rbSec1A and B Colocalize with Syntaxin 1 and SNAP-25 throughout the Axon, but Are Not in a Stable Complex with Syntaxin

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Abstract. rbSec1 is a mammalian neuronal protein homologous to the yeast *SEC1* gene product which is required for exocytosis. Mutations in Sec1 homologues in the nervous systems of *C. elegans* and *D. melanogaster* lead to defective neurotransmitter secretion. Biochemical studies have shown that recombinant rbSec1 binds syntaxin 1 but not SNAP-25 or synaptobrevin/VAMP, the two proteins which together with syntaxin 1 form the synaptic SNARE complex. In this study we have examined the subcellular localization of rbSec1 and the degree of interaction between rbSec1 and syntaxin 1 in situ. rbSec1, which we show here to be represented by two alternatively spliced isoforms, rbSec1A and B, has a widespread distribution in the axon and is not restricted to the nerve terminal. This distribution parallels the localization of syntaxin 1 and SNAP-25 along the entire axonal plasmalemma. rbSec1 is found in a soluble and a membrane-associated form. Although a pool of rbSec1 is present on the plasmalemma, the majority of membrane-bound rbSec1 is not associated with syntaxin 1. We also show that rbSec1 is not part of the synaptic SNARE complex or of the syntaxin 1/SNAP-25 complex we show to be present in non-synaptic regions of the axon. Thus, in spite of biochemical studies demonstrating the high affinity interaction of rbSec1 and syntaxin 1, our results indicate that rbSec1 and syntaxin 1 are not stably associated. They also suggest that the function of rbSec1, syntaxin 1, and SNAP-25 is not restricted to synaptic vesicle exocytosis at the synapse.

NEURONS release neurotransmitters at synapses via a highly regulated process involving the fusion of synaptic vesicles with the presynaptic plasma membrane (Südhof and Jahn, 1991; Bennett and Scheller, 1994; Mundigl and De Camilli, 1994). Recently a convergence of genetic and biochemical studies from a variety of systems led to the formulation of a hypothetical model for the molecular interactions leading to vesicle fusion in general, and synaptic vesicle exocytosis in particular. This model, referred to as the SNARE hypothesis, proposes that vesicles dock to a target membrane through the interaction of vesicular and target membrane proteins referred to as SNAREs (v- and t-SNAREs, respectively) (Rothman, 1994). In the case of synaptic vesicle exocytosis, synaptobrevin/VAMP (Trimble et al., 1988; Baumert et al., 1989) is the v-SNARE, and SNAP-25 (Oyler et al., 1989) and syntaxin 1 (Bennett et al., 1992) are the t-SNAREs (Söllner et al., 1993*b*). Formation of the SNARE complex is followed by the recruitment of cytosolic proteins required for fusion including α/β-SNAP, γ-SNAP (Clary et al., 1990; Whiteheart et al., 1993), and NSF (Wilson et al., 1989). The extremely short delay between nerve terminal depolarization and synaptic vesicle exocytosis suggests that formation of the synaptic SNARE complex leads to a pool of docked synaptic vesicles (visible by electron microscopy at active zones) which are arrested at a stage preceding full fusion by a negative clamp. Elevations in cytosolic Ca^{2+} would then release this clamp allowing the fusion reaction to proceed (Rothman, 1994).

While the SNARE model is consistent with a large body of experimental evidence (Schiavo et al., 1992; Blasi et al., 1993*a*, *b*; Protopopov et al., 1993; Brennwald et al., 1994), the precise sequence of the molecular interactions that lead to fusion are still unclear. Furthermore, the events which precede and promote the assembly of the SNARE complex are still unknown. Yeast genetics has suggested that several other proteins, in addition to the yeast homologues of the v- and t-SNAREs, NSF, and SNAPs, participate in exocytosis (Novick et al., 1981) and may therefore play a role in the regulation of the assembly of the SNARE complex. They include members of the Rab (Novick and Brennwald, 1993; Nuoffer and Balch, 1994) and Sec1 families (Aalto et al., 1992). Yeast Sec1 and Sec4 (a rab GTPase) participate in exocytosis (Novick et al., 1981), while other members of both families (Wada et al., 1990; Ossig et al., 1991; Dascher

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et al., 1991; Cowles et al., 1994; Piper et al., 1994) function in other intracellular vesicular transport steps. In fact, the identification of the Rab and Secl families were among the first pieces of experimental evidence indicating a similarity of the membrane fusion machinery at all stations of the secretory and endocytic pathways (Aalto et al., 1992; Novick and Brennwald, 1993).

Secl homologues have been identified in the nervous system of *C. elegans* (Hosono et al., 1992; Genyo-Ando et al., 1993), *Drosophila* (Salzberg et al., 1993; Harrison et al., 1994), and mammals (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994*a*; Hodel et al., 1994) where they appear to be involved in synaptic vesicle exocytosis. Secl interacts directly with a t-SNARE, syntaxin, as first suggested by genetic work in yeast (Aalto et al., 1993), and then confirmed biochemically with mammalian proteins (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994*a*; Hodel et al., 1994). Thus, Secl may control the presynaptic SNARE complex through an interaction with syntaxin 1. However, the precise role of neuronal Secl in the formation or regulation of the fusion complex remains unknown and no information is yet available on the intracellular localization of neuronal Secl relative to that of presynaptic v- and t-SNAREs.

As a step toward the elucidation of the function in synaptic vesicle exocytosis of Secl (henceforth referred to as rbSecl [Garcia et al., 1994], but also known by other names; munc-18 [Hata et al., 1993], n-Secl [Pevsner et al., 1994*a*], and mSecl [Hodel et al., 1994]), we have now investigated its subcellular localization in neurons and endocrine cells. We report that rbSecl, like syntaxin 1 and SNAP-25, has a widespread distribution throughout the axon, raising the possibility that the role of these proteins may not be limited to synaptic vesicle exocytosis. Although a pool of rbSecl is present on the plasmalemma, the bulk of rbSecl is not associated with syntaxin 1. rbSecl is not part of the synaptic SNARE complex nor of the syntaxin 1/SNAP-25 complex observed in non-synaptic regions of the axon. Additionally, we demonstrate that rbSecl exists in two alternatively spliced isoforms which differ at their COOH termini and are differentially expressed in the brain.

Materials and Methods

Materials

Male Sprague-Dawley rats (~ 150–200 g) were obtained from Charles River Laboratories (Wilmington, MA). Fresh bovine brains were obtained from a local slaughterhouse. A cDNA encoding histidine-tagged syntaxin 1A (a.a. 1–265) was a kind gift from E. Chapman and R. Jahn (Chapman et al., 1994).

