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Alternative splicing affecting the SH3A domain controls the binding properties of intersectin 1 in neurons

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ABSTRACT

Intersectin 1 (ITSN1) is a conserved adaptor protein implicated in endocytosis, regulation of actin cytoskeleton rearrangements and mitogenic signaling. Its expression is characterized by multiple alternative splicing. Here we show neuron-specific expression of ITSN1 isoforms containing exon 20, which encodes five amino acid residues in the first SH3 domain (SH3A). *In vitro* binding experiments demonstrated that inclusion of exon 20 changes the binding properties of the SH3A domain. Endocytic proteins dynamin 1 and synaptojanin 1 as well as GTPase-activating protein CdGAP bound the neuron-specific variant of the SH3A domain with higher affinity than ubiquitously expressed SH3A. In contrast, SOS1, a guanine nucleotide exchange factor for Ras, and the ubiquitin ligase Cbl mainly interact with the ubiquitously expressed isoform. These results demonstrate that alternative splicing leads to the formation of two pools of ITSN1 with potentially different properties in neurons, affecting ITSN1 function as adaptor protein.

Intersectin 1 (ITSN1) is an evolutionarily conserved adaptor protein involved in clathrin-mediated endocytosis, apoptosis, signal transduction, and regulation of cytoskeletal rearrangements [1–4]. Expression of the ITSN1 gene is characterized by multiple alternative splicing events [5–7]. The major ITSN1 protein isoforms described in mammals are ubiquitously expressed short form, ITSN1-S, and the long form, ITSN1-L, that is mainly expressed in neurons. The short form consists of two N-terminal Eps15 homology domains (EH1 and EH2), a coiled-coil region and five Src homology domains (SH3 A-E). The long form contains C-terminal extension with a Dbl homology (DH), a pleckstrin homology (PH), and a C2 domains [6,7].

ITSN1 has been shown to interact with several essential endocytic proteins, including dynamin, epsin, Eps15, and synaptojanin [1,7–9]. Studies of loss-of-function mutations of Dap160 (*Drosophila* homolog of human ITSN) demonstrated that Dap160 is directly involved in synaptic vesicle trafficking and is required for the localization of several endocytic proteins [10]. The DH domain of ITSN1-L functions as a guanine nucleotide exchange factor for Cdc42 GTPase and modulates regulation of the actin cytoskeleton [4]. Furthermore, ITSN1 was shown to associate with the ubiquitin protein ligase Cbl and coordinate the ubiquitination and degradation of the epidermal growth factor receptor [11]. Silencing of ITSN expression by shRNA and siRNA demonstrated the activation of apoptosis [2,12].

Previously, we found seven additional splice variants of the human and mouse ITSN1 genes [5]. Expression analysis of these alternatively spliced transcripts revealed brain- and development-specific expression of ITSN1 isoforms containing exon 20 which encodes five amino acid residues (VKGEW) in the SH3A domain. This domain binds a number of proteins, including Ras exchange factor SOS1, dynamin 1, synaptojanin 1, Cbl, a GTPase-activating protein CdGAP, and the neuronal Wiskott–Aldrich syndrome protein (N-WASP) [1,3,4,7,9,11,13].

In view of the potential link between alternative exon 20 usage and the binding properties of ITSN1, we have investigated the interaction of two SH3A domain splice variants with the main ITSN1 protein partners. The results presented here highlight differences in the binding properties of alternatively spliced SH3A domains of ITSN1 and showed how alternative splicing can regulate the functions of ITSN1 as adaptor protein.

