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

Yankun Li, Lih-Shen Chin, Charlotte Weigel, and Lian Li‡

From the Department of Pharmacology and Cell and Molecular Physiology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

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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 Ca2+-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 Ca2+-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) 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 syntaptobrevin), 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 Ca2+ 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 Ca2+-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 AZAPII (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 (DDRGLRGFPKRNVR) of Spring.
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-SNP-25 (SMI 81, Sternberger Monoclonals, Inc.); anti-syntactin 1 (HPC-1, Sigma); anti-VAMP2 (Wako Pure Chemical Industries, Ltd.); and anti-synaptophysin (SVP-58, Sigma); and anti-Mouse Protein A (Southern Biotech). The Triton X-100 extract of rat brains. For binding experiments, brain homogenates were fractionated into crude synaptosome fractions (PG3) which 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. 

**Protein Expression and Purification**—GST or His6-tagged fusion proteins were expressed in *Escherichia coli* BL21 cells as described previously (16). GST fusion proteins were affinity-purified using the glutathione-agarose beads (Sigma). His6-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 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 cloned into the mammalian expression vectors pCDNA3.1(+), or other expression vectors such as pCDNA3.1(+) or pET28c (Novagen) for transient transfection studies. 

**In Vitro Binding Assays**—For binding experiments (Figs. 4 A and 4B), 100 nM His6-SNAP-25 was incubated with soluble His-Spring (50 nM) in the PBS buffer and processed as above. The EC50 was defined as the effective concentration of the RBCC motif is unclear, this tripartite motif is found in a large number of proteins involved in diverse cellular functions 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. 

**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. 

**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. 

**Transfection of PC12 Cells and Assays of GH Secretion**—Exponentially growing PC12 cells were harvested and transfected with 5 µg of pXGH5 encoding human growth hormone and 30 µg of test plasmid as described previously (18). Measurements of GH secretion were performed 24 h after transfection. PC12 cells were washed with a physiological salt solution (PSS (in mM): 145 NaCl, 5.6 KCl, 2.2 CaCl2, 0.5 MgCl2, 10 glucose, 15 HEPES, pH 7.4). The cells were then incubated with 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). 

**Subcellular Fractionations**—Subcellular fractions of rat brain membrane and cytosol fractions were performed as described (19). The synaptosomal membranes were subfractionated by Triton X-100, 4 µm urea, 1.5 mM NaCl, or 100 mM Na2HPO4, 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 (PS2) 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-SNP-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-SNP-25 complexes, the clarified supernatant was incubated with anti-SNAP-25. The supernatant was subjected to immunoprecipitation by anti-SNP-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 transfected with 5 µg of pXGH5 encoding human growth hormone and 30 µg of test plasmid as described previously (18). Measurements of GH secretion were performed 24 h after transfection. PC12 cells were washed with a physiological salt solution (PSS (in mM): 145 NaCl, 5.6 KCl, 2.2 CaCl2, 0.5 MgCl2, 10 glucose, 15 HEPES, pH 7.4). The cells were then incubated with 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).
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. 1B). 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. 2A). 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. 2B).
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. 2C). 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. 2D), which is consistent with the pattern of Spring mRNA expression (Fig. 2A).

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. 3A). 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 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. 3A), 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. 3B). 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. 3C). The presence of Spring in these fractions suggests that it is involved in synaptic vesicle trafficking.
immunoreactivity was detected in the myelin (PG1) and mitochondrial (PG4) fractions. To characterize further the distribution of Spring in synaptosomes, the purified synaptosome fraction (PG3) was subfractionated into the synaptosomal plasma membrane (LP1), synaptic vesicle (LP2), and cytosol (LS2) fractions (Fig. 3C). As expected, synaptophysin was highly enriched in the synaptic vesicle fraction, whereas SNAP-25 was found primarily in the synaptosomal 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 synaptosomal 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. 4A, His-tagged Spring bound selectively to GST-SNAP-25 but not to GST alone or other SNARE proteins, such as SNAP-23/SNAP-25. These in vitro binding data demonstrate a direct and specific association between Spring and SNAP-25, which is consistent with the results 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. 4B). 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 approximately 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. 4C). 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. 4D). 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. 5A). 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. 5B). 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. 5A, 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. 6A). The results showed that as the concentration of syntaxin 1 increased, the binding of Spring to 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. 6B).

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 SNARE 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. 7A). 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 Spring with SNAP-25 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. 7B). 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. 7C). The results demonstrated that the association of Spring with SNAP-25 completely prevented the assembly of the ternary SNARE complex.

Role of Spring in Ca2+-dependent Exocytosis—To determine whether Spring is involved in Ca2+-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 Ca2+-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 analysis of cell lysates confirmed that exogenous Spring proteins and syntaxin 1 were expressed at similar levels in transfected cells (Fig. 8A). 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
high K⁺-induced GH release (Fig. 8B), 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. 8B) (37, 38). In contrast, overexpression of SpringΔ2, a fragment of Spring (residues 1–138) that is unable to bind SNAP-25 (Fig. 5B), did not have any significant effect on basal GH release nor on stimulated GH release (Fig. 8B). Together, these data suggest that Spring has a functional role in modulating Ca²⁺-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 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 Ca²⁺-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
immobilized SNAP-25 (100 nM) with 500 nM 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-Syntaxin 1. A, the association of Spring with SNAP-25 abolishes the ability of SNAP-25 to interact with other SNAREs. B, 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-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).

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. 4B) 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 consensuseserine/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, complexes, CIRL/iatrophilin, tomosyn, and synaptophysin (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, Rab, 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 may lead to the dissociation of SNAP-25 from Spring, thereby allowing SNAP-25 to interact with other components of the SNARE fusion machinery.

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