Antibodies

Two rabbit anti-rbSecl antibodies were raised: EGM1 is directed against aa #102–255 fused to maltose-binding protein (Garcia et al., 1994), and EGM2 is directed against full-length rbSeclA fused to glutathione-*S*-transferase (GST). Rabbit anti-SNAP-25 antibodies were raised by the protocol described by Oyler et al. (1989), and anti-synapsin antibodies were described previously (De Camilli et al., 1983*a*). The following antibodies were generous gifts: HPC-1 mouse monoclonal anti-syntaxin 1 from C. Barnstable (Yale University) (Barnstable et al., 1985); 78.1 and 78.2 mouse monoclonal anti-syntaxin 1, rabbit anti-rab3A antiserum (Matteoli et al., 1991), anti-synaptophysin (Navone et al., 1986), and rabbit anti-syntaxin 1 antiserum (R31) from R. Jahn (Yale University); affinity-purified rabbit anti-synaptotagmin and rabbit anti-rbSecl raised against amino acids #575–594 of rbSeclA (Pevsner et al., 1994*a*) from R. Scheller; mouse monoclonal anti-Na^+/K^+ ATPase from M. Caplan (Yale University) (Pietrini et al., 1992); mouse monoclonal anti-secretogranin I from W. Hüttner (Heidelberg, FRG). Anti-syntaxin antibodies 78.1, 78.2, and R31 will be described elsewhere (Hanson, P., and R. Jahn, manuscript in preparation). Indocarbocyanine (Cy3)-conjugated goat anti-rabbit IgGs and fluorescein (DTAF)-conjugated goat anti-mouse IgGs were from Jackson Laboratories (West Grove, PA), nonimmune rabbit IgGs and rabbit anti-mouse IgGs from Cappel (Malvern, PA). Protein A-gold conjugates (6 nm) were prepared as described (Slot and Gueze, 1985). Goat anti-mouse IgG-gold conjugates (5 nm) were obtained from Amersham Corp. (Arlington Heights, IL).

Immunocytochemistry

Immunofluorescence. Rat brains or pancreas were fixed by transcardial perfusion (4% formaldehyde in 0.12 M Na-phosphate buffer). Preparation of frozen sections and immunostaining was carried out as described (De Camilli et al., 1983*a*; Takei et al., 1992).

Immunoelectron Microscopy. Immunogold staining of agarose embedded tissue fragments was carried out as described (De Camilli et al., 1983*b*; Takei et al., 1992). Rabbit antibodies were detected with protein A-gold conjugates. Mouse monoclonal antibodies were revealed with goat anti-mouse IgG-gold conjugates. Alternatively, rabbit anti-mouse IgGs were used as bridges, and then labeled with protein A-gold conjugates.

Subcellular Fractionation of Rat Brains

Synaptic fractions were prepared from rat brain as described (Hüttner et al., 1983) with minor modifications. To obtain S_3 and P_3, the S_2 fraction from Hüttner et al. (1983) was centrifuged at 165,000 g_{av} for 2 h. The fraction of the eluate from the controlled-pore glass (CPG) column (Hüttner et al., 1983) containing synaptic vesicles is referred to as CPG-3. To study the membrane association of rat brain proteins, postnuclear supernatants were prepared from fresh rat brains as described for PC12 cells. Four separate aliquots were centrifuged at 165,000 *g* for 20 min in a TLA.100.2 (Beckman Instrs., Fullerton, CA). Each pellet was resuspended in one of the following: 10 mM Hepes (pH 7.4) or Hepes containing 0.2 M KCl, 1 M KCl, or carbonate buffer at pH 11.5. The mixtures were then recentrifuged as above, pellets were collected, and analyzed by SDS-PAGE and Western blotting for their protein content.

Sucrose Density Gradients of PC12 Cells

Approximately 1.65×10^8 confluent PC12 cells (Tischler and Greene, 1975) were homogenized in 0.32 M sucrose, 10 mM Hepes (pH 7.4), 0.83 mM benzamidine, and 2 mM PMSF (1:10, wt/vol) and centrifuged at 1,000 *g* for 5 min. The resulting postnuclear supernatant was separated on 0.4–2 M sucrose gradients as described (Navone et al., 1989) and aliquots of gradient fractions were analyzed by SDS-PAGE and Western blotting.

Sucrose Density Gradients of Solubilized Brain Extracts

Postnuclear supernatants were prepared from fresh rat brains as described above for PC12 cells except that sucrose was omitted. One aliquot was centrifuged in a TLA.100.2 rotor (Beckman Instrs.) at 165,000 *g* for 15 min to produce a soluble and particulate fraction. The particulate fraction was resuspended in 140 mM KCl, 10 mM Hepes (pH 7.4). Triton X-100 and KCl were then added to the soluble fraction, the resuspended particulate fraction, and the original postnuclear supernatant to a final concentration of 0.5% and 140 mM, respectively, and the resulting mixtures were incubated with gentle mixing at 4°C. All three fractions (soluble, particulate, and postnuclear supernatant) were centrifuged in a TLA.100.2 rotor at 100,000 *g* for 30 min to pellet Triton X-100 insoluble material. The supernatants from each sample were loaded onto separate 5–20% sucrose gradients containing 10 mM Hepes (pH 7.4), 140 mM KCl and 0.5% Triton X-100, centrifuged at 140,000 *g* for 4 h in a VAC-50 rotor (Beckman Instrs.), and collected as 20 2-ml fractions. Aliquots of fractions 1–19 were analyzed by SDS-PAGE and Western blotting. Selected fractions were used for immunoprecipitation experiments (see below).

1. *Abbreviations used in this paper*: CPG, controlled-pore glass; GST, glutathione-S-transferase.

Immunoprecipitation

Samples to be immunoprecipitated were precleared by incubation with Protein G Fastflow beads (Pharmacia LKB Biotechnology, Piscataway, NJ) precoated with preimmune IgGs for 1 h, followed by another round of Protein G Fastflow beads. Antibodies directed against various proteins of interest were prebound to protein G Fastflow beads, which were then added to selected fractions and incubated at 4°C with continuous mixing. Beads were then washed three times with PBS containing 0.5% Triton X-100. Bound proteins were eluted by boiling in Laemmli buffer (Laemmli, 1970). Samples were analyzed by SDS-PAGE and Western blotting.

PCR Cloning of an Alternatively Spliced rbSec1 Isoform

DNA fragments were amplified from a rat brain cDNA library (Burton et al., 1993) by PCR techniques using a forward primer 5'-CCACCAAATTCCTCATGGAC-3' derived from the COOH-terminal sequence of clone #24 identified in the initial screen for rbSec1, and a reverse primer 5'-CACAGGAGAAGACTC-3', that is derived from 3' untranslated sequences of rbSec1 (Garcia et al., 1994).

Amplified DNA fragments were separated on 1.8% agarose gels, transferred to Zeta-Probe nylon filters under alkaline conditions, baked at 80°C for 30 min under vaccum, and prehybridized in 5× SSC (150 mM NaCL/15 mM trisodium citrate, pH 7.0) at 68°C for 1 h. A PvuII–EcoRI fragment containing 3'-untranslated sequences from rbSec1 cDNA was radiolabeled with [γ-^{32}P]dCTP (Amersham Corp.) by random priming (Boehringer Mannheim Corp., Indianapolis, IN) and hybridized to the filter overnight under the prehybridization conditions described above. The filter was washed at 56°C with 2× SSC containing 0.1% SDS. Filters were exposed overnight to XAR film at −70°C. A 320-P DNA fragment that hybridized to the probe under these conditions was subcloned into pBlueScript SK$^-$ (Stratagene, La Jolla, CA) and sequenced in both direction by standard methods (Ausubel et al., 1990; Sambrook et al., 1989).

Construction of Recombinant GST-rbSec1B

Primer FOR5 (5'-GTATGAAGCATTACCAAGGC-3') and mCES16 (5'-TTAAGTGCTTATTTCTTCG-3') were used to PCR amplify a 3' fragment of rbSec1B cDNA which contains the SacI site common to both rbSec1A and rbSec1B (nucleotide position +1035). The PCR fragment was digested with SacI and subcloned in the proper orientation into pGEX-2T-rbSec1A (Garcia et al., 1994) also digested to completion with SacI, resulting in an in frame-fusion of the alternatively spliced form of rbSec1B to the GST-rbSec1 fusion protein.

