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Short Communication

Endocytic adaptor protein intersectin 1 forms a complex with microtubule stabilizer STOP in neurons

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ABSTRACT

Intersectin 1 (ITSN1) is a multidomain adaptor protein that functions in clathrin-mediated endocytosis and signal transduction. This protein is highly abundant in neurons and is implicated in Down syndrome, Alzheimer's disease and, possibly, other neurodegenerative disorders. Here we used an *in vitro* binding assay combined with MALDI-TOF mass spectrometry to identify novel binding partners of ITSN1. We found that the neuron-specific isoform of the stable tubule-only polypeptide (STOP) interacts with SH3A domain of ITSN1. STOP and ITSN1 were shown to form a complex *in vivo* and to partially co-localize in rat primary hippocampal neurons. As STOP is a microtubule-stabilizing protein that is required for several forms of synaptic plasticity in the hippocampus, identification of this interaction raises the possibility of ITSN1 participation in this process.

1. Introduction

Intersectin 1 (ITSN1) is a multidomain endocytic adaptor protein that was shown to act at different stages of clathrin-mediated endocytosis from initiation of clathrin coat formation to vesicle fission (Henne et al., 2010; Simpson et al., 1999). Additionally, it is implicated in various processes such as signal transduction, apoptosis, receptor tyrosine kinase ubiquitylation and regulation of actin cytoskeleton rearrangements (reviewed in O'Bryan, 2010; Tsyba et al., 2011). ITSN1 functions as a scaffold for the organization of molecular complexes via protein–protein interactions performed by its domains. The most ubiquitous short isoform of ITSN1 contains two Eps15 homology domains (EH1 and EH2), a coiled-coil region and five Src homology 3 domains (SH3A-E). The long isoform (ITSN1-L), which is generated by alternative splicing, contains an extended C-terminus consisting of a Dbl homology (DH), a Pleckstrin homology (PH) and a C2 domain. In addition, numerous alternative splicing events affecting domain composition of ITSN1 have been reported (reviewed in Tsyba et al., 2011).

ITSN1 plays an important role in neuronal functioning. Immunohistochemical analysis revealed high level of *ITSN1* expression in rat brain with particular enrichment in hippocampus, globus pallidus, subthalamic nucleus, substantia nigra and layer III of neocortex (Ma et al., 2003). Studies of loss-of-function mutations of *Dap160* (*Drosophila* ortholog of human *ITSN1*) demonstrated that Dap160 is required for proper formation of synaptic boutons and organization of pre-synaptic endocytic zones in neuro-muscular junctions (Koh et al., 2004; Marie et al., 2004). Additionally, disrupting the expression of ITSN1 in mice leads to alterations in vesicle trafficking in neuronal cells (Yu et al., 2008). Moreover, neurons are characterized by a specific set of ITSN1 isoforms. ITSN1-L was shown to be expressed predominantly in neurons and not in glia (Hussain et al., 1999; Ma et al., 2003). More recently, insertion of exon 20 in the *ITSN1* mRNA, which leads to inclusion of 5 amino acid residues into the SH3A domain and alteration of its binding properties, was reported to be neuron-specific (Dergai et al., 2010).

The functional role of ITSN1 in neurons is not fully understood. Several studies indicate involvement of ITSN1 in synaptic vesicle endocytosis in various organisms (reviewed in Pechstein et al., 2010). However, other data suggest that ITSN1 is not required for synaptic vesicle recycling in rat hippocampal neurons (Thomas et al., 2009). ITSN1 was also shown to act at the post-synapse affecting dendritic spine development by regulation of small GTPase Cdc42 activity, that promotes actin nucleation and formation of spine head (Nishimura et al., 2006). Moreover, ITSN1 was shown to be associated with the development of several pathologies that affect the nervous system. The human *ITSN1* gene was mapped to chromosome 21 and its expression is upregulated in patients with Down syndrome (DS) (Hunter et al., 2011; Pucharcós et al., 1999). As association between

Abbreviations: ITSN1, intersectin 1; STOP, stable tubule-only polypeptide; MALDI-TOF, matrix-assisted laser desorption/ionization–time-of-flight; DIV, days-in-vitro; FP, fluorescent protein.

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endocytic abnormalities and pathological processes of DS and early onset of Alzheimer's disease (AD) was clearly stated, ITSN1 is considered to be associated with these disorders (Keating et al., 2006).

In the current report we attempted to extend our understanding of the interaction network of ITSN1 in neurons. We identified a stable tubule-only polypeptide (STOP) as an ITSN1-binding protein using affinity chromatography followed by matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry. We have also found that these proteins form a complex *in vivo* and partially co-localize in rat primary hippocampal neurons.

2. Materials and methods

2.1. Expression constructs and antibodies

Expression constructs containing GST-fused SH3 domains of ITSN1 were described previously (Nikolaienko et al., 2009). To obtain fluorescent protein (FP)-tagged proteins, full-length coding sequences of STOP and ITSN1-L were amplified by PCR and inserted into pmCherry-N1 (Clontech) and pGFP-C1 (Clontech) vectors, respectively. pmCitrine-N1 plasmid was obtained by replacing GFP in pGFP-N1 (Clontech) with mCitrine from mCitrine-SNAP25 construct (obtained from the lab of Dr. Robert Zucker) via digestion with AgeI and BsrGI restriction enzymes and subsequent ligation. Rabbit polyclonal antibodies against the EH2 domain of ITSN1 were described previously (Nikolaienko et al., 2009). Mouse monoclonal anti-STOP antibodies (MAB5524), secondary horseradish peroxidase-labeled (HRP) goat anti-rabbit antibodies and anti-mouse antibodies were purchased from Millipore, Promega and Sigma, respectively. Rabbit anti-β-tubulin III antibodies (T2200) were purchased from Sigma. For immunocytochemistry, goat anti-rabbit Alexa 405 (Invitrogen) and horse anti-mouse Texas Red (Vector Laboratories) antibodies were used.

