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Spring, a Novel RING Finger Protein That Regulates Synaptic Vesicle Exocytosis*

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The synaptosome-associated protein of 25 kDa (SNAP-25) interacts with syntaxin 1 and vesicle-associated membrane protein 2 (VAMP2) to form a ternary soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) complex that is essential for synaptic vesicle exocytosis. We report a novel RING finger protein, Spring, that specifically interacts with SNAP-25. Spring is exclusively expressed in brain and is concentrated at synapses. The association of Spring with SNAP-25 abolishes the ability of SNAP-25 to interact with syntaxin 1 and VAMP2 and prevents the assembly of the SNARE complex. Overexpression of Spring or its SNAP-25-interacting domain reduces Ca^{2+}-dependent exocytosis from PC12 cells. These results indicate that Spring may act as a regulator of synaptic vesicle exocytosis by controlling the availability of SNAP-25 for the SNARE complex formation.

At synapses, neurotransmitters are released via Ca^{2+}-triggered exocytotic fusion of synaptic vesicles with the presynaptic plasma membrane. Recent genetic and biochemical studies have revealed that this highly regulated fusion process involves a cascade of protein-protein and protein-lipid interactions (1, 2). Among them, a ternary protein complex known as the soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE)[1] complex is of fundamental importance for synaptic vesicle exocytosis (3). The SNARE complex is assembled by three neuronal SNAREs, vesicle-associated membrane protein 2 (VAMP2, also called synaptobrevin), and presynaptic plasma membrane proteins syntaxin 1 and synaptosome-associated protein of 25 kDa (SNAP-25). Structural studies have demonstrated that the SNARE complex consists of a parallel four-stranded helical bundle formed by two helices from SNAP-25 and one helix each from VAMP2 and syntaxin 1 (4). Interference with the integrity of such a superhelical structure by various mutations in the SNAREs has been shown to inhibit membrane fusion (5, 6). Moreover, specific cleavage of each SNARE by the clostridial neurotoxins prevents the assembly of a stable SNARE complex and blocks neurotransmitter release without affecting the docking of synaptic vesicles (7, 8). Thus, the formation of the SNARE complex is a crucial event in the synaptic vesicle fusion process.

Although the functional importance of the SNARE complex in membrane fusion is well established, its precise role in the fusion process remains unclear. It has been proposed that the formation of the SNARE complex in a *trans* configuration pulls the apposing membranes into close contact and provides a driving force for membrane fusion (9). Consistent with this view, the assembly of SNARE complexes has been shown to serve as the minimal machinery for membrane fusion in reconstituted liposomes (10). Furthermore, the SNARE complex formation in permeabilized PC12 cells is triggered by Ca^{2+} and coupled directly to exocytosis (11). On the other hand, evidence from studies of yeast vacuole fusion suggests that the SNARE complex does not act at the fusion step (12). Rather, the complex formation occurs at an upstream step to signal other proteins to execute fusion (13). In addition, a recent study using synaptosomes suggests that SNARE complexes assemble at the priming step prior to neurotransmitter release and may regulate the amount of synaptic vesicle to undergo exocytosis (14).

Whereas the SNAREs and the SNARE complex seem to be universally required for all fusion reactions, synaptic vesicle exocytosis is several orders of magnitude faster and more tightly regulated than any other form of membrane fusion (15). To achieve the extraordinary speed, precision, and plasticity of neurotransmission, additional proteins have to be involved to regulate the function of these SNAREs and control temporal and spatial formation of SNARE complexes. In an effort to identify additional proteins that regulate neurotransmitter release, we have performed a search in rat brain for SNAP-25-binding proteins using a yeast two-hybrid screen. We report here the isolation of a novel RING finger protein, termed Spring, that specifically interacts with SNAP-25 and modulates the SNARE complex formation and Ca^{2+}-dependent exocytosis.

EXPERIMENTAL PROCEDURES

Identification and cDNA Cloning of Spring—Yeast two-hybrid screens to identify novel SNAP-25-interacting proteins were performed as described previously (16). Prey plasmids from positive clones were rescued and re-transformed into fresh yeast cells with the pPC97-SNAP-25 bait or various control baits to confirm the specificity of the interactions. For cloning of full-length Spring, a partial Spring cDNA probe from the prey clone was used to screen a rat hippocampal cDNA library in λZAPII (Stratagene), according to standard procedures (17). The cDNA inserts from positive Spring clones were sequenced multiple times on both strands by an Applied Biosystems 373A DNA sequencer.

Antibodies—A polyclonal anti-Spring antibody was raised in rabbit against amino acid residues 138–151 (DDRGLRGFPKNRVL) of Spring.

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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) AF350422.

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[1] The abbreviations used are: SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; GH, growth hormone; GST, glutathione *S*-transferase; HA, hemagglutinin; SNAP-25, synaptosome-associated protein of 25 kDa; RBCC, RING-B box coiled-coil; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; BBC, B box C-terminal coiled-coil; VAMP2, vesicle-associated membrane protein 2.

The antibody was affinity-purified using the immunogen peptide coupled to an Aminolink Immobilization column (Pierce). Other antibodies used in this study are as follows: anti-SNAP-25 (SMI 81, Sternberger Monoclonals, Inc.); anti-syntaxin 1 (HPC-1, Sigma); anti-VAMP2 (Wako Pure Chemical Industries, Ltd.); anti-synaptophysin (SVP-38, Sigma); and anti-HA (3F10, Roche Molecular Biochemicals).

Northern and Western Blot Analyses—Northern blot analysis of Spring mRNA expression was performed on a rat Multiple Tissue Northern (MTN™) blot and a human Multiple Tissue Expression (MTE™) Array (CLONTECH), using a ^{32}P-labeled Spring cDNA fragment from the prey clone as probe (17). 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 anti-Spring and other antibodies. Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence were used to visualize the results.

Expression Constructs—Conventional molecular biological techniques (17) were used to generate the constructs in this study. DNA fragments encoding full-length and truncated forms of Spring were subcloned into the following vectors: the pPC97 and pPC86 vectors for yeast two-hybrid interaction studies; the prokaryotic expression vectors pGEX-5X-2 (Amersham Pharmacia Biotech) and pET28c (Novagen) for the production of GST- and His_6-tagged fusion proteins; and the mammalian expression vectors pCDNA3.1(+) (Invitrogen) and pCHA (16) for transfection into CHO and PC12 cells.

Protein Expression and Purification—GST or His_6-tagged fusion proteins were expressed in *Escherichia coli* BL21 cells as described previously (16). GST fusion proteins were affinity-purified by using the glutathione-agarose beads (Sigma). His_6-tagged proteins were purified using the His·Bind Resin and Buffer kit (Novagen). Protein concentrations were estimated by Coomassie Blue staining of protein bands following SDS-PAGE, using bovine serum albumin as standard.

Rat Brain GST Pull-down Assays—Rat brain extracts were prepared by homogenizing the brains in a homogenization buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 300 mM sucrose, plus protease inhibitors). Triton X-100 was added to the homogenates to a final concentration of 1% and incubated at 4 °C for 30 min. Insoluble material was removed by centrifugation at 100,000 × *g* for 1 h at 4 °C, and the supernatant was used as the Triton X-100 extract of rat brains. For binding experiments, brain extracts (100 μl) were incubated at 4 °C for 1 h with various GST-Spring fusion proteins immobilized on the glutathione-agarose beads. After extensive washes, bound proteins were eluted by boiling in the Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting.