Miscellaneous Procedures

Triton X-114 detergent phase separation of brain postnuclear supernatants was performed as described (Bordier, 1981; Solimena et al., 1993). Affinity chromatography of rat brain extracts onto GST-rbSec1 Sepharose beads was performed as previously reported (Garcia et al., 1994). SDS-PAGE and Western blotting were carried out according to Laemmli (1970) and Towbin et al., (1979).

Results

rbSec1 Is Not Restricted to Nerve Terminals

The localization of rbSec1 in rat brain sections was investigated by immunofluorescence using affinity-purified anti-rbSec1 antibody, EGM1. These antibodies selectively recognize rbSec1 (Garcia et al., 1994) (see also Fig. 9). Under low power observation, rbSec1 immunoreactivity was found to have a widespread distribution throughout the gray and white matter. A pool of the protein was clearly detectable in the cytoplasmic matrix of many neurons. It was present at higher concentration in axons than in perikarya and dendrites but was not confined to presynaptic nerve terminals. A heterogeneity in the staining pattern observed in various brain regions suggested variable levels of expression of rbSec1 in different neuronal subpopulations. The pattern of rbSec1 immunoreactivity visible at low magnification was strikingly similar to that of syntaxin 1 and SNAP-25 immunoreactivities which were also widely distributed in the axon. Representative examples of immunofluorescence patterns are shown in Fig. 1 and 2.

In the cerebellar cortex intense staining for rbSec1 (Fig. 1 *a*), extremely similar to staining for syntaxin 1(Fig. 1 *b*), was observed in the molecular layer, where both immunoreactivities were accounted for primarily by parallel fiber axons, and also by axons surrounding the base of Purkinje cells. rbSec1 and syntaxin 1 were not enriched in presynaptic terminals. Synaptophysin, a synaptic vesicle protein, and therefore a marker for presynaptic terminals (Navone et al., 1986), is shown for comparison in Fig. 1 *c*. In the hippocampus, very intense immunoreactivity for rbSec1 and syntaxin 1 was observed in the mossy fiber axons originating from granule cells of the dentate gyrus which are shown in cross-section (Fig. 1, *d* and *e*). Again, the pattern of immunoreactivity for rbSec1 and syntaxin 1 was distinct from that produced by antibodies directed against synaptophysin (Fig. 1 *f*). It was consistent with a localization of the two proteins along the entire axon and not restricted to the nerve terminal (see also Fig. 2 *b*). In the brainstem (Fig. 1, *g* and *h*), rbSec1 and syntaxin 1 had a widespread distribution in the neuropile, were enriched in axons, but were not restricted to synaptophysin-enriched presynaptic terminals (Fig. 1 *i*). The presence of a pool of rbSec1 in the cytoplasmic matrix of neuronal perikarya was visible in large neurons of this brain region (Fig. 1 *g*). The striking colocalization of syntaxin 1 and SNAP-25 throughout the axon of hippocampal mossy fibers is shown in Fig 2, *a* and *b*. This localization is distinct from that of the nerve terminal specific protein synaptophysin (Fig. 2, *c* and *d*).

Lack of precise colocalization between rbSec1 and the two t-SNAREs is clearly visible in the high power micrographs of cross-sectioned axon bundles shown in Fig. 2, *e–h*. In contrast to syntaxin 1 and SNAP-25 immunoreactivities, which primarily outline the axonal plasmalemma, rbSec1 was localized throughout the cytosolic matrix of axons. The presence of a large cytosolic pool of rbSec1 was also apparent in immunofluorescent staining of endocrine cells, such as pancreatic islets (Fig. 2 *i*) which, like neurons, express rbSec1 and the neuronal t-SNAREs, syntaxin 1, and SNAP-25. SNAP-25 and syntaxin 1 immunoreactivities in these cells are primarily localized to the cell surface and the Golgi complex area (Fig. 2, *j* and *k*).

A Pool of rbSec1 Is Membrane-associated but Not Concentrated on Secretory Organelles

It was reported that rbSec1 is concentrated in the membrane of secretory organelles (Hodel et al., 1994). The lack of any obvious enrichment of rbSec1 in nerve terminals (Figs. 1 and 2) did not support the presence of rbSec1 on synaptic vesicles. To further investigate the localization of rbSec1 on nerve terminal membranes, we performed electron microscopy and subcellular fractionation.

For electron microscopy, fragments of total rat brain were labeled for rbSec1 by an immunogold labeling procedure designed to maximize labeling of the cytoplasmic surfaces of cell membranes. This technique involves lysis of cells before

