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Identification of SNAP-47, a Novel Qbc-SNARE with Ubiquitous Expression*[S]

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The SNARE proteins are essential components of the intracellular fusion machinery. It is thought that they form a tight four-helix complex between membranes, in effect initiating fusion. Most SNAREs contain a single coiled-coil region, referred to as the SNARE motif, directly adjacent to a single transmembrane domain. The neuronal SNARE SNAP-25 defines a subfamily of SNARE proteins with two SNARE helices connected by a longer linker, comprising also the proteins SNAP-23 and SNAP-29. We now report the initial characterization of a novel vertebrate homologue termed SNAP-47. Northern blot and immunoblot analysis revealed ubiquitous tissue distribution, with particularly high levels in nervous tissue. In neurons, SNAP-47 shows a widespread distribution on intracellular membranes and is also enriched in synaptic vesicle fractions. *In vitro*, SNAP-47 substituted for SNAP-25 in SNARE complex formation with the neuronal SNAREs syntaxin 1a and synaptobrevin 2, and it also substituted for SNAP-25 in proteoliposome fusion. However, neither complex assembly nor fusion was as efficient as with SNAP-25.

Eukaryotic cells are compartmentalized into membrane-enclosed organelles that communicate with each other by membrane traffic. Each trafficking step involves three basic steps: the formation of an organelle by budding from a precursor, transport of the vesicle to its destination along cytoskeletal tracks, and finally docking and fusion of the organelle with its target membrane (reviewed in Refs. 1 and 2). Docking and fusion are mediated by the ordered assembly of multimolecular protein complexes that form upon organelle contact and that are disassembled after fusion is completed. Tethering and docking require large arrays of proteins, many of which are specific for a given transport step. Fusion itself, however, is catalyzed by a set of conserved proteins termed soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs)[4] (3, 4).

SNARE proteins comprise a superfamily of mostly membrane-bound proteins with at least 24 members in yeast and 35 members in mammals (5, 6). The characteristic feature of all SNAREs is the presence of a stretch of ~60 amino acids arranged in heptad repeats that is referred to as a SNARE motif. In most SNAREs, SNARE motifs are positioned adjacent to a transmembrane domain at the C terminus. Exceptions include SNAREs that lack a transmembrane domain and contain two SNARE motifs, instead of one, which are connected by a flexible linker, such as mammalian SNAP-25, SNAP-23, and SNAP-29/GS32. SNAP-25 and SNAP-23 contain multiple palmitate residues attached to cysteine residues in the linker region (7, 8), whereas SNAP-29 lacks a membrane anchor altogether (9, 10).

SNARE proteins undergo an assembly-disassembly cycle that is mediated by the SNARE motifs. Each of the membranes destined to fuse contains sets of SNAREs that are complementary to each other. Before membrane merger, the SNAREs are thought to connect with each other in a "trans-configuration," forming an α-helical bundle of four SNARE motifs. It is thought that "zippering" of the helix bundle proceeds from the N-terminal to the membrane-adjacent C-terminal ends, thus clamping the membranes together and initiating fusion (11). The crystal structures of two SNARE complexes revealed that each helical bundle contains four SNARE motifs, which each belong to an evolutionarily conserved subfamily (12, 13). Inspired by a highly conserved layer of interacting amino acid side chains in the center of the SNARE complex, in which a glutamine (Q) or arginine (R) is contributed by each of the proteins, these subfamilies are classified as Qa-, Qb-, Qc-, and R-SNAREs, respectively (5, 14).

In the present study, we report on a novel mammalian SNARE, termed SNAP-47. During proteomic analysis of synaptic vesicles, we found a peptide mapping to a novel protein that had been previously registered in the data base as containing a single SNARE motif and termed SVAP1. More thorough sequence analysis, however, revealed two SNARE motifs belonging to the Qb and Qc subfamilies, respectively, that are connected by a linker region, thus identifying the protein as a new member of the SNAP-25 subgroup (Qbc-SNARE). Here we report a preliminary characterization of this novel SNARE with respect to tissue and subcellular distribution. Furthermore, we have investigated whether SNAP-47 enters SNARE complexes and is capable of functioning as a SNARE in the fusion of proteoliposomes.

EXPERIMENTAL PROCEDURES

Identification and Cloning of SNAP-47—Subcellular fractionation of rat brain, allowing the preparation of highly purified synaptic vesicles by controlled pore glass size exclusion chromatography was performed as described (15). Approximately 500 μg of synaptic vesicle proteins were separated by 16BAC/SDS-PAGE (16). After Coomassie Blue staining, all visible spots were excised, cut into ~1-mm^2 pieces, and subjected

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[4] The abbreviations used are: SNARE, *N*-ethylmaleimide-sensitive factor attachment protein receptor; SNAP, soluble *N*-ethylmaleimide attachment protein; TMR, transmembrane region; BoNT, botulinum neurotoxin; Syb, synaptobrevin 2; Syx, syntaxin; MS, mass spectrometry; aa, amino acids; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline; NBD, *N*-(7-nitro-2,1,3-benzoxodiazole-4-yl); PE, phosphatidylethanolamine.

to in-gel trypsinization (17). The extracted peptides were analyzed by liquid chromatography-coupled tandem MS (liquid chromatography-MS/MS) on a Q-ToF Ultima mass spectrometer (Waters), and proteins were identified in the National Center for Biotechnology Information nonredundant data base using MASCOT software (Matrix Science, London, UK) as a search engine.

Based on the nucleotide sequence, full-length SNAP-47 cDNA was amplified by PCR from a mouse fetal brain cDNA library using the following primers 5′-AAGCTT(HindIII)ATGAGTTCTGATATGC-GTGTCC-3′ and 5′-CTCGAG(XhoI) CTACATCAGCTTTCTCAT-ACGC-3′, followed by subcloning into pCR2.1 (Invitrogen). Full-length SNAP-47 (amino acids (aa) 1–412), N-terminally EGFP-tagged full-length SNAP-47 (aa 1–412), and His-tagged N-terminally truncated SNAP-47 (aa 115–412) were amplified by PCR and subcloned into pcDNA3.1, pEGFP-C3, and pET28a vectors, respectively.