In Vitro Binding Assays—For binding experiments (Figs. 4*A* and 5*A*), 100 nM GST or various GST-SNARE fusion proteins immobilized on glutathione-agarose beads were incubated with soluble His-Spring (50 nM) in the PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4 plus protease inhibitors) at 4 °C for 3 h under gentle rocking. Bound proteins were analyzed by SDS-PAGE and immunoblotting. For saturation experiments (Fig. 4*B*), 100 nM immobilized GST-SNAP-25 were incubated with increasing amounts of soluble His-Spring or His-syntaxin 1 (residues 1–261) in the PBS buffer and processed as above. The EC_{50} was defined as the effective concentration of each soluble protein at half-maximal binding. For competition experiments (Fig. 6, *A* and *B*), 50 nM immobilized GST-SNAP-25 were incubated with a constant amount of His-Spring (50 nM) and increasing amounts of His-syntaxin 1 or His-VAMP2. To study the effects of the association of Spring with SNAP-25 on the ability of SNAP-25 to interact with other SNAREs (Fig. 7, *A–C*), immobilized Spring-SNAP-25 binary complexes were assembled by incubation of 100 nM immobilized GST-Spring with His-SNAP-25 (200 nM). After extensive washes to remove unbound SNAP-25, the immobilized Spring-SNAP-25 complexes were incubated with increasing amounts of His-Syntaxin 1, His-VAMP2 or both His-Syntaxin 1 and His-VAMP2 (1:1 molar ratio) in the PBS buffer and processed as above. Control binding experiments were performed by incubation of immobilized GST-SNAP-25 (100 nM) with His-Syntaxin 1, His-VAMP2 or both His-Syntaxin 1 and His-VAMP2 (500 nM each).

Subcellular Fractionations—Subcellular fractionations of rat brain into membrane and cytosol fractions were performed as described (16). The membrane fractions were subjected to extraction by 4% Triton X-100, 4 M urea, 1.5 M NaCl, or 100 mM Na_2CO_3, pH 11.5. For cytoskeleton association studies, rat brains 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 a standard procedure (18). For synaptosomal localization studies, rat brain homogenates were fractionated into crude synaptosome fractions as described (19). The washed crude synaptosome (P2′) pellet fraction was then fractionated on a three-step Percoll gradient into myelin, mitochondria, and purified synaptosome fractions (20, 21). The purified synaptosome fraction (PG3) was further fractionated into the synaptosomal membranes (LP1), synaptic vesicle (LP2), and cytosol (LS2) fractions (19). All protein samples were subjected to SDS-PAGE and immunoblotting.

Coimmunoprecipitation—Extracts were prepared from CHO cells transiently transfected with pCHA-Spring and pCDNA3.1-SNAP-25, and immunoprecipitations were performed as described previously (22), using rat monoclonal anti-HA antibody (3F10) or control rat IgG. For detection of endogenous Spring-SNAP-25 complexes, the clarified supernatant of solubilized P2′ synaptosome fraction was subjected to immunoprecipitation by anti-SNAP-25 antibody (SMI81) or control mouse IgG. The immunocomplexes were recovered by incubation with protein A/G-agarose beads (Santa Cruz Biotechnology) for 1 h at 4 °C. After extensive washes, the immunocomplexes were analyzed by SDS-PAGE and immunoblotting.

Cotransfection of PC12 Cells and Assays of GH Secretion—Exponentially growing PC12 cells were harvested and then cotransfected with 5 μg of pXGH5 encoding human growth hormone and 30 μg of test plasmid as described previously (16). Measurements of GH secretion were performed 48 h after transfection. 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 or PSS containing 56 mM KCl and 95 mM NaCl. The amounts of GH released into the medium and retained in the cells were determined by using a radioimmunoassay kit (Nichols Institute).

RESULTS

Identification of Spring, a SNAP-25-Interacting RING Finger Protein—To identify novel proteins that regulate SNARE function in neuronal cells, we used the full-length mouse SNAP-25b as bait to screen a two-hybrid rat hippocampal/cortical cDNA library. One of the positive clones was shown to encode part of a novel protein that we referred to as Spring because it is a SNAP-25-interacting RING finger protein. Retransformation experiments confirmed that Spring interacts specifically with SNAP-25 but not with irrelevant baits such as synaptophysin nor with other neuronal SNAREs such as syntaxin 1 and VAMP2 (data not shown; see Fig. 4*A*). Moreover, our yeast two-hybrid interaction studies (not shown) and *in vitro* binding data (Fig. 4*A*) reveal that, unlike several other known SNAP-25-interacting proteins such as syntaxins, SNIP, and intersectin, Spring does not interact with SNAP-23/syndet, a ubiquitously expressed SNAP-25 homologue (23). Because the t-SNARE coiled-coil domains of SNAP-25 share significant homology with the t-SNARE domains in SNAP-23/syndet, syntaxin 1, and VAMP2 (24), the inability of Spring to interact with these other SNARE proteins further confirms the specificity of observed Spring-SNAP-25 interaction.

By screening a λZAPII rat hippocampal cDNA library, we isolated three full-length and nine independent overlapping partial Spring cDNA clones. The full-length Spring cDNA contains an in-frame stop codon upstream of the initiator ATG codon with a Kozak consensus sequence and a single open reading frame encoding 710 amino acids in length, with a calculated molecular mass of 79.2 kDa (Fig. 1*A*). Spring is a hydrophilic protein with a theoretical isoelectric point (pI) of 6.54 and a high percentage (22%) of charged amino acids over the entire length. It contains neither a signal sequence nor a potential transmembrane domain.

Analysis of Spring protein sequence reveals the presence of a RING finger domain followed by two B box motifs and a coiled-coil domain (Fig. 1), indicating that Spring is a new member of the RING-B box coiled-coil (RBCC) subfamily of RING finger proteins (25, 26). The RING finger and the B box motifs are cysteine/histidine-rich Zn^{2+}-binding domains that are thought to mediate protein-protein interactions (27). Although the function of the RBCC motif is unclear, this tripartite motif is found in a growing number of proteins involved in diverse cellular

