, CGNs or re-aggregates were plated on poly-D-lysine (PLY)-treated chamber slides and incubated with 250 μ g/ml Tag-1 blocking antibody (Denaxa et al. 2001), control antibody (anti-LacZ, Cortex Biochem, San Leandro, CA), or pre-immune serum for 24 h. Axons were stained with anti-pNFL antibody and their lengths were quantified by computer-assisted microscopy (Wang et al. 2007). For re-aggregates, the area occupied by all axons was normalized to the area of each cell cluster, and only clusters of similar size were compared (Wang et al. 2007). Eight to ten re-

aggregates were examined per field within five randomly selected fields per experiment. Cerebellar slices were incubated with Tag-1 blocking antibody (250 $\mu\text{g}/\text{ml}$) or pre-immune serum for 60 h. A total of five separate slices were examined with 300–400 GFP(+) cells analyzed per treatment group. Virus-transduced cells were identified using GFP antibody. The total number of GFP(+) cells in slices did not significantly vary for different antibody treatments (data not shown).

CGN Migration

Migration assays were performed using Transwell membrane filters (pore size 5 μm) (Wang et al. 2007) with the under surfaces coated with PLY for 2 h at 37°C. Dissociated CGNs (10^5 per well) were incubated in the upper chamber with Tag-1 blocking antibody (250 $\mu\text{g}/\text{ml}$) or pre-immune serum in NeurobasalTM/B27 medium.

Statistical Tests

A minimum of three independent experiments were performed and data were analyzed using the Student's *t*-test. Results were expressed as the average $\pm$ SE and *P* values <0.05 were considered significant.

Results

To address endogenous Tag-1 activity during mouse CGN axon formation, we employed blocking antibodies that were previously used to examine the role of Tag-1 in other neuronal populations (Denaxa et al. 2001; Kyriakopoulou et al. 2002; Morante-Oria et al. 2003). In re-aggregate cultures, neurite extension was dramatically inhibited by Tag-1 antibodies, while control serum and LacZ antibodies were without effect (Fig. 1a). Interestingly, Tag-1 antibodies did not interfere with axon extension by dissociated CGN cultures (control: $66.5 \pm 3.6 \mu\text{m}$; anti-Tag-1: $68.2 \pm 2.4 \mu\text{m}$; Fig. 1b). Thus, Tag-1 action on neurite extension in vitro was only apparent under conditions of homotypic cell–cell contact.

To examine the effects of Tag-1 interfering antibodies in situ within the developing cerebellar cortex, P5 cerebellar slices were infected with GFP-expressing retrovirus to monitor parallel fiber extension. In coronal slices incubated with control antibody, most GFP(+) processes were present as long parallel projections at the interface between the PMZ and ML (Fig. 2a). In contrast, slices treated with Tag-1 blocking antibody contained only immature GFP(+) CGNs with very short bipolar axons, and few processes were evident at the PMZ/ML junction (Fig. 2a). Thus, Tag-1 interference blocked the initial emergence of parallel fibers within the mouse EGL.

The impact of Tag-1 inhibitory antibodies on radial migration from the EGL to the ML/IGL was also assessed using sagittal cerebellar slices. GFP(+) CGN cell bodies were present within all three cortical layers using control antibody. However, Tag-1 blocking antibodies greatly diminished the departure of CGNs to lower layers and cells remained localized to the EGL (Fig. 2b).

Tag-1 expressed on the surface of Chinese hamster ovary (CHO) cells was reportedly unable to promote CGN neurite extension by itself (Buttiglione et al. 1998). Based on the above studies, we revisited this question by testing the ability of a Tag-1 substrate to promote CGN axon formation. Tag-1 increased the average lengths of CGN neurites in a concentration-dependent manner by as much as 20-fold relative to BSA (Fig. 3). Thus, extracellular Tag-1 stimulates axon extension from maturing mouse CGNs, consistent with antibody blocking studies.

Modified Boyden chamber assays were used to directly assess Tag-1 migratory function in maturing CGNs. Tag-1 interference caused a ~2-fold increase in CGN migration relative to that observed with pre-immune serum (Fig. 3b). This suggested that endogenous Tag-1 exerts an inhibitory effect on the migratory behavior of immature CGNs.

