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The Susd2 protein regulates neurite growth and excitatory synaptic density in hippocampal cultures

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ABSTRACT

Complement control protein (CCP) domains have adhesion properties and are commonly found in proteins that control the complement immune system. However, an increasing number of proteins containing CCP domains have been reported to display neuronal functions. Susd2 is a transmembrane protein containing one CCP domain. It was previously identified as a tumor-reversing protein, but has no characterized function in the CNS. The present study investigates the expression and function of Susd2 in the rat hippocampus. Characterization of Susd2 during development showed a peak in mRNA expression two weeks after birth. In hippocampal neuronal cultures, the same expression profile was observed at 15 days in vitro for both mRNA and protein, a time consistent with synaptogenesis in our model. At the subcellular level, Susd2 was located on the soma, axons and dendrites, and appeared to associate preferentially with excitatory synapses. Inhibition of Susd2 by shRNAs led to decreased numbers of excitatory synaptic profiles, exclusively. Also, morphological parameters were studied on young (5DIV) developing neurons. After Susd2 inhibition, an increase in dendritic tree length but a decrease in axon elongation were observed, suggesting changes in adhesion properties. Our results demonstrate a dual role for Susd2 at different developmental stages, and raise the question whether Susd2 and other CCP-containing proteins expressed in the CNS could be function-related.

1. Introduction

In the nervous system, adhesion molecules participate in neuronal migration, neurite outgrowth, synapse formation, maintenance and plasticity, their diversity and patterns of expression allowing the elaboration of complex neuronal networks (Togashi et al., 2009). Altered functions of such proteins may lead to neurological developmental disorders (Ye et al., 2010; Redies et al., 2012), reflecting sometimes subtle defects in neuronal circuitry. Adhesion molecules are often part of large protein families, characterized by a specific protein domain with adhesive properties. This is the case for proteins of the immunoglobulin superfamily, for integrins and N-cadherins, and for the newly identified leucin rich repeat (LRR) superfamily (Linhoff et al., 2009; Nam et al., 2011). Recently, the proteins containing the complement control protein (CCP) domain have also been shown to be involved in several aspects of neuronal development in invertebrates and vertebrates (Nakayama and Hama, 2011).

CCP domains, also named Sushi or SCR (for a short consensus repeat), contain approximately 60 amino acids and are characterized by a consensus sequence that includes four invariant cysteines participating in an ovoid ternary structure (Schmidt et al., 2008). The CCP domains support protein–protein and protein–carbohydrate interactions. They were first identified in a number of plasmatic proteins of the immune system, in particular proteins regulating activation of the complement system (Sjöberg et al., 2009; Trouw and Daha, 2011). In *Drosophila*, the *Hikaru genki* gene codes a secreted protein with five CCP domains, identified in a screen for mutants exhibiting reduced locomotion (Hoshino et al., 1996). Location in the synaptic cleft and specific requirement of protein expression during development for correct behavioral phenotype suggested a role in synapse formation. In *Caenorhabditis elegans*, LEV-9, a secreted protein containing eight CCP domains, was shown to localize at the neuromuscular junction and to be necessary for the clustering of nicotinic acetylcholine receptors (Gendrel et al., 2009). In humans, three genes coding CCP-containing proteins were found to be related to clinical conditions: *CSMD1/2*, *Srpx2*, and *Sez6*. CSMD1/2 proteins are expressed in the nervous system and were identified as genetic susceptibility factors in schizophrenia (Håvik et al., 2011). *Srpx2* was found to be mutated in two different families suffering from rolandic seizures, mental

Abbreviations: aa, amino acid(s); AMOP, adhesion-associated domain in Muc-4 and other proteins; CCP, complement control protein; DIV, days in vitro; HA, human influenza hemagglutinin; HEK, human embryonic kidney; HRP, horseradish peroxidase; LRR, leucine rich repeat; SCR, short consensus repeat; SO, somatomedin B-like domain; VWD, von Willebrand factor type D domain.

