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# SNIP, a Novel SNAP-25-interacting Protein Implicated in Regulated Exocytosis*

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**Synaptosome-associated protein of 25 kDa (SNAP-25) is a presynaptic membrane protein that has been clearly implicated in membrane fusion in both developing and mature neurons, although its mechanisms of action are unclear. We have now identified a novel SNAP-25-interacting protein named SNIP. SNIP is a hydrophilic, 145-kDa protein that comprises two predicted coiled-coil domains, two highly charged regions, and two proline-rich domains with multiple PP*X*Y and P*XX*P motifs. SNIP is selectively expressed in brain where it co-distributes with SNAP-25 in most brain regions. Biochemical studies have revealed that SNIP is tightly associated with the brain cytoskeleton. Subcellular fractionation and immunofluorescence localization studies have demonstrated that SNIP co-localizes with SNAP-25 as well as the cortical actin cytoskeleton, suggesting that SNIP serves as a linker protein connecting SNAP-25 to the submembranous cytoskeleton. By using deletion analysis, we have mapped the binding domains of SNIP and SNAP-25, and we have demonstrated that the SNIP-SNAP-25 association is mediated via coiled-coil interactions. Moreover, we have shown that overexpression of SNIP or its SNAP-25-interacting domain inhibits $Ca^{2+}$-dependent exocytosis from PC12 cells. These results indicate that SNIP is involved in regulation of neurosecretion, perhaps via its interaction with SNAP-25 and the cytoskeleton.**

Membrane fusion is a fundamental process that is essential to cellular organization and function of all eukaryotic cells (1). In developing neurons, fusion of plasmalemmal precursor vesicles with the plasma membrane is thought to mediate membrane expansion at the growth cone (2). At mature nerve terminals, exocytotic fusion of synaptic vesicles with the plasma membrane releases neurotransmitters and initiates synaptic transmission (3, 4). Activity-dependent modulation of neurotransmitter release is an important mechanism underlying the processes of learning and memory, whereas dysregulated neurotransmitter release has been linked to several disorders of the nervous system such as depression and schizophrenia. Thus, elucidation of the molecular mechanisms that mediate and regulate neuronal exocytosis is crucial to our understanding of neuronal function and dysfunction as well as to the understanding of membrane trafficking in general.

Synaptosome-associated protein of 25 kDa (SNAP-25)[1] was identified 10 years ago as a brain-specific protein that is localized in the plasma membrane of nerve terminal (5). SNAP-25 has been implicated in neurotransmitter release as well as neurite outgrowth by the following studies. Specific proteolysis of SNAP-25 by botulinum neurotoxins A and E inhibits neurotransmitter release (6–9). The blockade of neurotransmitter release by botulinum neurotoxin E can be reversed using a C-terminal fragment of SNAP-25 (10). Furthermore, introduction of antibodies or synthetic peptides specific to SNAP-25 into synaptosomes or PC12 cells blocks $Ca^{2+}$-dependent exocytosis (11, 12). In *Drosophila*, SNAP-25 null mutation results in a complete loss of the evoked release, whereas the spontaneous release is unaffected, implicating a role for SNAP-25 in the stimulation-secretion coupling (13). In mice, coloboma (*Cm*/+) mutation that deletes a fragment of chromosome 2 encompassing the *SNAP-25* gene leads to impaired neurotransmitter release, profound spontaneous hyperactivity, and learning deficits (14–18). These functional impairments can be rescued by introducing the *SNAP-25* transgene into the coloboma mutant mice (15, 17). During development, interfering with SNAP-25 expression using antisense oligonucleotides or botulinum neurotoxins prevents neurite elongation (19, 20).

Despite the overwhelming evidence linking SNAP-25 to membrane fusion in both developing and mature neurons, it is not understood how SNAP-25 actually facilitates the fusion process. SNAP-25 (a t-SNARE) interacts with syntaxin (another t-SNARE) and synaptobrevin/VAMP (a v-SNARE) to form a ternary complex referred to as the core complex or the SNARE complex (21, 22). According to the SNARE hypothesis, the core complex formation between cognate v- and t-SNAREs underlies the specificity of membrane fusion (21–23). However, this view has been challenged by the localization studies demonstrating that the t-SNAREs SNAP-25 and syntaxin are not localized exclusively at the release sites of target membranes (24–27). Rather, they are distributed along the entire plasma membrane of axons (24, 25), and some of them are even localized on synaptic vesicles (26, 27). Furthermore, it has been shown that various v- and t-SNAREs can form stable ternary complexes rather promiscuously (28–30). Recent structural studies revealed that the core complex consists of a parallel four-

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF156981 and AF156982.*

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[1] The abbreviations used are: SNAP-25, synaptosome-associated protein of 25 kDa; SNIP, SNAP-25-interacting protein; SNAP, soluble *N*-ethylmaleimide-sensitive fusion attachment protein; SNARE, SNAP receptor; GST, glutathione *S*-transferase; GH, growth hormone; HA, hemagglutinin; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

stranded helical bundle formed by one helix each from syntaxin and synaptobrevin and two helices from SNAP-25 (31–34). The core complex is extremely stable and resistant to denaturation by SDS and high temperature (35, 36). It has been proposed that the energy released upon formation of such an unusually stable complex provides a driving force for membrane fusion (31, 37). Consistent with this view, *in vitro* studies have shown that assembly of the core complex from recombinant synaptobrevin, SNAP-25, and syntaxin reconstituted in liposomes leads to spontaneous mixing of lipid bilayer membranes (38). Most recently, it was reported that the core complex formation is triggered by $Ca^{2+}$ and coupled directly to the membrane fusion process in PC12 cells (10). However, evidence from studies in other fusion systems, namely the yeast vacuole fusion and the cortical vesicle fusion in sea urchin eggs, argues against such a direct association of the core complex with the fusion step (39–41). Despite these controversies, it is generally agreed that additional proteins and events are required to regulate and catalyze the fusion reaction (38–40).

To identify regulatory proteins that control the activity of SNAP-25 and to determine the molecular mechanisms by which SNAP-25 mediates membrane fusion, we performed a search in rat brain for proteins that interact with SNAP-25 using a yeast two-hybrid screen. We report here the isolation and characterization of a novel protein called SNIP (SNAP-25-interacting protein). SNIP is a brain-specific protein of about 145 kDa that specifically interacts with the N-terminal t-SNARE coiled-coil domain of SNAP-25. Our data suggest that SNIP may be involved in anchoring SNAP-25 to the membrane cytoskeleton and have a role in regulating neurotransmitter release.

## EXPERIMENTAL PROCEDURES

*Yeast Two-hybrid Screens and Interaction Assays*—The bait plasmid, pPC97-SNAP25, was constructed by subcloning the entire open reading frame of mouse SNAP-25b (5) into the pPC97 vector (42, 43). For the two-hybrid screen, the yeast strain CG-1945 (CLONTECH) was transformed sequentially with pPC97-SNAP25 and a rat hippocampal/cortical two-hybrid cDNA library (43), using the lithium acetate method (44). Positive clones were selected on 3-aminotriazole (5 mM, Sigma)-containing medium lacking leucine, tryptophan, and histidine and confirmed by a filter assay for β-galactosidase activity (45). Prey plasmids from positive clones were rescued and re-transformed into fresh yeast cells with the SNAP-25 bait or various control baits to confirm the specificity of the interaction. For analysis of SNIP-SNAP-25 interaction, deletion constructs of SNAP-25 were made by polymerase chain reaction and were subcloned into the pPC97 vector. The interactions of SNAP-25 deletion mutants with SNIP were tested in the yeast two-hybrid assay by using the *HIS3* and β-galactosidase as the reporter genes. Quantitative β-galactosidase assay was performed on the yeast extracts by using the substrate chlorophenol red β-D-galactopyranoside as described previously (46).