Materials and methods

Expression constructs. The cDNA sequences corresponding to the proline-rich region (PRD) of dynamin 1 (residues 742–851, GenBank Accession No. NP_0010053360), SOS1-IsfI (residues

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1070–1284, Accession No. EAX00353), SOS1-IsfII (residues 1070–1299, Accession No. NP_005624), c-Cbl (residues 355–472, Accession No. X57110), and Cbl-b (residues 482–982, Accession No. BC032851) were produced by PCR using human embryonal brain and muscle cDNAs and High Fidelity PCR enzyme mix (Fermentas). The PCR products obtained were cloned in bacterial expression vector with a GST tag, pGEX-4T-3 (GE Healthcare). N-WASP cDNA (residues 22–459, Accession No. NP_003932), cDNA fragments encoding the PRD of synaptojanin 1 (SYNJ1) (residues 1003–1558, Accession No. NP_003886) and the central domain of CdGAP (residues 174–683, Accession No. AAI12166) were amplified from human embryonal brain cDNA and cloned into the pcDNA4/His-Max vector (Invitrogen). The cDNA fragments encoding the neuron-specific and ubiquitously expressed forms of the SH3A domain of ITSN1 (residues 739–807, Accession No. NP_003015) were amplified from human 12-week embryonal brain and liver cDNAs and cloned into the pET24a vector (Novagen) and pGEX-4T-3. All PCR-generated DNA fragments were sequenced to confirm fidelity. The cloned fragment of SYNJ1 corresponds to the alternatively spliced isoform (GenBank Accession No. DQ421853).

Recombinant protein expression, pull-down assays, and Western-blot analysis. The recombinant GST-fused and His-tagged proteins were expressed in *Escherichia coli* BL21(DE3)pLysE and purified using glutathione–Sepharose 4B (GE Healthcare) or HisLink Protein purification resin (Promega) according to the manufacturer's instructions. Mouse tissues lysates, lysates of BJAB cells, and transiently transfected CHO-K1 and COS-1 cells were prepared in extraction buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100 and complete EDTA-free protease inhibitor cocktail (Roche)) and centrifuged for 20 min at 12,000g at 4 °C. For pull-down assays 2–5 μg of purified GST fusion proteins or GST alone were bound to 30 μl of 50% glutathione–Sepharose 4B beads and incubated with 0.5–1 μM purified SH3A(+)-6xHis and SH3A(−)-6xHis proteins or tissue and cell lysates for 1 h at 4 °C. The beads were extensively washed and boiled in Laemmli sample buffer. Eluted proteins were resolved by SDS–PAGE and transferred to nitrocellulose membranes. The blots were blocked 1 h in 5% non-fat milk, 1× PBS, 0.1% Tween 20, incubated with corresponding primary antibodies for 1 h at room temperature and washed. Detection was performed by horseradish peroxidase-labeled secondary antibodies (Promega).

Antibodies. Polyclonal antibodies against the SH3A domain of ITSN1 were raised in rabbits using the His-tag fusion protein containing amino acids 742–804 of ITSN1. The SOS1 (C-23): sc-256, Cbl (C-15): sc-170, Dynamin 1 (C-16): sc-16, Cbl-b (H-121): sc-1704 and anti-Omni (D-8): sc-7270 antibodies were purchased from Santa Cruz Biotechnology.

Cell culture and transfection. The B-cell line BJAB was cultured at 37 °C in 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 200 units/ml penicillin and 200 μg/ml streptomycin. The adherent cell lines COS-1 and CHO-K1 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transient transfection of COS-1 and CHO-K1 cells was carried out with the use of FuGENE 6 (Roche).

Acute isolation of neurons and RT-PCR. Parts of the thalamus of 30–35 day-old BALB/c x C57BLF1 mice were dissected into 0.5–1 mm^3 slices and incubated for 20 min in carbogen-saturated (95% O_2, 5% CO_2) solution containing 135 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 10 mM D-glucose and 10 mM HEPES pH 7.4 then treated with 2 mg/ml protease XXIII (Sigma) for 20 min at 33 °C and washed (all steps were carbogenated). To collect single-cells, slices were pipetted with fire-polished Pasteur pipettes and the cell suspension obtained was transferred to a microscope chamber. Two neurons were expelled into a tube containing 50 pmoles oligo$(dT)_{15}$ and 4 μl DEPC-water. The tube was heated at 65 °C for 10 min and then cooled on ice. cDNA was synthesized using Expand Reverse Transcriptase (Roche) for 2 h at 42 °C. PCR was performed using external primers within exon 18 (5′-CATCCGCAT CAGGAGCCAGCTA-3′) and exon 22 (5′-CCGTTTCTGGCTTCTGTTT GAGC-3′) of the mouse ITSN1 cDNA (GenBank Accession No. AF132481). Five microliters of the resulting product were used for the second amplification with nested sense primers to exon 20 (5′-AGTCATGGTTAAAGGGGAATGG-3′) or to the exon 19/21 junction (5′-AGCCAGGAGACATAGTCATGGTGG-3′). Expression of the ± exon 20 ITSN1 isoforms in the human glioblastoma cell line U-251 MG was determined using the primers indicated above for exon 20 or the exon 19/21 junction, and the antisense primer within the human ITSN1 exon 23: 5′-CTTGTAGACTTCCTTATG-3′ (GenBank Accession No. AF114487). Total RNA from U-251 MG was kindly provided by Dr. K. Shostak (Institute of Molecular Biology and Genetics, Ukraine).