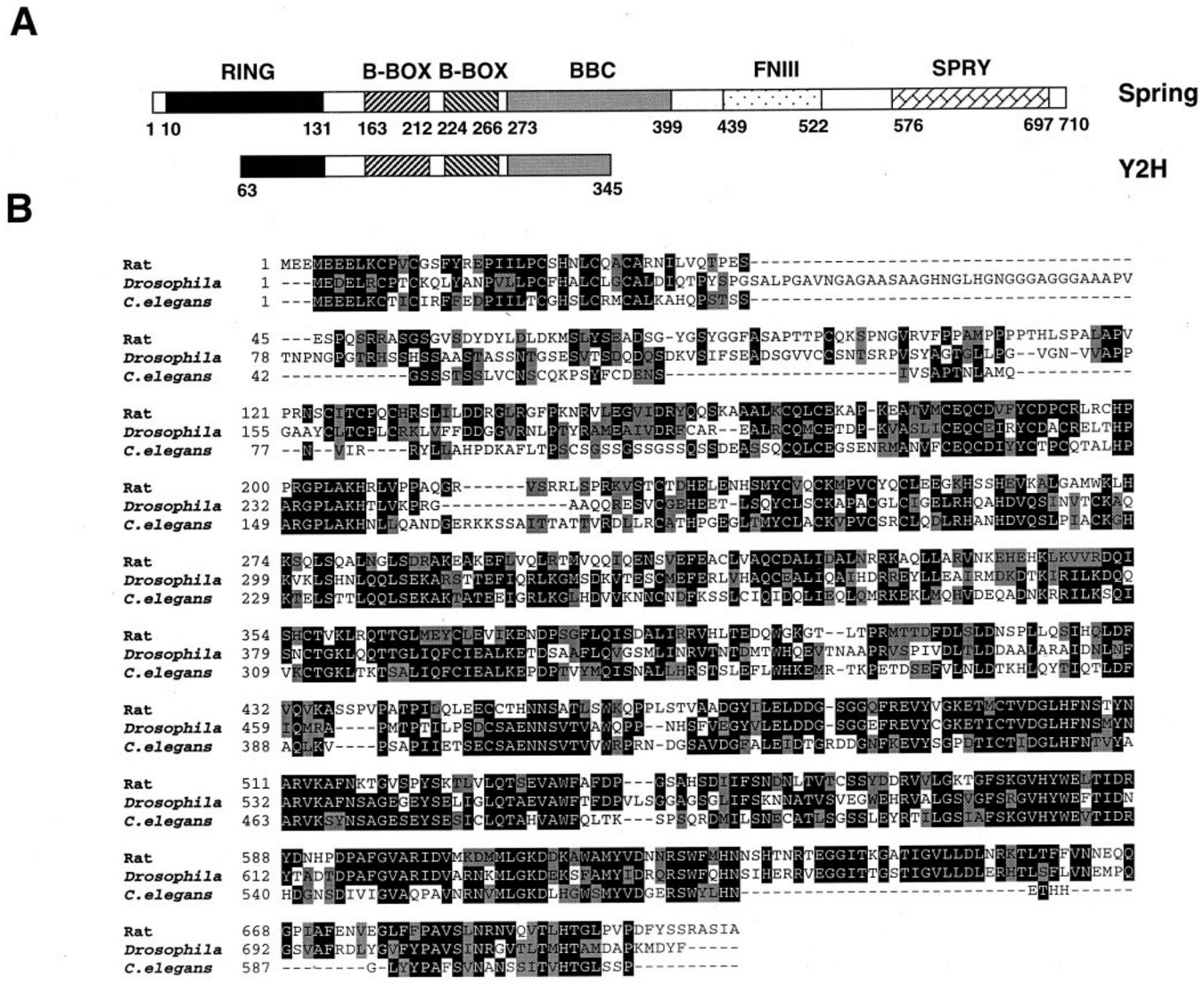
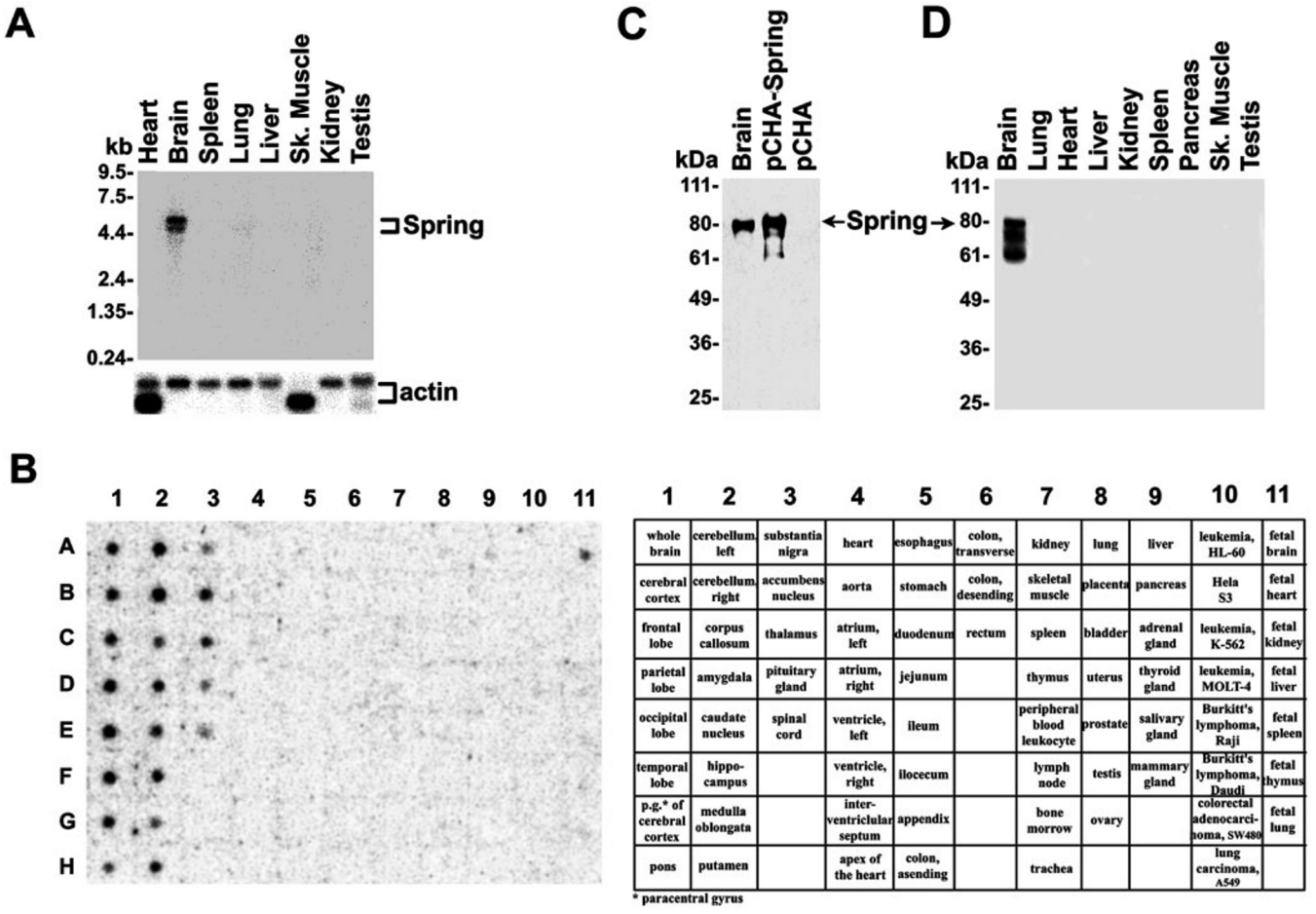


FIG. 1. **Structure of Spring.** *A,* domain structure of Spring. Rat Spring is a 710-amino acid protein that contains an N-terminal RBCC tripartite motif consisting of a RING finger, two B boxes, and a BBC domain, a fibronectin type III domain, and a C-terminal SPRY domain. *Numbers* indicate the amino acid residues that define the boundaries of these domains. The location of the SNAP-25-interacting clone isolated from the yeast two-hybrid screen (*Y2H*) is indicated *below* the domain structure. *B,* alignment of the rat Spring amino acid sequence with homologous proteins deduced from the genomic sequences of *Drosophila* and *C. elegans*.

processes, from gene transcription and signal transduction to organelle transport (25, 26, 28). It has been proposed that the RBCC motifs may act as molecular building blocks in formation of large macromolecular scaffolds for these complex biological processes (26). In addition to the RBCC motif, Spring contains a fibronectin type III domain, an autonomously folded protein module that is thought to mediate protein-protein interactions in both intracellular and extracellular compartments (29). At the C terminus, Spring has an SPRY domain, a putative protein-protein interaction module that was originally identified in the spla kinase and the ryanodine receptor (30). The SPRY domain has been found at the C terminus of several RBCC proteins with a conserved spacing between these two domains (31), although the significance of such a domain organization is not understood.

Data base searches revealed the presence of Spring homologues as uncharacterized cDNAs or open reading frames obtained from genome projects in a number of organisms, including human, mouse, *Drosophila*, *Caenorhabditis elegans*, and zebrafish. The amino acid sequence of rat Spring is 98% identical to a recently published human sequence TRIM9, one of the 37 RBCC tripartite motif-containing proteins identified by dbEST data base searches with a consensus of the B box domain (32). Moreover, the overall sequence of Spring protein shares 41 and 47% identity with *C. elegans* hypothetical protein C39F7.2 (GenBank™ accession number T33778 and AC006906) and a putative protein deduced from the genomic sequence of *Drosophila* (a splice product of CG13145 and CG6256 genes, GenBank™ accession number AE003629), respectively (Fig. 1*B*). The conspicuous homology and conserved domain structure among Spring homologues from different species indicate that Spring is an evolutionarily conserved protein.

Brain-specific Expression of Spring mRNA and Protein—Northern blot analysis of Spring mRNA expression revealed the presence of a major Spring transcript of 5.6 kilobase pairs and a minor form of 4.8 kilobase pairs, which may represent the products of alternative splicing or differential polyadenylation (Fig. 2*A*). Spring mRNAs were prominently expressed in rat brain but were undetectable in the other tissues examined. Consistent with this result, analysis of human Spring mRNA expression using a Multiple Tissue Expression Array showed that Spring mRNA(s) was exclusively expressed in fetal and adult human brain where it was widely distributed in all brain regions tested (Fig. 2*B*).