*cDNA Cloning*—For cloning of the full-length SNIP, a rat hippocampal cDNA library in λZAPII (Stratagene) was screened using a partial cDNA probe of SNIP from the prey clone C53, according to standard procedures (47). The cDNA inserts from positive SNIP clones were sequenced multiple times on both strands, using an Applied Biosystems 373A DNA sequencer.

*Antibodies*—An anti-SNIP antibody was raised in chicken against the synthetic peptide CPSRGSPVRQSFRKD, corresponding to amino acids 533–546 of SNIP. The N-terminal cysteine residue was added for the coupling purposes. The antibody was affinity purified using the immunogen peptide coupled to a SulfoLink column (Pierce). Other antibodies that were used in this study are as follows: anti-SNAP-25 (SMI 81, Sternberger Monoclonals, Inc.); anti-synaptophysin (clone SVP-38, Sigma) and anti-syntaxin 1 (HPC-1, Sigma); anti-actin (clone C4, Roche Molecular Biochemicals); anti-HA (HA.11, Berkeley Antibody Co.); and anti-FLAG (M5, Eastman Kodak Co.). The anti-synapsin antibody (G304) was a generous gift from A. Czernik (Rockefeller University).

*Northern and Western Blot Analyses*—Total RNAs were extracted from various rat tissues using the TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Poly(A)$^+$ mRNAs were isolated using the Oligotex mRNA purification system (Qiagen). Northern blot analysis was performed according to standard procedures (47), using a partial SNIP cDNA probe from the prey clone C53. For Western blot analysis, rat tissues were homogenized in 1% SDS and subjected to SDS-PAGE. The proteins were transferred onto nitrocellulose membranes and probed with the anti-SNIP and other antibodies. Antibody binding was detected by using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

*Interaction of SNIP Proteins with Endogenous SNAP-25*—The full-length SNIP-a and its fragments were subcloned in frame into the expression vector pGEX-5X-2 (Amersham Pharmacia Biotech). After transformation of these fusion constructs into *Escherichia coli* BL21 cells, synthesis of fusion proteins was induced with isopropyl β-D-thiogalactopyranoside (0.1 mM) during the mid-logarithmic phase of bacteria growth ($A_{600}$ of 0.6). After 4 h of induction, bacteria were harvested and lysed. GST-SNAP-25 fusion protein was purified by affinity chromatography using the glutathione-agarose beads (Sigma). For binding experiments, rat brain homogenates were prepared by homogenizing the brains in 4 mM HEPES-NaOH, pH 7.4, and 1 mM phenylmethylsulfonyl fluoride followed by addition of an equal volume of 2× solubilization buffer (0.2 M NaCl, 4 mM HEPES-NaOH, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100). After 4 h incubation at 4 °C under gentle rocking, insoluble material was removed by 30 min centrifugation at 120,000 × *g*. The brain homogenates were then incubated for 2 h at 4 °C under gentle rocking with various GST-SNIP fusion proteins immobilized on glutathione-agarose beads. After extensive washes with 1× solubilization buffer, the bound proteins were eluted by boiling in the Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting.

*Expression Constructs and Transfections*—Conventional molecular biological techniques (47) were used to generate the following expression constructs: pcDNA3.1-SNIP-a and pcDNA3.1-SNIP-b, which contain the full-length (including the 5′- and 3′-untranslated regions) SNIP-a and SNIP-b in the pcDNA3.1 vector, respectively; pCHA-SNIP-a, pCHA-SNIP-b, and pCHA-SNIP (C53), which direct the expression of N-terminal HA epitope-tagged, full-length SNIP-a, SNIP-b, and a SNIP fragment (residues 337–779), respectively; and pFLAG-SNIP-a and pFLAG-SNIP-b, which direct the expression of N-terminal FLAG epitope-tagged, full-length SNIP-a and SNIP-b, respectively. All recombinant sequences were determined to be free of polymerase chain reaction errors by DNA sequencing analysis. Transfections of HEK293 cells with various expression constructs were carried out using LipofectAMINE (Life Technologies, Inc.). Transfected cells were either harvested at 48 h post-transfection for Western blot analysis or selected with 0.5 mg/ml G418 (Geneticin, Life Technologies, Inc.) and grown until sufficient cells were obtained for subsequent subcellular fractionation experiments.

*Immunofluorescence Microscopy*—PC12 cells were grown on poly-L-lysine-coated glass coverslips and differentiated for 1–2 days with nerve growth factor (50 ng/ml). The cells were fixed with 4% paraformaldehyde and processed for indirect immunofluorescence microscopy as described previously (48, 49). The following antibodies were used: the chicken anti-SNIP antibody, a mouse anti-SNAP-25 antibody (SMI 81, Sternberger Monoclonals, Inc.), and secondary antibodies coupled with fluorescein or Texas Red (Jackson ImmunoResearch Labs, Inc.). Cells were stained simultaneously for F-actin with Texas Red-labeled phalloidin (Molecular Probes). Stained cells were analyzed by using a Leica TCS-NT confocal microscope, and the images were processed using Adobe Photoshop 5.0 (Adobe Systems, Inc.).

*Subcellular Fractionations*—Subcellular fractionations of rat brain or SNIP-transfected HEK293 cells into membrane and cytosol fractions were performed as described (50). Brain membranes were subjected to extraction studies as described (50, 51). The following extraction solutions were used: 1.5 M NaCl, 3 M KSCN, 1% CHAPS, 1% CHAPS with 1 M NaCl, 4% Triton X-100, 2% SDS, 2% *N*-lauroylsarcosine (Sarkosyl), 4 M urea, 8 M urea, or 0.1 M $Na_2CO_3$ at pH 11.5. For sucrose gradient flotation analysis, brain membranes were resuspended in 55% sucrose in gradient buffer (20 mM HEPES, pH 7.4, 150 mM NaCl and 1 mM dithiothreitol) in a volume of 0.4 ml. The resuspended membranes were placed at the bottom of an ultracentrifuge tube and overlaid with 4.8 ml of a linear 25–52.5% sucrose gradient. Flotation was carried out for 16 h at 165,000 × *g* in a Beckman SW50.1 rotor. Following centrifugation, the gradient was divided into 18 fractions and analyzed by Western blot analysis. For cytoskeleton association studies, rat brains or SNIP-transfected HEK293 cells were lysed in a cytoskeleton stabilizing buffer and separated into a low speed cytoskeleton fraction, a high speed cytoskeleton fraction, and a soluble fraction, according to the procedure of Stam *et al.* (52). The cytoskeleton fractions were further analyzed by

extraction studies using 1.5 M NaCl, 4% Triton X-100, 4 M urea, or 0.1 M $Na_2CO_3$ at pH 11.5 as described (52). For synaptosomal localization studies, rat brain homogenates were fractionated into crude synaptosome fractions according to the procedure of Huttner *et al.* (53). The washed crude synaptosome (P2′) pellet fraction was then subfractionated on a three-step Percoll gradient into myelin, mitochondria, and purified synaptosome fractions as described previously (54, 55). The purified synaptosome fraction (PG3) was further fractionated into the synaptosomal membranes (LP1), synaptic vesicle (LP2), and cytosol (LS2) fractions as described (53). All protein samples were subjected to SDS-PAGE and Western blot analysis.

*Co-transfection of PC12 Cells and Assays of GH Secretion*—Exponentially growing PC12 cells were harvested and resuspended at a density of $2 \times 10^7$ cells/ml in the Opti-MEM medium (Life Technologies, Inc.). The cell suspension (0.4 ml) was then co-transfected with 10 $\mu$g of pXGH5 (56) encoding human growth hormone and 30 $\mu$g of test plasmid by electroporation using a Bio-Rad Gene Pulser at 250 V and 960 microfarads in a 0.4-cm cuvette. Following electroporation, the cells were placed into the growth medium (46) and transferred to the collagen-coated 12-well dishes at a density of $0.8 \times 10^6$ cells/well. Release experiments were performed 48 h after electroporation. PC12 cells were washed with a physiological salt solution (PSS (in mM): 145 NaCl, 5.6 KCl, 2.2 $CaCl_2$, 0.5 $MgCl_2$, 10 glucose, 15 HEPES, pH 7.4). The cells were then incubated for 15 min at 37 °C in PSS, PSS containing 56 mM KCl and 95 mM NaCl, or PSS containing 300 $\mu$M ATP. The amounts of GH released into the medium and retained in the cells were determined by radioimmunoassay (Nichols Institute).

