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L1 syndrome mutations impair neuronal L1 function at different levels by divergent mechanisms

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ABSTRACT

Mutations in the human L1CAM gene cause neurodevelopmental disorders collectively referred to as L1 syndrome. Here, we investigated cellular pathomechanisms underlying two L1 syndrome mutations, R184Q and W1036L. We demonstrate that these mutations cause partial endoplasmic reticulum (ER) retention of L1, reduce L1 cell surface expression, but do not induce ER stress in neuronal NSC-34 cells. We provide evidence that surface trafficking of mutated L1 is affected by defective sorting to ER exit sites and attenuated ER export. However, in differentiated neuronal cultures and long-term cultured hippocampal slices, the L1-R184Q protein is restricted to cell bodies, whereas L1-W1036L also aberrantly localizes to dendrites. These trafficking defects preclude axonal targeting of L1, thereby affecting L1-mediated axon growth and arborization. Our results indicate that L1 syndrome mutations impair neuronal L1 function at different levels, firstly by attenuating ER export and secondly by interfering with polarized neuronal trafficking.

Introduction

The neural cell adhesion molecule L1 plays a critical role in neuronal migration, axon growth and synapse formation in the developing nervous system (Maness and Schachner, 2007). The importance of L1 is reflected by gene mutations that cause clinically variable neurological disorders jointly referred to as L1 syndrome (Panicker et al., 2003). This X-linked syndrome comprises a broad phenotypic spectrum including hydrocephalus, mental retardation, aphasia, spastic paraplegia and adducted thumbs (Weller and Gärtner, 2001; Schäfer and Altevogt, 2010; Vos and Hofstra, 2010). Missense mutations account for over one-third of pathological L1 mutations described, and those affecting extracellular domains result in more severe clinical consequences than those affecting the cytoplasmic part of L1 (Kenwrick et al., 2000). Domain modeling studies predicted that some missense mutations affect structurally important amino acid sites in the extracellular domains which may cause protein misfolding (Bateman et al., 1996). We and others have shown that such missense mutations interfere with homo- and heterophilic ligand binding, intracellular trafficking, neurite growth as well as neurite branching and reduce L1 cell surface expression *in vitro*, which may correlate to their pathogenicity in L1 syndrome patients (De Angelis et al., 1999; Moulding et al., 2000; De Angelis et al., 2002; Michelson et al., 2002; Cheng and Lemmon, 2004). In support of this view, many pathological features of L1 syndrome patients are also observed in L1-deficient mice (Dahme et al., 1997; Cohen et al., 1998). Notably, transgenic mice engineered to express the L1 missense mutation C264Y display a phenotype indistinguishable from L1 knockout mice (Runker et al., 2003). In L1-C264Y mice, the mutant protein accumulates in cell bodies, probably in the ER, and is not detectable at neuronal cell surfaces (Runker et al., 2003). Together, these studies indicate that a loss of function is responsible for the severe forms of L1 syndrome.

ER retention of misfolded transmembrane proteins has been implicated in various disorders such as cystic fibrosis (Ward et al., 1995) and Kallmann syndrome (Monnier et al., 2009). The pathogenicity of these mutations results most likely from disrupted cell surface trafficking and thus functional loss of the gene products. In addition, as

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shown for several neurological disorders, ER accumulation of mutated protein also induces ER stress that renders cells more vulnerable for apoptosis (Oyadomari and Mori, 2003; Lindholm et al., 2006).

The mechanisms underlying ER accumulation and reduced cell surface expression of human pathogenic L1 proteins as well as possible consequences for cellular homeostasis are not known. Studies of the molecular mechanisms involved in neuronal (mis) trafficking of L1 may therefore help to better understand L1 syndrome pathogenesis. We here investigated two different L1 missense mutations that reduce cell surface expression, cause partial ER retention and attenuate ER export of L1 but do not induce ER stress. Transfection experiments in differentiated neuronal cultures and organotypic hippocampal slice cultures demonstrate that both mutations impair axonal targeting of L1 and thereby interfere with L1-mediated axon growth and arborization. Our study provides evidence that L1 syndrome mutations can affect neuronal L1 function at different levels, firstly by impairing ER export and secondly by interfering with polarized axonal targeting of L1.