Results

Neuron-specific expression of ITSN1 mRNA containing exon 20

Alternative splicing of ITSN1 exon 20 that encodes the amino acids VKGEW of the SH3A domain (Fig. 1A and B) is observed in humans, mice, and rats [5,7]. Expression of transcripts with exon 20 occurred exclusively in brain tissues, while transcripts lacking exon 20 were detected in all tissues tested [5]. Moreover, the frequency of the +exon 20 variant of ITSN1 increased during brain development whereas the level of the transcript lacking exon 20 decreased correspondingly. To examine whether both isoforms are expressed in neurons and glial cells we performed RT-PCR on isolated neurons from mouse brain and found that both RT-PCR products corresponding to ITSN1 transcripts with or without exon 20 were detected in isolated neurons (Fig. 1C). Similar RT-PCR reactions were carried out on total RNA from the human glioblastoma cell line U-251 MG, but only isoforms lacking exon 20 were detected.

Sequence alignment of different SH3 domains showed that the five amino acid residues encoded by exon 20 of ITSN1 are located within the n-Src loop of the SH3A domain (Fig. 1B, [5]). It is known that SH3 domains bind to proline-rich motifs in target proteins. The specificity in SH3 domain target sequence recognition is often provided by the amino acid residues of the n-Src and RT loops [14]. To evaluate the effect of exon 20 splicing on the binding specificity of the SH3A domain a series of *in vitro* binding experiments were carried out. We used bacterially expressed pure recombinant proteins to avoid the contribution of other proteins in interaction as well as endogenous or overexpressed in mammalian cells proteins.

Dynamin 1, synaptojanin 1, and CdGAP preferentially bind the neuron-specific isoform of the SH3A domain of ITSN1

Intersectin 1 (Dap160) was initially isolated in *Drosophila* by co-fractionation with dynamin [8]. Dynamin 1 is a neuronal GTPase that regulates late events in clathrin-coated vesicle formation and fission during endocytosis. ITSN1 was shown to control the amount of dynamin 1 released from the cluster of synaptic vesicles to the periactive zone of synapse [15]. To examine whether both isoforms of the ITSN1 SH3A domain interact with dynamin 1 we at first performed pull-down experiments with purified GST-fused proline-rich domain (PRD) of dynamin 1 and His-tagged SH3A+ (neuron-specific) and SH3A− (ubiquitously-expressed) domains of ITSN1. The amount of SH3A domains specifically bound to the PRD of dynamin 1 was assessed using Western blot analyses with anti-SH3A domain antibodies and normalized relative to the GST