1	2	3	4	5	6	7	8	9	10	11
whole brain	cerebellum left	substantia nigra	heart	esophagus	colon, transverse	kidney	lung	liver	leukemia, HL-60	fetal brain
cerebral cortex	cerebellum right	accumbens nucleus	aorta	stomach	colon, desending	skeletal muscle	placenta	pancreas	Hela S3	fetal heart
frontal lobe	corpus callosum	thalamus	atrium, left	duodenum	rectum	spleen	bladder	adrenal gland	leukemia, K-562	fetal kidney
parietal lobe	amygdala	pituitary gland	atrium, right	jejunum		thymus	uterus	thyroid gland	leukemia, MOLT-4	fetal liver
occipital lobe	caudate nucleus	spinal cord	ventricle, left	ileum		peripheral blood leukocyte	prostate	salivary gland	Burkitt's lymphoma, Raji	fetal spleen
temporal lobe	hippo-campus		ventricle, right	ilocecum		lymph node	testis	mammary gland	Burkitt's lymphoma, Daudi	fetal thymus
p.g.* of cerebral cortex	medulla oblongata		inter-ventricular septum	appendix		bone morrow	ovary		colorectal adenocarci-noma, SW480	fetal lung
pons	putamen		apex of the heart	colon, asending		trachea			lung carcinoma, A549	

* paracentral gyrus

FIG. 2. **Expression and distribution of Spring mRNA and protein.** *A,* Northern blot analysis of Spring mRNA shows a high level of Spring mRNA expression in rat brain. The loading of poly(A)$^+$ RNA in each lane was confirmed by hybridization of the same blot with a ^{32}P-labeled β-actin cDNA probe (*lower panel*). *B,* a Multiple Tissue Expression Array (CLONTECH) containing poly(A)$^+$ RNA from various human tissues was hybridized with a ^{32}P-labeled Spring cDNA probe. The diagram of the RNA sources is shown on the *right. C,* specificity of the rabbit anti-Spring antibody. Homogenates of rat brain and transfected CHO cells were analyzed by immunoblotting using the affinity-purified anti-Spring antibody. *D,* Western blot analysis of Spring protein distribution. Equal amounts of homogenates (100 μg of protein per lane) from the indicated rat tissues were analyzed by immunoblotting using the anti-Spring antibody. Sk., skeletal.

To characterize Spring protein, a rabbit anti-Spring antibody was generated against a 14-amino acid peptide of Spring. The anti-Spring antibody, but not preimmune serum, specifically recognized an 80-kDa protein in cells transfected with HA-tagged, full-length Spring cDNA, whereas no immunoreactivity was detected in vector-transfected control cells (Fig. 2*C*). The same 80-kDa band was also detected using the anti-HA antibody (data not shown). In rat brain, the anti-Spring antibody recognized a major 80-kDa band of endogenous Spring (Fig. 2, *C* and *D*). Occasionally, some minor bands of lower molecular weights were observed in brain homogenates as well as in cells expressing recombinant Spring protein (Fig. 2, *C* and *D*). These minor bands are likely to be the degradation products of the Spring protein because their relative intensity as compared with the 80-kDa band varied from preparation to preparation. Pre-absorption of the anti-Spring antibody with recombinant Spring protein completely eliminated its immunoreactivity to both recombinant and endogenous Spring protein (data not shown), confirming the specificity of the antibody. Western blot analysis of multiple rat tissues showed that Spring was expressed exclusively in brain (Fig. 2*D*), which is consistent with the pattern of Spring mRNA expression (Fig. 2*A*).

Subcellular Distribution of Spring in Brain—To determine the intracellular distribution of Spring, rat brain postnuclear supernatant was separated into cytosol and membrane particulate fractions and then subjected to Western blot analysis with the anti-Spring antibody (Fig. 3*A*). Although the primary structure of Spring does not contain any transmembrane domain, a large pool of Spring was found in the membrane fraction. The membrane-associated Spring could be extracted by 1.5 M NaCl, 8 M urea, or a pH 11.5 solution, suggesting that it is peripherally associated with membranes. Surprisingly, the membrane-associated Spring was resistant to solubilization by 4% Triton X-100 (Fig. 3*A*), suggesting that it may be associated with cytoskeleton. To examine this possibility, we used a well established protocol to isolate directly the cytoskeleton fractions from brain (18). The integrity of these fractions was confirmed by immunoblotting with antibodies against actin, synaptophysin, and SNAP-25 (Fig. 3*B*). Immunoblot analysis of these fractions with the anti-Spring antibody revealed the presence of a substantial amount of Spring in the cytoskeleton fractions, indicating that a significant percentage of Spring is associated with brain cytoskeleton.

To examine the subcellular distribution of Spring in more detail, synaptosome fractions were isolated and further fractionated using standard procedures (19–21). Spring was found to copurify with synaptophysin and SNAP-25 in crude synaptosomes (fraction P2′) as well as in the light membrane fraction (P3) that contained a considerable percentage of synaptic vesicles and plasma membranes (Fig. 3*C*). Subsequent fractionation of crude synaptosomes (P2′) revealed that Spring was enriched in purified synaptosomes (PG3), whereas no Spring

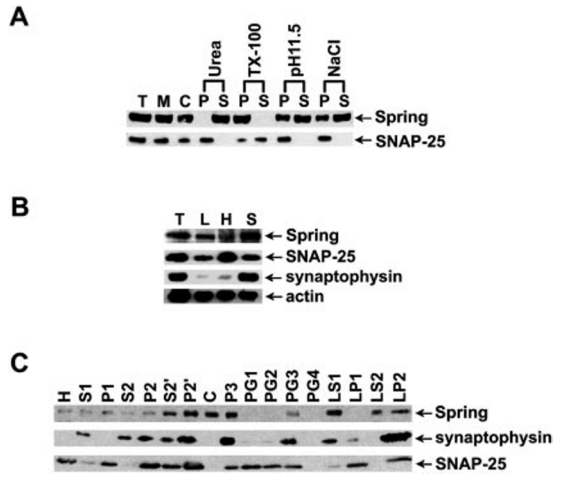


FIG. 3. **Subcellular distribution of Spring in brain.** *A,* postnuclear supernatant (*T*) from rat brain was separated into cytosol (*C*) and membrane (*M*) fractions. The membranes were then extracted with 1.5 M NaCl, 4% Triton X-100 (*TX-100*), 0.1 M Na_2CO_3, pH 11.5, or 4 M urea, and separated into soluble (*S*) and pellet (*P*) fractions. Aliquots representing an equal percentage of each fraction were analyzed by immunoblotting. *B,* total lysate (*T*) from rat brain was 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 fraction were analyzed by immunoblotting. *C,* rat brain homogenates were fractionated as described under "Experimental Procedures." The fractions are as follows: *H*, homogenate; *S1*, 800 × *g* supernatant; *P1*, 800 × *g* pellet; *S2*, 9,200 × *g* supernatant; *P2* (crude synaptosomes), 9,200 × *g* pellet; *S2′*, 10,200 × *g* supernatant; *P2′* (washed synaptosomes), 10,200 × *g* pellet; *C* (cytosol), 165,000 × *g* supernatant; *P3* (light membranes), 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* (synaptic plasma membranes), 25,000 × *g* pellet; *LS2* (cytosolic synaptosomal fraction), 165,000 × *g* supernatant; *LP2* (crude synaptic vesicles), 165,000 × *g* pellet. Equal amounts of proteins from each fraction were analyzed by immunoblotting.

immunoreactivity was detected in the myelin (PG1) and mitochondria (PG4) fractions. To characterize further the distribution of Spring in synaptosomes, the purified synaptosome fraction (PG3) was subfractionated into the synaptic plasma membrane (LP1), synaptic vesicle (LP2), and cytosol (LS2) fractions (Fig. 3*C*). As expected, synaptophysin was highly enriched in the synaptic vesicle fraction, whereas SNAP-25 was found primarily in the synaptic plasma membrane fraction and to a lesser extent in the synaptic vesicle fraction (33). Spring was co-enriched with synaptophysin in the synaptic vesicle fraction. In addition, Spring was present in the synaptosomal cytosol fraction but was absent in the synaptic plasma membrane fraction. Together, these data suggest that Spring is enriched at synaptic terminals where it exists in a soluble form and a synaptic vesicle-associated form.

