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Synaptic scaffolding molecule (S-SCAM) membrane-associated guanylate kinase with inverted organization (MAGI)-2 is associated with cell adhesion molecules at inhibitory synapses in rat hippocampal neurons

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Abstract

Synaptic scaffolding molecule (S-SCAM) is a synaptic protein, which harbors five or six PSD-95/Discs large/ZO-1 (PDZ), a guanylate kinase and two WW domains. It interacts with NMDA receptor subunits, neuroligin and β-catenin, and is involved in the accumulation of neuroligin at excitatory synapses. In this study, we have demonstrated S-SCAM is localized at inhibitory synapses in rat primary cultured hippocampal neurons. We have identified β-dystroglycan (β-DG) as a binding partner for S-SCAM at inhibitory synapses. WW domains of S-SCAM bind to three sequences of β-DG. We have also revealed that S-SCAM can interact with neuroligin 2, which is known to be exclusively localized at inhibitory synapses. The WW domains and the second PDZ domain of S-SCAM are involved in the interaction with neuroligin 2. β-DG, neuroligin 2 and S-SCAM form a tripartite complex *in vitro*. Neuroligin 2 is detected in the immunoprecipitates by anti-β-DG antibody from rat brain. S-SCAM, β-DG and neuroligin 2 are partially co-localized in rat hippocampal neurons. These data suggest that S-SCAM is associated with β-DG and neuroligin 2 at inhibitory synapses, and functions as a linker between the dystrophin glycoprotein complex and the neurexin–neuroligin complex.

Keywords: dystroglycan, inhibitory synapse, neuroligin.
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Pre- and post-synaptic architectures are organized through numerous protein–protein interactions. Recent studies have suggested that cell adhesion precedes the complete assembly of pre- and post-synaptic components, and plays an important role in the basic organization of neuronal synapses (Washbourne *et al*. 2004; Craig *et al*. 2006). The experiments using gene-targeted mice, RNA interference and dominant negative proteins demonstrate that several cell adhesion molecules, such as cadherins, immunoglobulin superfamily proteins and Eph/Ephrin, are involved in synaptogenesis (Dalva *et al*. 2000; Biederer *et al*. 2002; Togashi *et al*. 2002; Henkemeyer *et al*. 2003; Saghatelyan *et al*. 2004). Cultures of neurons with non-neuronal cells expressing key molecules more directly indicate that synCAM, neuroligin and neurexin indeed have synaptogenic activity (Graf *et al*. 2004; Chih *et al*. 2005; Levinson *et al*. 2005; Nam and Chen 2005; Sara *et al*. 2005). SynCAM and

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Abbreviations used: ADH1, alcohol dehydrogenase 1; BHK, baby hamster kidney; BSA, bovine serum albumin; β-DG, β-dystroglycan; DEAE, diethylaminoethyl; Div, day *in vitro*; GFP, green fluorescent protein; GK, guanylate kinase; GST, glutathione-*S*-transferase; MAGI, membrane-associated guanylate kinase with inverted organization; MBP, maltose-binding protein; MDCK, Madine–Darby canine kidney; NL2, neuroligin 2; NR1, NMDA receptor subunit 1; PBS, phosphate-buffered saline; PDZ, PSD-95/Discs large/ZO-1; PSD-95, postsynaptic density-95; SAPAP1, Synapse-associated protein 90/PSD-95-associated protein 1; SH3, src homology 3; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; S-SCAM, synaptic scaffolding molecule; VGAT, vesicular GABA transporter; YFP, yellow fluorescent protein; ZO-1, zonula occludens-1.

neuroligin induce pre-synaptic differentiation, whereas neurexin induces post-synaptic differentiation.

Neurexin is the only identified cell adhesion molecule to induce inhibitory synapses. Neurexin has two binding partners at inhibitory synapses. One is α-dystroglycan (Sugita *et al*. 2001; Levi *et al*. 2002); the other is neuroligin 2 (Ichtchenko *et al*. 1996; Varoqueaux *et al*. 2004). Neurons deficient of dystroglycan can form GABAergic synapses, suggesting that neurexin–neuroligin *trans*-synaptic interaction may play a more dominant role in the basal GABA post-synaptic differentiation. Neuroligin 2 is expressed only at inhibitory synapses, but its overexpression induces not only inhibitory but also excitatory pre-synaptic contacts (Graf *et al*. 2004; Chih *et al*. 2005). Moreover, the co-expression of post synaptic density-95 (PSD-95) shifts neuroligin 2 to excitatory synapses and increases the ratio of excitatory pre-synaptic contacts (Prange *et al*. 2004; Levinson *et al*. 2005). These observations suggest that under physiological conditions, there is some mechanism to compete with PSD-95 and selectively recruit neuroligin 2 to inhibitory synapses. An additional link may also be necessary to subsequently connect neuroligin 2 with other molecules of matured inhibitory synapses.

Synaptic scaffolding molecule (S-SCAM) has a molecular organization similar to that of PSD-95 (Hirao *et al*. 1998). Both proteins have multiple PSD-95/Discs large/ZO-1 (PDZ) domains and a guanylate kinase (GK) domain. S-SCAM has two WW domains, whereas PSD-95 has an src homology 3 (SH3) domain. Both S-SCAM and PSD-95 interact with neuroligin 1 (Irie *et al*. 1997; Hirao *et al*. 1998). We previously reported that neuroligin 1 binds to PSD-95 only through the PDZ-binding motif, but can interact with the WW domains of S-SCAM by an additional sequence (Iida *et al*. 2004). Although S-SCAM has so far been studied in the context of excitatory synapses, we report here the localization of S-SCAM at inhibitory synapses and show the possibility that S-SCAM is a molecular link between the neurexin–neuroligin complex and the dystrophin glycoprotein complex.

Experimental procedures

Construction of expression vectors

cDNA of human dystroglycan precursor (gene accession number, BC012740) and mouse neuroligin 2 (gene accession number, BC056478) were purchased from Open Biosystems (Huntsville, AL, USA). pClneoMyc S-SCAM (1–1277) (1–301) (295–578) (573–1277) and pBTM116 KM vector were described previously (Hirao *et al*. 1998; Hirabayashi *et al*. 2005). A yeast three-hybrid vector, pBTM116KM Bridge, was constructed as follows. A linker H-1289/H-1290 (5′-GGCCATGGTACCAGATCTGAGCTCACTAGTTAACGCTAGCGGCCGC-3′ and 5′-GATCGCGGCCGCTAGCGTTAACTAGTGAGCTCAGATCTGGTACCAT-3′) was ligated into *Not*I/*Bgl*II sites of pBridge vector (BD Biosciences, San Jose, CA, USA) in order to generate pBridge H-1289/H-1290 with additional cloning sites. PCR was performed using primers, H-1291 (5′-GACGTCAACTTCTTTTCTTTTTTTTTC-3′) and H-1292 (5′-AAGCTTGTCGACTCGAGTTGATTGTATGCTTGGTA-3′) on pBridge to amplify the alcohol dehydrogenase 1 (ADH1) promoter, and the product was subcloned into pCR®4-TOPO® (Invitrogen, Carlsbad, CA, USA) to generate TA H-1291/H-1292. PCR was performed using primers, H-1293 (5′-GTCGACAATGGGCCATATGGCTTC-3′) and H-1294 (5′-AAGCTTGACGTCATTAATGCAGGAAGATCC-3′) on pBridge H-1289/H-1290 to obtain hemagglutinin (HA)-tag followed by multiple cloning sites, and the product was digested by *Sal*I/*Hind*III and ligated into *Xho*I/*Hind*III sites of TA H-1291/H-1292 to generate TA H-1291/H-1292/H-1293/H-1294. An *Aat*II fragment from TA H-1291/H-1292/H-1293/H-1294 was ligated into the *Aat*II site of pBTM116 KM. The resulting pBTM116 KM Bridge has two ADH1 promoters. pBTM116 KM Bridge S-SCAM-*N*-SAPAP1 encodes the N-terminal region of rat S-SCAM (the amino acid residue 1–442) fused with the Lex A DNA-binding domain and the full length of rat SAPAP1 under each ADH1 promoter. SAPAP1 is a long isoform of a guanylate kinase-associated protein (Kim *et al*. 1997; Takeuchi *et al*. 1997). pClneoMyc SAPAP1 covers the full-length of rat SAPAP1. Various glutathione-*S*-transferase (GST) fusion constructs of S-SCAM were prepared using pGex4T-1 vector (GE Healthcare, Piscataway, NJ, USA). pSinRep4YFP vector was prepared from pSp2S-MCS vector (a gift from Dr Pavel Osten, Max-Planck-Institute for Medical Research, Heidelberg, Germany) as follows (Kim *et al*. 2004). PCRs were performed using primers H-1222 (5′-GGGCCCTCGAGCGGCCGCAATGATCCGACCAGCAAAAC-3′) and H-1235 (5′-GTCGACTTAATTAATCGAGGAATTCCCCTTTTTTT-3′) on pSinRep5 vector (Invitrogen, Carlsbad, CA, USA) and primers H-1529 (5′-TCTAGACCGGTCGCCACCATGGTGA-3′) and H-1530 (5′-ACGCGTAGATCTGAGTCCGGACTTGTA-3′) on pEYFPC1 vector (BD Biosciences). The former product was digested by *Apa*I and *Sal*I and ligated into the *Apa*I and *Xho*I sites of pSp2S-MCS vector to generate pSinRep4. The latter product was digested by *Xba*I and *Mlu*I and ligated into pSinRep4 to obtain pSinRep4YFP vector. Various expression constructs of β-dystroglycan (β-DG) and neuroligin 2 were prepared using pFLAG, pSinRep4YFP, pGex4T-1 and pET32a (Novagen, Darmstadt, Germany) and pMalC2 (New England BioLabs, Ipswich, MA, USA) vectors. PCR was performed using primers H-470 (5′-GCTAGCCCGGGATCCACCGGTCGCC-3′) and H-471 (5′-GCATGCACGTGAATTCACGCGTCAGGAACAGGTGGTGGCGGCC-3′) on pDsRed2-N1 (BD Biosciences). The product was digested with *Nhe*I and *Eco*RI and ligated into the same sites of pClneo vector to generate pClneoDsRed vector. pClneoDsRed NL2 (702–835) covered the cytoplasmic region of neuroligin 2. Constructs of S-SCAM, β-DG and neuroligin 2 were summarized in each figure. Residues in the putative WW-binding sequences of β-DG were substituted by PCR methods. pClneoMyc membrane-associated guanylate kinase with inverted organization-3 MAGI-3 covers the full-length of rat Slipr/MAGI-3. The pFLAG-dystroglycan precursor contains the amino acid residues 29–895 of human dystroglycan.