Recombinant Protein Purification—The expression constructs for cysteine-free SNAP-25A (C84S, C85S, C90S, C92S; aa 1–206), for the syntaxin 1a SNARE motif (SyxH3; aa 180–262), and for synaptobrevin 2 (Syb; aa 1–96) have been described before (18, 19). SNAP-47 was used as either the full-length protein (aa 1–412) or the N-terminal deletion mutant (ΔN-SNAP-47, aa 115–412; see Fig. 1), which contained both entire SNARE helices and was less prone to precipitate. Recombinant SNARE proteins were isolated from *Escherichia coli* and purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography, followed by ion exchange chromatography on an ÄKTA system (GE Healthcare), essentially as described (19). The TMR-containing constructs, syntaxin 1a SNARE motif (aa 183–288), and full-length synaptobrevin 2 (aa 1–116) were purified in the presence of 20 mM cholate or 15 mM Chaps, respectively. Ternary SNARE complexes were assembled overnight and purified using a Mono Q-column (GE Healthcare). Protein concentration was determined by absorption at 280 nm. Native PAGE was carried out as described (18).

Antibodies—Rabbits were immunized to raise antisera against SNAP-47, using as antigen either recombinant ΔN-SNAP-47 or a synthetic peptide corresponding to the amino acid sequence AASR-PKGCTPHRELPT, which is derived from the linker region between the two SNARE motifs, coupled to keyhole limpet hemocyanin via an N-terminal cysteine. The resulting sera were affinity-purified using ΔN-SNAP-47 coupled to CNBr-Sepharose (Sigma) as the solid phase.

Other antibodies used were as follows: monoclonal antibodies against synaptobrevin 2 (clone 69.1), Rab5 (clone 621.3), polyclonal antibody against SNAP-29 (SySy); and monoclonal antibodies against protein-disulfide isomerase and γ-adaptin (BD Biosciences). Peroxidase-labeled Cy3 and Cy5, and Alexa-dyed labeled secondary antibodies were purchased from Sigma, Dianova, and Molecular Probes, respectively.

Tissue Distribution Analysis—A mouse multiple tissue Northern blot (Clontech) was hybridized at high stringency according to the manufacturer's protocol. [^{35}S]dATP was used to produce uniformly labeled probes derived from the full coding sequence (1242 bp) of SNAP-47 cDNA.

In situ hybridization was performed on paraformaldehyde-fixed 12-μm-thick cryostat sections from freshly frozen adult mouse brains. Antisense oligonucleotides representing bp 438–482 and bp 986–1030 of mouse SNAP-47 cDNA were chosen as probes and labeled with [^{35}S]dATP by using terminal transferase. Both generated probes gave identical labeling. In control experiments, hybridizations were performed with a 1000-fold excess of the respective unlabeled oligonucleotide (20). Sections were exposed to Kodak Biomax films for 2 weeks.

For immunoblot analysis, mouse brains from different developmental stages and tissues and brain regions from adult mice were taken out, protein samples were prepared, and Western blots were performed as previously described (21).

Immunoautoradiography was performed as described (22). In brief, sagittal sections of rat brain were fixed with 4% paraformaldehyde and incubated with the SNAP-47 anti-peptide antiserum (1:1000) and then with ^{125}I-labeled goat anti-rabbit IgG (0.25 μCi/ml; Amersham Biosciences). Sections were exposed to Biomax films for 5 days.

Cell Culture and Transfection—Primary neurons were prepared from newborn mouse striata and maintained in culture as described (23). After 3 weeks *in vitro*, neurons were fixed with 4% paraformaldehyde in phosphate buffer for 10 min on ice and processed for immunostaining. Briefly, neurons were incubated overnight at 4 °C in primary antibodies diluted in 0.1 M phosphate buffer, 5% normal goat serum, and 0.1% Triton X-100, washed thoroughly, further incubated for 1 h with fluorescent secondary antibodies, washed again, and mounted with Mowiol on gelatin-coated slides. All fluorescently labeled preparations were observed at high magnification with an inverted confocal laser-scanning microscope and corresponding software (Axiovert 200-LSM510; Zeiss) or with an upright epifluorescence Olympus BX-61 microscope coupled to a digital camera (F-ViewII) and the AnalySIS image acquisition software (Soft-Imaging Systems).

Transfection of GFP-SNAP-47 in HEK293 cells was performed using the calcium phosphate method (24). Thirty-six hours post-transfection, the cells were labeled by immunofluorescence (as above), or protein was extracted using 1% Triton X-100 solubilization and subsequently used for immunoblot analysis.

Protein Spectroscopy—SyxH3, containing a single cysteine residue at position 197 (25), was labeled with the sulfhydryl-reactive fluorophore Oregon Green 488 iodoacetamide essentially as described ($SyxH3^{197OG}$). The concentration of the labeled protein was determined by measuring absorption at 488 and 280 nm, using the molar absorption coefficients of the dye and protein, respectively. Fluorescence anisotropy measurements were carried out using a Fluorolog-3 spectrometer equipped with autopolarizers (Jobin Yvon), essentially as described (26). All measurements were performed at 25 °C in 1-cm quartz cuvettes (Hellma) using phosphate-buffered saline (PBS) buffer (20 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol). Protein concentrations used were as follows: 500 nM $SyxH3^{197OG}$; 1 μM SNAP-25 or (ΔN)-SNAP-47; 1 μM Syb (aa 1–96).

CD spectroscopy was performed using a Jasco model J-720 instrument. All experiments were carried out in phosphate-buffered saline buffer. For thermal denaturation experiments about 10 μM purified ternary SNARE complexes were heated in Hellma quartz cuvettes, with a path length of 0.1 cm. The ellipticity at 222 nm was recorded between 25 and 95 °C with a temperature increment of 30 °C/h.

Fusion of Proteoliposomes—Liposome fusion by lipid dequenching was carried out as described (27–29). In brief, two populations of liposomes were prepared using SyxH3 with TMR (aa 183–288) and full-length synaptobrevin (aa 1–116), with a final lipid/protein ratio of 100:1 (*n*/*n*). The SyxH3 containing liposomes were reconstituted together with a mixture of fluorescent phospholipids, NBD-PE and rhodamine-PE. Fluorescence dequenching upon fusion with unlabeled synaptobrevin containing liposomes was measured using 460 nm for excitation and 538 nm for emission in a Fluoromax-2 (Jovin Yvon) at 30 °C. The total reaction volume was 75 μl, with liposomes added to a buffer containing 100 mM KCl, 25 mM Hepes, pH 7.4. After 90 min, Triton X-100 was added to completely solubilize the liposomes and dequench the fluorescence of NBD. To normalize the experiments, the lowest NBD fluores-