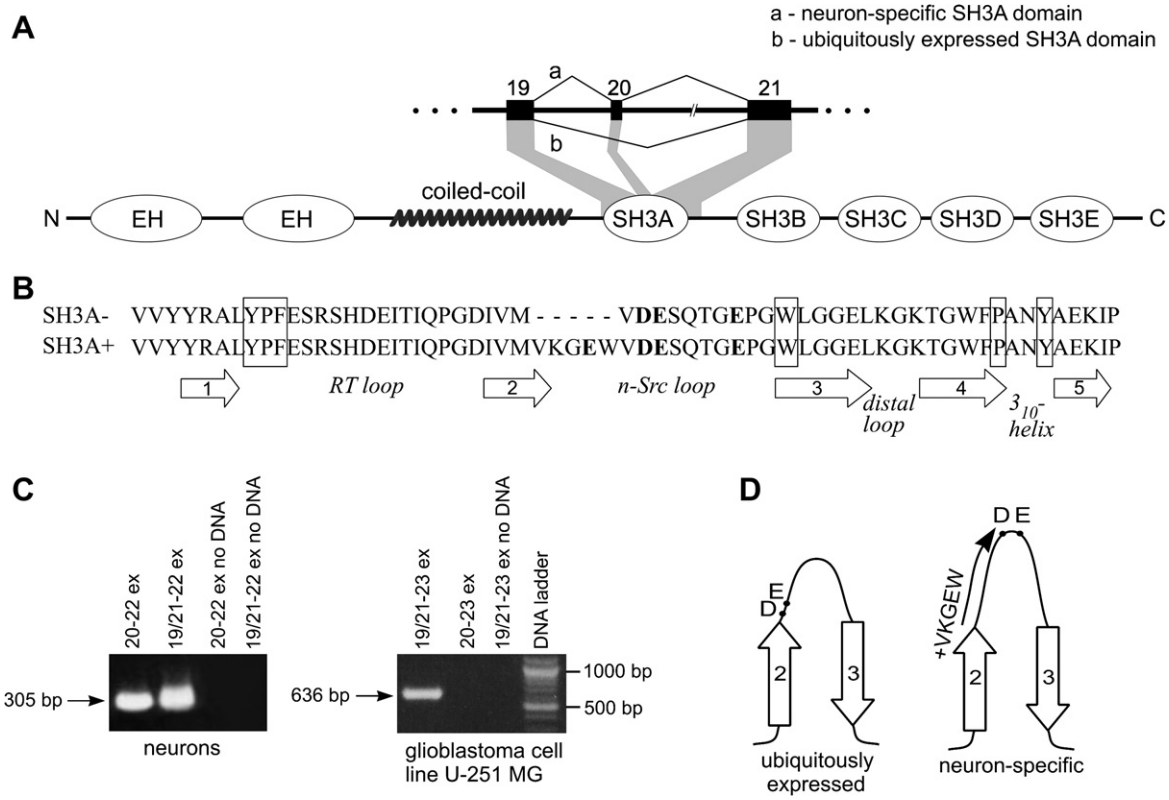

Fig. 1. Neuron-specific expression of ITSN1 isoform containing exon 20. (A) Schematic representation showing the alternative splicing event affecting the SH3A domain of ITSN1. Black boxes represent exons with corresponding numbers above. The domain structure of the short form ITSN1 is shown. (B) Sequence alignment of neuron-specific (SH3A+) and ubiquitously expressed (SH3A−) isoforms of the ITSN1 SH3A domain. Conserved hydrophobic amino acids involved in binding proline residues are boxed. Negatively charged residues located in the n-Src loop are in bold. The secondary structural elements (β strands) in the SH3 domains predicted by PHDsec (www.predictprotein.org) are shown below the sequence as arrows. (C) RT-PCR analysis of ITSN1 transcripts produced by alternative splicing of exon 20 in neurons and glioblastoma cell line. The primer pairs used for each amplification are indicated on the top of each lane. Control experiments on plasmids containing ITSN1 cDNAs with and without exon 20 proved that primers to exon 20 and to the exon 19/21 junction can discriminate two types of ITSN1 isoforms. (D) Scheme of possible effect of a 5 amino acid insertion on translocation of the charged group in the n-Src loop of the neuron-specific SH3A domain.

fusion proteins (Fig. 2A). The ability of anti-SH3A antibodies to recognize both variants of SH3A domain was verified. In addition endogenous dynamin 1 was pulled down from mouse brain lysates with immobilized GST fusion isoforms of the SH3A domain and detected using anti-dynamin 1 antibodies (Fig. 2B). The results showed that dynamin 1 bound the neuron-specific SH3A domain with higher affinity than ubiquitously expressed SH3A.