## RESULTS

*Identification and Cloning of SNIP*—To identify proteins that interact with SNAP-25, we screened a rat hippocampal/cortical cDNA library by yeast two-hybrid selection, using the full-length mouse SNAP-25b as bait. DNA sequencing analysis revealed that some of the positive clones encode known SNAP-25-interacting proteins such as syntaxin 1B and syntaxin 4 (data not shown), confirming the validity of the two-hybrid screen. One of the positive clones, clone 53, was shown to encode part of a new protein called SNIP (Fig. 1*A*). Re-transformation experiments confirmed that SNIP interacts specifically with SNAP-25 but not with irrelevant baits (data not shown). Since SNAP-25 shares similar structure and function with a ubiquitously expressed protein named SNAP-23/syndet (57–60), we tested in the yeast two-hybrid system whether SNIP also interacts with SNAP-23/syndet. The results revealed that, similar to other SNAP-25-binding proteins such as syntaxins and EHSH1/intersectin (57, 59, 61, 62), SNIP is able to bind SNAP-23/syndet (data not shown). Furthermore, like EHSH1/intersectin (62), SNIP is unable to bind a more distantly related t-SNARE, syntaxin 1 (data not shown). Deletion studies revealed that SNIP specifically interacts with the N- but not the C-terminal coiled-coil domain of SNAP-25 (Fig. 2). The N- and C-terminal coiled-coil domains of SNAP-25 and the most C-terminal coiled-coil domain of syntaxin 1, also referred to as the t-SNARE domains, are homologous to each other (63). The inability of SNIP to interact with syntaxin 1 as well as with the C-terminal coiled-coil domain of SNAP-25 further confirms the specificity of SNIP-SNAP-25 interaction.

cDNA cloning and sequencing analysis revealed that SNIP has at least two alternatively spliced isoforms referred to as SNIP-a and SNIP-b, which contain identical 5′ sequences and diverge in their 3′-oding sequences and 3′-untranslated regions (GenBank™ accession numbers AF156981 and AF156982). The open reading frame of SNIP-a and SNIP-b encodes a protein of 1173 and 1197 amino acids in length, with a calculated molecular mass of 127.1 and 129.7 kDa, respectively (Fig. 1). The sequence surrounding the initiator methionine codon of SNIP-a and SNIP-b conforms well to the translation initiation consensus sequence (64) and is preceded by an in-frame stop codon in the 5′-untranslated region. Furthermore, the coding sequences of SNIP-a and SNIP-b starting with this methionine initiator can be expressed in heterologous cells to yield recombinant proteins with apparent molecular weight similar to that of endogenous SNIP proteins in rat brain (Fig. 3*A*), confirming that the cloned SNIP sequences contain the entire coding region. SNIP-a and SNIP-b are highly hydrophilic and contain neither a signal sequence nor a potential transmembrane domain. Their calculated isoelectric point (pI value) is 9.39, with a high percentage (25%) of charged amino acids over the entire length, including two highly charged regions near the C terminus (Fig. 1). Both proteins contain two proline-rich regions with multiple PP*X*Y and P*XX*P motifs, some of which overlap (Fig. 1). The proline-containing motifs PP*X*Y and P*XX*P are involved in protein-protein interactions with the WW domain (65) and the SH3 domain (66), respectively. By using the algorithm of Lupas *et al.* (67), we identified two regions with high probability of forming a coiled-coil structure (Fig. 1). The coiled-coil feature of these regions was supported by their sequence similarity to the coiled-coil regions of various filamentous cytoskeleton-associated proteins such as myosin heavy chains and tropomyosin.

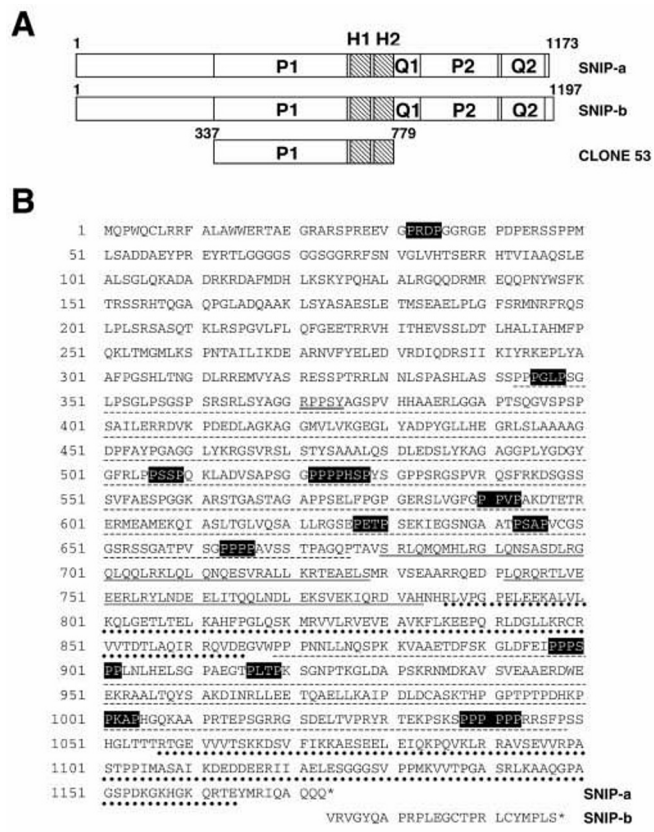


FIG. 1. **Structures of SNIP-a and SNIP-b.** *A,* domain structures of SNIP-a and SNIP-b. The following domains are indicated: P1 and P2, proline-rich domain 1 (residues 343–676, 15% proline content in 334 amino acids) and proline-rich domain 2 (residues 869–1048, 18% proline content in 180 amino acids); H1 and H2, predicted coiled-coil domain 1 (residues 680–728) and coiled-coil domain 2 (residues 742–783); Q1 and Q2, charged sequence 1 (residues 786–865, 35% charged amino acids in 80 amino acids) and charged sequence 2 (residues 1057–1164, 33% charged amino acids in 108 amino acids). The location of the SNAP-25-interacting clone isolated from the yeast two-hybrid screen is indicated *below* the domain structures. *B,* sequences of SNIP-a and SNIP-b. The nucleotide sequences of SNIP-a and SNIP-b (not shown) were deposited in GenBank™ with accession numbers AF156981 and AF156982. The deduced amino acid sequences of SNIP-a and SNIP-b are shown in *single-letter code* and are *numbered* on the *left*. In frame stop codons are denoted with *asterisks*. Indicated are the predicated coiled-coil domains (*underline*), the proline-rich domains (*dashed underline*), the charged sequence (*dotted line*), potential WW domain interaction motif (*double underline*), and SH3 domain interaction motifs (*black boxes*).