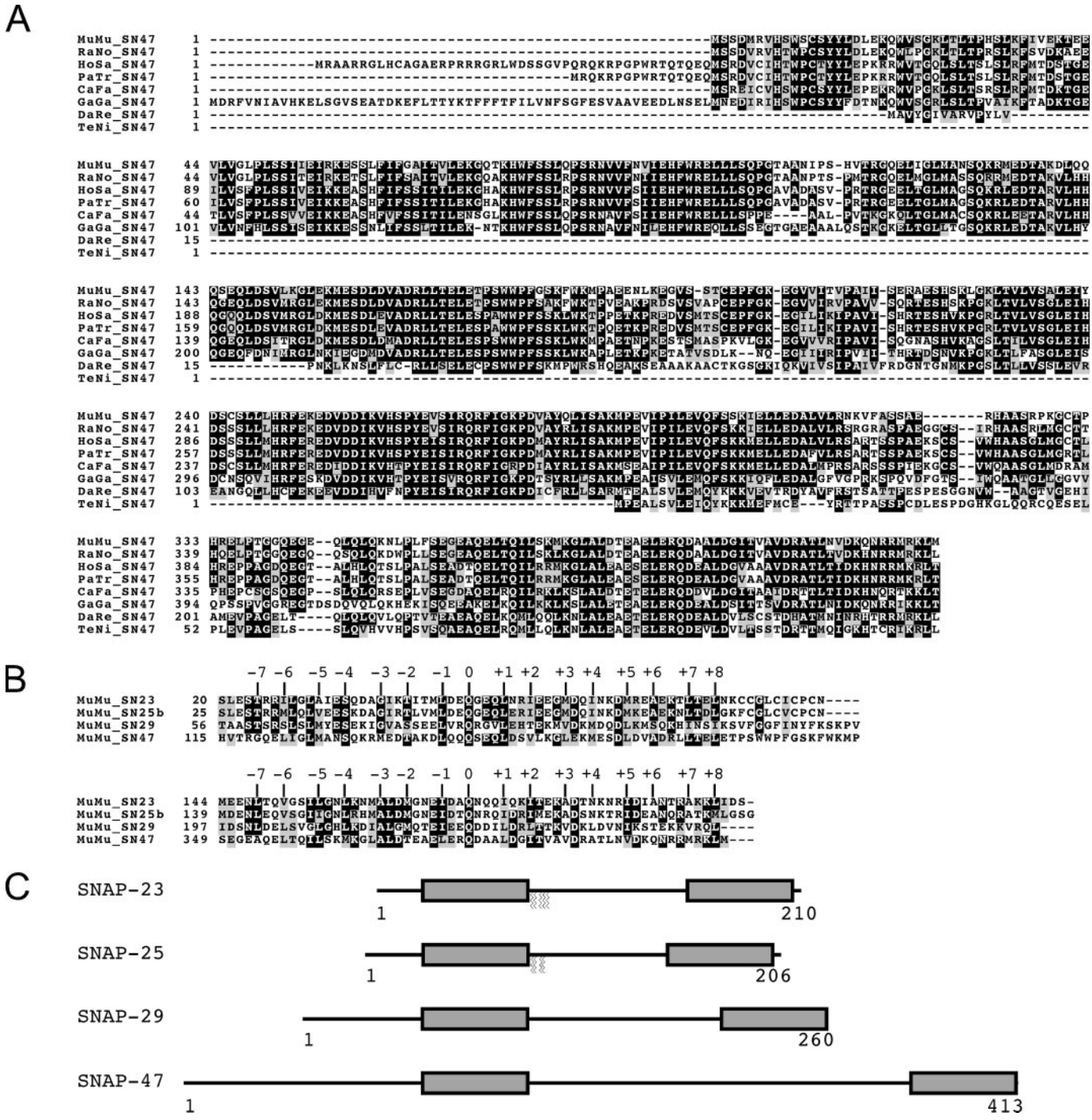

FIGURE 1. **SNAP-47 is a novel SNAP-25-like (Qbc) SNARE protein.** *A*, sequence alignment of SNAP-47 from different species. SNAP-47 was found in *M. musculus* (*MuMu*; gi:21362303; GeneID: 67826), *R. norvegicus* (*RaNo*; gi:40786477; GeneID: 303183), *Homo sapiens* (*HoSa*; gi:20270215, GeneID: 116841; C1orf142), pantroglodytes (*PaTr*; gi:55589472; GeneID: 457794), *Canis familiaris* (*CaFa*; gi:73975430; GeneID: 475181), and *Gallus gallus* (*GaGa*; gi:50732155; GeneID: 420399). In addition, two partial sequences were found in the fishes *Danio rerio* (*DaRe*; gi:68404785) and *Tetraodon nigroviridis* (*TeNi*). The program Muscle (45) was used for initial alignment, and minor adjustments were then made manually. The *black boxes* indicate identical residues, and *gray boxes* show conserved exchanges. *B*, alignment of the two SNARE motifs of the Qbc-SNAREs SNAP-23 (gi:6678049), SNAP-25b (gi:675558), SNAP-29 (gi:31543752), and SNAP-47 from mouse. The well conserved heptad repeat layers −7 to +8 are indicated. *C*, schematic view of the domain structure of Qbc-SNAREs. All SNAP-25 homologues contain tandem SNARE motifs (*boxed*). Note that SNAP-47 has an extended N-terminal domain and linker region compared with other Qbc-SNAREs. Sequences are illustrated at scale. Note the palmitoylation sites in the linker region indicated on SNAP-23 and SNAP-25.

cence signal was set to 0%, and the maximal signal, reached after detergent addition, was set to 100% fluorescence.

RESULTS

SNAP-47 Is a Novel SNAP-25 Homologue—During the course of a comprehensive proteomic analysis of synaptic vesicles, we separated a highly purified fraction of rat brain synaptic vesicles by means of 16BAC/two-dimensional SDS-PAGE. In two spots that migrated at ~47 kDa, we sequenced two tryptic peptides by liquid chromatography-coupled electrospray ionization MS/MS (tandem mass spectrometry) that matched previously uncharacterized gene products when we searched the fragment spectra of the peptides against all entries in the NCBI nr data base using Mascot