Materials and methods

Antibodies and reagents

The following primary antibodies and dilutions were used for immunocytochemistry (ICC) and immunoblot (IB) analyzes: rabbit anti-human L1 (2.6 mg/ml; 1:2000 ICC; 1:5000 IB), anti-KDEL (10C3, Santa Cruz, 1:300 ICC), anti-N-cadherin (BD Transduction Laboratories, 1:1000, ICC), anti-HSP90 (SPA-846, StressGen, 1:10,000, IB), anti-actin (AC-40, Sigma, 1:2000, IB), anti-phospho-PERK (16F8, Cell Signaling, 1:1000, IB), anti-phospho-EIF2α (119A11, Cell Signaling, 1:1000, IB), anti-CHOP (F-168, Santa Cruz, 1:1000, IB), anti-c-myc (9E10, Santa Cruz, 1:2000, ICC and IB), anti-GFP (mab3580, Millipore, 1:1000, ICC), anti-MAP2 (Leinco, 1:1000, ICC), anti-calnexin (Abcam, 1:800, IB) and anti-p58K (Sigma-Aldrich, 1:500, IB). Fluorochrome- or horseradish peroxidase-conjugated secondary antibodies were from Invitrogen or Jackson ImmunoResearch. Reagents were from the following suppliers: PBS, trypsin–EDTA, culture media and supplements (Invitrogen), Fluoromount (Southern Biotechnology), 4′-6-diamidino-2-phenylindole (DAPI), complete protease inhibitors (Roche), poly-L-lysine (Biochrom AG), MG132 and staurosporine (Tocris). All other chemicals and reagents were purchased from Roth unless otherwise indicated.

Expression plasmids

The construction of L1 cDNAs containing individual missense mutations or encoding for L1-Fc protein was previously described (De Angelis et al., 1999, 2002). The cDNA for wild-type Sar-1 (Sar1-wt) was amplified from mouse spinal cord with the Pfu ultra high-fidelity DNA polymerase (Stratagene) using the sense primer: 5′-GTCGACATGTCT TTCATCTTTGAGTGGATC-3′ and antisense primer: 5′-GGATCCTCAGT-CAATATACT GGGAAAGCCAG-3′. The PCR product was cloned into the pCR2.1 vector using TA-cloning kit (Invitrogen). The H79G, GTP-restricted, mutant form of Sar-1 (Sar1-GTP) was generated by site-directed mutagenesis using pCR2.1-Sar1-wt as a template with the following primer sequence (sense): 5′-TCTCGGTGGGGGTGAGCAAG-CACG-3′. Sar1-wt and Sar1-GTP were then cloned into *Xba*I/*Eco*RI sites of pCS2+-myc expression vector to incorporate in frame a myc-tag sequence at the 5′ end of both cDNA. GFP-ARF1 constructs were kindly provided by Carol Gaillard-Baron. The DsRed–galactosyltransferase construct was purchased from Clontech. The probity of each construct was verified by sequencing.