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retardation and speech disorder (Roll et al., 2006). Involvement in the migration of cortical neurons in rodents has been suggested for Srpx2 (Royer-Zemmour et al., 2008) and, more recently, it was demonstrated to regulate excitatory synapse formation and vocalization in mice (Sia et al., 2013). Finally, mutation of the *Sez6* gene was identified as a risk factor for childhood febrile seizures (Yu et al., 2007). Manipulation of Sez6 protein levels in the mouse cortex was shown to affect dendritic arborization and excitatory synaptogenesis (Gunnersen et al., 2007). Hence, several CCP-containing proteins expressed in the CNS appear to play a role on its development and/or function.

After considering the expression of CCP-containing proteins in the CNS according to the Allen Brain Atlas (http://mouse.brain-map.org) and their described function in the literature, we identified a new candidate gene, of unknown function in the nervous tissue. The *Sushi domain containing 2* (*Susd2*) gene codes an 822 amino acid type 1 membrane protein with one extracellular CCP domain along SO, AMOP and VWD domains. Initial studies suggested a role for *Susd2* as a tumor-reversing gene (Sugahara et al., 2007) and showed an activity of the protein on cell attachment and migration. These authors also showed that Susd2 is expressed in the rodent brain. Because Susd2 mRNA expression levels are high in areas of the brain such as the hippocampus (Allen Brain Atlas), we hypothesized that Susd2 might play a specific role in such areas. Using primary hippocampal cell cultures, we investigated the expression and function of the protein in this model. We show in the present paper that Susd2 protein levels control neuritic outgrowth and excitatory synapse numbers.

1.1. Experimental methods

1.1.1. Construction of vectors

Three RT-PCRs targeting overlapping cDNA fragments of *Susd2* from adult rat hippocampus were necessary to obtain a full-length Susd2 PCR amplified fragment. Primers used were (from Nter to Cter; 5′–3′): (1) forward GGCAATCTCTGAGCCACTGT, reverse GGAAGTCATCACCC AGATGC; (2) forward GGCCCTCTGGACCAATGAT, reverse CAGAAACA TGCCCTTCAGGT; and (3) forward TGAACAAAATTGGATGGACCT, reverse GTCAAGGGTGCTGTGGTGT. Full-length Susd2 was cloned into the mammalian expression vector pEGFP-N1 (Clontech) to obtain a Susd2-GFP construct. Full-length Susd2, with an insertion of an N-terminal HA tag between the signal peptide and the SO domain, was similarly cloned to obtain the HA-Susd2-GFP construct. ShRNA#1 and shRNA#2 against Susd2 were obtained according to the manufacturer's instructions (pSUPER from Clontech) using the following oligonucleotides: shRNA#1 forward GATCCCCTGGGAACACTCAACGAT AATTCAAGAGATTATCGTTGAGTGTTCCCATTTTTGGAAA, shRNA#1 reverse AGCTTTTCCAAAAATGGGAACACTCAACGATAATCTCTTG AATTAT CGTTGAGTGTTCCCAGGG, shRNA#2 forward GATCCCCCTGCGTTCT TGAC GTGATATTCAAGAGATATCACGTCAAGAACGCAGTTTTTGGAAA, shRNA#2 reverse AGCTTTTCCAAA AACTGCGTTCTTGACGTGATATCTCT TGAATATCACGTCAAGAACG CAGGGG.

1.1.2. RT-PCR and quantitative RT-PCR

For standard RT-PCR, total RNA was isolated using the Qiagen RNAeasy Mini Kit. Equivalent amounts of total RNA (800 ng) were reverse-transcribed according to the protocol of Super ScriptII Reverse Transcriptase (Invitrogen). Negative controls were prepared by incubation of samples without reverse transcriptase. Primers used for Susd2 (5′–3′) were: forward CTATGACTCTCGGCCTCTGG, reverse ACTTCAAG CCTTCCTTGTGC; for GAPDH: forward ACAGCAACAGGGTGGTGGAC, reverse TTTGAGGGTGCAGCGAACTT. PCR was performed with an annealing temperature of 60 °C, for 34 cycles, using the manufacturer's instructions (Taq polymerase, New England Biolabs).