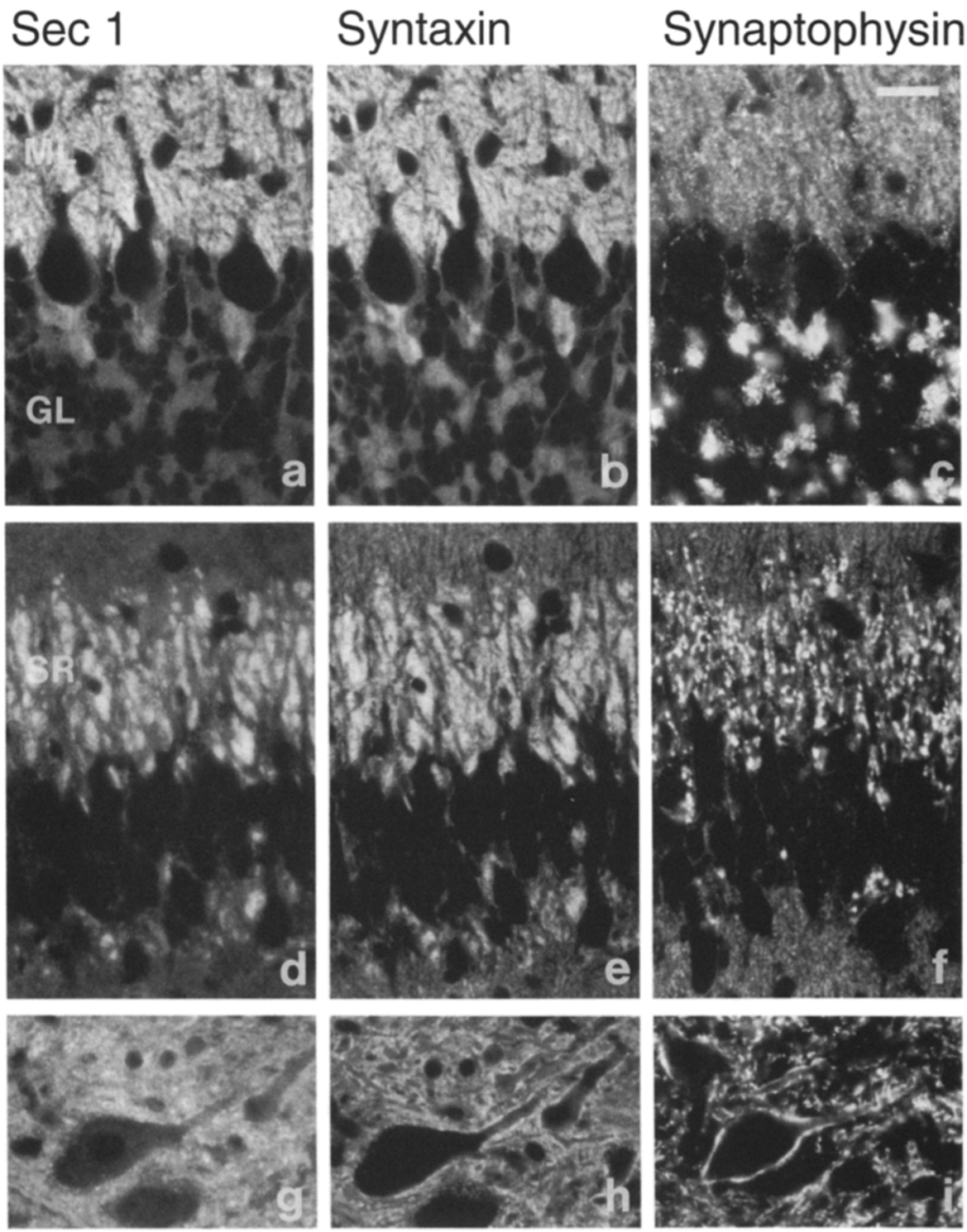

Figure 1. Close colocalization of rbSecl and syntaxin 1 in brain by low power immunofluorescence microscopy. The two left fields of each row are double immunofluorescence images of the same section stained for rbSecl and for syntaxin 1. The right field is from a similar section stained for synaptophysin. (*a–c*) Cerebellar cortex; rbSecl and syntaxin 1 are concentrated in axons of the molecular layer (*ML*) which are cut in cross-section. Lower levels of these proteins are present in the granule cell layer (*GL*). Synaptophysin appears as bright dots representing synaptic terminals and axonal varicosities. (*d–f*) CA3 region of the hippocampus; rbSecl and syntaxin 1 are enriched in the axons of mossy fibers which are seen in cross-section above the layer of pyramidal neurons. The nerve terminals of these axons are intensely positive for synaptophysin immunoreactivity. (*g–i*) Brain stem; rbSecl and syntaxin 1 have a similar localization. rbSecl, but not syntaxin 1, is also present in the cytoplasmic matrix of these brain stem neurons. Synaptophysin is restricted to nerve terminals which appear as small dots which outline the profiles of perikarya and dendrites. Bar, 20 μm.

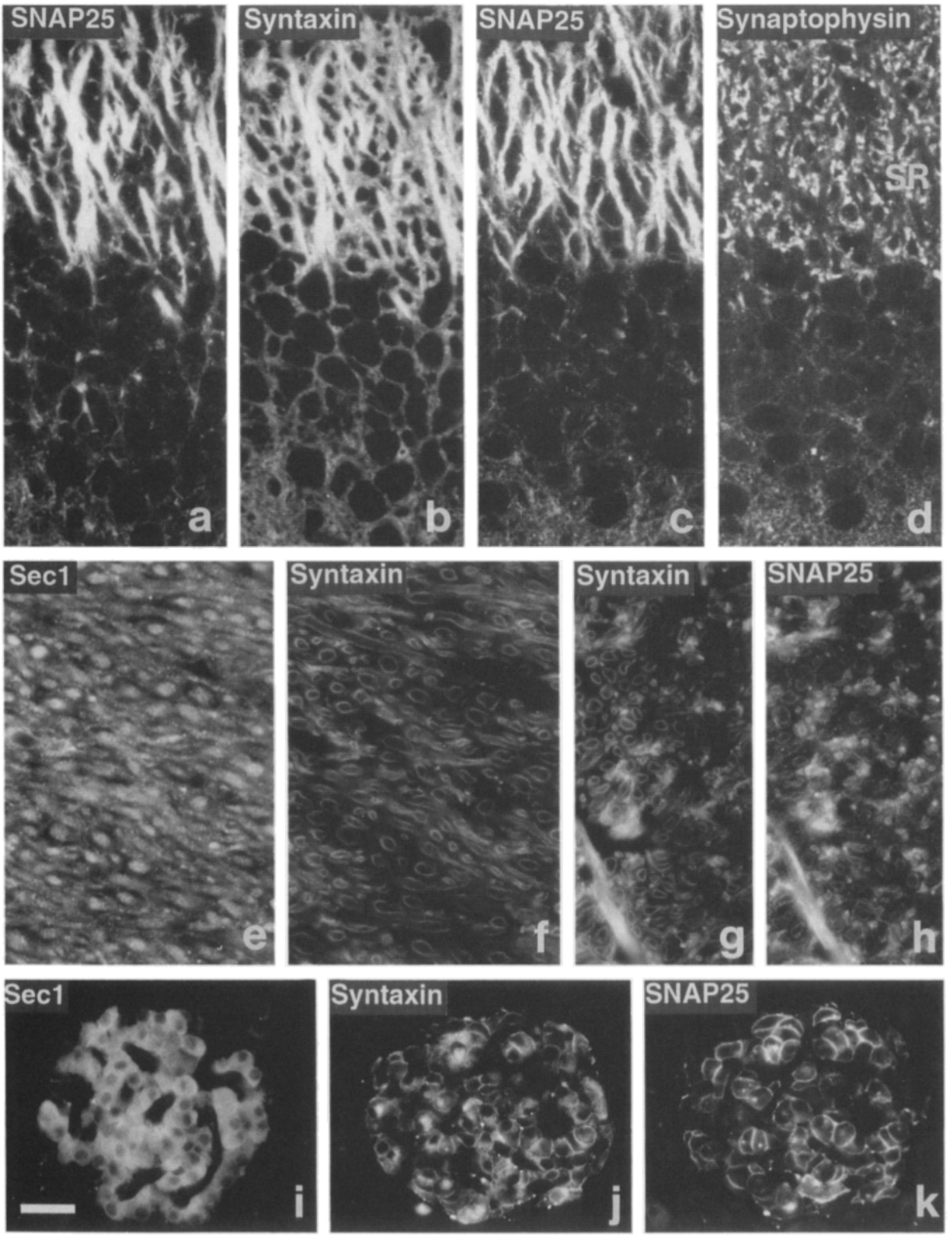

Figure 2. Comparative analysis of the distribution of rbSec1, syntaxin I, and SNAP-25 in neurons and endocrine cells. (*a–d*) CA3 region of the hippocampus sectioned along a plane parallel to the mossy fiber axons and double stained for SNAP-25 and syntaxin I (*a* and *b*) and for SNAP-25 and synaptophysin (*c* and *d*). SNAP-25 and syntaxin I are localized along the axons (*bright, white, vertical bundles*), while synaptophysin is concentrated in nerve terminals. (*e–h*) Sections of myelinated axon bundles in the brain stem. rbSec1 is present throughout the axonal cytomatrix. Syntaxin 1 and SNAP-25 immunoreactivities outline the plasma membrane of the axons which appears as white ovals and circles in cross section, and railroad tracks longitudinally. *e* and *f*, *g* and *h* represent double staining images. (*i–k*) Endocrine pancreas; rbSec1 is predominately cytosolic, whereas syntaxin 1 and SNAP-25 are primarily localized to the plasma membrane. *j–k* are a double staining of the same section. Bars:(*a–d*) 20 μm; (*e–h*) 16 μm; (*i–k*) 30 μm.