Antibodies and reagents

Rabbit polyclonal anti-S-SCAM antibody was raised against the WW domains of S-SCAM [anti-S-SCAM (303–405)] and the N-terminal region of S-SCAM [anti-S-SCAM (1–421)] covering the PDZ0, GK and WW domains (Hirao *et al*. 1998). The

anti-S-SCAM (1–421) serum was affinity-purified using maltose-binding protein (MBP)-S-SCAM 16–64 to obtain an antibody that specifically recognizes S-SCAM [anti-S-SCAM (16–64)]. Recombinant proteins were purified from *Escherichia coli* transformed with pET32 and pGex4T-1 constructs using Ni-NTA agarose (Qiagen, Hilden, Germany) and glutathione Sepharose 4B beads (GE Healthcare). Anti-β-DG antibody was raised against the product of pGex4T-1 (826–895) using rabbits. Mouse monoclonal NMDA receptor subunit 1 (NR1) was a kind gift from Dr Nils Brose (Max-Planck-Institute for Experimental Medicine, Göttingen, Germany). The anti-SAPAP antibody was described previously (Takeuchi *et al*. 1997). Other antibodies were obtained from commercial sources: mouse monoclonal anti-Myc 9E10 (American Type Culture Collection, Rochville, MD, USA); mouse monoclonal anti-FLAG-M2, mouse monoclonal anti-GST, mouse monoclonal anti-MBP, anti-HA, rabbit polyclonal anti-MAGI-1, anti-MAGI-2 and anti-MAGI-3 antibodies (Sigma-Aldrich, St Louis, MO, USA); rabbit polyclonal anti-His (Medical and Biological Laboratories, Nagoya, Japan); mouse monoclonal anti-green fluorescent protein (GFP), mouse monoclonal β-DG and goat polyclonal anti-neuroligin 2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-gephyrin and anti-vesicular GABA transporter (VGAT) antibodies (Synaptic System, Göttingen, Germany); mouse monoclonal anti-β-DG (Novocastra, Newcastle, UK); mouse monoclonal anti-PSD-95-family (BD Biosciences); rhodamine-, fluorescein isothiocyanate- and Cy5-conjugated secondary antibodies for dual labeling (Chemicon International Inc., Temecula, CA, USA).

Primary cell cultures and immunocytochemistry

Hippocampal neuron cultures were obtained from rat embryonic day-18 (E18) embryos as described previously (Goslin and Banker 1991). Primary astrocyte cultures were obtained from newborn Wistar rats (Goslin *et al*. 1991). Hippocampal neurons at div. 21 were fixed with phosphate-buffered saline (PBS) containing 4% (w/v) paraformaldehyde and 4% (w/v) sucrose for 15 min. For MAGI antibodies, cells were fixed with cold methanol for 15 min at −20°C. Cells were permeabilized with 0.25% (w/v) Triton X-100 in PBS for 5 min, blocked with PBS containing 10% (w/v) bovine serum albumin (BSA) and were incubated with the first antibody in PBS containing 3% (w/v) BSA overnight at 4°C. Samples were washed with PBS, incubated with the secondary antibody in PBS containing 3% (w/v) BSA for 2 h at 25°C, washed with PBS and embedded in 95% (w/v) glycerol in PBS. For the immunocytochemistry of COS-7 cells, cells were fixed with 4% (w/v) formaldehyde in PBS for 15 min and subsequently incubated with 50 mM glycine in PBS for 30 min, 0.2% (w/v) Triton X-100 in PBS for 15 min and 1% (w/v) BSA in PBS for 30 min at room temperature. Cells were incubated with various first antibodies in PBS containing 1% (w/v) BSA and 0.1% (w/v) Triton X-100 overnight at 4°C. Samples were washed with PBS, incubated with the secondary antibody in PBS containing 1% (w/v) BSA and 0.1% Triton X-100 for 2 h at room temperature, washed with PBS and embedded in 95% (w/v) glycerol in PBS. Images were obtained by Olympus IX71 CCD microscope with a 40× objective (Olympus Corporation, Tokyo, Japan). For analysis of clusters in neurons, we used Scion Image (Scion Corporation, Frederick, MD, USA) and defined the signals with the following properties as being clusters: peak fluorescence levels 50% greater than the maximal fluorescence levels of diffuse dendritic signals in the vicinity; and 3–50 pixels in size (Yao *et al*. 2002). Clusters were counted from seven neurons from each independent preparation and three preparations were used. Madine–Darby canine kidney (MDCK) cells were immunostained as described previously (Hirabayashi *et al*. 2003).

Yeast three-hybrid screening

Yeast three-hybrid screening was performed using pBTM116 KM Bridge S-SCAM-N-SAPAP1, human brain cDNA library (BD Biosciences) and yeast strain L40. Histidine selection plates contained 8 mM 3-amino-1,2,4-triazole and 360 mg/L of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. After 6 days of incubation, blue colonies were picked up for further analysis.

Pull-down assay

Sindbis virus was produced using baby hamster kidney (BHK) 21 cells as described previously (Yao *et al*. 2002). Various yellow fluorescent protein (YFP)-tagged β-DG proteins were expressed in BHK21 cells by using the Sindbis virus. Cells from one 10-cm plate were homogenized in 250 μL of the lysis buffer containing 25 mM Tris/HCl (pH 8.0), 100 mM NaCl and 1% (w/v) Triton X-100 and centrifuged at 100 000 *g* for 15 min at 4°C. A volume of 400 μL of 25 mM Tris/HCl (pH 8.0) and 100 mM NaCl was added to 200 μL of the supernatant. Each diluted supernatant was incubated with 250 pmol of various GST proteins fixed on 7.5 μL of glutathione Sepharose 4B beads. Various Myc-tagged S-SCAM and FLAG-tagged neuroligin 2 proteins were expressed in COS-7 cells. Cell lysates were prepared and diluted as described above for BHK21 cells, except that Tris/HCl (pH 7.4) was used. Diluted supernatant was incubated with 250 pmol of His-S-NL2 fixed on either 20 μL of *S*-agarose beads or 250 pmol of various GST proteins fixed on 7.5 μL of glutathione Sepharose 4B beads. After the beads were washed, the precipitates were analyzed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with appropriate antibodies.

Immunoprecipitation from rat brain and COS-7 cells

The synaptic membrane fraction was prepared from five rat brains as described (Takeuchi *et al*. 1997). The sample was homogenized in 3 mL of 25 mM Tris/HCl (pH 7.4) containing 50 mM NaCl, 1% (w/v) Nonidet P-40 and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and centrifuged at 100 000 *g* for 30 min at 4°C to collect the supernatant. The extract was diluted by adding 6 mL of 25 mM Tris/HCl (pH 7.4) containing 50 mM NaCl and complete protease inhibitor cocktail. A 4-mL volume of the diluted sample was incubated with 1 μL of polyclonal anti-β-DG antibody fixed on 10 μL of protein G Sepharose 4 fast flow beads (GE Healthcare). After the beads were washed, the precipitates were analyzed in SDS–PAGE and immunoblotted with anti-β-DG (Novocastra), anti-MAGI-2 (Sigma-Aldrich) and anti-neuroligin 2 antibodies. COS-7 cells were transfected with various pClneoMyc S-SCAM and pFLAG β-DG constructs using the diethylaminoethyl (DEAE) dextran method. Cells from one 10-cm plate were homogenized in 250 μL of the lysis buffer containing 25 mM Tris/HCl (pH 7.4), 100 mM NaCl and 1% (w/v) Triton X-100 and centrifuged at 100 000 *g* for 15 min at 4°C. A 400-μL volume of 25 mM Tris/HCl (pH 7.4) and 100 mM NaCl was added to 200 μL of the supernatant. Each diluted supernatant was incubated with

1 μL of anti-Myc antibody fixed on 10 μL of protein G Sepharose 4 fast flow beads. After the beads were washed, the precipitates were analyzed in SDS–PAGE and immunoblotted with either anti-Myc or anti-FLAG antibody. For western blotting, ECL western blotting detection reagents (GE Healthcare) and SuperSignal West Femto (Pierce Biotechnology Inc., Rockford, IL, USA) were used.