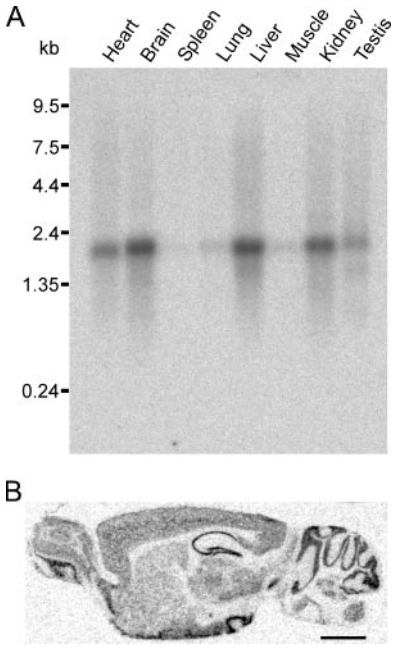

FIGURE 2. **Northern blot and *in situ* hybridization of SNAP-47 mRNA.** *A*, SNAP-47 mRNA was ubiquitously expressed, with the strongest levels in the brain and liver and lower expression levels in the kidney, heart, and testis. The RNA load was normalized to the β-actin hybridization signal (data not shown). *B*, *in situ* hybridization on mouse brain gave the strongest signal in the cortex, principal cells of the hippocampus (CA layers and dentate gyrus), and the cerebellar granule cell layer. Strong expression was also found in the olfactory bulb, the thalamus, and the brain stem, whereas the signal was weaker in the striatum. *Scale bar*, 2 mm.

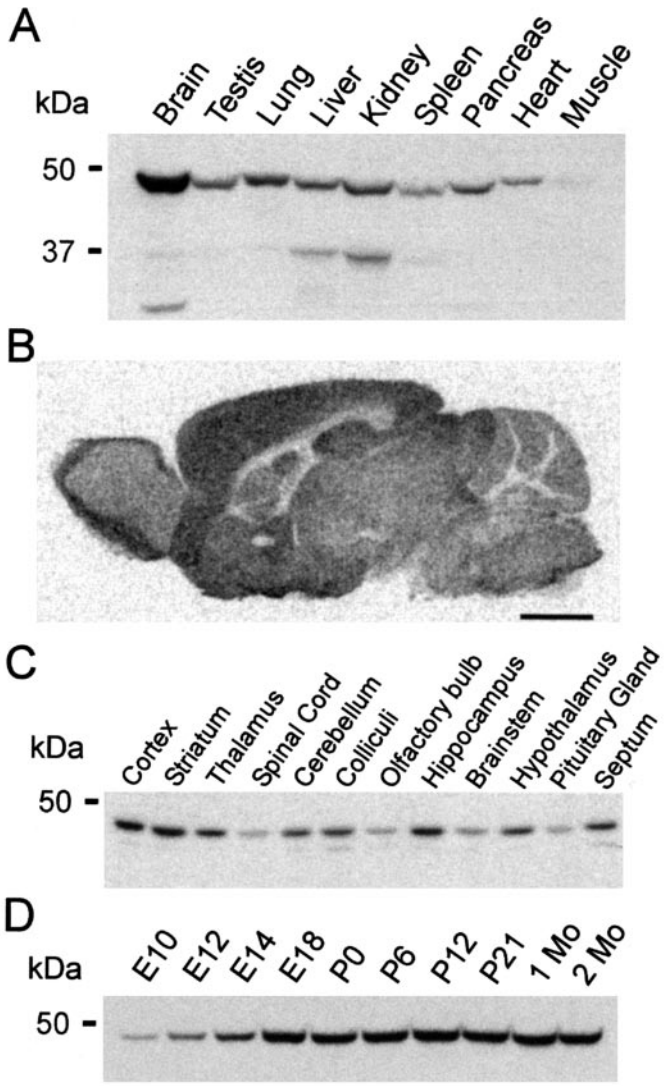

FIGURE 3. **Tissue distribution and developmental profile of the SNAP-47 protein.** *A*, SNAP-47 is ubiquitously expressed but is particularly enriched in the brain. In addition, it is abundant in liver, kidney, lung, spleen, and testis. The protein was not detected in skeletal muscle. *B* and *C*, SNAP-47 is expressed in all brain regions as determined by immunoautoradiography on sagittal mouse brain sections (*B*) and Western blotting on various brain areas (*C*). *Scale bar*, 2 mm. *D*, SNAP-47 was detected as early in development as embryonic day 10 (*E10*), with maximal levels of expression reached at embryonic day 18 (*E18*). These levels were maintained into early adulthood. For all Western blots, an equal amount of protein was loaded in each lane (20 μg). When possible, actin was used as a loading control (data not shown).

as the search engine. One peptide (MESDLDVADR, sequenced in its oxidized and non-oxidized form) appeared in both the RIKEN cDNA 1110031B06 (*Mus musculus*) (gi:21362303) and in the rat hypothetical protein (*Rattus norvegicus*) (gi:40786477), whereas a second peptide, VPAVVSQR, was found only in the latter.

BLAST homology searches revealed sequence similarity to a human protein containing a single SNARE motif and which had been deposited in the data base as SVAP1 (gi:56206865) (30). SVAP1 exhibits a high similarity to its rodent orthologues (~70% amino acid identity). Other orthologues were identified in several vertebrate species (Fig. 1*A*). Using PCR with a mouse fetal brain cDNA library as a template, we isolated the corresponding mouse cDNA clone of mSVAP1. The nucleotide sequence was identical to that of the RIKEN cDNA clone 1110031B06. Based on the nucleotide sequence, a protein with a theoretical molecular mass of 46,524 Da and a pI of 5.54 was predicted.

The closest mammalian homologue of this protein appeared to be SNAP-29, a SNARE protein that contains two SNARE motifs connected by a linker (Fig. 1*B*). Whereas the data base predicted only a single SNARE motif at the C terminus in mSVAP1, another SNARE motif toward the N terminus of the protein was identified when we compared the sequence with SNAP-23, SNAP-25, and SNAP-29 (Fig. 1*B*). Both SNARE motifs contain a glutamine in the position aligning with the highly conserved "0" layer. Based on the predicted molecular weight, which is in good agreement with the apparent M_r determined by SDS-PAGE, and the characteristic tandem SNARE motifs, we propose to rename the novel Qbc-SNARE SNAP-47.

Sequence comparison of SNAP-47 with other SNAP-25 homologues shows that SNAP-47 has a longer N-terminal stretch and a markedly extended loop between its two SNARE motifs (Fig. 1*C*). As in the other SNAP-25 homologues, a typical transmembrane domain is missing. However, unlike SNAP-25 and SNAP-23, SNAP-47 lacks a conserved stretch of cysteine residues in the linker region connecting the two SNARE motifs. In SNAP-25 and SNAP-23 these cysteines are palmitoylated to allow for stable membrane association of the proteins, and they are characterized by flanking hydrophobic and basic residues, respectively (31). In agreement with this observation, hydroxylamine treatment, which is known to remove esterified palmitate residues (32), did not induce any noticeable change in the apparent molecular mass (data not shown), suggesting that SNAP-47, similar to its closest relative SNAP-29, is not palmitoylated. Furthermore, SNAP-47 is not cleaved by botulinum neurotoxins (BoNT) A or E light chains, two bacterial proteases that are known to cleave SNAP-25 but not SNAP-29 (33) (data not shown).