We next examined whether alternative splicing alters the binding efficiency of the SH3A domain to another endocytic protein synaptojanin 1 (SYNJ1) and two proteins, CdGAP and N-WASP, implicated in regulation of actin polymerization. Omni-tagged N-WASP, PRD of SYNJ1, and central domain of CdGAP that are known to interact with ITSN1 SH3 domains [4,9,13] were expressed in CHO-K1 cells and used for binding experiments with GST-fused SH3A+ and SH3A− proteins. The amount of proteins specifically bound to the beads was assessed using Western blots with anti-Omni antibodies. The results revealed that central domain of CdGAP and PRD of SYNJ1 bind the neuron-specific variant of the ITSN1 SH3A domain with higher affinity when compared to the ubiquitously expressed isoform (Figs. 2C and 3A). No significant difference in N-WASP binding specificity to the alternatively spliced isoforms of the SH3A domain was detected (Fig. 3B).

SOS1 and Cbl preferentially interact with the ubiquitously expressed isoform of the ITSN1 SH3A domain

Several lines of evidence suggest that ITSN1 regulates Ras activation through interaction with SOS1, a guanine nucleotide exchange factor for Ras [3], and forms a complex with two members of the Cbl ubiquitin ligases protein family, c-Cbl and Cbl-b, implicated in the negative regulation of receptor tyrosine kinases [11]. Pull-down experiments were performed to assay the ability of endogenous SOS1, c-Cbl, and Cbl-b interact with SH3A domain isoforms of ITSN1. GST-fused SH3A+ and SH3A− domains were incubated with mouse brain and kidney lysates as well as with BJAB cell lysate. The proteins bound to the beads were analyzed by Western blots with antibodies to the SOS1, c-Cbl, and Cbl-b, and normalized relative to the GST fusion protein (Fig. 4). The results demonstrated that SOS1 and c-Cbl bind with higher affinity to GST constructs containing the ubiquitously expressed SH3A domain of ITSN1 and bind weakly to the neuron-specific variant. No significant differences in the binding of Cbl-b between GST-fused SH3A+ and SH3A− were observed (Fig. 4C). Pull-down experiments in the reverse orientation showed that GST-fused PRDs of both c-Cbl and Cbl-b interact more strongly with the ubiquitously expressed SH3A domain and bound considerably less efficiently to the neuron-specific variant (Supplementary 1A). Differences in the results obtained with endogenous and recombinant Cbl-b could be explained by involving other cell proteins in interaction of the SH3A domain and Cbl-b.

Previously, it was found that two alternatively spliced isoforms of SOS1 possess PRDs of different lengths that differ in their ability to bind the SH3 domain of the adaptor protein Grb2 [16]. Our results revealed that both isoforms of SOS1 interacted more strongly with the ubiquitously expressed SH3A domain. No significant differences between the isoforms of SOS1 in binding efficiency to the ITSN1 SH3A domain were found (Supplementary 1B).