Co-localization experiments in COS-7 cells

COS-7 cells were transfected with various combinations of pFLAG-dystroglycan precursor, pClneoMyc S-SCAM and pClneoDsRed NL2 (702–835) using the DEAE dextran method. Cells were fixed with 4% (w/v) formaldehyde in PBS and immunostained with the appropriate antibodies.

***In vitro* binding assay**

Recombinant GST, MBP and *His-S*-protein fusion proteins were prepared from *E. coli* (BL21DE3). To analyze the interaction between the WW domains of S-SCAM and neuroligin 2, 100 pmol of recombinant neuroligin 2 proteins, *His-S*-NL2 (702–835) and MBP-NL2 (766–814), were incubated with 100 pmol of various GST-S-SCAM proteins fixed on 7.5 μL of glutathione Sepharose 4B beads. For the complex formation of S-SCAM, β-DG and neuroligin 2, 100 pmol of GST-S-SCAM (295–578) and GST-β-DG (826–895) were incubated with 10 μL of either *S*-protein or 100 pmol of *His-S*-NL2 (702–835) fixed on 10 μL of *S*-protein beads. After the beads were washed with 25 mM Tris/HCl (pH 7.4), 50 mM NaCl and 1% (w/v) Triton X-100, the precipitates were analyzed in SDS–PAGE and immunoblotted with the appropriate antibodies.

Results

Anti-S-SCAM antibody recognizes signals at inhibitory synapses

We originally identified S-SCAM as a protein interacting with SAPAP and reported the interaction with NMDA receptor subunits (Hirao *et al*. 1998). Both proteins are components of post-synaptic density associated with excitatory synapses. Since then, we have focused on S-SCAM at excitatory synapses. However, we observed signals along dendritic shafts as well as in dendritic spines in immunocytochemistry using affinity purified anti-S-SCAM (303–405) antibody (Fig. 1a). Some signals were not overlapped by NR1 (Fig. 1a, arrowheads). This observation led us to question whether S-SCAM is localized not only at excitatory synapses but also at inhibitory synapses. To investigate, further we doubly immunostained rat primary cultured hippocampal neurons at div. 21 with anti-S-SCAM (303–405) and anti-VGAT antibodies (Fig. 1b, upper panel). We found that 32.1 ± 5.4% of S-SCAM clusters were apposed to VGAT clusters, whereas 69.8 ± 5.8% of VGAT clusters were apposed to S-SCAM clusters. We also immunostained cells with anti-gephyrin antibody (Fig. 1b, lower panel). We found that 35.4 ± 7.4% of S-SCAM clusters were co-localized with gephyrin clusters, whereas 62.7 ± 10.9% of gephyrin clusters were co-localized with S-SCAM clusters. These findings suggest that almost one-third of signals detected by anti-S-SCAM antibody are localized at inhibitory synapses.

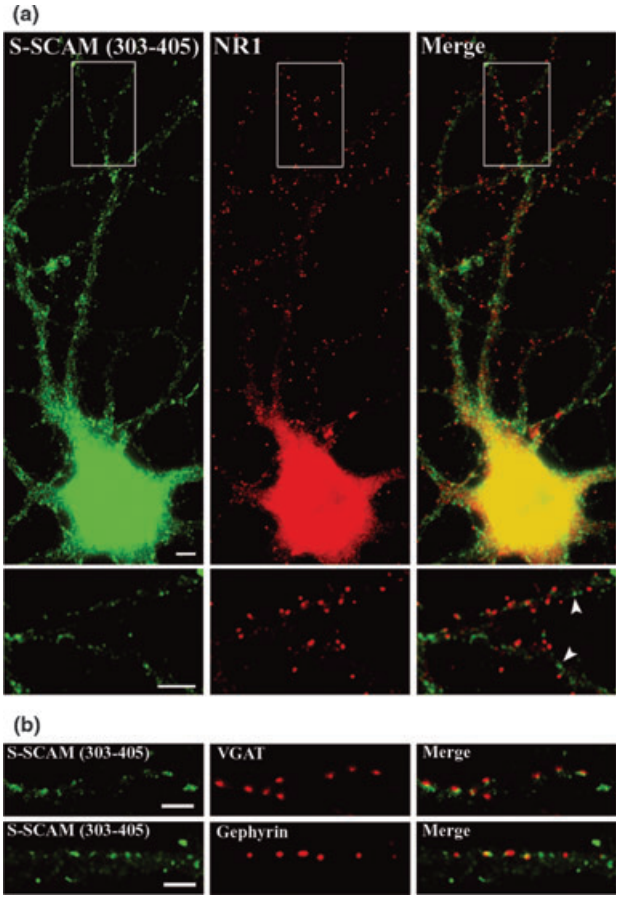

Fig. 1 Anti-synaptic scaffolding molecule (anti-S-SCAM) (303–405) antibody detects signals that are overlapped by vesicular GABA transporter (VGAT) and gephyrin signals. (a) Rat primary cultured hippocampal neurons (div. 14) were immunostained with rabbit anti-S-SCAM and mouse anti-NMDA receptor subunit 1 (anti-NR1) antibodies. Arrowheads indicate signals detected by anti-S-SCAM antibody (303–405) that are not overlapped by NR1. Scale bars, 5 μm. (b) Rat primary cultured hippocampal neurons (div. 21) were immunostained with rabbit anti-S-SCAM (303–405) and either mouse anti-VGAT or mouse anti-gephyrin antibodies. Scale bars, 5 μm.

MAGI family proteins have the same molecular structures composed of PDZ, GK and WW domains. For the initial immunocytochemistry, we used the antibody raised against the WW domains of S-SCAM (303–405). Among 103 amino acid residues of this region, 68 and 64 amino acids are shared by MAGI-1 and MAGI-3, respectively. The antibody cross-reacts with both MAGI-1 and MAGI-3 (Fig. 2a, top panel). Thereby we could not exclude the possibility that the antibody recognizes either MAGI-1 or MAGI-3, and not S-SCAM, at inhibitory synapses. To determine which isoform of the MAGI proteins is present at inhibitory synapses, we made use of MAGI-1- and MAGI-3-specific antibodies. We

found that neither anti-MAGI-1 nor anti-MAGI-3 antibodies cross-reacted with S-SCAM (Fig. 2a, third and fourth panels). Both antibodies detected proteins in the lysates of rat brain. We next examined whether these antibodies are applicable for immunocytochemistry. We detected signals with both antibodies at cell contacts in MDCK cells and primary cultured rat glia, supporting the idea that these antibodies can be used for immunocytochemistry (data not shown). However, in the immunofluorescence with either MAGI-1 or MAGI-3 antibodies, we could not find any signals that formed clusters apposed to VGAT clusters in neurons (Fig. 2b, middle and bottom panels). Next, we performed the affinity purification from the antiserum against GST-S-SCAM (1–421) using GST-S-SCAM (16–64). Among 49 amino acid residues, only 14 amino acids are shared by MAGI-1 and MAGI-3 (Fig. 2c). As expected, the affinity purified antibody recognized only S-SCAM (Fig. 2a, second panel). This affinity purified S-SCAM-specific antibody recognizes signals apposed to VGAT clusters, whereas either MAG-I-1 or MAG-I-3-specific antibody does not (Fig. 2b). We found that 37.2 ± 9.0% of these signals were apposed to VGAT clusters. These data indicate that S-SCAM, but neither MAGI-1 nor MAGI-3, is localized at inhibitory synapses in rat primary cultured hippocampal neurons.