SNAP-47 Exhibits a Widespread Tissue and Subcellular Localization—Unlike SNAP-25, whose expression is limited to neurons and neuroendocrine cells and tissues, SNAP-23 and SNAP-29 are expressed in a wide variety of tissues (8, 9). To learn about the expression pattern

of SNAP-47, we investigated SNAP-47 mRNA and protein distribution in a variety of mouse tissues.

In Northern blot analysis, SNAP-47 mRNA was always detected as a single transcript of ~2 kb, suggesting that it is not alternatively spliced. The signal was particularly strong in brain, followed in abundance by liver, kidney, heart, and testis. Low levels of mRNA were detectable in the lung, skeletal muscle, and spleen (Fig. 2*A*). Since the transcript was comparatively highly expressed in brain, we performed *in situ* hybridizations on sagittal mouse brain sections (Fig. 2*B*). SNAP-47 mRNA was broadly distributed throughout all brain regions, with particularly strong signals obtained in the cortex, the principal cell layers of the hippocampus (CA layers and dentate gyrus), and the cerebellar granule cell layer. Strong expression was also observed in the olfactory bulb, the thalamus, and the brain stem, with relatively weaker expression in the striatum.

For the characterization of SNAP-47 protein distribution, we generated polyclonal antibodies to SNAP-47, using as antigens either a recombinant fragment lacking part of the N terminus (ΔN-SNAP-47) or a peptide corresponding to a stretch in the linker region between the two SNARE motifs. All antibodies were affinity-purified against recombinant ΔN-SNAP-47. The antibodies recognized a major band with an approximate M_r of 47,000 (Figs. 3–4 and supplemental Fig. 1). No significant difference in labeling pattern was found between these antibodies. We next assayed the tissue distribution of SNAP-47 by immunoblotting. The protein was found to be prominently expressed in brain, liver, and kidney (Fig. 3*A*). Whereas the signal was weak on the Northern blot, SNAP-47 was clearly detectable in tissues such as lung and spleen. Thus, SNAP-47 appears to be ubiquitously expressed, since slight discrepancies between the signal strength in the Northern blot (standardized to actin content) and Western blot (standardized to total protein) are expected. In the brain, the protein was ubiquitously expressed, with strongest expression in the glomerular layer of the olfactory bulb, the cortex, striatum, hippocampus, and colliculi, as shown by immunoautoradiography and Western blot (Fig. 3, *B* and *C*). Furthermore, we investigated the expression levels of SNAP-47 during mouse brain development. SNAP-47 is detectable as early as embryonic day 10 (*E10*) and reaches maximal levels at embryonic day 18 (*E18*) (Fig. 3*D*) (*i.e.* well before synaptogenesis peaks and before presynaptic proteins reach maximal expression levels) (34, 35).

Since SNAP-47 was originally identified during a proteomic screen of purified synaptic vesicles, we investigated whether the protein copurifies with synaptic vesicles during subcellular fractionation. As shown in Fig. 4*A*, the distribution pattern of SNAP-47 resembled that of synaptobrevin 2 (also referred to as VAMP2), a known synaptic vesicle-resident R-SNARE that plays a role in neuronal exocytosis (4). The similarity was particularly evident in purification steps after lysis of synaptosomes (P2′) that enrich for small vesicles in three consecutive purification steps, including differential centrifugation (LP2), sucrose gradient centrifugation, and finally size exclusion chromatography in which a fraction containing exclusively small vesicles (CPG) is separated from a fraction that contains additional larger membrane fragments (PK1; see also Ref. 36). Intriguingly, SNAP-29, the closest relative of SNAP-47, showed a very similar distribution. In contrast, markers of the endoplasmic reticulum, including protein-disulfide isomerase (Fig. 4*B*) and the SNAREs Use1 and Sec22 (data not shown), were reduced or absent in the CPG fraction, as was the *trans*-Golgi marker γ-adaptin. Furthermore, it is noteworthy that SNAP-47 was absent from the soluble fractions S3 and LS2, suggesting that the protein is exclusively membrane-bound. We conclude that SNAP-47 has a widespread intracellular distribution that includes synaptic vesicles but also other intracellular membrane pools.