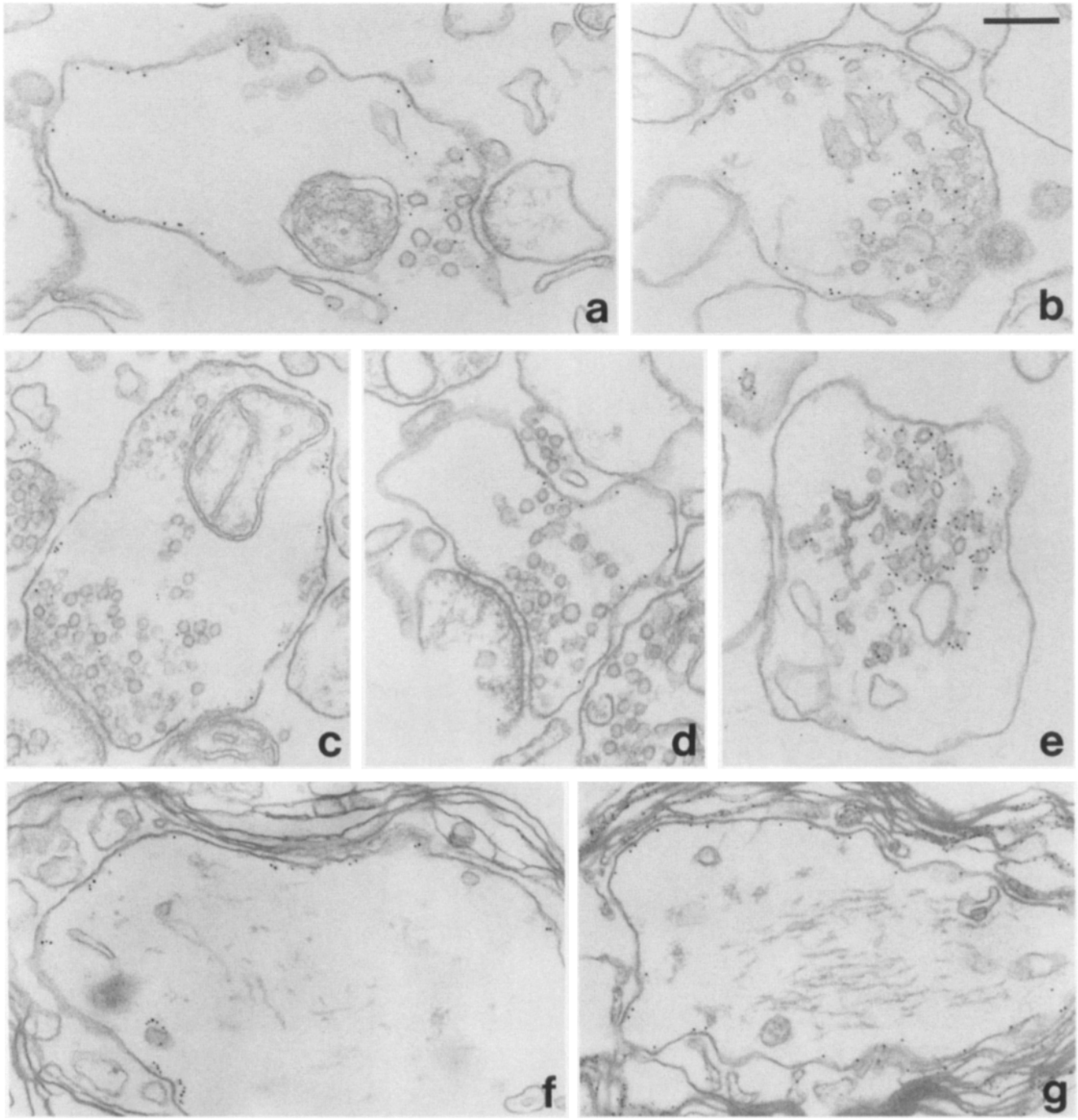

Figure 3. Presence of a pool of rbSecl in the axonal plasmalemma demonstrated by immunogold electron microscopy. Brain tissue was mildly homogenized in hypotonic conditions to elute the bulk of cytoplasmic proteins before immunolabeling. (*a* and *b*) rbSecl immunoreactivity. Note the presence of gold particles on the plasmalemma and scattered gold particles in regions which still contain cytoplasmic matrix. (*c* and *d*) Syntaxin 1 and SNAP-25 immunoreactivity, respectively. Gold particles are selectively associated with the cytoplasmic surface of the plasmalemma. (*e*) Synaptophysin immunoreactivity. Gold particles are concentrated on synaptic vesicles. (*f* and *g*) Labeling for syntaxin 1 and SNAP-25 in myelinated axons. Note the presence of gold particles decorating the cytoplasmic surface of the axonal plasma membrane. Fine heterogeneous black grains visible in the myelin of field *g* represent dye microprecipitates and not gold particles. Bars: (*a*, *b*, and *e*) 200 nm; (*c* and *d*) 220 nm; (*f* and *g*) 230 nm.

fixation which results in a partial elution of the cytoplasmic matrix. It is therefore not suited to detect cytosolic rbSecl. As shown by the distribution of gold particles in Fig. 3, *a* and *b*, a pool of rbSecl was localized along the axonal plasmalemma. A few gold particles were also observed in the proximity of synaptic vesicles, but rbSecl was not enriched on synaptic vesicle membranes. Some of these gold particles, which are localized in the matrix surrounding the vesicles, may represent incompletely eluted cytosolic rbSecl. Syntaxin 1 (Fig. 3, *c* and *f*) and SNAP-25 (Fig. 3, *d* and *g*)

were localized on the plasmalemma, while staining for synaptophysin and for synaptobrevin I and II, carried out as a control, resulted in labeling of synaptic vesicles but no labeling of the plasma membrane (Fig. 3 *e* and not shown). Immunolabeling for rbSecl, syntaxin 1, and SNAP-25 was not restricted to synaptic portions of the plasmalemma and was clearly seen on the axonal plasmalemma underlying myelin (e.g., Fig. 3, *f* and *g*) in agreement with the immunofluorescence results of Fig. 2. In fact, no gold particles were seen at presynaptic active zones (where exocytosis occurs) most likely due to the inaccessibility of this region to gold particles because of the dense cytoplasmic matrix.

The possible presence of rbSecl on synaptic vesicles was further investigated by subcellular fractionation using a procedure (Hüttner et al., 1983) which yields a highly purified synaptic vesicle preparation (Fig. 4). The effectiveness of the fractionation was demonstrated by the enrichment of the synaptic vesicle protein synaptotagmin in fractions LP_2 (crude synaptic vesicles) and CPG-3 (glass bead purified synaptic vesicles). A plasmalemma marker, Na^+/K^+ ATPase (Pietrini et al., 1992), had a widespread distribution in most particulate fractions in agreement with the presence of plasmalemma elements in most membrane fractions, but was significantly de-enriched in highly purified synaptic vesicles (CPG-3). Syntaxin 1 and SNAP-25 had a distribution similar to that of the Na^+/K^+ ATPase although they were relatively more enriched in the CPG-3 fraction consistent with the presence of a pool of these two proteins in synaptic vesicles (Walch-Solimena et al., 1994). A protein concentrated in patches of postsynaptic membranes, the NMDA receptor (NMDA-R), was remarkably absent from fractions which contain only slowly sedimentable membranes (LP_2 and CPG-3). rbSecl had a distribution similar to syntaxin 1 and SNAP-25, but, like Na^+/K^+ ATPase, was clearly de-enriched in highly purified synaptic vesicles. In addition, a pool of rbSecl was also recovered in LS_2, a fraction containing only soluble proteins. It is unlikely that rbSecl detected in LS_2 is due to a loss of rbSecl from the membrane during fractionation as a large cytosolic pool of rbSecl is demonstrated by immunofluorescence in brain and endocrine cells.