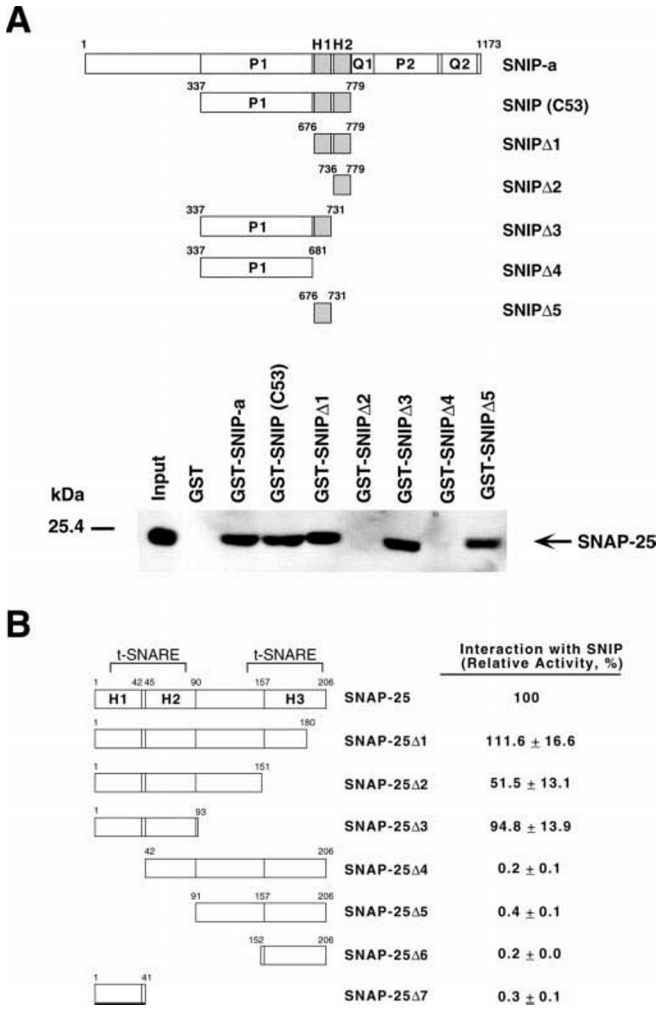


FIG. 2. **Biochemical characterization of the SNIP-SNAP-25 interaction.** *A,* binding of endogenous SNAP-25 to immobilized GST-SNIP fusion proteins. Schematic representation of SNIP proteins encoded by the GST-fusion cDNA constructs is shown on the *top*. Rat brain homogenate (*Input*) was incubated with GST or GST-SNIP fusion proteins immobilized on glutathione-agarose beads. After extensive washes, the bound proteins were eluted and analyzed by SDS-PAGE and immunoblotting using a monoclonal antibody against SNAP-25. *B,* identification of the SNIP-interacting domain of SNAP-25. A domain structure of full-length SNAP-25 (residues 1–206) is illustrated, including three predicted coiled-coil domains, H1 to H3 (69). The position of the recently described t-SNARE domain (63) is indicated at the *top* of the sequence representation. A series of SNAP-25 deletion mutants that were generated and tested in this study is shown diagrammatically on the *left*. The interactions of these SNAP-25 deletion mutants with SNIP were tested using a yeast two-hybrid assay. The β-galactosidase activity was determined using the substrate chlorophenol red β-D-galactopyranoside, normalized to the protein content, and expressed as a percentage of the activity of the full-length SNAP-25. Data are shown as mean ± S.E. of the results from triplicate determinations.

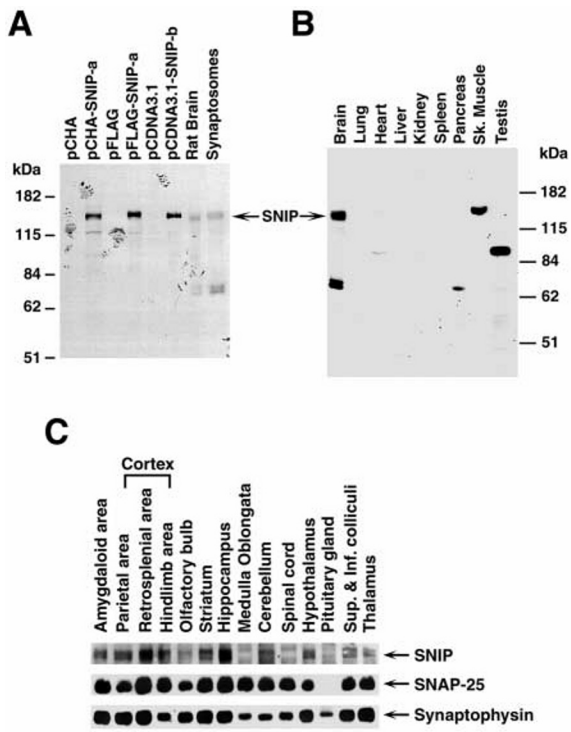


FIG. 3. **Expression and distribution of SNIP proteins.** *A,* specificity of the anti-SNIP antibody. Lysates of transfected HEK293 cells were analyzed along with homogenates from rat brain and crude synaptosomes by immunoblotting with the affinity-purified anti-SNIP antibody. A specific band of 145 kDa was detected in rat brain and crude synaptosomes as well as in HEK293 cells transfected with SNIP cDNAs but not in cells transfected with vector alone. *B,* Western blot analysis of SNIP expression in rat tissues. Equal amounts of homogenates (60 μg protein per lane) from the indicated rat tissues were analyzed by immunoblotting using the anti-SNIP antibody. *Sk.*, skeletal. *C,* regional distribution of SNIP proteins in rat brain, compared with SNAP-25 and synaptophysin. Equal amounts of homogenates (30 μg of protein per lane) from the indicated rat tissues were analyzed by SDS-PAGE and immunoblotting for SNIP, SNAP-25, and synaptophysin. *Sup.*, superior; *Inf.*, inferior.

Data base searches revealed that SNIP-a and SNIP-b do not share significant homology with known proteins. However, they are homologous to an unpublished mouse sequence P140 (GenBank™ accession number AF040944). Mouse P140 appears to be a different isoform of rat SNIP-a and SNIP-b, with a conserved central region and divergent N- and C-terminal sequences as well as different 5′- and 3′-untranslated regions. Furthermore, two putative *Drosophila* proteins (GenBank™ accession numbers AL030993 and AL031025) share significant sequence homology with SNIP, suggesting that the SNIP proteins are evolutionarily conserved. It is also interesting to note that the residues 714–966 of SNIP that includes H2, C1, and part of P2 exhibited weak homology (21% identity and 43% similarity) with the cytoskeletal membrane linker protein ezrin (68). Whether this homology has any functional relevance remains to be determined.

*Interaction of SNIP with SNAP-25*—To obtain independent evidence for the interaction between SNIP and SNAP-25, GST fusion proteins containing various portions of SNIP were immobilized on glutathione beads and used to affinity purify ("pull down") endogenous SNAP-25 from brain homogenates. As shown in Fig. 2*A*, the GST fusion proteins bearing the full-length SNIP-a or the SNIP fragment from the two-hybrid prey clone (C53) were able to bind endogenous SNAP-25. In contrast, control GST protein was unable to pull down SNAP-25 (Fig. 2*A*), confirming the specificity of the interaction. Moreover, control immunoblot with an anti-syntaxin 1 antibody revealed that the GST-SNIP fusion proteins could not pull down syntaxin (data not shown), indicating that SNIP specifically binds to SNAP-25 but not syntaxin 1 nor syntaxin 1-SNAP-25 complexes. To delineate the region of SNIP involved in binding SNAP-25, we generated the N- and C-terminal deletions of C53, SNIPΔ1 to SNIPΔ5 (Fig. 2*A*). SNAP-25 only bound to the fusion proteins that contain the predicted coiled-coil domain H1 (GST-SNIPΔ1, GST-SNIPΔ3, and GST-SNIPΔ5) but not to the fusion proteins lacking the H1 domain (GST-SNIPΔ2 and GST-SNIPΔ4). These data indicate that the SNAP-25-binding site of SNIP lies within the H1 domain, between amino acid residues 681 and 731.

