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The cell adhesion molecule Tag1, transmembrane protein Stbm/Vangl2, and Lamininα1 exhibit genetic interactions during migration of facial branchiomotor neurons in zebrafish

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ABSTRACT

Interactions between a neuron and its environment play a major role in neuronal migration. We show here that the cell adhesion molecule Transient Axonal Glycoprotein (Tag1) is necessary for the migration of the facial branchiomotor neurons (FBMNs) in the zebrafish hindbrain. In *tag1* morphant embryos, FBMN migration is specifically blocked, with no effect on organization or patterning of other hindbrain neurons. Furthermore, using suboptimal morpholino doses and genetic mutants, we found that *tag1*, *lamininα1* (*lama1*) and *stbm*, which encodes a transmembrane protein Vangl2, exhibit pairwise genetic interactions for FBMN migration. Using time-lapse analyses, we found that FBMNs are affected similarly in all three single morphant embryos, with an inability to extend protrusions in a specific direction, and resulting in the failure of caudal migration. These data suggest that *tag1*, *lama1* and *vangl2* participate in a common mechanism that integrates signaling between the FBMN and its environment to regulate migration.

Introduction

Migration of newborn neurons from the germinal zone to their final positions is a critical step in the development of a functional nervous system. Defective neuronal migration can lead to severe impairments including mental retardation, epilepsy and learning disabilities (Marin and Rubenstein, 2003). Neuronal migration is largely dependent on the ability of the migrating neurons to interact with adjacent cells to sense and respond to migration cues. Cell migration involves the protrusion of a leading edge process and the formation of adhesion sites at the front while simultaneously contracting and releasing adhesions at the rear (Porcionatto, 2006). Adhesion sites are generated by interactions between cell adhesion molecules (CAMs) and extracellular matrix (ECM) molecules. CAMs play critical roles in various developmental processes, including axon pathfinding and neuronal migration (Sobeih and Corfas, 2002). Transient Axonal Glycoprotein 1 (Tag1), a glycophosphatidylinositol-anchored glycoprotein CAM, is a well-studied member of the immunoglobulin superfamily (Furley et al., 1990) with six immunoglobulin (Ig) domains followed by four fibronectin type III domains (Freigang et al., 2000). The Tag1 CAM plays a significant role in the tangential migration of neurons in the caudal medulla (Denaxa et al., 2005), and the neocortex in mouse (Denaxa et al., 2001). Tag1 also functions in the superficial migratory stream that produces the lateral reticular and external cuneate nuclei in mouse (Kyriakopoulou et al., 2002), and regulates growth cone behaviors of sensory neurons and interneurons in zebrafish (Liu and Halloran, 2005; and Wolman et al., 2008). *tag1* is expressed in the migrating facial branchiomotor neurons (FBMNs) in the mouse and zebrafish hindbrain (Chandrasekhar et al., 1997; Warren et al., 1999; and Garel et al., 2000); however, its role in tangential migration of FBMNs has not been examined.

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ECM molecules also regulate neuronal migration. Laminins are a major family of extracellular matrix glycoproteins that typically function as permissive cues for axon outgrowth and neuronal migration (Liesi et al., 1992, 1995; Kuhn et al., 1995, 1998; Adams et al., 2005; and Paulus and Halloran, 2006). Laminins are heterotrimeric protein complexes consisting of α, β and γ subunits, each of which has several isoforms (Colognato and Yurchenco, 2000; Libby et al., 2000; and Miner and Yurchenco, 2004). Laminins are required for cerebellar granule cell migration (Selak et al., 1985; and Liesi et al., 1995). Importantly, laminins can modulate a neuron's response to extracellular guidance molecules (Hopker et al., 1999; and Weinl et al., 2003). In zebrafish, *laminin*α*1* (*lama1*) is required for guidance of retinal ganglion and nucMLF growth cones (Karlstrom et al., 1996; Paulus and Halloran, 2006; and Wolman et al., 2008). In addition, *lama1* plays a role in facial branchiomotor neuron (FBMN) migration (Paulus and Halloran, 2006).

Branchiomotor neurons are generated in specific rhombomeres of the vertebrate hindbrain, and innervate muscles of facial expression, chewing, and vocalization (Lumsden and Keynes, 1989; and Chandrasekhar, 2004). In zebrafish, facial branchiomotor neurons (FBMNs) are born in rhombomere 4 (r4) and migrate caudally (tangentially) into r6 and r7 (Chandrasekhar et al., 1997; Higashijima et al., 2000; and Chandrasekhar, 2004). Several membrane proteins (Stbm/Vangl2, Celsr2, and Fzd3a) have been identified as necessary for FBMN migration (Bingham et al., 2002; and Wada et al., 2006). Interestingly, all of these molecules function non-cell autonomously for FBMN migration (Jessen et al., 2002; and Wada et al., 2006), and little is known about how these molecules function on cells surrounding the FBMNs to regulate their migration. We report here that the cell surface protein Tag1 is necessary for FBMN migration. Furthermore, *tag1*, *stbm*/*vangl2* and *lama1* exhibit strong genetic interactions for FBMN migration, and FBMN migratory behaviors are affected in a similar fashion in *tag1*, *stbm*, and *lama1* morphants. These results indicate that *tag1*, *stbm*, and *lama1* may regulate a common pathway in migrating FBMNs, and offer an approach to elucidate cell autonomous mechanisms underlying FBMN migration.