Cell culture, transfection and immunocytochemistry

NSC-34 cells (Cashman et al., 1992) were cultured in Minimum Essential Medium (MEM) supplemented with 10% FCS and transfected with cDNA constructs using cationic lipid reagents according to the manufacturer's protocol (Invitrogen). Twenty hours later, cells were fixed by addition of an equal volume of 4% PFA directly into the medium for 15 min at 37 °C, washed in PBS and postfixed for 20 min in 4% PFA at room temperature (RT). The cells were permeabilized in 0.2% Triton X-100 (TX-100) in PBS for 5 min, blocked with 5% goat serum, 0.1% BSA in PBS for 1 h and incubated o/n with the primary antibodies diluted in blocking solution. After washing, cells were incubated with fluorochrome-conjugated secondary antibodies, 0.2 µg/ml DAPI/PBS, and mounted in Fluoromount. To quantitatively assess cell surface expression of wild-type and mutant human L1, live NSC34 cells were incubated with specific antibodies against the extracellular part of human L1 (De Angelis et al., 1999, 2002) for 30 min at 17 °C, washed with MEM/2% FCS, incubated with Cy3-conjugated secondary antibodies for 30 min at 17 °C, washed and fixed in 2% PFA for 20 min at RT. Cells were then blocked and permeabilized in PBS containing 5% goat serum, 0.1% BSA, 0.025% TX-100 for 1 h, incubated o/n with specific antibodies against GFP and processed as described above. Images were taken with identical acquisition parameters using a 20× objective and analyzed by ImageJ software using the "analyze particles" function. For cell surface labeling in other experiments, NSC-34 cells and primary neurons were fixed in 1.5% PFA for 20 min at RT, blocked and incubated with specific antibodies against human L1. Cells were then washed and permeabilized in 0.2% TX-100 for 5 min, followed by immunostaining with antibodies against GFP, MAP2 or myc-tag, respectively, and processed as described above. Primary neuronal cortical cultures were prepared from E15.5 mice embryos essentially as described (Bock and Herz, 2003) and 7×10^4 cells seeded on 12 mm glass coverslip coated with poly-L-lysine or L1-Fc protein. L1-Fc protein was produced and purified as described (De Angelis et al., 1999). Coverslips were preincubated with goat anti-human Fc-specific antibody (Jackson ImmunoResearch) followed by incubation with 5 µg/ml L1-Fc protein according to (Alberts et al., 2003). The medium of cultured neurons was changed after 20 h to Neurobasal medium, supplemented with 2% B27, 1% penicillin/streptomycin and 1 mM Ultraglutamine (Lonza). DNA transfection of neuronal cultures was performed at 2 div or 6 div by magnetofection, according to the method of Igor Medina's lab (Buerli et al., 2007), and processed for cell surface labeling as described above. Images were acquired by confocal microscopy with an inverted LSM 510 microscope using 63×, 40× or 20× objectives (Zeiss).

Immunoblots, ER stress induction and glycosidase digestion

NSC-34 cells were seeded into coated 6-well plates at a density of 200,000 cells/well. The next day, cells were transfected with cDNA constructs, cultured for 24 to 72 h and collected in lysis buffer, and equal amounts of proteins, as determined using BCA kit (Pierce), were subjected to SDS–PAGE and immunoblotting. Immunoblots were revealed using Pierce ECL kit (Pierce). For ER stress induction, cells were treated for 4 h with 1 µM thapsigargin (Calbiochem) or transfected with pCS2+-myc incorporating the human $SOD1^{A4V}$ mutant form (Raoul et al., 2005). For deglycosylation of proteins, NSC-34 cells were harvested 24 h posttransfection, lysed, denatured and then incubated overnight at 37 °C with or without Endoglycosidase H (EndoH) or N-Glycosidase F (PNGaseF) following the instructions of the manufacturer (New England Biolabs).

Subcellular fractionation

NSC-34 cells were cultured and transfected in 10 cm culture dishes as described above. After 2 days, cells from five dishes for each condition were homogenized through a 27-gauge needle in 1.5 ml of buffer containing 0.25 M sucrose, 1 mM EDTA, 5 mM NaCl, 10 mM KCl, 10 mM Tris–HCl, pH 7.4, and centrifuged for 10 min at 900×*g* to remove nuclei. The postnuclear supernatant was adjusted to 5% Optiprep (Axis Shield) and loaded on the top of a discontinuous 5% step OptiPrep gradient (10–