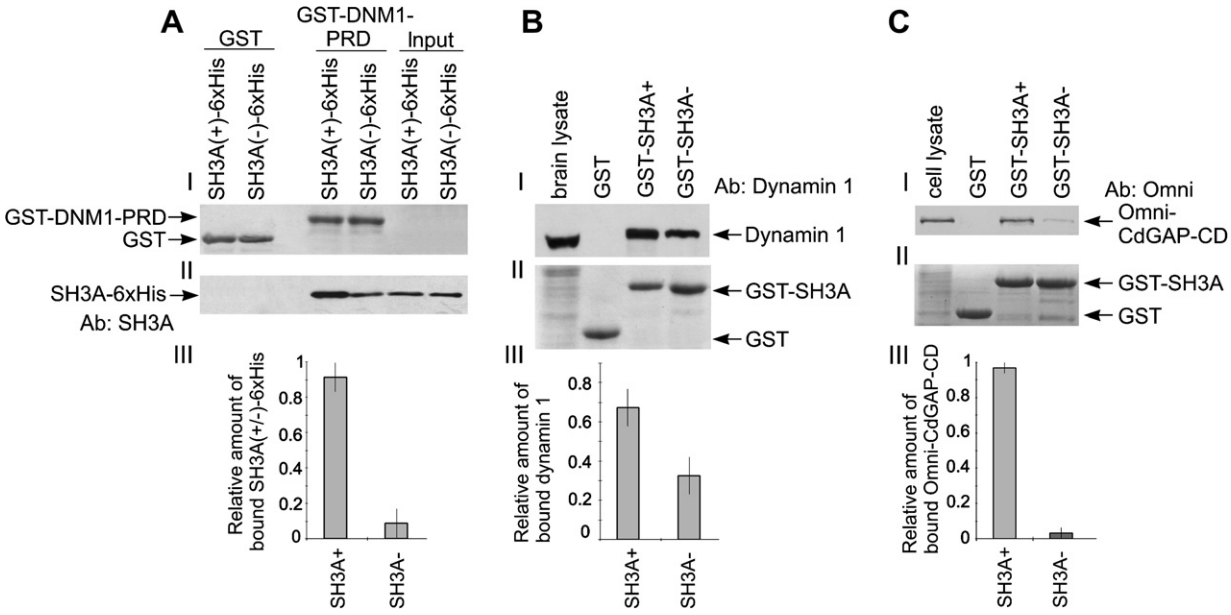

Fig. 2. Dynamin 1 and CdGAP preferentially bind to the neuron-specific isoform of the SH3A domain of ITSN1. (A) Glutathione Sepharose 4B beads were loaded with purified GST (control) and GST-fusion PRD of dynamin 1 (GST-DNM1-PRD), washed and incubated with His-tagged SH3A+ or SH3A− domains. 2.5% of SH3A(+/−)-6×His proteins used for GST pull-down experiments were loaded as a control (input lane). The beads were washed extensively, the bound proteins were eluted from the beads and resolved by SDS–PAGE. The amount of GST-fused proteins bound to the beads was checked by Coomassie staining (I). SH3A domains from the lower part of gel (II) were detected by Western blotting with antibodies against the SH3A domain of ITSN1. (III) Quantification of pull-down results. The amount of SH3A domains bound to the GST-DNM1-PRD was normalized to the amount of GST fusion protein in the assay using the ImageJ program. The histogram represents the average and SD of three separate assays. (B,C) Recombinant proteins GST-SH3A+, GST-SH3A− and GST alone were immobilized on glutathione beads and incubated with equal amounts of mouse brain lysate or lysate of CHO-K1 cells transfected with the expression plasmids encoding central domain of CdGAP (Omni-CdGAP-CD). 2% of brain lysate and lysate of CHO-K1 cells used for pull-downs were loaded as a control. Pulled down proteins were analyzed by immunoblotting with anti-dynamin 1 or anti-Omni antibodies (I). The Coomassie-stained lower part of gel (II) shows the amount of GST and GST fusion proteins in the assay. (III) Quantification of pull-down results. The amount of dynamin 1 and Omni-CdGAP-CD bound to the GST-SH3A+/− was normalized to the amount of GST fusion protein in the assay. The histogram represents the average and SD of three separate experiments.