S-SCAM interacts with β-DG

S-SCAM consists of multiple modular domains and is considered to provide a synaptic scaffold at excitatory synapses. We investigated whether S-SCAM plays a similar role at inhibitory synapses. Toward this aim, we searched for putative S-SCAM-binding partners localized at inhibitory synapses. We attempted to perform yeast two-hybrid screenings using various regions of S-SCAM as baits. However, as the N-terminal region of S-SCAM containing the first PDZ (PDZ0), GK and WW domains showed an autonomous activation on reporters in yeast, we could not use this region as bait. To overcome this problem, we generated a yeast three-hybrid vector to co-express SAPAP1 and succeeded to suppress the autonomous activation caused by the bait. The mechanism by which the co-expression of SAPAP1 blocks the self-activation by the bait is not clear. The N-terminal region of S-SCAM was accumulated in the nucleus when expressed alone in COS-7 cells (Fig. 3a). Myc-SAPAP1 was distributed in the cytosol (Fig. 3b). As Myc-SAPAP1 can interact with HA-S-SCAM (1–421) through the GK domain, it recruited HA-S-SCAM (1–421) from the nucleus to the cytosol (Fig. 3c). Thereby, we speculate that SAPAP1 may inhibit the nuclear localization of the bait covering the N-terminal region of S-SCAM in yeast and suppress the reporter activation by the bait itself. We obtained nine positive clones from a human brain cDNA library. Among them, we found one clone encoding the C-terminal 70 amino acids of β-DG. A recent study has revealed that β-DG is localized at inhibitory synapses and not at excitatory synapses (Levi *et al.*

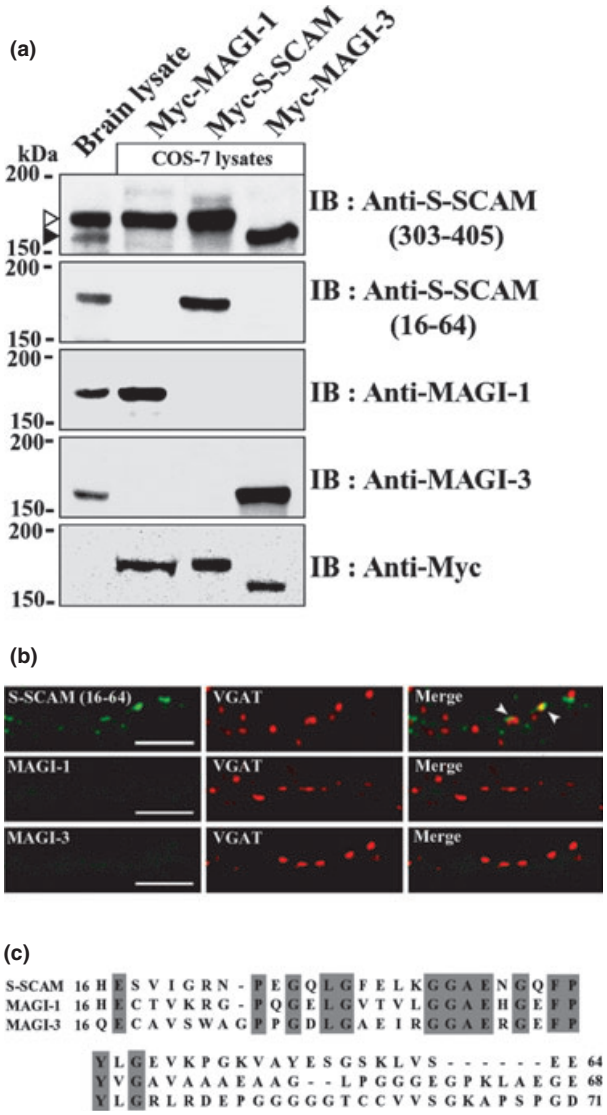

Fig. 2 S-SCAM-specific antibody detects signals at inhibitory synapses but neither anti-membrane-associated guanylate kinase with inverted organization-1 (anti-MAGI-1)- nor anti-MAGI-3-specific antibody detects signals in neurons. (a) Reactivity of anti-synaptic scaffolding molecule (anti-S-SCAM), anti-MAGI-1 and anti-MAGI-3 antibodies. Lysates of rat brain and COS-7 cells expressing Myc-tagged S-SCAM, MAGI-1 and MAGI-3 were immunoblotted by anti-S-SCAM (303–405), anti-S-SCAM (16–64), anti-MAGI-1, anti-MAGI-3 and anti-Myc antibodies. The open arrowhead indicates the expected band of MAGI-1 and S-SCAM in rat brain lysate. The closed arrowhead corresponds to the expected band of MAGI-3. Protein standards are indicated on the left. (b) Rat primary cultured neurons were immunostained with anti-S-SCAM (16–64), anti-MAGI-1 and anti-MAGI-3 antibodies. Neurons were also immunostained with mouse anti-vesicular GABA transporter (anti-VGAT) antibody. The anti-S-SCAM (16–64) antibody detected signals apposed to VGAT clusters (arrowheads), but neither anti-MAGI-1 nor anti-MAGI-3 antibody detected any signals. Scale bars, 10 μm. (c) Sequence alignment of MAGI-1 and MAGI-3 against the amino acid residues 16–64 of S-SCAM. 14 amino acids (shaded) are shared by MAGI-1 and MAGI-3.

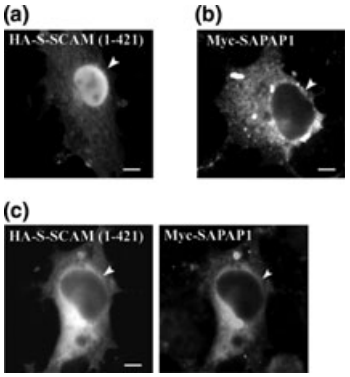

Fig. 3 The nuclear localization of the N-terminal region of synaptic scaffolding molecule (S-SCAM) and its recruitment by synapse-associated protein 90/PSD-95-associated protein-1 (SAPAP1) to the cytosol. COS-7 cells were transfected with pCMV HA-S-SCAM (1–421) and pClneoMyc SAPAP1. Cells were immunostained with mouse monoclonal anti-HA and rabbit anti-SAPAP antibodies. (a) HA-S-SCAM (1–421) alone; (b) Myc-SAPAP1 alone; (c) HA-S-SCAM (1–421) and Myc-SAPAP1; HA-S-SCAM (1–421) was recruited by Myc-SAPAP1 from the nucleus to the cytosol, although some HA-S-SCAM (1–421) was still detected in the nucleus. Arrowheads indicate nuclei. Scale bars, 10 μm.

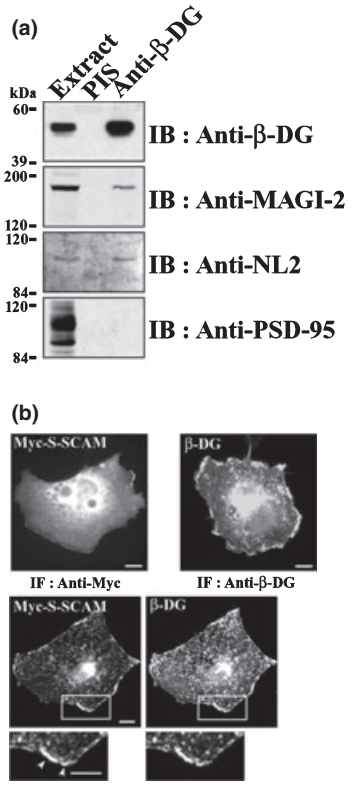

Fig. 4 The interaction of β-dystroglycan (β-DG) and synaptic scaffolding molecule (S-SCAM). (a) Immunoprecipitation by anti-β-DG antibody from rat brain lysates. Rat brain lysates were incubated with either pre-immune serum (PIS) or rabbit anti-β-DG antibody (anti-β-DG) immobilized on protein G Sepharose beads. After the beads were washed, the precipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with mouse anti-β-DG (top panel) (Novocastra), anti-MAGI-2 (second panel) (Sigma-Aldrich), anti-neuroligin 2 (third panel) and anti-PSD-95 (fourth panel) antibodies. The lysate used for immunoprecipitation (15 μg of total protein) was run as an input control (Extract). Protein standards are indicated on the left. (b) Recruitment of Myc-S-SCAM to the cell periphery in COS-7 cells expressing β-DG. COS-7 cells were transfected with pClneoMyc S-SCAM, pFLAG dystroglycan precursor and pClneoMyc S-SCAM/pFLAG dystroglycan precursor. Cells were immunostained with anti-Myc antibody and/or rabbit anti-β-DG antibody. Myc-S-SCAM was diffusely distributed when expressed alone (upper left panel). β-DG was accumulated at the cell periphery (upper right panel). Myc-S-SCAM was recruited to the cell periphery and co-localized with β-DG when both proteins were expressed together (lower panels). The demarcated area is demonstrated at a higher magnification at the bottom and arrowheads show Myc-S-SCAM accumulated at the cell periphery. Scale bars, 10 μm.

2002). If S-SCAM interacts with β–DG, this interaction is likely to take place at inhibitory synapses. To confirm the interaction between S-SCAM and β-DG *in vivo*, we immunoprecipitated β-DG from rat brain and detected S-SCAM in the immunoprecipitates (Fig. 4a, top and second panels). Here we used anti-MAGI-2 antibody from Sigma-Aldrich, which is applicable for western blotting but not for immunocytochemistry. PSD-95 was not detected in the immunoprecipitates (Fig. 4a, bottom panel). We next exogenously expressed Myc-tagged S-SCAM (1–1277) and FLAG-tagged dystroglycan precursor in COS-7 cells. S-SCAM was diffusely distributed in the cytosol (Fig. 4b, upper left panel). When the FLAG-tagged dystroglycan precursor was expressed in COS-7 cells, α-dystroglycan was expressed as a protein with an N-terminal FLAG-tag, whereas β-DG was expressed without a tag and mainly localized at the cell periphery (Fig. 4b, upper right panel). The co-expression of FLAG-dystroglycan precursor with S-SCAM induced the recruitment of S-SCAM to the cell periphery (Fig. 4b, lower panel, arrowheads). This finding also supports the interaction between S-SCAM and β-DG.