To understand further the structural requirements that un-

derlie the interaction between SNIP and SNAP-25, we used deletion analysis to map the specific region of SNAP-25 responsible for interaction with SNIP. Based on the analysis using the algorithm of Lupas *et al.* (67), SNAP-25 contains three coiled-coil domains (Fig. 2*B*) (69). According to a more recent study using a very sensitive computer method called generalized profile technique, SNAP-25 has two t-SNARE coiled-coil homology domains (Fig. 2*B*) (63). The N- and C-terminal t-SNARE domains of SNAP-25 are homologous to each other and are thought to be derived from an internal duplication event during evolution (63). To define the SNIP-binding domain of SNAP-25, we made a series of SNAP-25 deletion mutants that were expressed in yeast as fusion proteins to the GAL4 DNA binding domain (Fig. 2*B*). The abilities of these SNAP-25 deletion mutants to bind SNIP were tested by using the yeast two-hybrid interaction assay (Fig. 2*B*). The results demonstrated that the fusion proteins containing the N-terminal t-SNARE domain (SNAP-25Δ1 to SNAP-25Δ3) were capable of interacting with SNIP. In contrast, the fusion proteins containing the C-terminal t-SANRE domain (SNAP-25Δ5 and SNAP-25Δ6) were unable to bind SNIP. Deletion of the N-terminal 41 amino acids (SNAP-25Δ4) abolished the ability of SNAP-25 to interact with SNIP, indicating that the N-terminal 41 amino acids of SNAP-25 are necessary for binding SNIP. However, the N-terminal 41 amino acids of SNAP-25 by itself (SNAP-25Δ7) is not sufficient for binding SNIP. These data, together with the results from deletion studies of SNIP, suggest that the association of SNIP with SNAP-25 is mediated through a coiled-coil interaction between the H1 domain of SNIP and the N-terminal t-SNARE domain of SNAP-25.

To test further whether SNIP interacts with SNAP-25 *in vivo*, we have attempted to co-immunoprecipitate the SNIP-SNAP-25 complex. Unfortunately, SNIP is exclusively and tightly associated with insoluble particulate fractions (data not shown, see Figs. 4 and 5). Neither high salt conditions, alkaline conditions, nonionic detergents, such as Triton X-100, nor zwitterionic detergents such as CHAPS (even in the combination with high salts) were able to solubilize SNIP from the particulate fractions of brain or of transfected cells (data not shown). Although SNIP could be partially solubilized by chaotropic reagents such as potassium thiocyanate and urea, and completely solubilized by the ionic detergents such as SDS and Sarkosyl, these denaturing conditions also dissociated the SNIP-SNAP-25 complex (data not shown). Due to these difficulties, we are currently unable to provide direct evidence for an *in vivo* SNIP-SNAP-25 interaction. However, the co-localization of SNIP and SNAP-25, as demonstrated by subcellular fractionation and immunofluorescence localization studies (Figs. 4–6), does suggest that a least a subset of these two proteins are localized together and have the chance to interact with each other *in vivo*.

*Distribution of SNIP Expression in Rat Tissues and Brain Region*—To analyze the expression of SNIP protein, a chicken anti-SNIP antibody was generated against a 14-amino acid peptide of SNIP. To characterize this antibody, HEK293 cells were transfected with the full-length SNIP cDNAs, some of which were tagged with a sequence encoding the HA or FLAG epitope at the 5′ end (Fig. 3*A*). Western blot analysis of cell lysates revealed that the chicken anti-SNIP antibody, but not the preimmune chicken IgY fraction (data not shown), specifically recognized a protein of approximately 145 kDa in the cells transfected with SNIP cDNAs but not in the cells transfected with the control vectors (Fig. 3*A*). The same 145-kDa band was also detected using the anti-HA or anti-FLAG antibody (data not shown). In rat brain as well as in crude synaptosomes, the anti-SNIP antibody recognized a triplet of proteins at 145 kDa

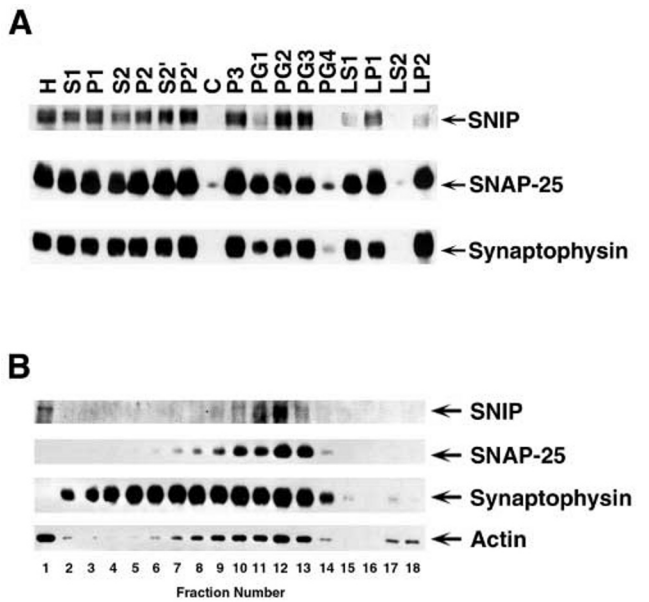


FIG. 4. **Subcellular localization of SNIP in rat brain.** *A,* co-distribution of SNIP with SNAP-25 in synaptosomal fractions. Rat brain homogenates were fractionated as described (53–55). The fractions are as follows: *H*, homogenate; *S1*, 800 × *g* supernatant; *P1*, 800 × *g* pellet; *S2*, 9,200 × *g* supernatant; *P2* (crude synaptosome fraction), 9,200 × *g* pellet; *S2′*, 10,200 × *g* supernatant; *P2′* (washed synaptosome fraction), 10,200 × *g* pellet; *C* (cytosolic fraction), 165,000 × *g* supernatant; *P3* (light membrane fraction), 165,000 × *g* pellet; *PG1*, myelin fraction; *PG2,* myelin-synaptosome fraction; *PG3*, purified synaptosome fraction; *PG4*, mitochondria fraction; *LS1*, 25,000 × *g* supernatant of lysed *PG3* synaptosomes; *LP1* (lysed synaptosomal membrane fraction), 25,000 × *g* pellet; *LS2* (cytosolic synaptosomal fraction), 165,000 × *g* supernatant; *LP2* (crude synaptic vesicle fraction), 165,000 × *g* pellet. Equal amounts of proteins from each fractions were analyzed by SDS-PAGE and immunoblotting for SNIP, SNAP-25, and synaptophysin. *B,* co-fractionation of SNIP with SNAP-25 and membrane skeletal actin on a sucrose gradient. Brain membranes were placed at the bottom of a sucrose gradient and subjected to a flotation analysis. The gradient was divided into 18 fractions, with Fraction 1 corresponding to the bottom of the gradient. Equal volumes of each fractions were analyzed by SDS-PAGE and immunoblotting for SNIP, SNAP-25, synaptophysin, and actin.

and a doublet of proteins at 70 kDa (Fig. 3*A*). The 145-kDa triplet may represent alternatively spliced SNIP isoforms, SNIP-a, SNIP-b, and P140, because it co-migrated with the full-length recombinant SNIP proteins. The 70-kDa doublet may result from proteolysis of the 145-kDa SNIP proteins since the relative intensity of the doublet as compared with the 145-kDa triplet varied from preparation to preparation. Preabsorption of the anti-SNIP antibody with the peptide immunogen or GST-SNIP (C53) fusion proteins completely eliminated its immunoreactivity to the recombinant as well as endogenous SNIP proteins (the 70- and 145-kDa bands) (data not shown), confirming the specificity of the antibody.