Direct and Specific Association of Spring with SNAP-25—To determine whether the Spring-SNAP-25 interaction detected in yeast actually takes place *in vitro*, we performed *in vitro* binding assays using recombinant proteins. As shown in Fig. 4*A*, His-tagged Spring bound selectively to GST-SNAP-25 but not to GST alone or other SNARE proteins, such as SNAP-23/syndet, syntaxin 1, and VAMP2. These *in vitro* binding data demonstrate a direct and specific association between Spring and SNAP-25, which is consistent with the result of yeast two-hybrid interaction analysis. To further characterize biochemically the interaction between Spring and SNAP-25, a series of *in vitro* binding assays were performed by incubation of increasing concentrations of soluble Spring with immobilized GST-SNAP-25 (Fig. 4*B*). The results showed that Spring bound to GST-SNAP-25 in a dose-dependent and saturable manner, with an EC_{50} (the effective concentration at half-maximal binding) of approximate 20 nM Spring. As a control, we analyzed the *in vitro* binding of syntaxin 1 to GST-SNAP-25 in parallel experiments (data not shown). Under the same experimental conditions, syntaxin 1 bound to SNAP-25 with an EC_{50} of 400 nM syntaxin 1. Thus, Spring seems to bind SNAP-25 with higher apparent affinity than syntaxin 1. Furthermore, the complex formed between Spring and SNAP-25 has a stoichiometry of 1:1, as determined by scanning of Coomassie Blue-stained gels and by comparison with the titration curves of recombinant Spring and SNAP-25 (data not shown but see Fig. 7).

The strong interaction between Spring and SNAP-25 observed *in vitro* suggests that these two proteins may associate with each other *in vivo*. To examine this possibility, we first performed coimmunoprecipitation experiments using lysates of CHO cells cotransfected with SNAP-25 and HA-tagged Spring (Fig. 4*C*). Spring and SNAP-25 were coimmunoprecipitated by the anti-HA antibody but not by the IgG control, confirming a specific association of Spring with SNAP-25 in mammalian cells. We then performed additional coimmunoprecipitation experiments to examine the association of endogenous Spring and SNAP-25 in rat brain synaptosomes (Fig. 4*D*). Anti-SNAP-25 antibody, but not the mouse IgG control, was able to coimmunoprecipitate SNAP-25 and Spring from solubilized synaptosomes, indicating the existence of endogenous Spring-SNAP-25 complexes.

Identification of the Binding Domains of SNAP-25 and Spring—To understand the structural requirements that underlie the interaction between Spring and SNAP-25, we used deletion analysis to map the specific domains of SNAP-25 and Spring required for their association. A series of SNAP-25 deletion mutants were generated as GST fusion proteins and tested for their ability to bind recombinant Spring in the *in vitro* binding assays (Fig. 5*A*). The results demonstrated that only the fusion proteins containing the N-terminal t-SNARE domain (SNAP-25Δ1 to SNAP-25Δ3) were capable of binding Spring, whereas the C-terminal t-SNARE domain and the central domain of SNAP-25 were not required for the binding. The entire N-terminal t-SNARE domain (SNAP-25Δ3) was both necessary and sufficient for binding Spring, because further truncations of this domain (SNAP-25Δ7 or SNAP-25Δ8) abolished its ability to bind Spring.

To delineate the region of Spring involved in binding SNAP-25, we generated a series of GST fusion proteins containing various truncations of Spring, and we analyzed their interaction with endogenous SNAP-25 in rat brain extracts (Fig. 5*B*). The B box C-terminal coiled-coil (BBC) domain of Spring was found to be solely responsible for binding SNAP-25, whereas the RING finger, two B box domains, and the C-terminal fibronectin type III and SPRY domains were not involved. These results, together with those of Fig. 5*A*, demonstrate that the association of Spring with SNAP-25 is mediated by the BBC domain of Spring and the N-terminal t-SNARE domain of SNAP-25.

Spring Competes with Syntaxin 1 and VAMP2 for Binding to SNAP-25—Previous studies (34, 35) have shown that the N-terminal t-SNARE coiled-coil domain of SNAP-25 directly binds syntaxin 1, whereas both N- and C-terminal t-SNARE domains are required for binding VAMP2. Thus, the binding of Spring to the N-terminal t-SNARE domain of SNAP-25 may affect the binding of syntaxin 1 and/or VAMP2 to SNAP-25. To test these possibilities, we performed a series of *in vitro* binding experiments by incubation of immobilized GST-SNAP-25 with a constant amount of Spring and increasing amounts of syntaxin 1 (Fig. 6*A*). The results showed that as the concentration of syntaxin 1 increased, the binding of Spring to SNAP-25

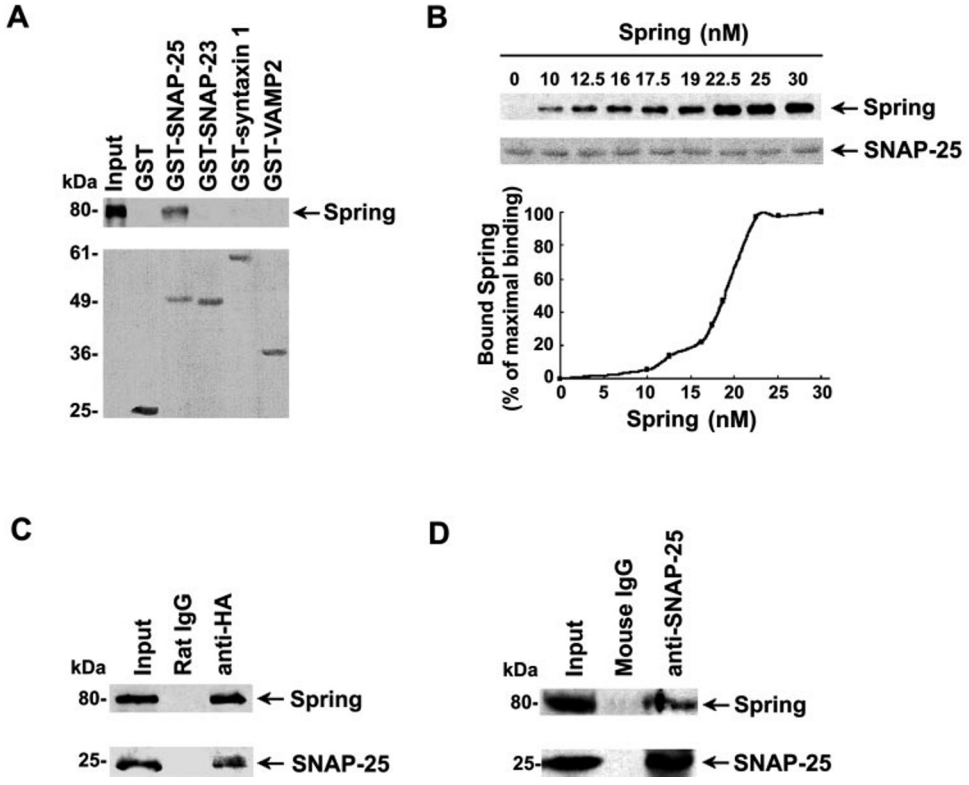


FIG. 4. **Specific interaction of Spring with SNAP-25.** *A,* soluble His-tagged Spring proteins (50 nM) were incubated with 100 nM immobilized GST or GST fusion proteins (*lower panel,* Ponceau S staining). Bound Spring was detected by immunoblotting (*upper panel*). *B,* immobilized GST-SNAP-25 fusion proteins (100 nM) were incubated with increasing concentrations of soluble Spring as indicated. Quantification of the amount of bound Spring demonstrates that Spring binds to GST-SNAP-25 in a dose-dependent and saturable manner. *C,* coimmunoprecipitation of Spring with SNAP-25 from transfected CHO cells. Extracts from CHO cells cotransfected with pCHA-Spring and pcDNA3.1-SNAP-25 were subjected to immunoprecipitation with anti-HA antibody (3F10) or control rat IgG. *D,* association of endogenous Spring with SNAP-25 in rat brain. Detergent extracts of rat synaptosomes (P2′ fractions) were subjected to immunoprecipitation with anti-SNAP-25 antibody or control mouse IgG. The immunoprecipitates in *C* and *D* were analyzed by immunoblotting for Spring and SNAP-25.

diminished gradually, indicating that Spring and syntaxin 1 compete with each other for binding to SNAP-25. Similarly, when immobilized GST-SNAP-25 was incubated with a constant amount of Spring and increasing amounts of VAMP2, Spring and VAMP2 were found to bind to SNAP-25 in a competitive manner (Fig. 6*B*).