β-Dystroglycan binds to the WW domains of S-SCAM

To determine which region of S-SCAM is involved in the interaction with β-DG, we expressed FLAG-β-DG with various Myc-tagged S-SCAM proteins in COS-7 cells and performed the immunoprecipitation with anti-Myc antibody (Fig. 5a). FLAG-β-DG was detected as two bands on SDS–PAGE of the lysates of COS-7 cells transfected with pFLAG β-DG (Fig. 5b, inputs). Both bands were detected in the immunoprecipitates by an anti-Myc antibody with Myc-S-SCAM (295–578), but were neither detected with Myc-S-SCAM (1–301) nor (573–1277). Myc-S-SCAM (295–578) consists of two WW domains and the PDZ1 domain. To test which domain is involved in the interaction we prepared various GST proteins (Fig. 5a). We pulled down YFP-tagged β-DG expressed in BHK21 cells using these GST proteins.

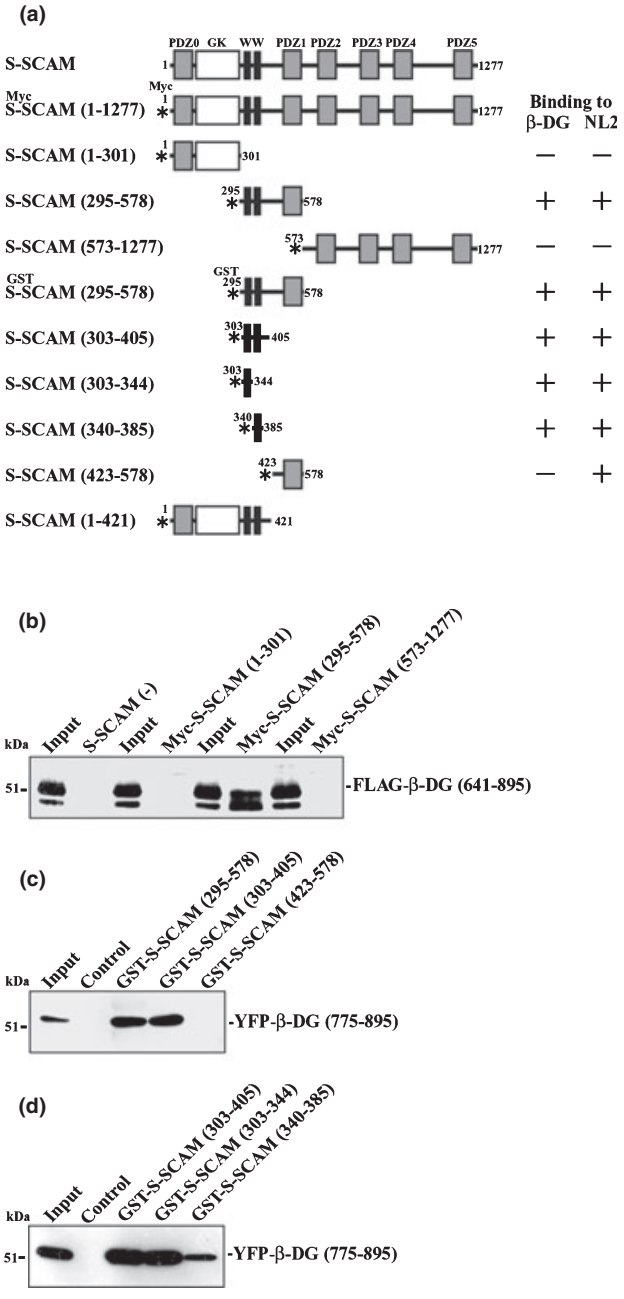

Fig. 5 *In vitro* binding analysis to determine the β-dystroglycan (β-DG)-binding region of synaptic scaffolding molecule (S-SCAM). (a) Schematic depiction of constructs of S-SCAM. The numbers correspond to those of the first and last amino acid residues of S-SCAM. White, black and gray rectangles show the guanylate kinase (GK), WW and PSD-95/Discs large/ZO-1 (PDZ) domains, respectively. Asterisks (*) represent Myc-, HA- and glutathione-*S*-transferase (GST)-tags. Marks on the right (+/–) represent the ability/inability of constructs to bind to the cytoplasmic regions of β-DG and neuroligin 2 (NL2). (b) Interaction of the middle region of S-SCAM with β-DG. Various Myc-tagged S-SCAM proteins and FLAG-β-DG (641–895) were expressed in COS-7 cells. The extracts of cells were incubated with anti-Myc ascites immobilized on protein G Sepharose 4 fast flow beads. After the beads were washed, the precipitates were immunoblotted with anti-FLAG antibody. FLAG-β-DG was co-immunoprecipitated with Myc-S-SCAM (295–578) containing the WW domains and the PDZ1 domain. Protein standards are indicated on the left. (c) and (d) Baby hamster kidney 21 (BHK21) cells were infected with Sindbis virus to express YFP-β-DG (775–895). The extracts of cells were incubated with control GST, GST-S-SCAM (295–578), GST-S-SCAM (303–405), GST-S-SCAM (423–578), GST-S-SCAM (303–344) and GST-S-SCAM (340–385) immobilized on glutathione Sepharose 4B beads. After the beads were washed, the precipitates were immunoblotted with anti-GFP antibody.

GST-S-SCAM (303–405) covering the WW domains trapped YFP-β-DG (775–895), whereas GST-S-SCAM (423–578) covering PDZ1 did not (Fig. 5c). Moreover, each of the first and second WW domains, GST-S-SCAM (303–344) and (340–385), could interact with YFP-β-DG (Fig. 5d). The result indicates that β-DG binds to the WW domains of S-SCAM.

β-Dystroglycan has three sequences to bind to the WW domains of S-SCAM

Detailed studies including the analysis of the crystal structure have revealed that the C-terminal 13 amino acids of β-DG bind the WW domain of dystrophin (Jung *et al*. 1995; Huang *et al*. 2000). We tested whether this sequence is involved in the interaction between β-DG and S-SCAM. We prepared a series of YFP-tagged proteins that contain various regions of the cytoplasmic tail of β-DG and tested for the interaction with GST-S-SCAM (303–405) (Fig. 6a). YFP-β-DG (775–882), which lacks the C-terminal 13 amino acids bound to GST-S-SCAM (303–405), as well as YFP-β-DG (775–895), which covers the whole cytoplasmic region, did react, but YFP-β-DG (775–819), which only contains the membrane-proximal region, did not (Fig. 6b). This result implies that S-SCAM binds to the C-terminal half region of the cytoplasmic domain of β-DG, but that this interaction does not solely depend on the extreme C-terminal 13 amino acids. As PPXY is regarded as a minimum consensus motif for the major group of the WW domain, we speculated that both PPEY (828–831) and PPPY (889–892) are involved in the interaction. To directly determine the S-SCAM-binding sequences, we substituted amino acid sequences in these putative binding sites. YFP-β-DG (878–895) was bound to GST-S-SCAM (303–405), but YFP-β-DG (878–895 mut) was not, indicating that PPPY (889–892) is a binding site. YFP-β-DG (775–862) interacted with GST-S-SCAM (303–405), whereas YFP-β-DG (775–862 mut) did not. The result means that PPEY (828–831) is also the binding site. However, unexpectedly, YFP-β-DG (775–895 mut), in which both of PPEY (828–831) and PPPY (889–892) are disrupted, still interacted with GST-S-SCAM (303–405). This finding suggests that there exists the third S-SCAM-binding site in addition to PPEY (828–831) and PPPY (889–892). Consistently, YFP-