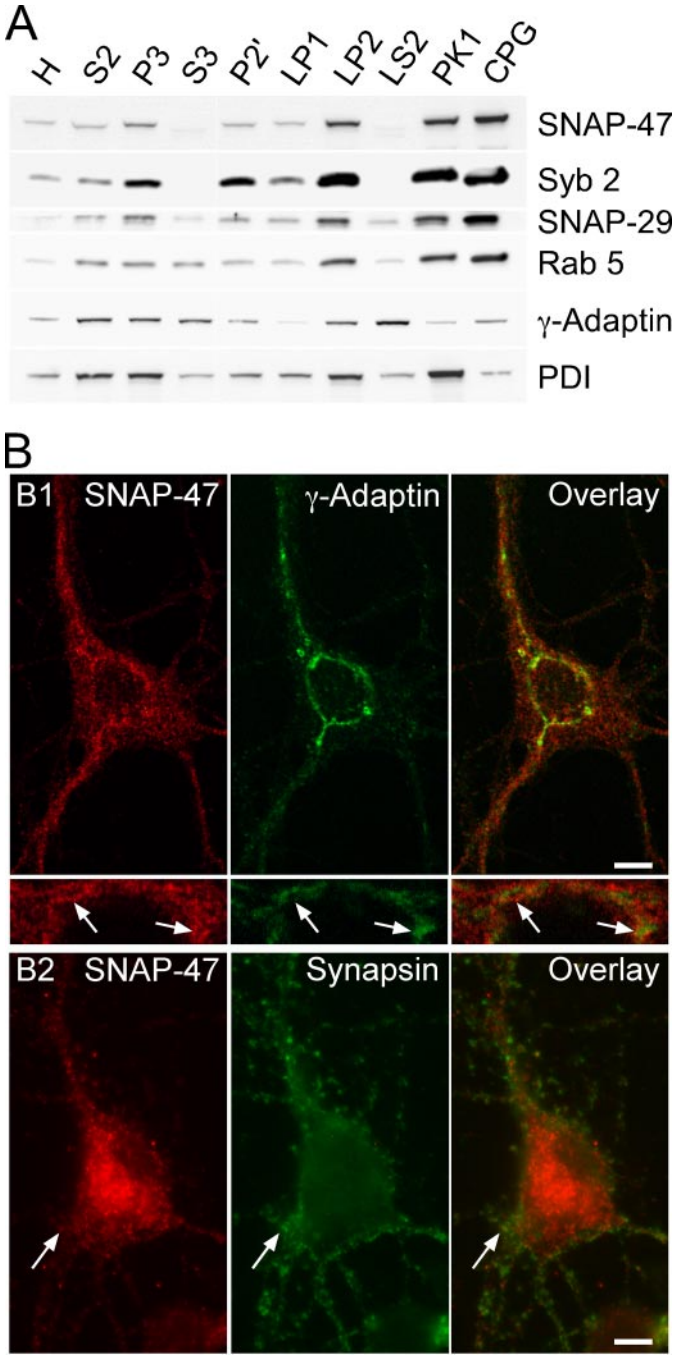

FIGURE 4. **Subcellular distribution of SNAP-47 in neurons.** *A*, fractions obtained during purification of synaptic vesicles were separated by SDS-PAGE and analyzed by immunoblotting. Fractions blotted represent increasing levels of purification (*left* to *right*) and include whole brain homogenate (*H*), 10,000 × *g* supernatant (*S2*), pellet (*P3*), and supernatant (*S3*) obtained after S2 centrifugation at 165,000 × *g*, washed synaptosomes (*P2′*), 25,000 × *g* pellet obtained after synaptosomal lysis (*LP1*), crude synaptic vesicles (*LP2*), first peak from the size exclusion column containing large membrane fractions (*PK1*), and purified synaptic vesicles (*CPG*). SNAP-47 copurifies with the synaptic vesicle protein synaptobrevin 2, with the highest enrichment in the synaptic vesicle (*CPG*) fraction. *B*, staining of mouse striatal neurons for SNAP-47 (Alexa 555; *red*) (*B1*, single-scan confocal image; *B2*, fluorescent micrograph) shows that it is widely expressed in both cell body and dendrites. SNAP-47 staining is punctate and enriched in the perinuclear and proximal dendritic areas. Double labeling with γ-adaptin, a protein of the *trans*-Golgi network (B1- Alexa 488; *green*) shows part of the SNAP-47 staining in close proximity to, but not overlapping with, the *trans*-Golgi network (*arrows* in the *middle panel* presenting a perinuclear area at higher magnification). Double labeling with synapsin (B2-Alexa 488; *green*) shows that SNAP-47 is not selectively localized to presynaptic nerve terminals, although colocalization was observed on rare occasions (*B2*, *arrows*). *Scale bars*, 25 μm in *B1* and 20 μm in *B2*.

Finally, we performed immunocytochemistry on primary neurons. SNAP-47 staining displayed a widespread punctate pattern in the cytoplasm, with prominent labeling of both neuronal cell bodies and dendrites (Fig. 4*B*). Interestingly, SNAP-47 immunoreactivity accumulated in the proximity of, but did not overlap with, the *trans*-Golgi network, visualized by staining for γ-adaptin (Fig. 4*B*, *B1*, *arrows* in the *higher magnification panel*). In contrast, SNAP-47-labeled *dots* only occasionally superimposed with nerve terminals (Fig. 4*B*, *B2*, *arrows*), suggesting that SNAP-47 is not selectively localized to presynaptic nerve terminals. Interestingly, the plasma membrane did not appear to be strongly labeled, which is in contrast to the localization of both SNAP-25 and SNAP-23 (37–39).

SNAP-47 Forms SNARE Complexes with Neuronal SNAREs and Catalyzes Fusion of Proteoliposomes—As outlined in the Introduction, membrane fusion reactions in the secretory pathway are dependent on the formation of SNARE complexes (also referred to as "core-complexes"). The best characterized set of SNAREs are those functioning in synaptic exocytosis: syntaxin 1 (Qa), synaptobrevin 2 (R), and SNAP-25 (Qbc) (4). Furthermore, our group and others have previously shown that in core-complex formation, SNAREs can substitute for each other, at least to a certain extent, as long as SNAREs belonging to the same subclass are exchanged (19, 40). Therefore, we asked whether SNAP-47 is capable of substituting for SNAP-25 in core-complex formation. Complex formation was then monitored by native polyacrylamide gel electrophoresis. Under these conditions, core-complexes do not dissociate and often can be separated from the unreacted SNAREs (18). As shown in Fig. 5*A*, a new protein band appeared when SNAP-47 was mixed with syntaxin 1a and synaptobrevin 2 (*arrowhead*), which was clearly separated from each of the single proteins. Furthermore, on SDS-PAGE, the ternary complex containing SNAP-47, syntaxin 1a, and synapaptobrevin 2 was partially SDS-resistant (Fig. 5*B*). This complex is reminiscent of the synaptic core-complex.

Thermal denaturation experiments have previously shown that SNARE complexes have unfolding transitions at temperatures well above 70 °C (41), and this thermal stability is considered one of the hallmarks of core-complexes. Due to the disruption of the α-helical structure, unfolding can be conveniently measured by CD spectroscopy. As shown in Fig. 6*A*, the purified ternary SNARE complex containing ΔN-SNAP-47 showed a thermal stability virtually identical to the synaptic complex, exhibiting a melt temperature of ~85 °C, as well as a similarly steep cooperative transition from the folded to unfolded states.

Considering that the SNAP-25 and SNAP-47 core-complexes are of similar stability, we next compared the kinetics of complex assembly. In order to monitor assembly kinetics, we measured anisotropy changes of Oregon Green-labeled syntaxin. Upon simultaneous mixing of all proteins, core-complex assembly with SNAP-25 was rapid and saturated after ~30 min (Fig. 6, *B* and *C*). In comparison, the rate of SNAP-47-mediated assembly was slower and did not reach saturation even after 2 h (Fig. 6, *B* and *C*).