Finally, to determine whether rbSecl is concentrated on peptide-containing secretory granules (large dense-core vesicles), we compared its distribution to secretogranin I in postnuclear supernatants of PC12 cells subjected to isopicnic centrifugation on sucrose density gradients. PC12 cells are a neuroendocrine cell line which, in addition to bona fide synaptic vesicles, contains high concentrations of large dense-core vesicles (Tischler and Greene, 1975). Secretogranin I, a secretory protein stored in large dense-core vesicles (Hüttner et al., 1991), was primarily recovered in the dense portion of the gradient which contains intact granules (Fig. 5) (Reetz et al., 1991). No enrichment of rbSecl was visible in these granule-containing fractions. A pool of rbSecl was present in the soluble material at the top of the gradient ("load fractions"), and another larger pool comigrated with a marker of the plasmalemma (Na^+/K^+ ATPase) (Fig. 5) (Cameron et al., 1991). Similar results were obtained in postnuclear supernatants of bovine adrenal medulla. Even in this material rSecl was concentrated in the light portions of the gradient with only very low amounts of the protein detected in granule fractions (Galli, T., T. J. Chilcote, E. P. Garcia and P. De Camilli, unpublished observations).

The Membrane Association of rbSecl Is Not Mediated by Syntaxin 1

Because rbSecl has been shown to bind syntaxin 1 in vitro (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994*a*; Hodel et al., 1994) and the experiments shown above indicate that a pool of rbSecl localizes to the plasmalemma, it was of interest to determine whether the plasma membrane-bound pool of rbSecl is bound to syntaxin 1. To this aim we investigated the sedimentation characteristics on sucrose density gradients of rbSecl and syntaxin 1 in Triton X-100–solubilized rat brain postnuclear supernatants.

Fig. 6 *A* shows gradient fractions analyzed by SDS-PAGE

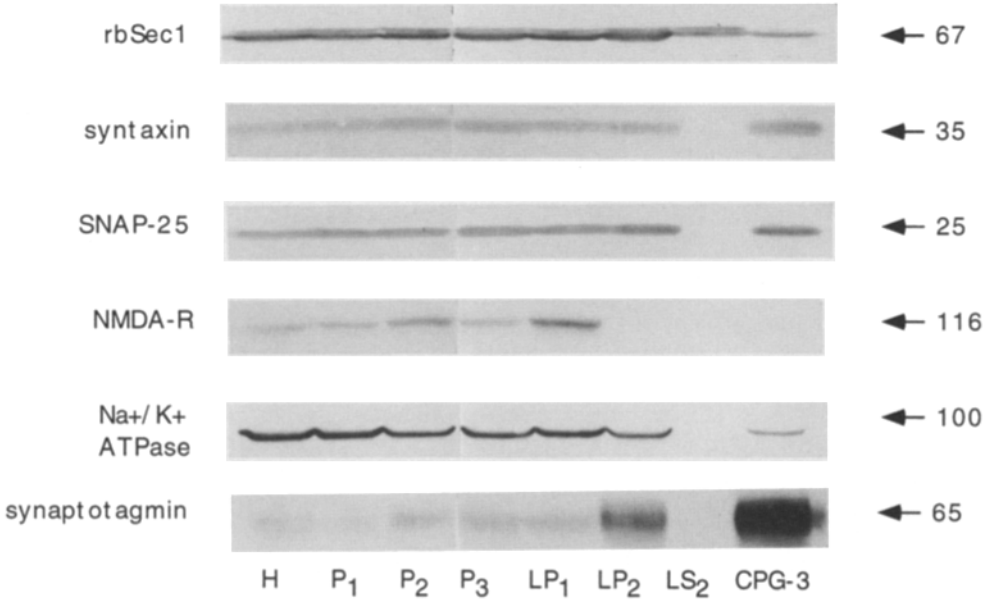

Figure 4. Comparison of the distribution of rbSecl and other neuronal proteins in subcellular fractions of rat brain. Fractions are defined as described in Materials and Methods and in Hüttner et al. (1983). Equal amounts of protein from each of the indicated fractions were analyzed by SDS-PAGE and Western blotting. rbSecl has a distribution similar to that of plasma membrane markers including syntaxin 1 and SNAP-25, but is de-enriched in purified synaptic vesicles and is present in the soluble fraction LS_2. Numbers at right indicate molecular weights.

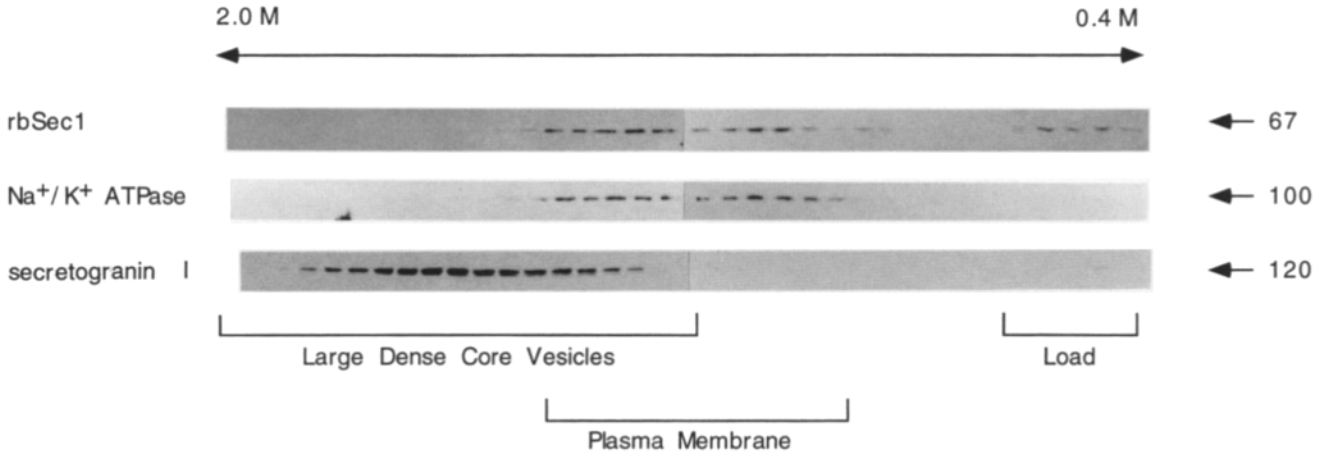

Figure 5. rbSecl is not concentrated on dense-core granules of PC12 cells. Postnuclear supernatants from PC12 cells were separated on a 0.4–2M sucrose density gradient. Equal volumes of each fraction were analyzed by SDS-PAGE and Western blotting. The peak fractions for large dense core granules, plasma membrane, and soluble (*LOAD*) material are indicated. Numbers at right indicate molecular weights (kD).

and Western blotting. The figure demonstrates that rbSecl from a postnuclear supernatant migrated as a roughly symmetrical peak around fractions #11 to #13. This migration, when compared to the sedimentation characteristics of protein markers, corresponds to the expected migration of monomeric rbSecl (67.5 kD). The bulk of syntaxin 1 (35 kD) had similar sedimentation characteristics. In spite of the similar migration of the two proteins, the close correspondence between the expected molecular weight of rbSecl and its position in the gradient speaks against a significant association between syntaxin 1 and rbSecl. Further supporting this conclusion, rbSecl from a brain soluble fraction devoid of syntaxin 1 migrated identically (Fig. 6 *a*; cytosolic rbSecl). A similar mobility was also reported previously for recombinant rbSecl/munc-18 (Hata et al., 1993). To determine whether some factor present in the cytosol may induce the dissociation of rbSecl from syntaxin 1 under the solubilization conditions used, we performed equivalent gradients using Triton X-100 extract of a total particulate fraction depleted of cytosol. Identical mobilities for rbSecl and syntaxin were observed (not shown) suggesting that the bulk of membrane-associated rbSecl does not form heteromeric complexes with syntaxin 1.

These findings do not rule out the possibility that a pool of rbSecl below detectability in the Western blot shown in Fig. 6 *A* could interact with syntaxin either alone or as a part of the SNARE complex. We therefore investigated the migration of other components of the SNARE complex present in our gradients and performed immunoprecipitation experiments from representative fractions (Fig. 6 *B*).