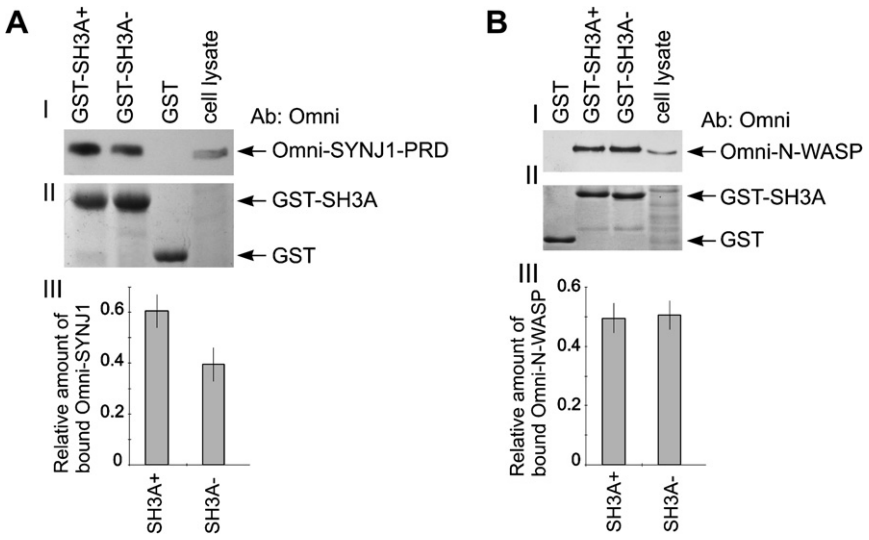

Fig. 3. Effect of exon 20 splicing on interaction of ITSN1 SH3A domain with PRD of synaptojanin 1 (A) and N-WASP (B). COS-1 and CHO-K1 cells were transiently transfected with expression plasmids encoding N-WASP (Omni-N-WASP) or PRD of synaptojanin 1 (Omni-SYNJ1-PRD). Whole-cell lysates were incubated with recombinant GST-SH3A+, GST-SH3A− or GST and pull-down experiments were performed as described in Fig. 2 B and C. Pulled down proteins were analyzed by immunoblotting with anti-Omni antibodies (I). The amount of GST-fused proteins bound to the beads was checked by Coomassie staining (II). The amount of Omni-SYNJ1-PRD and Omni-N-WASP bound to the GST-SH3A+/− was normalized to the amount of GST fusion proteins in the assay (III). The histogram represents the average and SD of three independent assays.

Discussion

SH3 domains that are often found in proteins implicated in cell signaling pathways, cytoskeletal organization and membrane traffic are important for the assembly of multiprotein complexes and as a consequence their specificity and affinity for particular targets often determine the efficiency and outcome of cell signals [17]. SH3 domains are highly adaptable. Phage library screens showed that substitution of two or three residues within an SH3 domain is sufficient to alter its specificity [18]. Our results demonstrate that alternative splicing can change the binding properties of SH3 domain of adaptor protein ITSN1. We have shown the presence of two isoforms of the ITSN1 SH3A domain in mammals, namely, neuron-specific and ubiquitously expressed, generated