Discussion

Based on its pattern of expression in the developing cerebellum, Tag-1 was postulated to participate in CGN axon extension and fasciculation (Pickford et al. 1989; Yamamoto et al. 1990). Our studies show that (1) Tag-1 promotes mouse CGN axon outgrowth as a substrate, and (2) Tag-1 interfering antibodies greatly impair axon emergence from CGNs in primary cultures as well as in situ. The effects in cultured cells were evident only under conditions of homotypic cell–cell contact, consistent with a cell adhesion function for Tag-1. Due to the early effects of blocking antibodies on axon emergence, we could not assess their impact on CGN axon fasciculation per se. The stimulatory actions of Tag-1 substrate on axon extension contrast with an earlier study in which Tag-1 expressed on CHO cells did not stimulate CGN neurite outgrowth (Buttiglione et al. 1998). These divergent results likely reflect differing surfaces used for measuring neurite outgrowth. In particular, CHO cells do not appear to be an optimal surface for observing Tag-1 substrate function (D. Karagogeos, unpublished results). Although not entirely ruled out here, indirect actions of Tag-1 on axon extension involving inhibition of CGN migration seem unlikely based on migratory studies in culture (see below). In total, the present findings strongly suggest the involvement of Tag-1 in axon emergence by developing mouse CGNs.

While Tag-1 is expressed in multiple neuronal populations and in glia, *Tag-1*-deficient mice show alterations in only a subset of neural cell types (Fukamauchi et al. 2001; Traka et al. 2003; Denaxa et al. 2005). Previous analyses of the cerebella of P2 *Tag-1* null mice found no overt granule neuron phenotype (Fukamauchi et al. 2001). Our own analyses of P10 cerebella from wild-type and *Tag-1*-deficient mice also found no significant differences in parallel fibers or the thicknesses of the EGL, ML, and IGL (data not shown). As previously suggested (Fukamauchi et al. 2001), this may reflect compensatory alterations during mouse development. Such mechanisms would not operate in acute function blocking studies as performed here. Similar differences between antibody blocking studies and *Tag-1* null mice also were reported for cortical interneuron migration (Denaxa et al. 2001, 2005).

Finally, the stimulatory effects of Tag-1 inhibition on CGN migration is consistent with an earlier proposed model in which down-regulation of Tag-1 within the PMZ promotes onset of CGN radial egress via dis-inhibition (Stottmann and Rivas 1998). Such effects were not apparent in slice studies, possibly due to the early arrest of CGN maturation within the EGL by Tag-1 antibodies.

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Fig. 1.

Inhibition of Tag-1 impairs axon extension by CGNs. **a** CGN re-aggregates on a PLY surface were treated with 250 µg/ml Tag-1 blocking antibody, anti-LacZ control antibody, or pre-immune serum for 24 h. Inhibition of Tag-1 function significantly reduced axon extension ($*P < 0.001$). Bisbenzimidazole staining of CGN nuclei is also shown. **b** Tag-1 blocking antibody does not affect axon formation in fully dissociated CGNs. Dissociated CGNs were treated with 250 µg/ml Tag-1 blocking antibody or pre-immune serum for 24 h. Axons were identified by neurofilament (NFL) staining in **a** and **b**. Scale bar 100 µm

Fig. 2.

Functional disruption of Tag-1 inhibits CGN parallel fiber formation in situ. Cerebellar slices prepared from P5 mice were transduced with GFP retrovirus and treated with 250 μ g/ml Tag-1 inhibitory antibody or pre-immune serum for 60 h. Virus-transduced cells were identified by immunostaining for GFP. **a** Tag-1 antibody significantly inhibits parallel fiber formation within the EGL. Note that bipolar processes are greatly truncated in anti-Tag-1-treated slices. Fasciculated parallel fibers in pre-immune serum-treated slices are indicated (*arrowhead*). **b** GFP(+) CGN migration from the EGL to lower layers was greatly reduced by Tag-1 antibody. Right panel shows numbers of GFP(+) CGNs in different layers.

*Significantly different from control antibody (Pre) ($P < 0.01$). Scale bar 100 μ m

Fig. 3.

Tag-1 promotes axon outgrowth and its inhibition stimulates migration by CGNs. **a** CGNs were plated on a Tag-1 surface at the indicated concentrations. The lengths of neurites were measured after 24 h. *Significantly different from control ($P < 0.01$). **b** Dissociated CGNs were assayed for migration using PLY-coated transwell plates. Addition of Tag-1 blocking antibody significantly increased the number of cells that migrated into the lower chamber relative to pre-immune serum (Pre)