Neuroscience 189 (2011) 68–78

CELLULAR DISTRIBUTION OF METASTASIS SUPPRESSOR 1 AND THE SHAPE OF CELL BODIES ARE TEMPORARILY ALTERED IN ENGRAILED-2 OVEREXPRESSING CEREBELLAR PURKINJE CELLS

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Abstract—Metastasis suppressor 1 (MTSS1, BEG4, MIM) is well described for its function as a metastasis suppressor gene and is expressed in a variety of tissues. However, only little is known about its expression in the central nervous system (CNS), and functions within the CNS have not been addressed so far. Here, we show that MTSS1 was expressed in postmitotic neurons of the cerebellar cortex. Within Purkinje cells, higher amounts of MTSS1 were temporarily localized in the axonal somatic compartment than in the dendritic compartment. In L7En-2 transgenic mice, in which the segment-polarity gene and regulator of neuronal maturation Engrailed-2 is overexpressed specifically in cerebellar Purkinje cells, MTSS1 was homogenously distributed within Purkinje cell somata throughout development. In parallel to the altered distribution of MTSS1 in L7En-2 Purkinje cells, L7En-2 Purkinje cell somata were distorted and in some cells invaginations of the plasma membrane were observed. These invaginations were only found in L7En-2 neurons, and displayed multiple synapses which could not be seen at the smooth surface of wildtype Purkinje cell somata. Current knowledge about MTSS1 function *in vitro* and the correlation between MTSS1 localization and the occurrence of membrane alterations in L7En-2 Purkinje cells described here suggest that MTSS1 might be involved in shaping neuronal membranes *in vivo*.

Key words: cerebellum, engrailed-2, actin cytoskeleton, membrane curvature, synaptogenesis, BAR domain.

Membranes within and around cells are typically bent and adopt a variety of shapes. Intracellularly, they form the endoplasmatic reticulum, the Golgi complex, or small vesicles. At the cell surface, membrane bending is often seen at the basal and/or apical poles of a cell giving rise to structures such as the basal folding of the proximal renal tubule or tubule like protrusions at the apical side of enterocytes (microvilli). Neurons of the peripheral and central nervous system also form membrane protrusions with various bending characteristics. Dendrites and axons are surrounded by less curved membranes while dendritic spines are highly curved structures. For energetical reasons lipid bilayers will tend to have a symmetric distribution of lipids and normally form a flat shape, hence every curvature within membranes is created by external forces. Such forces are generated by a complex interplay between lipids and proteins (Graham and Kozlov, 2010). In order to generate lipid asymmetries, either the lipid composition has to be changed, or proteins have to be integrated into the membrane. The most effective proteins for shaping cellular membranes are proteins containing a BAR domain. This BAR domain was originally described for Bin, Amphiphysin, and Rvs proteins (Sakamuro et al., 1996). The BAR domain forms amphipathic helices which induces dimerization and targets BAR domain-containing proteins to highly curved, negatively charged plasma membranes suggesting that BAR domains are sensors of membrane curvature (Ramjaun et al., 1999; Habermann, 2004; Peter et al., 2004; Madsen et al., 2010; Drin and Antonny, 2010). In addition, BAR domain-containing proteins drive membrane bending and participate in forming synaptic vesicles during endocytosis and exocytosis (Takei et al., 1999; Zhang and Zelhof, 2002; Habermann, 2004). A further characteristic of BAR domain-containing proteins is its association with the actin cytoskeleton mediating clathrin-mediated endocytosis (Galletta et al., 2010). Besides N-BAR domain-containing proteins, proteins containing an F-BAR domain (Itoh et al., 2005), an ENTH domain, and an I-BAR domain (Itoh and De, 2006) have been described.