In the final set of experiments, we investigated whether SNAP-47 is able to substitute for SNAP-25 in a liposome fusion assay. Proteoliposomes reconstituted with complementary sets of SNAREs spontaneously fuse with each other, thus providing a minimal system for testing SNARE function in membrane fusion (28). Syntaxin 1 and synaptobrevin were incorporated into two separate liposome populations. To monitor fusion, we used a standard lipid dequenching assay that results in an increase of donor fluorescence when the labeled membrane lipids are diluted in the membrane of the unlabeled acceptor liposome (see

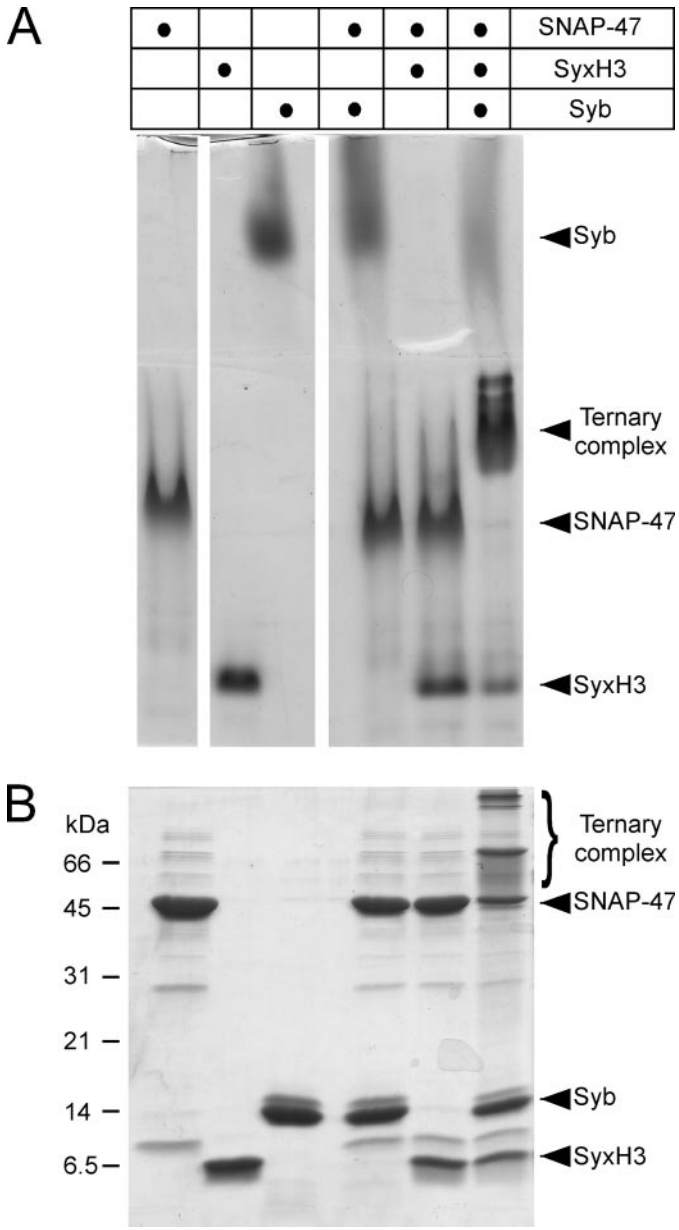

FIGURE 5. **SNAP-47 forms a ternary SNARE complex with syntaxin 1a and synaptobrevin 2.** *A*, SNAP-47 can form a complex (*arrowhead*) with the synaptic SNAREs syntaxin 1a (SyxH3) and Syb, as monitored by non-denaturing electrophoresis (*top*). No stable binary complex between SNAP-47 and either SyxH3 or Syb alone was detected. *B*, SDS-PAGE showing that SNAP-47, SyxH3, and Syb formed a partially SDS-resistant ternary complex. Note that the purified SNAP-47 contained some additional bands.

"Experimental Procedures"). Two observations prove that the reaction was SNARE-dependent. First, as previously reported, fusion of these liposomes depended on the presence of soluble SNAP-25 (Fig. 6*D*). Second, the reaction was dependent on SNARE proteins, with fusion being abolished after cleavage of synaptobrevin using BoNT/D light chain (29). When equimolar amounts of SNAP-47 were substituted for SNAP-25, fusion was observed, but initial observations suggest that over the time-scale used, both the rate and extent of fusion is lower than with SNAP-25 alone. SNAP-47 may also be able to compete with SNAP-25 in the fusion reaction, since mixing of the two SNAREs resulted in slower fusion kinetics than with SNAP-25 alone. Together, these results document that SNAP-47 does indeed function as a Qbc-SNARE both in the *in vitro* formation of SNARE complexes and in liposome fusion experiments.