2.2. Protein expression and pull-down assay

Recombinant GST and GST-fused SH3 domains were expressed in *Escherichia coli* BL21 (DE3) and purified on glutathione sepharose 4B beads (GE Healthcare) according to manufacturer's instructions. For lysate preparation, brains of 4 BALB/c mice were homogenized in 10 ml of extraction buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100 and complete EDTA-free protease inhibitor cocktail (Roche)) and centrifuged for 20 min at 12,000×*g* at 4 °C (all procedures with experimental animals were performed in accordance with the IMBG Bioethical Committee guidelines for care and use of animals in research). For pull-down assays, 3–5 μg of GST or GST-fused proteins were bound to 30 μl of 50% glutathione-sepharose 4B beads and incubated with mouse brain lysate for 1 h at 4 °C. After extensive washing the beads were boiled in Laemmli sample buffer (150 mM Tris–HCl pH 6.8, 2.5% glycerol, 10% SDS, 3% β-mercaptoethanol and 0.5% bromophenol blue). The eluted proteins were resolved by SDS-PAGE and either transferred to a nitrocellulose membrane for Western-blot analysis, or subjected to staining with Coomassie brilliant blue R250 for mass spectrometry. Blots were blocked for 1 h in 5% non-fat milk in 1× TBS-T (Tris-buffered saline: 20 mM Tris–HCl pH 8.0, 150 mM NaCl and 0.1% Tween 20) and incubated with the corresponding primary antibody for 1 h. Detection was performed using HRP-conjugated secondary antibodies.

2.3. MALDI-TOF mass spectrometry analysis

After staining with Coomassie brilliant blue R250, the bands of interest were accurately cut from the gel and fragmented to small pieces. Destaining and in-gel digestion of the samples were performed as described earlier (Shevchenko et al., 1996). Mass spectrometry of obtained peptides was performed using MALDI-TOF Reflex III mass spectrometer (Bruker). The proteins were identified by peptide mass spectra using Molecular Weight Search (MOWSE) algorithm provided by Mascot software (Matrix Science). Search criteria included one allowed missed cleavage, methionine oxidation and cysteine propionamidation as possible modifications. Peptide mass tolerance was set at the level of ±70 ppm.

2.4. Immunoprecipitations

For immunoprecipitation 2 μg of antibodies and 30 μl of protein A/G sepharose beads (Santa Cruz Biotechnology) were added to mouse brain lysate and incubated overnight at 4 °C. For negative controls, rabbit IgG were used instead of antibodies. After incubation, the beads were washed 4 times and boiled in Laemmli sample buffer. The proteins eluted were analyzed by SDS-PAGE and Western-blotting.

2.5. Cell culture and transfections

Dissociated cultures of hippocampal neurons were prepared and maintained as described (Dovgan et al., 2010). Transfections were performed after 13–17 days of cultivation using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Further steps were performed 2–3 days after transfection.

2.6. Immunocytochemistry and confocal imaging

Fourteen to twenty one days-in-vitro (14–21 DIV) neurons were washed with 1× HBSS (Hanks' Balanced Salt Solution) and fixed in 4% formaldehyde for 15 min. Fixed cells were washed in 1× PBS (phosphate buffered saline) supplemented with 0.2% Triton X-100 and blocked in 2% BSA and 0.2% TritonX-100 in 1× PBS for 30 min at room temperature, followed by incubation with appropriate antibodies diluted in blocking buffer. After completion of all steps, coverslips were mounted using PVA-DABCO (Fluka). Images were obtained on a Carl Zeiss LSM 510 META confocal microscope.

3. Results

In an attempt to identify new ITSN1-interacting proteins, we obtained recombinant GST-tagged SH3 domains of ITSN1 and used them as bait in GST pull-down assays with mouse brain total protein lysate. After resolving the bound proteins by SDS-PAGE, two bands were chosen for identification by MALDI-TOF mass spectrometry (Fig. 1). A major 100 kDa band was expectedly identified as dynamin 1, one of the best characterized interacting partners of ITSN1 (Roos and Kelly, 1998). The second band of approximately 120 kDa, observed exclusively among the SH3A domain-bound proteins, was identified as STOP, also known as Mtap6. Although the calculated molecular weight of the longest STOP isoform in the mouse proteome is 96 kDa, it has been reported that apparent molecular weight of STOP on SDS-PAGE is larger (Bosc et al., 1996), therefore we considered the results of mass spectrometry plausible.

To confirm the results of mass spectrometry, *in vitro* binding assays of the ITSN1 GST-SH3 domains with mouse brain lysate were performed. It was shown that STOP can bind only to the SH3A domain (Fig. 2A). To avoid the possibility of unspecific binding of STOP to the sepharose beads via interactions with elements of cytoskeleton, we checked the amount of neuronal β-tubulin in the reactions. The amount of bound STOP did not correlate with the amount of β-tubulin, indicating the specificity of this interaction (Fig. 2A). To determine if the interaction between ITSN1 and STOP really occurs *in vivo*, we performed co-immunoprecipitation of their complex from mouse brain lysate. Both ITSN1 and STOP were efficiently precipitated by either anti-ITSN1 or anti-STOP antibodies (Fig. 2B).

Glial and neuronal cells express STOP isoforms of different molecular weight (Galiano et al., 2004). Since the longest isoform which corresponds to 120 kDa on SDS-PAGE is expressed exclusively in