The I-BAR (inverse-Bin-Amphiphysin-Rvs) domain (also known as IRSp53-MIM-homology-domain or IMD domain in short) as found in IRSp53 and MTSS1 induces membrane tubulation and dynamic membrane bending as well, but has a straight shape reminiscent of a zeppelin rather than a curved shape typical for N-BAR domains (Millard et al., 2005; Suetsugu et al., 2006; Itoh and De, 2006; Lee et al., 2007). While many I-BAR domain-containing proteins do not bind directly to the actin cytoskeleton-they bind to actin-associated proteins such as Mena, N-WASP, mDia1, or Eps8 via their SH3 domain (Robens et al., 2010; Ahmed et al., 2010)—MTSS1 contains a WH2 domain (Wiskott–Aldrich-syndrome-protein-homology-2 domain; Scita et al., 2009) which is a small and widespread used actin-binding motif (Chereau et al., 2005) capable of sequestering actin mono-

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Abbreviations: CNS, central nervous system; I-BAR, inverse-Bin-Amphiphysin-Rvs; IGL, internal granule cell layer; PBS, phosphate buffered saline; PCs, Purkinje cells; RGV, relative gray value.

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2011.05.038

mers in an actin assembly reaction (Mattila et al., 2003; Machesky and Johnston, 2007). MTSS1 is thus an important linker molecule for the arrangement of actin filaments (Yamagishi et al., 2004). MTSS1 is localized in regions with highly dynamic actin-remodeling activities and its overexpression induces the formation of actin-rich protrusions similar to pseudopodia and micro spikes (Woodings et al., 2003; Yamagishi et al., 2004; Loberg et al., 2005). MTSS1 also has a function in cellular morphogenesis which is mediated by the cytoskeleton (Mattila et al., 2003; Yamagishi et al., 2004; Lin et al., 2005). In addition to the effect on normal cellular morphogenesis and motility, MTSS1 also affects the metastatic potential of tumor cells (Lee et al., 2002; Nixdorf et al., 2004; Loberg et al., 2005; Parr and Jiang, 2009).

While IRSp53 is well known to be expressed within the central nervous system, *mtss1* expression has only recently been shown by *in situ* hybridization to occur within the CNS in particular within the cerebellum (Glassmann et al., 2007). *Mtss1*-mRNA was transiently expressed in granule cells within 2 weeks after birth, and it was expressed throughout life in cerebellar Purkinje cells (PCs). In PCs, the expression of *mtss1* starts at a time when these neurons are settled within the cerebellar cortex and increases during the time of early dendritic development. Afterwards, *mtss1* expression remains at a rather high level in PCs until adulthood (Mattila et al., 2003; Glassmann et al., 2007). This expression pattern caused speculation that MTSS1 might be involved in process formation of cerebellar PCs. This was supported by a recent finding that *mtss1* expression was diminished in a transgenic animal model in which PC dendritogenesis was reduced (Holst et al., 2008). It has been reported that overexpressing the segment-polarity gene Engrailed-2 specifically in cerebellar PCs causes a retardation in the maturation of these cells in particular in the timed development of their dendritic tree (Jankowski et al., 2004). In these L7En-2 mice, *mtss1* was shown to be specifically down-regulated in PCs but not in neighboring granule cells.

In the present paper we investigated the detailed expression pattern and localization of MTSS1 in cerebellar Purkinje cells, and compared these findings to those obtained in L7En-2 mice. Since MTSS1 is involved in membrane bending we looked for changes in membrane curvatures of Engrailed-2 overexpressing PC neurons.

EXPERIMENTAL PROCEDURES

Animal husbandry and perfusion

Wildtype and transgenic mice were obtained by mating heterozygous L7En-2 (Baader et al., 1998) and wildtype FVB/N mice. With this method, L7En-2 mice were kept on a pure FVB/N background. To obtain timed pregnant mice, parents were mated around 5 PM and inseminal plugs were checked next morning around 9 AM. Afterwards, parental mice were separated. For staging of postnatal animals, the day of birth was defined as postnatal day 0 (P0). Mice designated as adult were at least 2 months and up to 1-year-old. For each experiment, at least two pairs of littermates derived from independent litters were used. All mice were handled in strict adherence to local governmental and institutional animal care regulations. Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local authorities.