Western blot analysis of various rat tissues demonstrated that the 145-kDa SNIP is expressed exclusively in brain (Fig. 3*B*), which is consistent with the result of Northern blot analysis demonstrating the presence of a brain-specific SNIP transcript of 9.5 kilobase pairs (data not shown). In testis, the anti-SNIP antibody detected a 95-kDa protein (Fig. 3*B*), which is probably the product of the 4.5-kilobase pair testis-specific SNIP transcript (data not shown). In addition, the antibody also cross-reacted with a 160-kDa protein in skeletal muscle and a 72-kDa protein in pancreas (Fig. 3*B*). However, unlike the specific protein bands in brain and testis, the immunoreactivity to these protein bands in skeletal muscle and pancreas could not be completely eliminated using the antibody preabsorbed with the antigen (data not shown). This result suggests

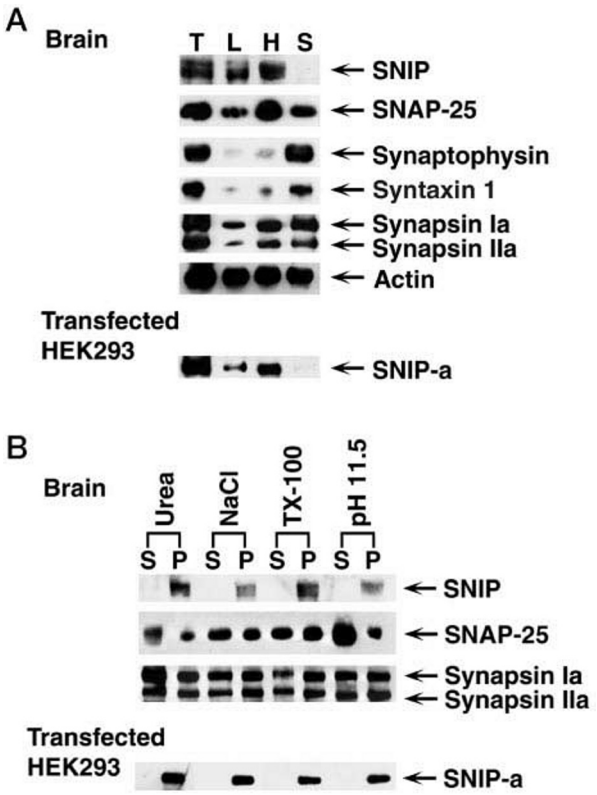


FIG. 5. **Co-fractionation of SNIP and SNAP-25 in cytoskeleton fractions.** *A,* total lysates (*T*) from rat brain or SNIP-a-transfected HEK293 cells were separated into a low speed cytoskeleton fraction (*L*), a high speed cytoskeleton fraction (*H*), and a soluble fraction (*S*). Aliquots representing an equal percentage of each fractions were analyzed by SDS-PAGE and immunoblotting for SNIP, SNAP-25, synaptophysin, syntaxin, synapsins, and actin. *B,* the high speed cytoskeleton fractions were extracted with 1.5 M NaCl, 4% Triton X-100 (*TX-100*), 4 M urea, or 0.1 M $Na_2CO_3$ at pH 11.5, and then separated into soluble (*S*) and pellet (*P*) fractions. Aliquots representing an equal percentage of each fractions were immunoblotted for SNIP.

that the 72- and 160-kDa immunoreactive bands are either nonspecific or they may represent the products of genes that share limited sequence homology with SNIP.

To characterize further the expression of SNIP in brain, various brain regions were dissected from adult rats and subjected to Western blot analysis using the anti-SNIP antibody (Fig. 3*C*). The results show that SNIP is relatively abundant in the telencephalon, including cerebral cortex, hippocampus, amygdaloid area, and striatum, and is expressed moderately in cerebellum, hypothalamus, thalamus, superior and inferior colliculi, and olfactory bulb. No SNIP proteins could be detected in medulla oblongata, spinal cord, and pituitary gland. Comparison of SNIP distribution pattern with that of SNAP-25 reveals that SNIP is co-expressed with SNAP-25 in most brain regions (Fig. 3*C*). The co-enrichment of SNIP and SNAP-25 proteins in the telencephalon suggests that these proteins and their interaction may have a role in the neuronal function and synaptic plasticity characteristic of this important brain structure.

*Co-distribution of SNIP and SNAP-25 in Synaptosomal Fractions*—To determine the subcellular localization of SNIP in brain, subcellular fractionation of rat brain homogenates was performed using the standard procedures (53–55) and analyzed by SDS-PAGE and Western blot analysis (Fig. 4*A*). No SNIP immunoreactivity was detected in the cytosolic fraction (fraction C), indicating that SNIP is not a soluble protein. SNIP was co-purified with synaptophysin and SNAP-25 in crude synaptosomes (fraction P2 or P2′) as well as in the light membrane fraction (P3) that contained considerable percentage of synaptic vesicles and plasma membranes (24). Subsequent fractionation of the washed crude synaptosome (P2′) pellet revealed an enrichment of SNIP proteins in synaptosome (fractions PG2 and PG3) relative to myelin (fraction PG1) and mitochondria (fraction PG4) fractions. To investigate the possible presence of SNIP proteins on synaptic vesicles, the purified synaptosome fraction (PG3) was further fractionated into the synaptic plasma membrane (LP1), synaptic vesicle (LP2), and cytosol (LS2) fractions. SNIP was detected only in the synaptosomal membrane fractions but not in the cytosolic synaptosomal fraction. Furthermore, SNIP was significantly deenriched in the synaptic vesicle fraction, in contrast to the enrichment of synaptic vesicle protein synaptophysin in this fraction. The distribution profile of SNIP was similar to that of SNAP-25 although SNAP-25 was more enriched in synaptic vesicle fraction due to the presence of a pool of this protein on synaptic vesicles (26, 27). Together, these data suggest that SNIP co-localizes with SNAP-25 on plasma membrane but not on synaptic vesicles. This view was further supported by the co-fractionation of SNIP and SNAP-25 in a sucrose gradient flotation analysis (Fig. 4*B*). A majority of SNIP floated up to the same region of the sucrose gradient as SNAP-25 and membrane skeletal actin, indicating that a large pool of SNIP is associated with either plasma membrane or membrane-associated cytoskeleton. The remaining SNIP stayed at the bottom of the gradient as a component of insoluble protein complexes, which may also contain cytoskeletal elements such as actin (Fig. 4*B*).

*Association of SNIP and SNAP-25 with the Cytoskeleton*—The insolubility of SNIP in various nondenaturing detergents such as Triton X-100 (data not shown) as well as the co-fractionation of SNIP with actin on sucrose gradient (Fig. 4*B*) suggested an association of SNIP with cytoskeletal elements. To confirm this association, rat brain was homogenized in a Triton X-100-containing cytoskeletion stabilizing buffer (52). The homogenates were then separated by differential centrifugation into a low speed cytoskeleton (15,000 × *g* pellet) fraction, a high speed cytoskeleton (200,000 × *g* pellet) fraction, and a soluble (200,000 × *g* supernatant) fraction (52). The low speed cytoskeleton fraction is known to contain large cytoskeletal structures, whereas the high speed cytoskeleton fraction contains submembranous cytoskeleton complexes (52). Western blot analysis (Fig. 5*A*) demonstrated that the integral membrane protein syntaxin 1 and synaptic vesicle membrane protein synaptophysin were predominantly localized to the soluble fraction, indicating that both membranes of synaptic vesicles and of the plasmalemma were adequately disrupted by Triton X-100. As expected, actin was distributed almost equally among the low speed cytoskeleton fraction, the high speed cytoskeleton fraction, and the soluble fraction, confirming the integrity of these fractions (70). Furthermore, the cytoskeleton fractions contained a substantial amount of synapsins, a family of presynaptic proteins known to interact with cytoskeletal elements (71). The association of synapsins with the cytoskeleton fractions confirmed that the fractionation procedure is capable of preserving cytoskeletal interactions. Western blot analysis using the anti-SNIP antibody revealed that SNIP is present in both the low speed and high speed cytoskeleton fractions and absent in the soluble fraction. Moreover, The SNIP in cytoskeleton fractions was resistant to extraction by 1.5 M NaCl, 4% Triton X-100, 4 M urea, or 0.1 M $NaHCO_3$ at pH 11.5 (Fig. 5*B*), indicating that SNIP is tightly associated with the cytoskeleton. Analysis of recombinant SNIP-a or SNIP-b expressed in transfected HEK293 cells using the same fraction and extraction protocols gave an identical result (Fig. 5; data not shown). These data suggest that SNIP is either a cytoskel-