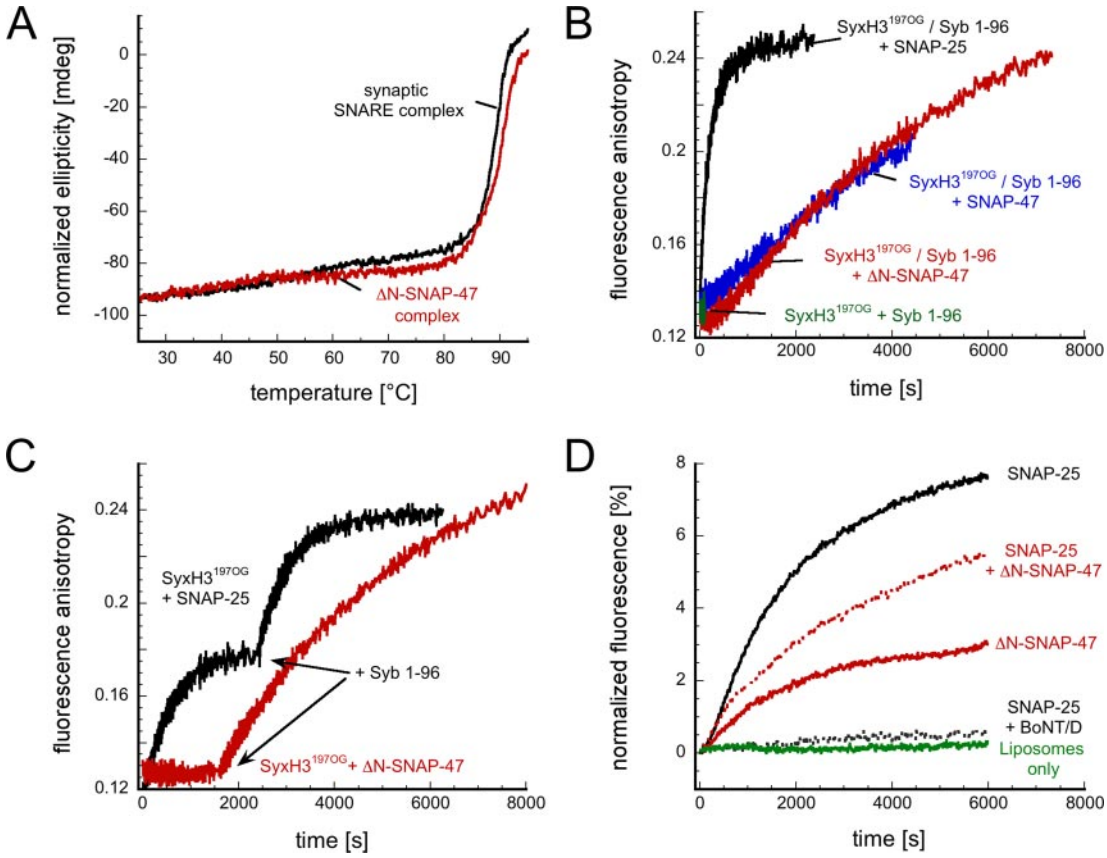

FIGURE 6. **SNAP-47 functions as a Qbc-SNARE that is able to substitute for SNAP-25.** *A*, the thermal stabilities of the SNAP-25- and ΔN-SNAP-47-containing core-complexes are essentially identical as assessed by CD spectroscopy. Both complexes unfolded at ~85 °C with similarly steep cooperative transitions. *B*, ternary SNARE complex formation is slower when SNAP-47 is used compared with SNAP-25. An increase in fluorescence anisotropy was seen upon assembly of SyxH3 labeled with Oregon Green at amino acid 197 (SyxH3^{197OG}) with synaptobrevin and either SNAP-47 or SNAP-25. No difference in the assembly rate was observed between full-length SNAP-47 and a version that lacked the N-terminal domain adjacent to the two SNARE helices, ΔN-SNAP-47. No increase in anisotropy was observed upon mixing of SyxH3^{197OG} and Syb. *B*, no increase in fluorescence anisotropy was observed when SyxH3^{197OG} and ΔN-SNAP-47 were mixed. After the addition of Syb, a similar slow increase occurs as in *B*. In contrast, mixing of SyxH3^{197OG} and SNAP-25 produced a clear change in anisotropy, which is due to the formation of a stable but unproductive 2:1 binary complex. The addition of Syb transformed the 2:1 complex into the ternary neuronal SNARE complex. *D*, ΔN-SNAP-47 can substitute for SNAP-25 in mediating liposome fusion. SyxH3 and Syb were incorporated into two separate liposome populations. Fusion was monitored by lipid dequenching. The reaction depended on the presence of SNAP-25 and was sensitive to BoNT/D, which cleaves Syb. When equimolar amounts of ΔN-SNAP-47 were substituted for SNAP-25, the rate and extent of fusion was lower than with SNAP-25.

DISCUSSION

In the present study, we have characterized a novel mammalian SNARE, named SNAP-47, that is related to the SNAP-25 subgroup of Qbc-SNAREs and that is ubiquitously expressed in many tissues. Initially, the protein was isolated from a fraction enriched in small vesicles, but it appears to have a widespread intracellular localization. SNAP-47 is capable of forming stable SNARE complexes with neuronal SNAREs *in vitro*, but assembly is slower, and the protein is not able to enter into stable binary complexes with syntaxin. Furthermore, SNAP-47 is capable of substituting for SNAP-25 in SNARE-driven fusion of liposomes, albeit with low efficiency.

In intracellular membrane trafficking, it is thought that each fusion step is mediated, at least to a large degree, by a specific SNARE complex containing one SNARE motif of each subfamily. However, it has been difficult to unequivocally assign a defined SNARE complex to each intracellular fusion step, because (i) some SNAREs participate in more than one complex (42), and (ii) SNAREs of the same subfamily are capable of substituting for each other *in vitro*, although this is often with limited efficiency (19, 40). A further complication arises from the fact that many SNAREs need to recycle by membrane trafficking after fusion to reach their original location, before being capable of engaging in another round of fusion (4). Thus, many SNAREs have a relatively widespread intracellular localization, and, therefore, localization alone does not suffice in determining during which fusion step a given SNARE is operating. Furthermore, SNAP-47 is unusual in that it does not appear to carry a membrane anchor domain, although more evidence is needed to safely exclude the presence of hydrophobic post-translational modifications. Thus, SNAP-47 resembles its closest relative SNAP-29, which also lacks membrane anchors (9). Despite the lack of a membrane anchor, SNAP-47 appears to be exclusively membrane-bound, with none detectable in the soluble fraction. Our results concerning the subcellular localization suggest that SNAP-47 is not confined to a single organelle. Immunocytochemistry revealed a widespread distribution in both neuronal cell bodies and neuronal processes. However, the nature of the immunoreactive structures could not be definitely identified, since the staining pattern was different from established markers for *trans*-Golgi network, endosomes, or synapses. Furthermore, the cofractionation pattern indicates that a pool of SNAP-47 resides on a population of small vesicles with a size and density reminiscent of synaptic vesicles, but the nature and origin of these vesicles remains to be established.

As a genuine SNARE, SNAP-47 is likely to participate in SNARE complex formation during intracellular fusion reactions. The fusion step(s), however, still need to be identified. Although SNAP-47 can form SNARE complexes with neuronal SNAREs, it is unlikely that the protein

plays a role in synaptic exocytosis, for the following reasons. First, as shown by our biochemical analysis and by liposome fusion experiments, SNAP-47 is a less effective partner for syntaxin 1a and synaptobrevin 2 than the cognate partner SNAP-25. Second, SNAP-47 does not appear to be enriched in the plasma membrane. This is in contrast to its two relatives SNAP-23 and SNAP-25, which are predominantly localized to the plasma membrane and are known to function in constitutive and regulated exocytosis. Indeed, plasma membrane association by palmitoylation has been shown to be essential for the function of SNAP-25 (43, 44). Third, overexpression of SNAP-47 is not capable of rescuing exocytosis in chromaffin cells derived from SNAP-25 knock-out mice.[5] Finally, SNAP-47 is expressed much earlier than synaptic proteins during brain development, displaying a pattern that is more compatible with a "housekeeping" role rather than with a specialized synaptic function. Thus, it is likely that (like its closest relative, SNAP-29) SNAP-47 participates in a basic intracellular fusion reaction that is common to all cells and that remains to be identified. It has been suggested that SNAP-29 acts as a "general utility" SNARE that is capable of providing Qb/Qc helices to a variety of SNARE complexes operating inside cells, and it is conceivable that SNAP-47 performs a similar function.

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