To perform quantitative RT-PCR (qRT-PCR), total RNA was isolated from three different Sprague–Dawley litters at different developmental stages. qRT-PCRs were performed using the reaction mixture SYBR Green I Master (Roche) in a LightCycler 480 (Roche). Susd2 mRNA was quantified using Pgk1 as the reference mRNA. Primers for Pgk1 (5′–3′) were: forward CTGACTTTGGACAAGCTGGACG, reverse GCAGCC TTGATCCTTTGGTTA. Efficiency (E) of each primer-specific PCR was calculated using serial dilutions. Crossing point (Cp) values for Susd2 were normalized to the Cp values of the Pgk1 control from the same experiment, as follows: $(E_{GAPDH}\hat{}Cp_{GAPDH}) / (E_{target}\hat{}Cp_{target})$. The size of the PCR products was confirmed on agarose gels.

1.1.3. Western blot

Transfected HEK cells were collected in a Laemmli buffer. Hippocampal cultured cells were resuspended in 50 mM Hepes, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100 and protease inhibitors (Complete Protease Inhibitor Cocktail, Roche). Equal amounts of proteins were separated by 12% SDS-PAGE and blotted onto polyvinyldifluoride membrane (Millipore). The membranes were hybridized with anti-GFP mouse antibody (1:2500, clone JL8, Clontech), anti-HA rat antibody (1:3000, Roche), anti-Susd2 rabbit antibody directed against extracellular amino acids 545–775 (1:300, gift from Toshiwo Andoh, Tokyo, Japan; characterized in Sugahara et al., 2007), and mouse anti-GAPDH antibody (1:3000, clone 6C5, Millipore). HRP-conjugated anti-rabbit or anti-mouse (1:50, DAKO) were used as secondary antibodies. Immunoblots were processed with a chemiluminescence detection system (ECL Plus Western blot detection reagent; GE Healthcare).

1.1.4. Coimmunoprecipitation

HEK cells were transfected with HA-Susd2-GFP and resuspended after 24 h in the same buffer as in the Western blot procedure. After homogenization and centrifugation, the supernatant was diluted in 0.2% Triton-X-100 to its final concentration. The solution was incubated with agarose beads (Chromotek) coated with anti-GFP antibodies at 4 °C for 4 h. After washing, beads were boiled at 95 °C in a Laemmli buffer to recover the eluate fraction. Immunoprecipitated (IP) and input (IN) samples were then used for Western blotting.

1.1.5. Cell culture

All research using animals in the present paper was carried according to the European Communities Council Directive of 24 November 1986 (86/609/EEC). Primary cultures of hippocampal neurons were prepared from E18 rat embryos as described in the literature by Goslin and Banker (1989) with some modifications of the protocol. HEK 293 cells were cultured at 37 °C in humidified 5% CO_2 incubators in DMEM (Gibco) containing penicillin, streptomycin, glutamin (Gibco) and 10% fetal bovine serum (v/v, Gibco).

1.1.6. Transfection procedure

Primary hippocampal cell cultures were transfected with 0.5–1 μg of total plasmid DNA per 20 mm-well. For the transfection of HEK cells, cultures were selected when showing 80% confluence in 9.6 cm^2 dishes and transfected with 2.5 μg of total plasmid DNA per dish. Lipofectamine 2000 (Invitrogen) was used for transfection in both cases.

1.1.7. In situ hybridization

In vitro transcription reactions of pGEMT vector (Promega) containing the Susd2 PCR-amplified fragment (see above) and incorporating digoxigenin-conjugated UTP (Digoxigenin-11-UTP, Roche) were used to obtain sense and antisense riboprobes. Products were purified using probeQuant (GE Healthcare). Hippocampal cells were fixed with paraformaldehyde (4% w/v, Serva Feinbiochemica) in PBS, pH 7.4. In situ hybridization (ISH) was adapted from Schaeren-Wiemers and Gerfin-Moser (1993), and Henrique et al. (1995). For riboprobe detection, an alkaline-phosphatase conjugated anti-digoxigenin antibody (1:3000, Roche) was used. Each experiment included an antisense control riboprobe.