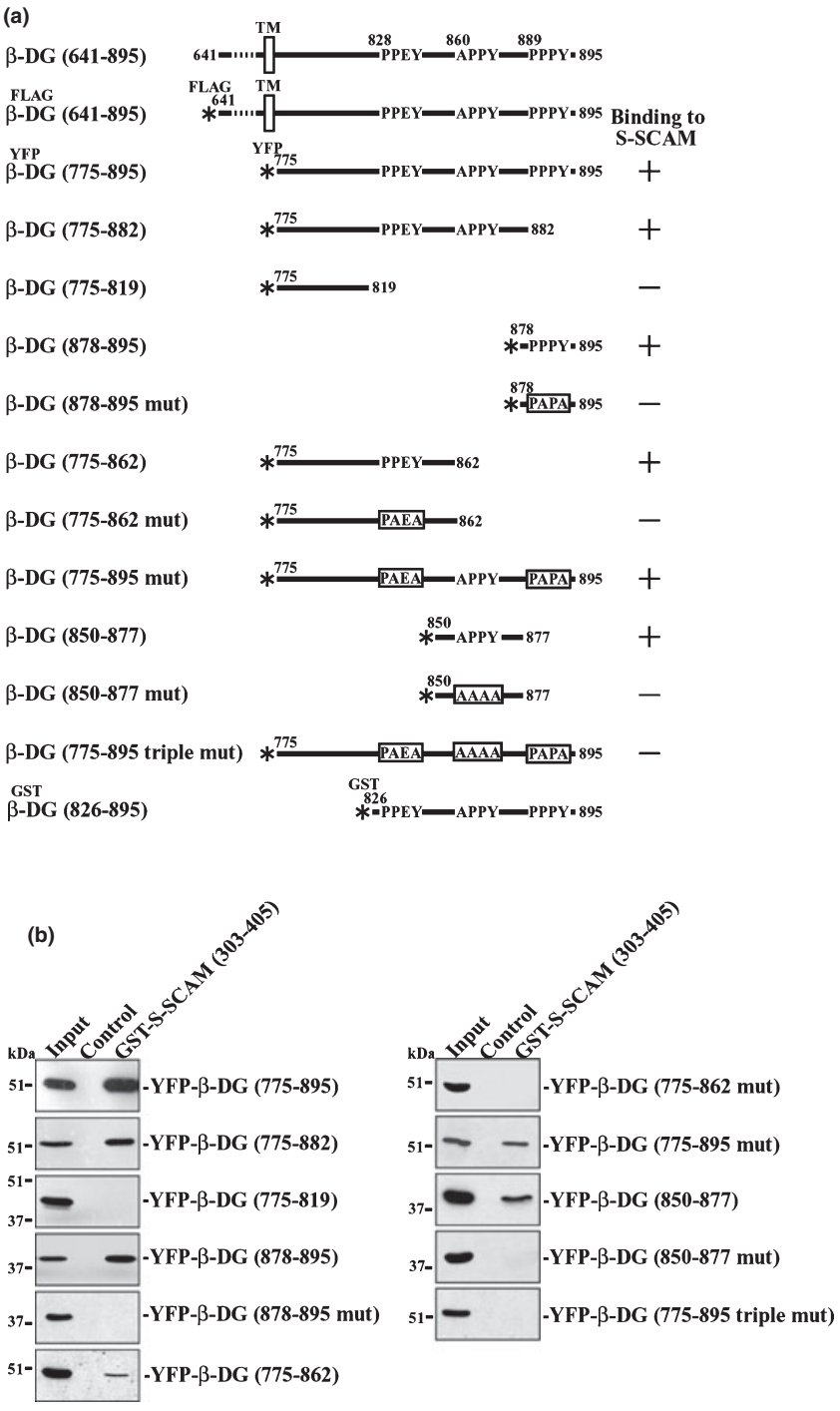

Fig. 6 *In vitro* binding analysis to determine the synaptic scaffolding molecule (S-SCAM)-binding region of β-dystroglycan (β-DG). (a) Schematic depiction of the constructs of β-DG. Numbers correspond to those of the first and last amino acid residues of the dystroglycan precursor covered by each construct. White rectangles indicate the transmembrane region of β-DG. Asterisks (*) represent FLAG-, YFP- and glutathione-*S*-transferase (GST)-tags. The amino acid residues of three putative S-SCAM-binding sequences are indicated as one-letter symbols. Mutated amino acids are bracketed. Marks on the right (+/–) represent the ability/inability of constructs to bind S-SCAM. (b) BHK21 cells were infected with Sindbis virus to express various YFP-β-DG proteins. The extracts of cells were incubated with either control GST or GST-S-SCAM (303–405) immobilized on glutathione Sepharose 4B beads. After the beads were washed, the precipitates were immunoblotted with anti-GFP antibody. Protein standards are indicated on the left.

β-DG (850–877) showed the interaction. APPY (860–863) does not fit with the consensus, but the disruption of this sequence in YFP-β-DG (850–877 mut) abolished the interaction. Finally, we tested YFP-β-DG (775–895 triple mut), in which all three sites are disrupted. This protein did not show the binding ability, indicating that these three sequences are responsible for the interaction. In conclusion, β-DG has three sequences that can interact with the WW domains of S-SCAM.

Neuroligin 2 can interact with S-SCAM

We previously demonstrated that PSD-95 binds only to the C-terminal PDZ-binding motif of neuroligin 1, whereas S-SCAM can interact with neuroligin 1 not only by the PDZ domain but also by the WW domains (Iida *et al*. 2004). Neuroligin 1 is localized at excitatory synapses and absent at inhibitory synapses, whereas neuroligin 2 is localized at inhibitory synapses (Song *et al*. 1999; Varoqueaux *et al*. 2004). The sequences between the

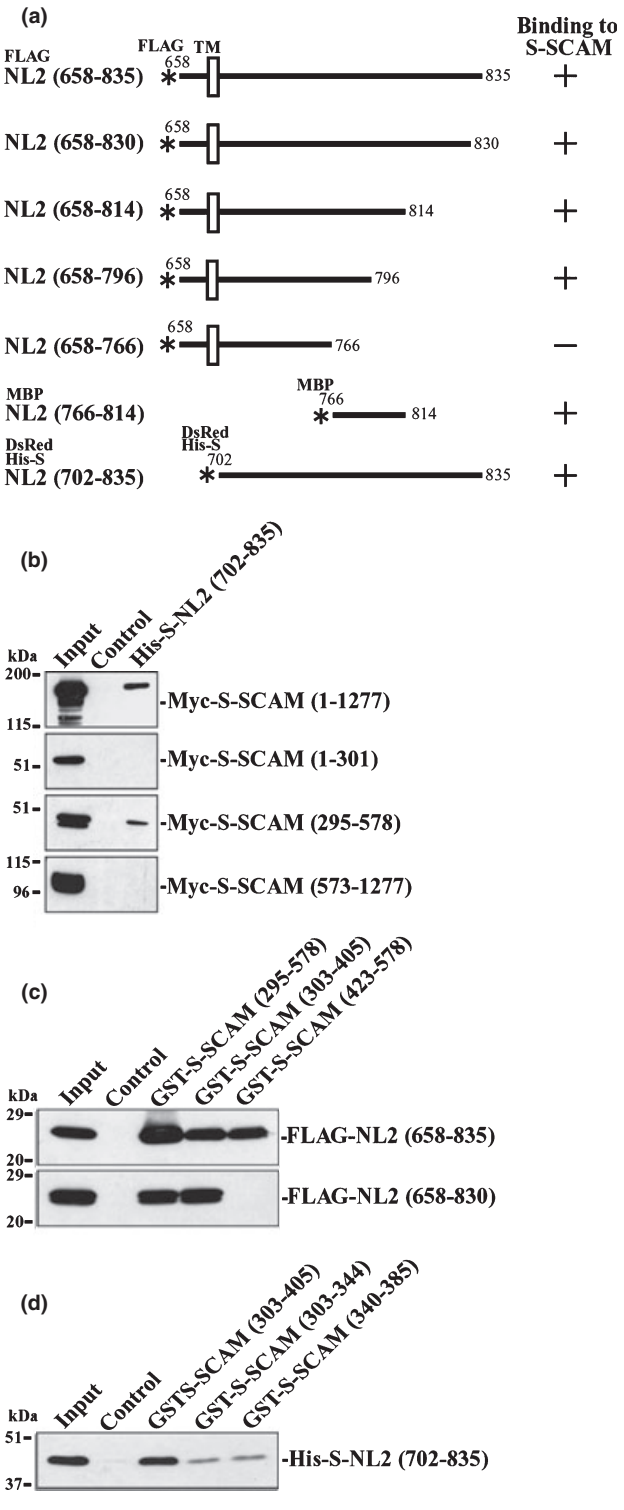

Fig. 7 The WW domains of synaptic scaffolding molecule (S-SCAM) interact with neuroligin 2. (a) A schematic depiction of the constructs of neuroligin 2. The numbers correspond to those of the first and last amino acid residues of neuroligin 2. White rectangles indicate the transmembrane region of neuroligin 2. Asterisks (*) represent FLAG-, maltose-binding protein (MBP)-, DsRed- and *His-S*-tag. Marks on the right (+/–) represent the ability/inability of constructs to bind S-SCAM. (b) Interaction of the middle region of S-SCAM with neuroligin 2. Various Myc-tagged S-SCAM proteins were expressed in COS-7 cells. The extracts of cells were incubated with *His-S*-NL2 (702–835) immobilized on *S*-agarose beads. After the beads were washed, the precipitates were immunoblotted with anti-Myc antibody. Myc-S-SCAM (1–1277) and Myc-S-SCAM (295–578) containing WW domains and PSD-95/Discs large/ZO-1 (PDZ1) domain were trapped by *His-S*-NL2 (702–835). Protein standards are indicated on the left. (c) The extracts of COS-7 cells expressing FLAG-NL2 (658–835) containing either the whole cytoplasmic region or FLAG-NL2 (658–830) lacking the C-terminal PDZ-binding motif were incubated with control glutathione-*S*-transferase (GST), GST-S-SCAM (295–578), GST-S-SCAM (303–405) and GST-S-SCAM (423–578) immobilized on glutathione Sepharose 4B beads. After the beads were washed, the precipitates were immunoblotted with anti-FLAG antibody. GST-S-SCAM (303–405) trapped both of FLAG-NL2 (658–835) and (658–830), whereas GST-S-SCAM (423–578) bound only FLAG-NL2 (658–835). Protein standards are indicated on the left. (d) Purified *His-S*-NL2 (702–835) protein was incubated with control GST, GST-S-SCAM (303–405), GST-S-SCAM (303–344) or GST-S-SCAM (340–385) immobilized on glutathione Sepharose 4B beads. After the beads were washed, the precipitates were immunoblotted with anti-*His* antibody. Protein standards are indicated on the left.