The majority of SNAP-25 (25 kD), peaked around fractions #14 to #16 (corresponding to a molecular mass of ~50 kD) and synaptobrevin I and II (18 kD) around fractions #16 to #18 (corresponding to a molecular mass of ~30 kD). Trails of both SNAP-25 and synaptobrevin were visible in overdeveloped blots in faster sedimenting fractions. Synaptotagmin (65 kD) migrated with an apparent molecular mass of ~68 kD.

The SNARE complex formed in the absence of exogenously added NSF and α-SNAP, was reported to comprise synaptobrevin, SNAP-25, and syntaxin 1 in stoichiometric ratios and synaptotagmin at a ratio of about 1:10 (Söllner et al., 1993*a*). This complex migrates as a 7-S particle (Söllner et al., 1993*a*). No substantial pools of either SNAP-25 or synaptobrevin were detectable at the 7-S position suggesting that under our experimental conditions, the bulk of the three SNAREs are not part of SNARE complexes. However, immunoprecipitation experiments with anti-syntaxin antibodies from selected fractions, followed by SDS-PAGE and Western blotting of the immunoprecipitates, revealed the presence of SNARE complexes. As shown in Fig. 6 *B*, immunoprecipitation with anti-syntaxin antibodies (HPC-1) from fractions #9, #12, and #15 resulted in the coprecipitation of SNAP-25 and synaptobrevin. The increasing amount of SNAP-25 and synaptobrevin coprecipitated with anti-syntaxin 1 antibodies in denser sucrose fractions (Fig. 6 *A*) demonstrates that the migration of syntaxin 1 with an S value higher than that predicted by its molecular mass (35 kD) is due, at least in part, to its interaction with other components of the 7-S synaptic SNARE complex (Söllner et al., 1993*b*). None of the immunoprecipitates, however, was significantly enriched in rbSecl. Parallel immunoprecipitations from the same fractions with two distinct polyclonal antibodies (EGM1 and EGM2) raised against rbSecl (Fig. 6 *B* and not shown) coprecipitated only a very small amount of syntaxin 1. Lack of substantial rbSecl-syntaxin 1 coprecipitation from fraction #12 confirms that rbSecl migrates as a monomeric protein and indicates that the similar sedimentation in the gradient of rbSecl and syntaxin 1 is not due to their association.

The inability to coprecipitate significant amounts of rbSecl/syntaxin 1 complexes may result from the inaccessibility of relevant rbSecl and syntaxin 1 epitopes in the heteromeric complex. Indeed, by performing immunoprecipitation experiments with recombinant rbSecl and syntaxin I proteins, we found that the epitope recognized by the anti-syntaxin 1 monoclonal antibody HPC-1 is masked in the rbSecl/syntaxin 1 complex. However, in contrast to HPC-1, both rbSecl antibodies used (EGM1 and EGM2) were found to coprecipitate an rbSecl/syntaxin 1 complex from a mix-

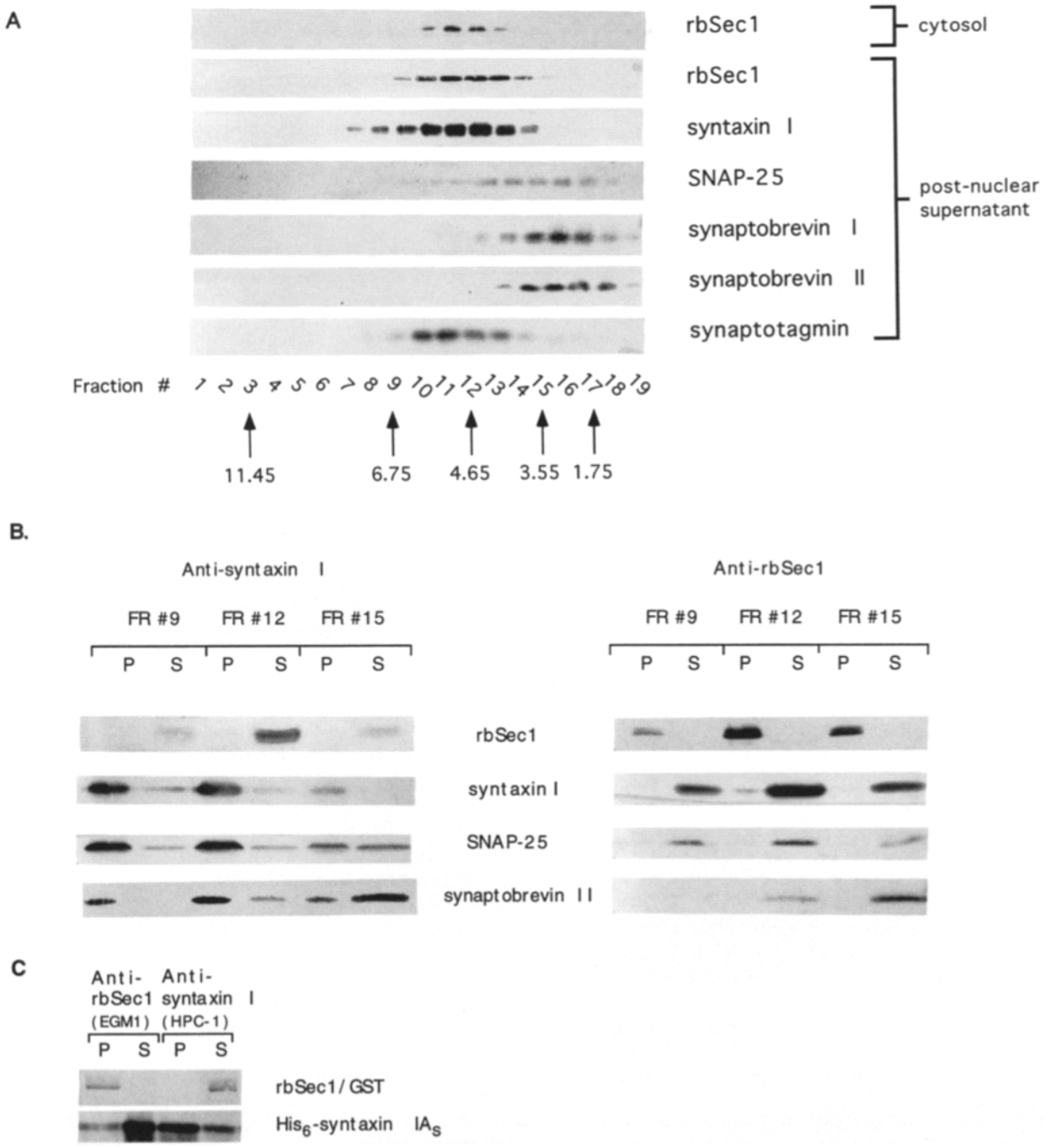

Figure 6. rbSecl behaves as a monomeric protein. (*A*) Brain cytosol and a Triton X-100 extact of a brain postnuclear supernatant were separated by velocity sedimentation on a sucrose gradient. Fractions were then analyzed by SDS-PAGE and Western blotting. Proteins used as markers for the gradients are as follows: cytochrome C (1.75 S; 29 kD), ovalbumin (3.55 S: 45 kD), bovine serum albumin (4.65 S; 68 kD), alcohol dehydrogenase (6.75 S; 150 kD), and tyroglobulin (11.45 S; 290 kD). The peak fraction for each of these markers is indicated. (*B*) Association of syntaxin 1 with SNAP-25 and synaptobrevin, but not with rbSecl. Anti-syntaxin (*HPC-1*) or anti-rbSecl (*EGMl*) antibodies were used to immunoprecipitate proteins from fractions 9, 12, and 15 of Fig. 6 *A*. The entire pellets (*P*) and supernatants (*S*) of the immunoprecipitates were analyzed by SDS-PAGE and Western blotting. (*C*) Demonstration that the epitope recognized by monoclonal antibody HPC-1, but not the epitope(s) recognized by EGM1 antibody, are masked in the rbSecl/syntaxin 1 complex. The two antibodies were used to immunoprecipitate the complex from a mixture of rbSecl/GST and histidine-tagged cytosolic portion of syntaxin IA (a.a. 1–265) referred to as syntaxin IA_s. Pellets (*P*) and supernatants (*S*) of the immunoprecipitates were analyzed by SDS-PAGE and Western blotting.