Association of Spring with SNAP-25 Inhibits the Assembly of SNARE Complexes—Because the N- and C-terminal t-SNARE domains of SNAP-25 are directly involved in formation of binary and ternary SNARE complexes, the binding of Spring to the N-terminal t-SNARE domain of SNAP-25 is likely to interfere with assembly of these SANRE complexes. To examine the effect of the association of Spring with SNAP-25 on the assembly of the binary SNAP-25-syntaxin 1 complex, immobilized GST-Spring was preincubated with soluble SNAP-25 to form binary Spring-SNAP-25 complexes. After extensive washes to remove unbound SNAP-25, the ability of the immobilized binary Spring-SNAP-25 complexes to bind syntaxin 1 was tested in a series of *in vitro* binding reactions with increasing concentrations of syntaxin 1 (Fig. 7*A*). The results demonstrated that the association of Spring with SNAP-25 was able to prevent the interaction of SNAP-25 with syntaxin 1. Conversely, when immobilized binary SNAP-25-syntaxin 1 complexes were incubated with increasing concentrations of Spring, no detectable binding of Spring to the SNAP-25-syntaxin 1 complexes was observed (data not shown). Thus, the association of SNAP-25 with Spring and the association of SNAP-25 with syntaxin 1 are mutually exclusive.

We also performed a similar series of binding experiments to examine the relationship between the association of SNAP-25 with Spring and the interaction of SNAP-25 with VAMP2. The association of Spring with SNAP-25 was found to abolish the ability of SNAP-25 to interact with VAMP2 (Fig. 7*B*). To further test whether the Spring/SNAP-25 interaction interferes with the ability of SNAP-25 to form the ternary SNARE complex, *in vitro* binding experiments were carried out by incubation of immobilized Spring-SNAP-25 complexes with increasing concentrations of both syntaxin 1 and VAMP2 (Fig. 7*C*). The results demonstrated that the association of Spring with SNAP-25 completely prevented the assembly of the ternary SNARE complex.

Role of Spring in Ca^{2+}-dependent Exocytosis—To determine whether Spring is involved in Ca^{2+}-dependent exocytosis, we investigated the effect of overexpression of Spring and its fragments on the regulated secretion of growth hormone (GH) from PC12 cells using a GH cotransfection secretion assay (36). This assay uses human GH expressed from the cotransfected plasmid as a reporter for regulated exocytosis and has been widely used for functional studies of presynaptic proteins (37, 38). The expressed GH is stored in dense core vesicles of the transfected PC12 cells and undergoes Ca^{2+}-dependent exocytosis in response to depolarization by high K^+ (39). To examine the effect of Spring on GH secretion, various Spring cDNA constructs were cotransfected with the pXGH5 encoding human GH. As a positive control, the cytoplasmic region of syntaxin 1 (residues 1–261) was cotransfected in parallel experiments. Western blot analysis of cell lysates confirmed that exogenous Spring proteins and syntaxin 1 were expressed at similar levels in transfected cells (Fig. 8*A*). Analysis of GH secretion revealed that overexpression of full-length Spring or a truncated form (SpringΔ7) of Spring containing the SNAP-25-interacting domain (residues 267–408) resulted in a large decrease in the

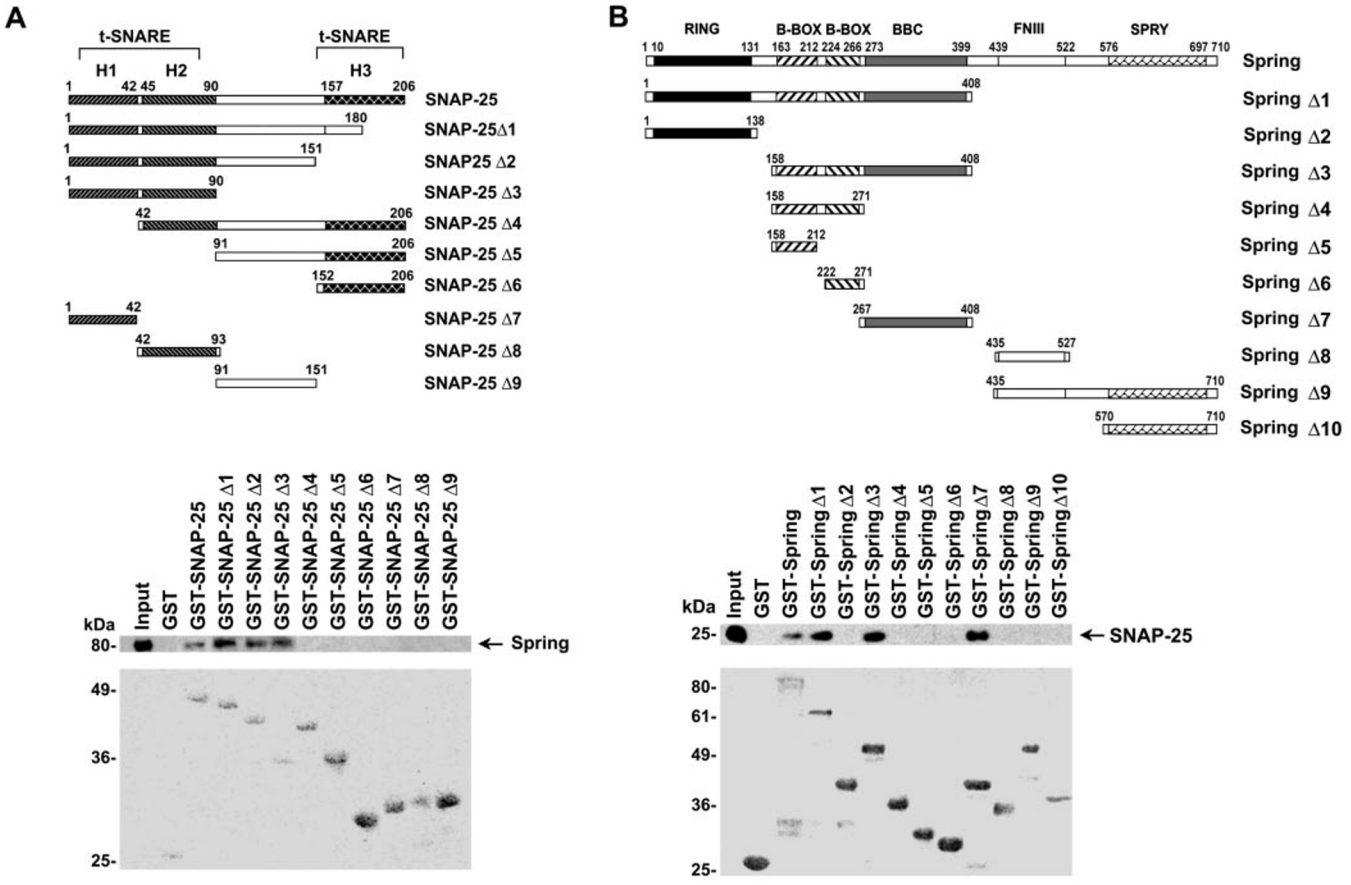


FIG. 5. **Identification of interacting domains of SNAP-25 and Spring.** *A,* mapping of the Spring binding domain of SNAP-25. Schematic representation of SNAP-25 and its deletion mutants encoded by the GST fusion cDNA constructs is shown on the *top*. These fusion proteins were immobilized on glutathione-agarose beads (*lower panel,* Ponceau S staining) and incubated with His-Spring. Bound Spring was detected by immunoblotting. *B,* mapping of the SNAP-25-binding domain of Spring. Rat brain homogenate (*Input*) was incubated with immobilized GST fusion proteins containing full-length or truncated forms of Spring as indicated. Bound SNAP-25 was detected by immunoblotting, and GST-Spring fusion proteins were shown as Ponceau S staining.