cytoplasmic regions of neuroligin 1 and 2 are well-conserved and 40% identical. Thereby, we next examined whether neuroligin 2 also binds to S-SCAM. We generated a set of recombinant neuroligin 2 proteins that cover various cytoplasmic regions of neuroligin 2 (Fig. 7a). We used *His-S*-tagged NL2 (702–835) protein for the pull-down assay. The protein interacted with Myc-S-SCAM (1–1277) and (295–578) but not with either Myc-S-SCAM (1–301) or (573–1277) (Fig. 7b). We subsequently performed reverse pull-down assays using GST-S-SCAM proteins and FLAG-tagged neuroligin 2 proteins. Both of GST-S-SCAM (303–405) and (423–578) trapped FLAG-tagged NL2 (658–835) (Fig. 7c, upper panel). Each WW domain could bind neuroligin 2 as it did for β-DG (Fig. 7d). As expected, GST-S-SCAM (423–578) containing only PDZ1 did not bind FLAG-NL2 (658–830) without the C-terminal PDZ-binding motif (Fig. 7c, lower panel). However, GST-S-SCAM (303–405) containing WW domains could interact with FLAG-NL2 (658–830). To determine which sequence of neuroligin 2 is involved in the interaction with the WW domains of S-SCAM, we further tested FLAG-NL2 (658–814) (658–796) and (658–766) for binding to GST-S-SCAM (303–405) (Fig. 8a). FLAG-NL2 (658–814) and (658–796) interacted with GST-S-SCAM (303–405), although the binding efficiency of FLAG-NL2 (658–796) was relatively low. FLAG-NL2 (658–766) did not bind to the WW domains. The results suggest that the sequences between 766 and 796 amino acids are involved in the interaction. MBP-NL2 (766–814) indeed interacted with GST-S-SCAM (303–405) (Fig. 8b).

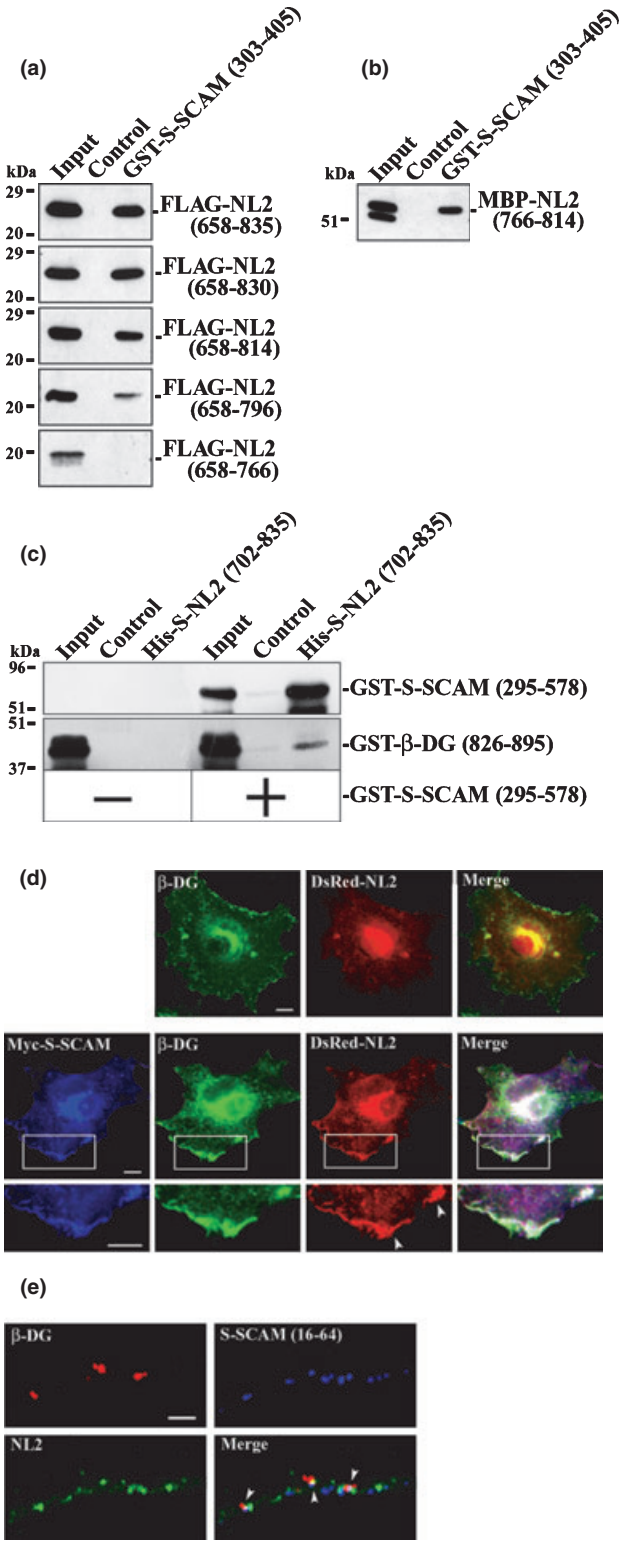

Fig. 8 Synaptic scaffolding molecule (S-SCAM), neuroligin 2 and β-dystroglycan (β-DG) form a complex and are partially co-localized at inhibitory synapses. (a) The extracts of COS-7 cells expressing various FLAG-neuroligin 2 proteins were incubated with either control glutathione-*S*-transferase (GST) or GST-S-SCAM (303–405) immobilized on glutathione Sepharose 4B beads. After the beads were washed, the precipitates were immunoblotted with anti-FLAG antibody. Protein standards are indicated on the left. (b) Purified maltose-binding protein (MBP)-NL2 (766–814) (100 pmol) was incubated with either control GST or GST-S-SCAM (303–405) immobilized on glutathione Sepharose 4B beads. After the beads were washed, the precipitates were immunoblotted with anti-MBP antibody. The lower band in the input lane has the same size as control MBP, suggesting that it is a side product caused by protein degradation. Protein standards are indicated on the left. (c) Tripartite complex formation of S-SCAM, neuroligin 2 and β-DG *in vitro*. Purified GST-fusion protein containing the cytoplasmic region of β-DG [GST-β-DG (826–895)] either without or with GST-S-SCAM (295–578) was incubated with *His-S*-NL2 (702–835) immobilized on *S*-agarose beads. After the beads were washed, the precipitates were immunoblotted with anti-GST antibody. Protein standards are indicated on the left. (d) Tripartite complex formation of S-SCAM, neuroligin 2 and β-DG in COS-7 cells. COS-7 cells were transfected with various combinations of pFLAG dystroglycan precursor, pCIneoMyc S-SCAM (1–1277) and pCIneoDsRed NL2 (702–835). Cells were immunostained with rabbit anti-β-DG and mouse monoclonal anti-Myc antibodies. DsRed NL2 was diffusely distributed when expressed with dystroglycan precursor (upper panel). The co-expression of Myc-S-SCAM accumulates DsRed NL2 to the cell periphery (lower panel, arrowheads) Scale bars, 10 μm. (e) Co-localization of S-SCAM, β-DG and neuroligin 2 at inhibitory synapses. Rat primary cultured hippocampal neurons were immunostained with rabbit anti-S-SCAM (14–64), mouse anti-β-DG (Santa Cruz Biotechnology) and goat anti-neuroligin 2 antibodies. Three proteins are partially colocalized (arrowheads). Scale bars, 5 μm.

The lower band in the input lane was estimated to be smaller than the upper band by about 5 kDa. MBP-NL2 (766–814) covers 49 amino acids of neuroligin 2. Thereby, the lower band apparently corresponded to a product of protein degradation that contains only MBP and did not interact with GST-S-SCAM (303–405). The result shown in Fig. 8(b) also indicates that the interaction between the cytoplasmic tail of neuroligin 2 and the WW domains of S-SCAM is direct and does not require any other protein.