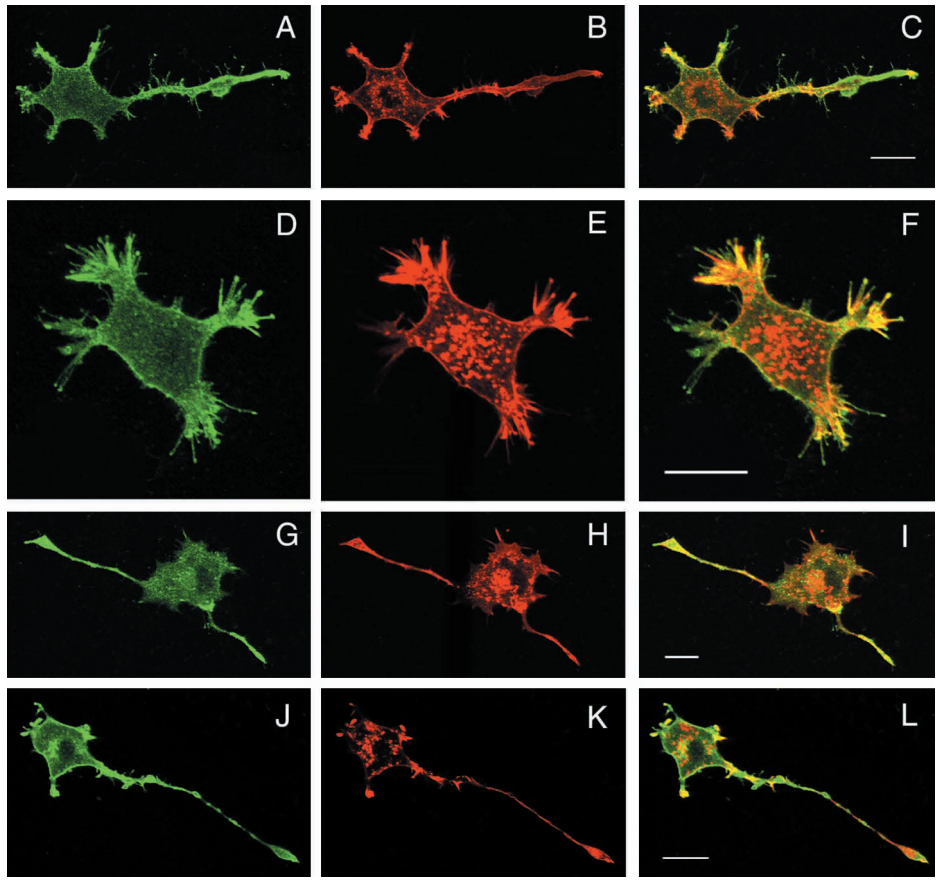


FIG. 6. **Co-localization of SNIP with SNAP-25 and F-actin in NGF-differentiated PC12 cells.** Distribution of endogenous SNIP (*A*, *D,* and *G*), SNAP-25 (*B* and *J*), and F-actin (*E*, *H,* and *K*) in differentiated PC12 cells is shown by double labeling in confocal images. Superimposed images (*C*, *F*, *I,* and *L*) demonstrate the overlapping distribution of these proteins. *Scale bar* = 20 $\mu$m.

etal protein or a membrane-cytoskeleton linker protein capable of interacting with the cytoskeletal elements in heterologous cells.

Interestingly, a substantial amount of SNAP-25 was found in the cytoskeleton fractions, indicating that a large pool of SNAP-25 is associated with the cytoskeleton (Fig. 5*A*). To our knowledge, such an association of SNAP-25 with the cytoskeleton has not been reported before, although the reports of SNAP-23 association with the cytoskeleton have recently appeared (60, 72). To investigate the nature of SNAP-25 association with cytoskeleton, brain cytoskeleton fractions were extracted with 1.5 M NaCl, 4% Triton X-100, 4 M urea, or 0.1 M $Na_2CO_3$ at pH 11.5 (Fig. 5*B*). In contrast to the membrane association of SNAP-25 that could not be dissociated by treatment with 0.1 M $NaHCO_3$ at pH 11.5 (data not shown) (73), the association of SNAP-25 with cytoskeleton could be disrupted by the high pH treatment (Fig. 5*B*). These results implicated an involvement of a hydrophilic protein-protein interaction in mediating the association of SNAP-25 with the cytoskeleton.

*Co-localization of SNIP with SNAP-25 and Actin Cytoskeleton in PC12 Cells*—To confirm further that the subcellular localization of SNIP is consistent with an *in vivo* association with SNAP-25, we used indirect immunofluorescence and confocal microscopy to analyze the distribution of SNIP in rat pheochromocytoma PC12 cells. PC12 is a well characterized neuroendocrine cell line that shares many characteristics of sympathetic neurons, such as secretion of neurotransmitters and the response to nerve growth factor (74). For immunofluorescence studies, PC12 cells were treated with NGF to induce the formation of neurites (49). Immunostaining of NGF-differentiated PC12 cells using the chicken anti-SNIP antibody revealed that SNIP immunoreactivity is enriched in filopodia, lamellipodia, neuritic processes, and the periphery of cell soma (Fig. 6, *A, D,* and *G*). In addition, some intracellular punctate staining was also observed. No staining was observed when the preimmune chicken IgY fraction was used or the anti-SNIP antibody was omitted (data not shown), confirming that the SNIP staining is specific. Double immunofluorescence analysis with an anti-SNAP-25 antibody demonstrated that the staining pattern of SNIP overlaps significantly with that of SNAP-25 (Fig. 6, *A–C*), which is consistent with the results of subcellular fractionation studies (Figs. 4 and 5). Since biochemical studies suggest SNIP and SNAP-25 are associated with cytoskeletal structure (Fig. 5), we sought to determine if these proteins are co-localized with the actin cytoskeleton. Double staining studies using phalloidin to label F-actin revealed that both SNIP and SNAP-25 appear to co-localize partially with the actin cytoskeleton, particularly in the filopodia, lamellipodia, and the neuritic extensions including the tips (Fig. 6, *D–L*).

*Role of SNIP in Regulated Secretion*—To determine whether SNIP is involved in $Ca^{2+}$-dependent exocytosis, we investigated the effect of overexpression of SNIP on regulated secretion from PC12 cells using a GH co-transfection secretion assay (75). This assay uses human GH expressed from the co-transfected vector as a reporter for regulated exocytosis and has been widely used for functional studies of presynaptic proteins (76–78). The expressed GH is stored in dense core vesicles of the transfected cells and undergoes $Ca^{2+}$-dependent exocytosis in response to a variety of stimuli including high $K^+$ and ATP (75, 79, 80). As shown in Fig. 7, overexpression of full-length SNIP-a resulted in a large decrease in the high $K^+$-induced GH release, whereas it had no effect on basal GH release. Similar extent of reduction in stimulated GH secretion was also observed when cells were treated with ATP (Fig. 7). These results suggest that SNIP is a negative regulator of $Ca^{2+}$-dependent exocytosis. Furthermore, overexpression of a SNIP fragment (amino acids 337–779) containing the SNAP-25-interacting domain (Fig. 2*A*) also led to a decrease in the high $K^+$-induced GH