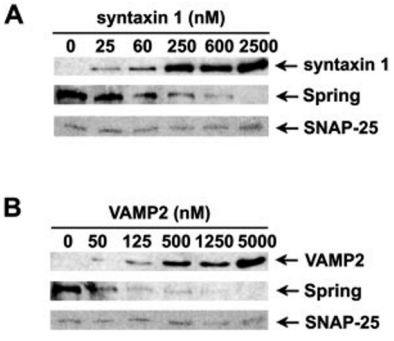


FIG. 6. **Spring competes with syntaxin 1 and VAMP2 for binding to SNAP-25.** *A,* binding competition between Spring and syntaxin 1 to SNAP-25. Immobilized GST-SNAP-25 (50 nM) was incubated with a constant amount of His-Spring (50 nM) and increasing amounts of His-syntaxin 1. Bound Spring and syntaxin 1 were detected by immunoblotting, and GST-SNAP-25 was shown as Ponceau S staining. *B,* binding competition between Spring and VAMP2 to SNAP-25. Immobilized GST-SNAP-25 (50 nM) was incubated with a constant amount of His-Spring (50 nM) and increasing amounts of His-VAMP. Bound Spring and VAMP2 were detected by immunoblotting, and GST-SNAP-25 was shown as Ponceau S staining.

high K^+-induced GH release (Fig. 8*B*), whereas the basal GH release was not affected (data not shown). The extent of reduction in the stimulated GH secretion was comparable with that caused by overexpression of the cytoplasmic region of syntaxin 1 (Fig. 8*B*) (37, 38). In contrast, overexpression of SpringΔ2, a fragment of Spring (residues 1–138) that is unable to bind SNAP-25 (Fig. 5*B*), did not have any significant effect on basal GH release nor on stimulated GH release (Fig. 8*B*). Together, these data suggest that Spring has a functional role in modulating Ca^{2+}-dependent exocytosis through its binding to SNAP-25.

DISCUSSION

In this study, we have identified and characterized Spring, a novel RING finger protein that interacts with SNAP-25, an essential component of neurotransmitter release machinery. Spring is exclusively expressed in brain and is concentrated at synapses. Spring interacts specifically with SNAP-25 but not with other SNAREs such as syntaxin 1 and VAMP2. Furthermore, unlike some other SNAP-25-binding partners such as syntaxins, SNIP, and intersectin, Spring does not interact with SNAP-23, a ubiquitously expressed isoform with 65% identity to SNAP-25, indicating that the interaction between Spring and SNAP-25 is highly specific. Moreover, Spring and SNAP-25 form a high affinity, stoichiometric complex that is unable to associate with syntaxin 1 and VAMP2 to form the SNARE fusion complex. The synaptic localization and binding properties of Spring suggest that Spring is well positioned to modulate synaptic vesicle exocytosis. Consistent with this notion, overexpression of full-length Spring or its SNAP-25-interacting domain leads to a significant inhibition of Ca^{2+}-dependent exocytosis from PC12 cells.

Our data support a model in which Spring, by interacting with monomeric SNAP-25, serves as a regulator of synaptic vesicle exocytosis. The association of Spring with the N-terminal t-SNARE domain of SNAP-25 may keep SNAP-25 in an

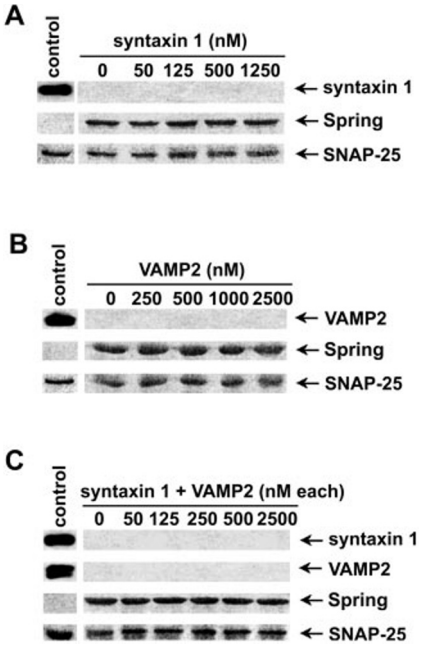


FIG. 7. **Association of Spring with SNAP-25 abolishes the ability of SNAP-25 to interact with other SNAREs.** *A,* the association of Spring with SNAP-25 prevents the interaction of SNAP-25 with syntaxin 1. Immobilized Spring-SNAP-25 binary complexes were assembled by incubation of GST-Spring (100 nM) with His-SNAP-25 (200 nM). After extensive washes, the immobilized Spring-SNAP-25 complexes (shown as Ponceau S staining) were incubated with increasing amounts of His-Syntaxin 1. Bound syntaxin 1 was detected by immunoblotting. In the *control lane*, a parallel binding experiment was performed by incubation of immobilized SNAP-25 (100 nM) with 500 nM His-Syntaxin 1. *B,* the association of Spring with SNAP-25 prevents the interaction of SNAP-25 with VAMP2. Immobilized Spring-SNAP-25 binary complexes (shown as Ponceau S staining) were assembled as described in *A* and then incubated with increasing amounts of His-VAMP2. Bound VAMP2 was detected by immunoblotting. In the *control lane*, a parallel binding experiment was performed by incubation of immobilized SNAP-25 (100 nM) with 500 nM His-VAMP2. *C,* the association of Spring with SNAP-25 prevents the assembly of the ternary SNARE complex. Immobilized Spring-SNAP-25 binary complexes (shown as Ponceau S staining) were assembled as described in *A* and then incubated with increasing amounts of His-syntaxin 1 and His-VAMP2 (1:1 molar ratio). Bound syntaxin 1 and VAMP2 were detected by immunoblotting. In the *control lane,* a parallel binding experiment was performed by incubation of immobilized SNAP-25 (100 nM) with His-syntaxin 1 and His-VAMP2 (500 nM each).