Materials and methods

Animals

Zebrafish (*Danio rerio*) were maintained following standard protocols and university ACUC guidelines as described previously (Westerfield, 1995; and Bingham et al., 2002). For analysis of facial branchiomotor neuron (FBMN) migration, *Tg(isl1:gfp)* fish, which expresses GFP in branchiomotor neurons (Higashijima et al., 2000), were crossed into mutant backgrounds. The following mutant lines were employed in these studies: *trilobite* (*tri*^{tc240a}, Hammerschmidt et al., 1996; and *tri*^{m209}, Solnica-Krezel et al., 1996); and *bashful* (*bal*^{b765}, L.M., unpublished data; and *bal*^{uw1}, Paulus and Halloran, 2006). Embryos were developed at 28.5 °C and staged by hours post fertilization (hpf) (Kimmel et al., 1995).

Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed according to standard protocols described previously (Chandrasekhar et al., 1997; and Bingham et al., 2002) using the following antibodies: acetylated α-tubulin (Chitnis and Kuwada, 1990; Sigma, 1:500 dilution), zn5 (Trevarrow et al., 1990, Developmental Studies Hybridoma Bank (DSHB), 1:10 dilution), tyrosine hydroxylase (Guo et al., 1999; Chemicon/Millipore, 1:500 dilution), GFP (Vanderlaan et al., 2005; Invitrogen, 1:1000 for DAB reaction and 1:4000 for FITC labeling), 3A10 (Hatta, 1992; DSHB, 1:500 dilution), Laminin1 (Paulus and Halloran, 2006; Sigma, 1:400 dilution) and TAG1 (Lang et al., 2001; 1:500 dilution). Fluorescent immunolabeling was performed using RITC-conjugated secondary antibody (for zn5, α-tubulin and TAG1 antibodies; Jackson Immunochemicals, 1:500), and FITC-conjugated secondary antibody (for GFP antibody; Invitrogen, 1:500). Synthesis of digoxygenin labeled probes and whole mount in situ hybridization were carried out using procedures described previously (Chandrasekhar et al., 1997; and Vanderlaan et al., 2005). Stained embryos were deyolked and mounted in glycerol, and imaged using DIC optics on an Olympus BX60 microscope. At least 10 wild-type and 10 mutant embryos were examined for all comparisons. For confocal imaging, embryos were mounted in glycerol and imaged using an Olympus IX70 microscope equipped with a BioRad Radiance 2000 confocal laser system. Images were processed in Adobe Photoshop to adjust brightness and contrast only.

Morpholino and mRNA injections

Morpholinos targeting *tag1* (MO1; Liu and Halloran, 2005), *stbm*/*vangl2* (Jessen et al., 2002) and *lama1* (MO1; Pollard et al., 2006) were obtained from Gene Tools (Corvallis, OR) or Open Biosystems (Huntsville, AL). For each MO, we performed at least two dose–response experiments to determine the doses that either resulted in a majority of embryos with normal or intermediate FBMN migration phenotypes (suboptimal dose; Figs. 2B, D) or completely blocked FBMN migration (optimal dose; Figs. 2C, D). Intermediate migration phenotypes spanned a spectrum of defects ranging from incomplete (partial) migration out of r4, with FBMNs found throughout the migratory pathway from r4 to r7 on both sides of the hindbrain (Figs. 2B, 5C, E) to relatively normal migration on one side of the hindbrain and an almost complete block of migration on the other side (Fig. 5B). We estimated the dose per embryo based upon the concentration of the MO solution, and the diameter (volume) of the injection bolus in the yolk cell. We typically injected 3–4 nl per embryo. The following doses (suboptimal, optimal) were used: *tag1* MO (6 ng; 12 ng); *stbm* MO (2 ng, 4 ng); and *lama1* MO (1 ng, 2 ng). For the rescue experiments with *tag1* RNA (Fig. 3), a dose of 9 ng *tag1* MO was used. For the genetic interaction experiments (Figs. 5–8), we co-injected two MOs at the sub-optimal doses. For single MO experiments, controls were either uninjected embryos or embryos injected with a standard control MO (7–10 ng) from Gene Tools (5′-CCTCTTACCTCAGTT-ACAATTTATA). Since the control MO did not affect FBMN migration (Fig. 8), many experiments included only uninjected embryos as controls. For the double MO experiments, controls included injection of single MOs with an appropriate amount of the control MO to match the total MO dose of the double MO-injected embryos. Embryos injected with a suboptimal dose of one MO alone or co-injected with control MO exhibited identical FBMN phenotypes (data not shown), indicating that the enhancement of FBMN migration defects seen in double MO-injected embryos (Fig. 8) is not a non-specific effect of increasing MO dose.

Since we observed increased cell death in *tag1* morphants at the optimal (12 ng) but not suboptimal (6 ng) dose, *p53* MO was co-injected with *tag1* MO in a 1.5:1 ratio to block *p53*-induced apoptosis (Robu et al., 2007). The FBMN migration defect was similar between *p53*+*tag1* MO-injected and *tag1* MO-injected embryos (data not shown), indicating that the migration defect is not a non-specific effect of the *tag1* MO dose. Data shown in Figs. 2 and 4 are from embryos injected with *tag1* MO alone and *tag1*+*p53* MOs, respectively.

Capped *tag1* mRNA was synthesized using the mMessage mMachine Kit (Ambion) from a template lacking the first 20 nucleotides of the 25 nt morpholino binding site in the 5′UTR. The mRNA was checked for purity and size by gel electrophoresis and estimated by UV spectrometry, and ~600 pg was injected into 1–2 cell stage embryos as described previously (Vanderlaan et al., 2005).