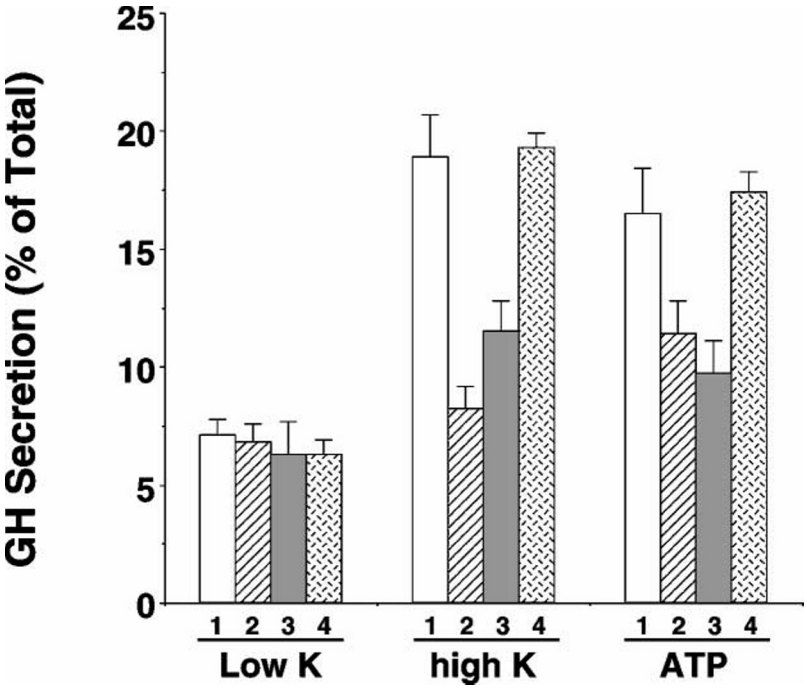


FIG. 7. **Effect of overexpression of SNIP and its fragments on $Ca^{2+}$-dependent secretion from PC12 cells.** PC12 cells were co-transfected with pXGH5 encoding human GH and a test plasmid as indicated below. *Bar 1*, pCHA; *bar 2*, pCHA-SNIP-a; *bar 3*, pCHA-SNIP (C53); and *bar 4*, pCHA-SNIPΔ4. GH secretion was induced with the low $K^+$ solution (5.6 mM KCl), the high $K^+$ solution (56 mM KCl), or ATP (300 μM), in the presence of extracellular $Ca^{2+}$ (2.2 mM). The extent of GH secretion is expressed as a percentage of total GH content. Data are mean ± S.E. (*error bar*) of the results from six independent determinations.

release as well as in the ATP-induced release without affecting the basal GH release (Fig. 7). In contrast, overexpression of SNIPΔ4, a SNIP fragment (amino acids 337–681) that cannot interact with SNAP-25 (Fig. 2*A*), did not have any significant effect on basal GH release nor on stimulated GH release induced either by high $K^+$ or ATP (Fig. 7). Together, these data suggest that SNIP interactions with SNAP-25 are involved in regulation of exocytosis.

## DISCUSSION

In this study, we have identified and characterized SNIP, a novel protein that interacts with SNAP-25. SNIP is a 145-kDa hydrophilic protein with an unusually high percentage of charged residues over the entire length, including two highly charged regions near the C terminus. In addition, SNIP contains two putative coiled-coil domains and two proline-rich regions with multiple PP*X*Y and P*XX*P motifs. Thus, SNIP could potentially interact with multiple proteins or be involved in the formation of multiprotein complexes via charge-charge interactions, coiled-coil interactions, and/or the interaction of its proline-rich motifs with the SH3 domain- or WW domain-containing proteins. The interaction of SNIP with SNAP-25 was demonstrated in the yeast two-hybrid system and confirmed by the GST fusion protein pull-down assays. Furthermore, we have mapped the minimal binding regions of SNIP and SNAP-25 and demonstrated that their interaction is likely to be mediated through a coiled-coil mechanism. Although due to the insolubility of SNIP in various nondenaturing detergents, we are presently unable to co-immunoprecipitate a SNIP-SNAP-25 complex, the following evidence supports a physiological significance of the observed interaction between SNIP and SNAP-25. 1) SNIP is specifically expressed in brain, with a regional distribution pattern similar to that of SNAP-25. 2) SNIP co-purified with SNAP-25 in synaptosomes, where it co-localized with SNAP-25 on synaptosomal membranes other than synaptic vesicles. 3) SNIP co-fractionated with SNAP-25 on sucrose gradient as well as in the cytoskeleton fractions. 4) Double immunofluorescence analysis demonstrated that the localization of SNIP and SNAP-25 overlaps in NGF-differentiated PC12 cells. 5) Overexpression of a SNAP-25-interacting domain of SNIP, but not a non-interacting SNIP fragment, had an inhibitory effect on regulated secretion from PC12 cells.

An interesting characteristic of SNIP is its extremely tight association with the brain cytoskeleton, as demonstrated by the following studies. SNIP was found to be exclusively associated with brain-insoluble particulate fractions and resistant to extraction by various nondenaturing detergents, high salt, and high pH solutions. Moreover, a majority of SNIP co-fractionated with membrane skeletal actin on sucrose gradient, suggesting that a large pool of SNIP is associated with membrane-associated cytoskeleton. Consistent with this notion, immunofluorescence studies of NGF-differentiated PC12 cells revealed that the localization of SNIP overlaps with the actin cytoskeleton in filopodia, lamellipodia, neuritic processes, and the cortex of cell soma. Furthermore, direct isolation of brain cytoskeleton demonstrated that SNIP is tightly associated with submembranous cytoskeleton complexes as well as with large cytoskeletal network structures. The strong association of SNIP with the brain cytoskeleton and the resistance to extraction by nondenaturing detergents and high salts are reminiscent of the presynaptic cytoskeleton-associated proteins Piccolo and Bassoon (51, 81). However, unlike these proteins, SNIP expression is not restricted to presynaptic terminals, but rather it exhibits a wider distribution pattern similar to SNAP-25 and syntaxin. SNIP is present both in synapses and outside of synapses, suggesting that it is a component of axonal membrane-associated cortical cytoskeleton instead of active zone-associated cytoskeleton (82).

In the course of characterization of the SNIP association with the cytoskeleton, we found that a large pool of SNAP-25 is associated with the cytoskeleton. In contrast, syntaxin 1, another neuronal t-SNARE, was present only in the detergent-soluble membrane fraction but not in the cytoskeleton fractions. These observations indicate that the SNAP-25 association with the cytoskeleton is a unique property of this t-SNARE. Although cytoskeletally associated SNAP-25 has not previously been reported, it has been recently shown that a substantial portion of SNAP-23, a ubiquitously expressed SNAP-25 homolog, is associated with the cytoskeleton in non-neuronal cells such as mast cells and 3T3-L1 adipocytes (60, 72). The association with the cytoskeleton is thought to func-

tion in sequestration of SNAP-23 in a reserve pool, and the relocation from this pool has been shown to regulate compound exocytosis in mast cells (60). By analogy, the SNAP-25 association with the cytoskeleton may play a similar role in neuronal exocytosis. The molecular mechanism that mediates the association of SNAP-25 or SNAP-23 with the cytoskeleton is not understood. Our results of the extraction studies have implicated an involvement of a hydrophilic protein-protein interaction in mediating the SNAP-25 association with the cytoskeleton. Since we have shown that SNIP is a cytoskeleton-associated hydrophilic protein that interacts and co-localizes with SNAP-25, it is likely that SNIP serves as a linker protein connecting SNAP-25 to the neuronal submembranous cytoskeleton.

The SNIP-SNAP-25 interaction may not only have a structure role to anchor SNAP-25 but may also have a functional role in regulated secretion. Consistent with this possibility, overexpression of full-length SNIP or its SNAP-25-interacting domain in PC12 cells leads to an inhibition of the $Ca^{2+}$-dependent exocytosis. Furthermore, we have demonstrated that the SNIP specifically interacts with the N-terminal coiled-coil domain of SNAP-25, a domain that also interacts with other key components of docking and fusion machinery, such as syntaxin, synaptobrevin, and $\alpha$-SNAP (35, 69, 83). Thus, the binding of SNIP to SNAP-25 is likely to interfere with the assembly as well as the disassembly of the SNARE complex. The molecular mechanism by which SNIP regulates $Ca^{2+}$-dependent exocytosis is unclear. One possible model is that SNIP keeps SNAP-25 in an inactive, cytoskeleton-associated state via its interaction with the N-terminal coiled-coil domain of SNAP-25. Phosphorylation or palmitoylation of SNAP-25 and/or of SNIP leads to a disruption of the SNIP-SNAP-25 interaction and thus dissociates SNAP-25 from the cytoskeleton. The dissociated SNAP-25 is in an active state, available to interact with other components of docking and fusion machinery to facilitate exocytosis. Future studies will test this model and determine at which stage of synaptic vesicle exocytosis the formation and/or dissociation of SNIP-SNAP-25 complex has a functional impact.

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