β-Dystroglycan is capable of forming a complex with neuroligin 2 in the presence of S-SCAM

As described above, neuroligin 2 and β-DG interact with S-SCAM using multiple sequences. This mode of interaction opens the possibility for neuroligin 2 and β-DG to simultaneously interact with S-SCAM. We directly confirmed this *in vitro*. We incubated GST-β-DG (826–895) with *His-S*-NL2 (702–835) fixed on *S*-agarose beads. These proteins did not interact with each other (Fig. 8c, left three lanes). However, when GST-S-SCAM (295–578) was added, GST-β-DG (826–895) was co-trapped with GST-S-SCAM (295–578) by *His-S*-NL2 (702–835) (Fig. 8c, right three lanes). The result indicates that the cytoplasmic region of neuroligin 2 can form a complex with the cytoplasmic region

of β-DG *via* S-SCAM. As the second assay, we expressed the cytoplasmic region of neuroligin 2 with the DsRed tag in COS-7 cells with various combinations of DG precursor and S-SCAM. DsRed-NL2 (702–835) showed a diffuse distribution when expressed either alone or with a DG precursor (data not shown and Fig. 8d, upper panel). However, DsRed-NL2 (702–835) was accumulated at the cell periphery when co-expressed with S-SCAM and DG precursor (Fig. 8d, lower panel, arrow heads). This result also supports the idea that neuroligin 2 forms a complex with β-DG *via* S-SCAM. Furthermore, neuroligin 2 was detected in the immunoprecipitates by anti-β-DG antibody (Fig. 4a, third panel). In the immunofluorescence of rat primary cultured hippocampal neurons, neuroligin 2 was partially overlapped with S-SCAM and β-DG (Fig. 8e). All these data support the idea that S-SCAM is a scaffold protein that links β-DG and neuroligin 2 at inhibitory synapses.

Discussion

In this study we have demonstrated the localization of S-SCAM at inhibitory synapses using rat primary cultured hippocampal neurons. In rat hippocampal neuron cultures, anti-S-SCAM antibody reveals signals on dendritic spines that are co-localized with excitatory synapse marker proteins including PSD-95 and NMDA receptor subunits. However, some signals are also detected by this antibody on dendritic shafts and are apposed to VGAT clusters. The signals are also partially overlapped by gephyrin clusters. These data suggest that proteins detected by our anti-S-SCAM antibody are localized at inhibitory synapses. As the sequences of the WW domains are well conserved among three members of the MAGI family of proteins, the antibody cross-reacts with MAGI-1 and MAGI-3. Therefore, we had to exclude the possibility that either MAGI-1 or MAGI-3 are detected by this antibody at inhibitory synapses. First, we made use of commercially available MAGI-1 and MAGI-3 antibodies and examined whether MAGI-1 and MAGI-3 are localized at inhibitory synapses. Western blottings confirmed that neither MAGI-1 nor MAGI-3 antibody reacts with S-SCAM. Both antibodies detected proteins with the approximately expected sizes of MAGI-1 and MAGI-3 in rat brain lysates. They showed signals concentrated at cell–cell contacts of MDCK cells and primary cultured astrocytes in immunocytochemistry. These observations support the view that MAGI-1 and MAGI-3 antibodies are applicable for immunocytochemistry. None of them, however, detected signals at inhibitory synapses in neurons. Next, we confirmed the localization of S-SCAM at inhibitory synapses using a specific antibody for S-SCAM. All these findings indicate that S-SCAM, but neither MAGI-1 nor MAGI-3, is localized at inhibitory synapses in primary cultured hippocampal neurons. The semiquantitative analysis suggests that almost 30% of S-SCAM is localized at inhibitory synapses. We did not detect S-SCAM at all of the inhibitory synapses. This may mean that S-SCAM is only localized at some distinct members of the inhibitory synapses.

Our next question was whether and how S-SCAM plays a role as a scaffold at inhibitory synapses. S-SCAM is a multimodular protein that interacts with various synaptic components. The researchers first identified a partial sequence of S-SCAM as a binding partner for atrophin-1 (Wood *et al*. 1998). Following this report, we reported the full-length of S-SCAM as a protein that interacts with the NMDA subunits neuroligin 1 and SAPAP at excitatory synapses (Hirao *et al*. 1998). Because the molecular organization of S-SCAM is similar to that of the canonical synaptic scaffold protein, PSD-95, the main efforts of researchers have focused on identifying which molecules interact with S-SCAM. As a consequence, the list of S-SCAM-binding proteins is continuously growing, supporting the concept that this protein functions as a scaffold at excitatory synapses. Our previous studies also indicate that S-SCAM is involved in the accumulation of neuroligin and PSD-95 at excitatory synapses in primary cultured hippocampal neurons (Iida *et al*. 2004). By analogy, we supposed that S-SCAM interacts with some molecules at inhibitory synapses. The first candidate is β-DG, which we obtained by a yeast three-hybrid screening. In the screening, we used the N-terminal region of S-SCAM as bait but had to co-express SAPAP1. Otherwise, the bait itself activated the reporter in yeast. The N-terminal region of S-SCAM shows a remarkable nuclear localization when expressed alone in COS-7 cells. SAPAP1 has an ability to recruit it from the nucleus to the cytosol. Although we have not determined the localization of these proteins in yeast, we speculate that SAPAP1 may similarly block the nuclear localization of the N-terminal region of S-SCAM and suppress the autonomous activation of the reporter in yeast. As the segregation of the N-terminal region of S-SCAM by SAPAP1 to the cytosol is not complete, the complex formation of LexA DNA-binding S-SCAM-N-bait with VP16 DNA-activating β-DG may enhance the reporter activation. Alternatively, the binding of β-DG to S-SCAM may attenuate the interaction between S-SCAM and SAPAP1 and inhibit the recruitment of S-SCAM by SAPAP1. In the later case, we may have detected the reporter activation caused by the N-terminal region of S-SCAM itself. The precise mechanism of how the screening worked is not yet clear, but the interaction between S-SCAM and β-DG was confirmed by co-immunoprecipitation from brain and *in vitro* binding assays. α-Dystroglycan and β-DG have been extensively studied in the context of muscle, but recent works have suggested a role of dystroglycan in brain (Mehler 2000; Durbeej and Campbell 2002; Michele *et al*. 2002; Moore *et al*. 2002; Montanaro and Carbonetto 2003). Most importantly, the abnormal glycosylation of α-dystroglycan causes brain dysfunction in humans and a targeted deletion of the dystroglycan gene results in defects of brain development in

mice. A recent study has revealed that β-DG is selectively associated with inhibitory GABAergic synapses (Levi *et al*. 2002). Therefore, we tried to confirm the interaction between S-SCAM and β-DG. The immunoprecipitation from rat brain and *in vitro* experiments using heterologous cells and recombinant proteins supported the interaction between S-SCAM and β-DG. The second candidate is neuroligin 2. Neuroligin 2 is exclusively localized at inhibitory synapses and is preferentially implicated in inhibitory synapse formation (Levi *et al*. 2002; Graf *et al*. 2004; Chih *et al*. 2005). In the preceding study we characterized the interaction of S-SCAM with neuroligin 1, which is a component of excitatory synapses (Hirao *et al*. 1998; Song *et al*. 1999; Iida *et al*. 2004). As the sequences between neuroligin 1 and 2 are highly conserved, we predicted that S-SCAM can bind neuroligin 2 and demonstrated by *in vitro* experiments that S-SCAM has a capacity to bind to neuroligin 2. Thereby, S-SCAM is likely to be associated with at least two membrane proteins, β-DG and neuroligin 2, at inhibitory synapses.

PPXY is a minimum consensus motif for the major group of the WW domain (Ilsley *et al*. 2002). The cytoplasmic tail of human β-DG harbors PPEY (828–831) and PPPY (889–892). Unexpectedly, during the course of detailed studies we found that APPY (860–863) is also involved in the interaction. Mouse neuroligin 2 contains PPDY (766–770). The WW domains of S-SCAM are likely to bind to this sequence. The interaction between S-SCAM and neuroligin 2 can additionally be mediated by the PDZ domain and the PDZ-binding motif. Moreover, each of the WW domains can bind to β-DG and neuroligin 2, although both WW domains are necessary for the efficient binding. These findings mean that multiple interaction interfaces among S-SCAM, neuroligin 2 and β-DG exist, and that these proteins may interact with each other in manifold ways to form a large complex.

Which role does S-SCAM play in GABAergic post-synaptic differentiation? As S-SCAM is localized at both of excitatory and inhibitory synapses, S-SCAM is not a molecular determinant to recruit neuroligin 2 exclusively to inhibitory synapses. At excitatory synapses, the truncated form of S-SCAM covering the neuroligin-binding region exhibits a dominant negative effect to block the synaptic accumulation of neuroligin, and subsequently reduces PSD-95 clustering (Iida *et al*. 2004). We do not yet have observations that exogenously expressed S-SCAM exerts a dominant effect on the structures of inhibitory synapses. GABAergic synapses, however, have diverse combinations of GABA receptor subunits (Moss and Smart 2001; Owens and Kriegstein 2002). They have different properties depending on the developmental stages. Therefore, it is obvious that further detailed studies are necessary to evaluate the role of S-SCAM in inhibitory post-synaptic differentiation. The basic organization of inhibitory synapses does not depend on dystrophin glycoprotein complex (Levi *et al*. 2002). Even so, the disorders related to this complex result in human brain dysfunction, suggesting that it has a physiological significance (Mehler 2000). S-SCAM may be important to link the modulatory machinery supported by dystrophin glycoprotein complex to the basic architecture provided by neurexin–neuroligin at inhibitory synapses.

Acknowledgements

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