Protein extracts and Western blot analysis

Protein extracts from cerebellar mouse tissue were prepared as described by Holst et al. (2008). Briefly, tissue was homogenized in lysis buffer containing 125 mM Tris/HCl, 0.1 g sodium dodecylsulfate (SDS), 10% sucrose, and 1 mM PefaBloc SC (Merck, Darmstadt, Germany) using glass homogenizers. After sonification, protein contents of the samples were determined by the BCA reagent from Pierce (Thermo Fisher Scientific, Bonn, Germany). Equal amounts of protein were loaded on to an SDS polyacrylamide gel and resolved according to the method of Laemmli (Laemmli, 1970). After electrophoresis, proteins were transferred to nitrocellulose by wet blotting (Biometra, Goettingen, Germany), and stained with Black Ink to ensure proper loading and blotting. Free protein binding sites on the membrane were blocked by incubation with 5% (w/v) skimmed milk in phosphate buffered saline (PBS: 10 mM Na_2HPO_4, and 150 mM NaCl; pH 7.4) containing 0.05% Tween 20 (PBST) for 1 h. Blocked membranes were then incubated with the primary antibody against MTSS1 (1:50; 6108A from Imgenex, San Diego, USA) over night at 4 °C, followed by incubation of membranes with a peroxidase-labeled secondary antibody against rabbit immunoglobulin (1:10,000; Sigma, Munich, Germany). Membranes were then washed in PBST, and bound antibodies were visualized using the ECL® detection system (GE Healthcare, Munich, Germany).

Immunohistochemistry on vibratome sections

Immunohistochemistry was done using perfused animals. Perfusions were performed by anesthetizing mice by i.p. injection of 2,2,2-tribromoethanol (Avertin; 2.5%, 0.03–0.06 ml/g body weight) before perfusing with mammalian Ringer's solution and 4% paraformaldehyde diluted in phosphate buffered solution (Jankowski et al., 2004).

Brains were dissected and postfixed in the same fixative for 6 h. After several rounds of washing in PBS, brains were embedded in 2% agar, trimmed and cut to 50 μm thick slices using a Leica VT1000S vibratome. After washing in PBS, sections were permeabilized by 0.5% Triton X-100 in PBS for 30 min. Blocking of unspecific binding sites was achieved by incubating sections in 0.2% gelatin/PBS for 30 min. Incubations with primary antibodies diluted in blocking solution containing 0.1% Triton X-100 were done at 4 °C over night. The following antibodies were used: MTSS1 (1:50; Imgenex), Synaptobrevin (1:50; Synaptic Systems, Goettingen, Germany), VGlut2 (1:500; Synaptic Systems), SMI36 (1:50, Covance, Muenster, Germany), Calbindin $D28K_{mouse}$ (1:1000; CB-955, Sigma), and Calbindin $D28K_{rabbit}$ (1:1000; CB-38, Swant, Bellinzona, Switzerland). After washing, sections were incubated with Alexa-labeled secondary antibodies (1:500 in blocking solution; Invitrogen, Darmstadt, Germany), and counterstained with Hoechst 34580 (1 μg/ml at room temperature for 10 min; Invitrogen). Sections were analyzed with a Leica TCS SP2 (Figs. 2 and 4D–F) or a Leica TCS SP5 (Figs. 1, 4A, B, and 6) confocal laser-scanning microscope (Leica, Wetzlar, Germany). The following parameters were used for imaging: images in Fig. 1 were acquired at a pinhole size of three airy discs and a resolution of 2048×2048 pixels (voxel size 0.53 μm×0.53 μm×0.30 μm); images in Fig. 2 were acquired at a pinhole size of one airy discs and a resolution of 2048×2048 pixels (voxel size: 0.12 μm×0.12 μm); Fig. 4A, B were acquired using three airy discs and the zooming in tool (2048×2048 pixel); images of Fig. 4D–F were acquired at a pinhole of one airy discs and a resolution of 4096×4096 pixels (voxel size: 0.09 μm×0.09 μm); and images of Fig. 6 were taken at a pinhole size of three airy discs and a