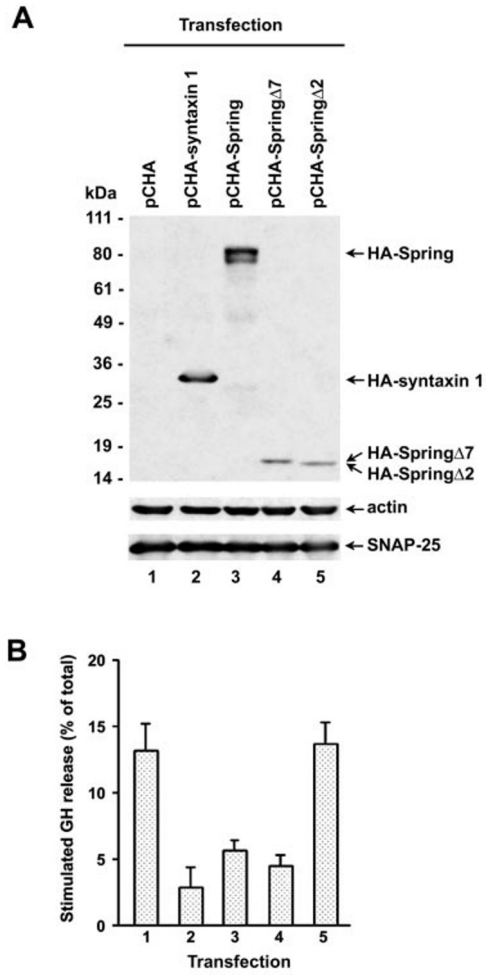


FIG. 8. **Effect of overexpression of Spring and its fragments on Ca^{2+}-dependent exocytosis from PC12 cells.** *A,* PC12 cells were cotransfected with pXGH5 encoding human GH and a test plasmid as indicated: *lane 1,* pCHA; *lane 2,* pCHA-syntaxin 1; *lane 3,* pCHA-Spring; *lane 4,* pCHA-SpringΔ7; and *lane 5,* pCHA-SpringΔ2. Cells were then lysed, and equal amounts of protein from each lysate (100 μg of protein per lane) were analyzed by sequential immunoblotting with anti-HA, anti-actin, and anti-SNAP-25 antibodies. *B,* the K^+-stimulated GH secretion was determined from PC12 cells transfected as described in *A* and expressed as a percentage of total GH content. Data are means ± S.E. (*error bars*) of the results from three independent determinations.

inactive state, unavailable to interact with other components of the SNARE fusion machinery. The binding characteristic of Spring to SNAP-25 is reminiscent of the binding of nSec1 (also known as Munc-18) to syntaxin 1, which makes syntaxin 1 inaccessible for interacting with other SNAREs to form the SNARE fusion complex (38, 40). Although based on these binding properties one would predict that nSec1 should negatively regulate vesicle exocytosis, accumulating evidence indicates that nSec1 plays both a positive and negative role in neurotransmitter release (41–43). Similarly, it is possible that Spring may also have dual functions in synaptic vesicle exocytosis. For example, the association of the cytosolic form of Spring with SNAP-25 localized on the plasma membrane may interfere with the formation of *trans*-SNARE complexes, thus negatively regulating synaptic vesicle fusion. On the other hand, the synaptic vesicle-associated form of Spring may be involved in sequestering vesicular SNAP-25 after it has been dissociated from *cis*-SNARE complexes by *N*-ethylmaleimide-sensitive factor in conjunction with soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) (44). This sequestration of vesicular SNAP-25 monomers would prevent reformation of *cis*-SNARE complexes on synaptic vesicles, thereby favoring the formation of *trans*-SNARE complexes and having a positive role in synaptic vesicle exocytosis.

The apparent affinity (EC_{50}) of Spring binding to SNAP-25 (Fig. 4*B*) is more than 20-fold higher than that of syntaxin 1 to SNAP-25 and more than 300-fold higher than that of VAMP2 to SNAP-25 (45, 46). Moreover, the complex formed between Spring and SNAP-25 is so stable that syntaxin 1 and VAMP2 are unable to dissociate SNAP-25 from the complex (Fig. 7, *A–C*). These findings suggest that the transition of SNAP-25 from a Spring-bound state to a SNARE (syntaxin 1 and VAMP2)-associated state is highly regulated. This regulation is unlikely to be mediated directly by a rise in intracellular Ca^{2+} concentration that triggers vesicle fusion, as our binding studies show that the interaction between Spring and SNAP-25 is insensitive to Ca^{2+} over a wide range (0–1 mM) of free Ca^{2+} concentrations (data not shown). Instead, this transition probably involves the regulation by protein phosphorylation. It has been proposed that phosphorylation of nSec1/Munc-18 by protein kinase C inhibits its association with syntaxin 1, thus facilitating the SNARE complex formation and synaptic vesicle fusion (47, 48). A similar mechanism may be involved in regu-

lating the interaction of Spring with SNAP-25. Consistent with this possibility, the sequence of Spring contains multiple consensus serine/threonine phosphorylation sites (9 sites for protein kinase C, 3 sites for cAMP-dependent protein kinase/protein kinase G, and 5 sites for Ca^{2+}/calmodulin-dependent protein kinase II) and 9 predicted tyrosine phosphorylation sites (49, 50). Phosphorylation of one or several of these sites may lead to the dissociation of SNAP-25 from Spring, thereby allowing SNAP-25 to interact with other components of the SNARE fusion machinery to facilitate synaptic vesicle exocytosis.

It is becoming increasingly clear that the function of synaptic SNAREs is regulated by other proteins, perhaps as a means to control the temporal and spatial formation of the SNARE fusion complexes. For example, more than a dozen regulators of syntaxin 1 have been identified, including nSec1/Munc-18, Munc-13, complexins, CIRL/latrophilin, tomosyn, and syntaphilin (37, 51–56). In comparison, the list of SNAP-25 regulators is fairly short. The present work has identified Spring as a potential neuron-specific regulator of SNAP-25. Interestingly, unlike nSec1 (a neuronal homologue of yeast Sec1 protein), Spring does not appear to have a yeast homologue, although there are Spring homologues in *Drosophila*, *C. elegans*, and zebrafish. In fact, despite a high degree of conservation from yeast to man in the basic components of the fusion machinery such as SNAREs, Rabs, and Sec1s, there is no evidence for the presence in yeast of a number of regulators of synaptic vesicle exocytosis, such as complexin, Munc-13, and synapsin. These neuron-specific proteins may have evolved to exert additional layers of regulation on neurotransmitter release, a highly specialized form of vesicular trafficking in multicellular organisms.

Spring is a novel member of the growing RING finger family. The RING finger is a cysteine/histidine-rich Zn^{2+}-binding motif that is found in a number of proteins involved in diverse cellular processes (25, 26). Although no unifying role has been identified for the RING function, the emerging common theme is that RING fingers appear to mediate the formation and architecture of large protein complexes that are critical for these cellular processes (25, 26). In addition to the RING finger, Spring contains other protein-protein interaction motifs, namely two Zn^{2+}-binding B boxes, a coiled-coil domain, a fibronectin type III domain, and a SPRY domain. Thus, Spring could potentially interact with multiple proteins or participate in the formation of multiprotein complexes. The RING finger, two B boxes, and the coiled-coil domain of Spring form a tripartite RBCC motif, whose domain organization is evolutionarily conserved. The RBCC motif has been found in a variety of proteins, some of which have been implicated in vesicular trafficking such as ARD1 (57) and BERP (28). It has been proposed that the RBCC motif may play a scaffolding role via homo-oligomerization and interactions with other proteins (25, 26). Our immunoprecipitation studies of HA- and FLAG-tagged Spring proteins revealed that Spring associates with itself to form homo-oligomers (data not shown). Furthermore, the RBCC motif in several proteins, such as MID1 and BERP, has been shown to interact with cytoskeletal elements, such as microtubules and actinin-4 (58, 59). In this study, we have shown that a significant pool of Spring is associated with brain cytoskeleton fractions (Fig. 3*B*). Together, these findings suggest that Spring may be involved in the formation of a multiprotein complex with cytoskeletal proteins, SNAP-25, and other proteins to regulate synaptic vesicle docking and fusion. Interestingly, two yeast RING finger proteins, Vps11 and Vps18, have recently been shown to form a multiprotein complex called the C-Vps complex with Vps33 (a Sec1 homologue), Vps16, Vps39, and Vps41 (60, 61). The C-Vps complex seems to direct the following three distinct reactions: activating Ypt7 (a rab GTPase), tethering vesicles to the vacuole, and interacting with unpaired/activated Vam3 (a t-SNARE) to facilitate the formation of the SNARE fusion machinery. By orchestrating these reactions, the RING finger C-Vps complex plays a key role to ensure the specificity and efficiency of the vacuole fusion process (61). Future studies will determine whether Spring forms a functionally similar multiprotein complex at nerve terminals to coordinate various protein-protein interactions important in synaptic vesicle docking and fusion.

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