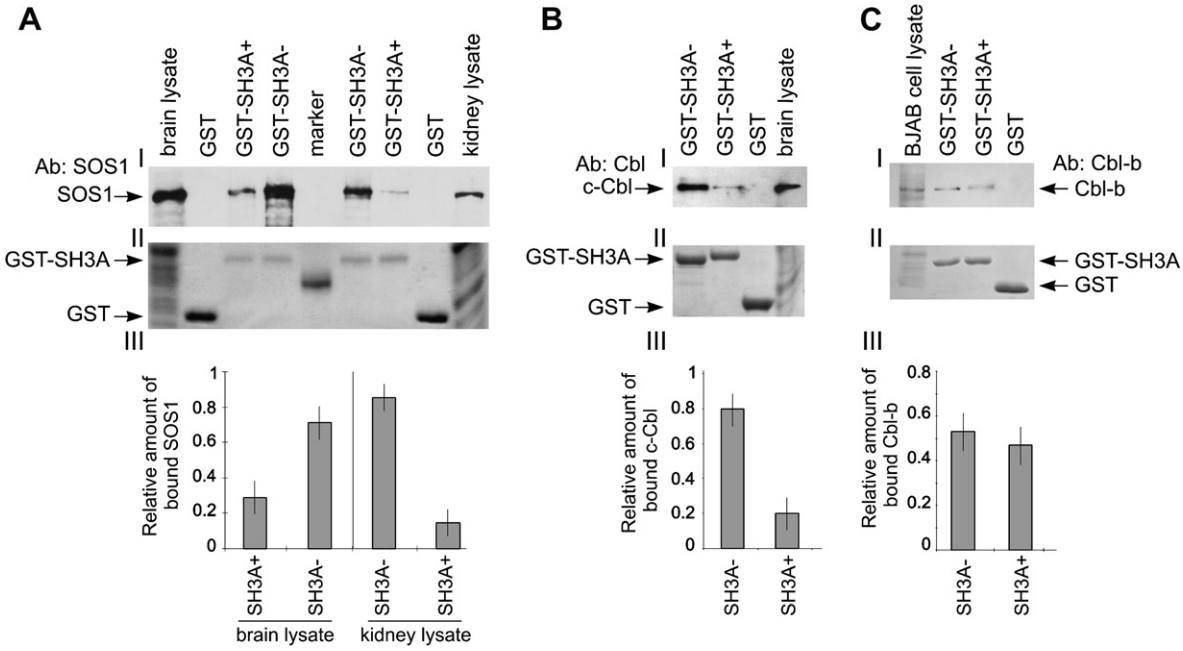

Fig. 4. SOS1 and c-Cbl preferentially interact with ubiquitously expressed isoform of the SH3A domain of ITSN1. Mouse brain and kidney lysates as well as BJAB cell lysate were incubated with GST-SH3A+, GST-SH3A− or GST proteins conjugated to glutathione beads and pull-down experiments were performed as described in Fig. 2B and C. Pulled down proteins were analyzed by immunoblotting with anti-SOS1 (A), anti-c-Cbl (B) or anti-Cbl-b (C) antibodies (I). (II) Coomassie-stained lower parts of gels. (III) Binding of SOS1, c-Cbl, and Cbl-b was normalized to GST fusion proteins. The histogram represents the average and SD of three (A,B) or four (C) assays.

by alternative splicing of exon 20. Inclusion of exon 20 extends the n-Src loop of the SH3A domain of ITSN1 (Fig. 1B) and changes binding properties of this domain.

Binding of SH3 domains to PRDs is mostly determined by two types of interactions. Hydrophobic residues form a core of interaction, while charged amino acids impact on the specificity of binding of proline-enriched motifs [14]. Owen et al. [19] showed that substrate specificity of the amphiphysin 2 SH3 domain, another SH3 domain with an unusually large n-Src loop, is determined by negative electrostatic potential of acidic residues mainly localized in the RT and n-Src loops. These acidic residues allow binding to the proline-rich motif of dynamin 1 containing two arginines. Changes in the n-Src loop structure of the SH3A domain of ITSN1 in neuronal isoform potentially involve rearrangement of charged groups engaged in the interacting interface (Fig. 1D). An additional 5 amino acids could move the pair of negatively-charged side chains to the interface influencing the interactions with the PRDs containing basic residues, e.g. PRD of dynamin 1.

Recently, it has been shown that small splice inserts of 5–19 amino acid residues have enormous consequences on the structure and function of proteins and it has been suggested that such small inserts may be functionally relevant to achieve the diversity of connections between neurons [20]. Our previous studies and novel unpublished results indicate that the ITSN1 gene is characterized by multiple alternative splicing events. Besides alternative splicing of exon 20, we and others revealed 6 alternative splicing events affecting the coding region of ITSN1 [5–7]. Different combinations of these events lead to the formation of 18 spliced isoforms of ITSN1. Due to the inclusion of exon 20, the number of ITSN1 isoforms doubles in neurons. Thus, two pools of ITSN1 isoforms with potentially different properties exist in these cells. We did not determine specific partner for each isoform of SH3A domain but showed differences in their ability to bind some proteins. Hence, we suppose that formation of the neuron-specific pool of ITSN1 isoforms does not alter binding partners but changes the equilibrium between them. Alterations of SH3A binding properties can also affect the time of complex assembly and its half-life.

ITSN1 belongs to the group of multifunctional adaptor and scaffold proteins that play crucial roles in mediating the temporal and spatial organization of macromolecular complexes [21]. Adaptor proteins are known to be regulated at the level of phosphorylation, oligomerization and cell distribution. Here we demonstrate how alternative splicing can contribute to tissue- and temporally-dependent regulation of adaptor proteins. Diversity in ligand specificity of the SH3A domain of ITSN1 due to the alternative splicing provides a mechanism capable of enhancing the plasticity and dynamics of ITSN1 function in endocytosis and signal transduction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.05.156.

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