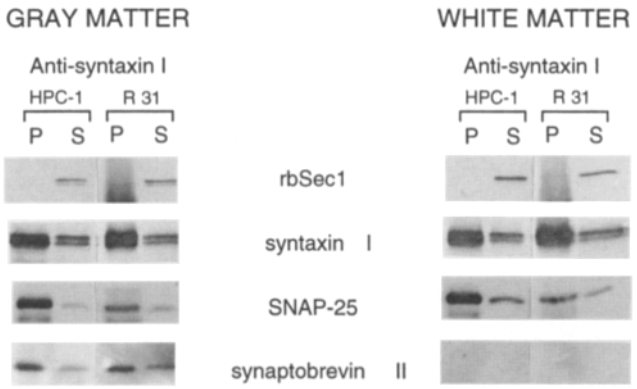

Figure 7. Association of syntaxin 1 with other SNAREs but not with rbSecl both in the gray and in the white matter of the brain. Triton X-100 solubilized gray or white matter extracts were immunoprecipitated with anti-syntaxin 1 antibodies (*HPC-1* or *R31*). Presence of relevant proteins in the pellets (*P*) and supernatants (*S*) was analyzed by SDS-PAGE and Western blotting. SNAP-25 and synaptobrevin are coprecipitated with syntaxin 1 from gray matter. SNAP-25 is also coprecipitated with syntaxin 1 from white matter which does not contain detectable amounts of synaptobrevin. rbSecl does not coprecipitate with syntaxin 1 from either the gray or white matter. Corresponding volumes of the pellet and supernatant were applied to the gel.

ture of the two recombinant proteins (Fig. 6 *C*). Two new anti-syntaxin 1 antibodies (78.2 [a monoclonal antibody] and R31 [a polyclonal antibody], both the kind gift of P. Hanson and R. Jahn) which coprecipitated a complex of the two recombinant proteins, also did not coprecipitate significant amounts of rbSecl/syntaxin 1 complexes from detergent solubilized brain extracts (not shown). Even these two new anti-syntaxin I antibodies did not coprecipitate significant amounts of rbSecl/syntaxin 1 complexes from detergent solubilized brain extracts (not shown). Finally, we investigated whether an rbSecl/syntaxin 1 complex could be preserved by solubilization in detergents other than Triton X-100. Immunoprecipitations from postnuclear supernatant extracts prepared in Triton X-100, CHAPS, or *n*-octylglucoside yielded similar results (not shown).

We also wished to rule out the possibility that rbSecl bound to syntaxin 1 could be artifactually displaced from syntaxin 1 by synaptobrevin after detergent solubilization. To this aim, immunoprecipitation experiments were performed from gray or white matter dissected from fresh bovine brain tissue. Gray matter is highly enriched in synapses and therefore, in synaptic vesicles and synaptobrevin. White matter contains axon tracts only and minimal levels of nerve terminal-specific proteins including synaptobrevin I and II. Fig. 7 shows that two different anti-syntaxin 1 antibodies (HPC-1 and R31) coprecipitated SNAP-25 from both gray and white matter, and synaptobrevin from the gray matter. However, neither anit-syntaxin 1 antibody coprecipitated substantial amounts of rbSecl from either gray or white matter. Similar results were obtained with anti-rbSecl antibodies (not shown).

The results discussed above do not support an interaction of rbSecl with the plasma membrane principally due to an interaction with syntaxin 1. The hydrophobic properties of rbSecl were therefore investigated using the Triton X-114 phase separation assay (Bordier, 1981). As shown by Fig. 8 *A*, a fraction of rbSecl present in a postnuclear supernatant and in the total particulate fraction derived from this supernatant, partitioned in the detergent phase, while all rbSecl present in a soluble cytosolic fraction was entirely recovered in the aqueous phase. Syntaxin 1 (an integral membrane protein) and synapsin I (a hydrophilic protein) (De Camilli et al., 1990) partitioned into the detergent and aqueous phase, respectively, confirming the validity of the separation procedure. These results suggest that posttranslational hydrophobic modifications may contribute to the membrane association of rbSecl. Accordingly, the pool of rbSecl recovered in membrane fractions could not be released by up to 1 M KCl, a condition which released a substantial amount of synapsin I—most likely all of the synapsin I not secluded in sealed compartments (Hüttner et al., 1983). rbSecl, however, was released from membranes by a carbonate wash at pH 11.5. The same treatment could not release a protein bound to membranes by a geranyl-geranyl tail such as rab3A (Fig. 8 *B*).

rbSecl Undergoes Alternative Splicing

In the Western blots shown rbSecl appears as a doublet. However, in a survey of the distribution of rbSecl in various tissues (Fig. 9 *A*) and brain regions (Fig. 9 *B*), we observed that the relative intensity of the two bands was variable. As shown in Fig. 9 *A*, rbSecl is detectable at a high level in brain and at lower levels in endocrine tissues and cell lines: pituitary, PC12 cells, and RINm5 cells (a cell line derived from the endocrine pancreas) (Gazdar et al., 1980), but not in skeletal muscle, heart, liver, kidney, lung, testis, or pancreas. The low proportion of endocrine cells in the latter tissue is probably insufficient to allow rbSecl detection (compare with Fig. 2) by Western blotting. In endocrine cells only the lower band was visible. The lower band was also the most abundant in whole brain and virtually the only protein detectable in striatum, hippocampus, olfactory bulb, and cerebral cortex (Fig. 9 *B*). The upper band was more intense in the brain stem, and in the cerebellum the two bands had a similar intensity. We considered the possibility that the doublet could represent alternatively spliced isoforms of rbSecl.

Of the three rbSecl clones we had originally isolated from a rat cDNA library (Garcia et al., 1994), two encoded an identical protein. One clone (#24) diverged after nucleotide +1702 and encoded an additional unique 45 nucleotide stretch that did not encode a stop codon. Therefore, this divergent clone could represent an alternatively spliced version of rbSecl. To determine whether the rbSecl doublet visible by Western blot analysis is represented by two rbSecl isoforms that differ at their COOH termini, we compared the reactivity of anti-rbSecl antibodies raised against a central portion of the protein (Garcia et al., 1994) or against its COOH terminus (Pevsner et al., 1994*a*). As shown in Fig. 9 *C*, the latter antibodies stained only the lower band of the doublet, confirming that the two bands do not share a common COOH terminus, but do share a central region.

We next used PCR primers designed to amplify a rbSecl cDNA fragment encoding the putative unique sequence at its 5′ end, and downstream untranslated sequences expected to be shared between the alternatively spliced isoforms of rbSecl. We obtained a PCR fragment that encodes at its 5′ end the unique sequence of clone #24 followed by a short

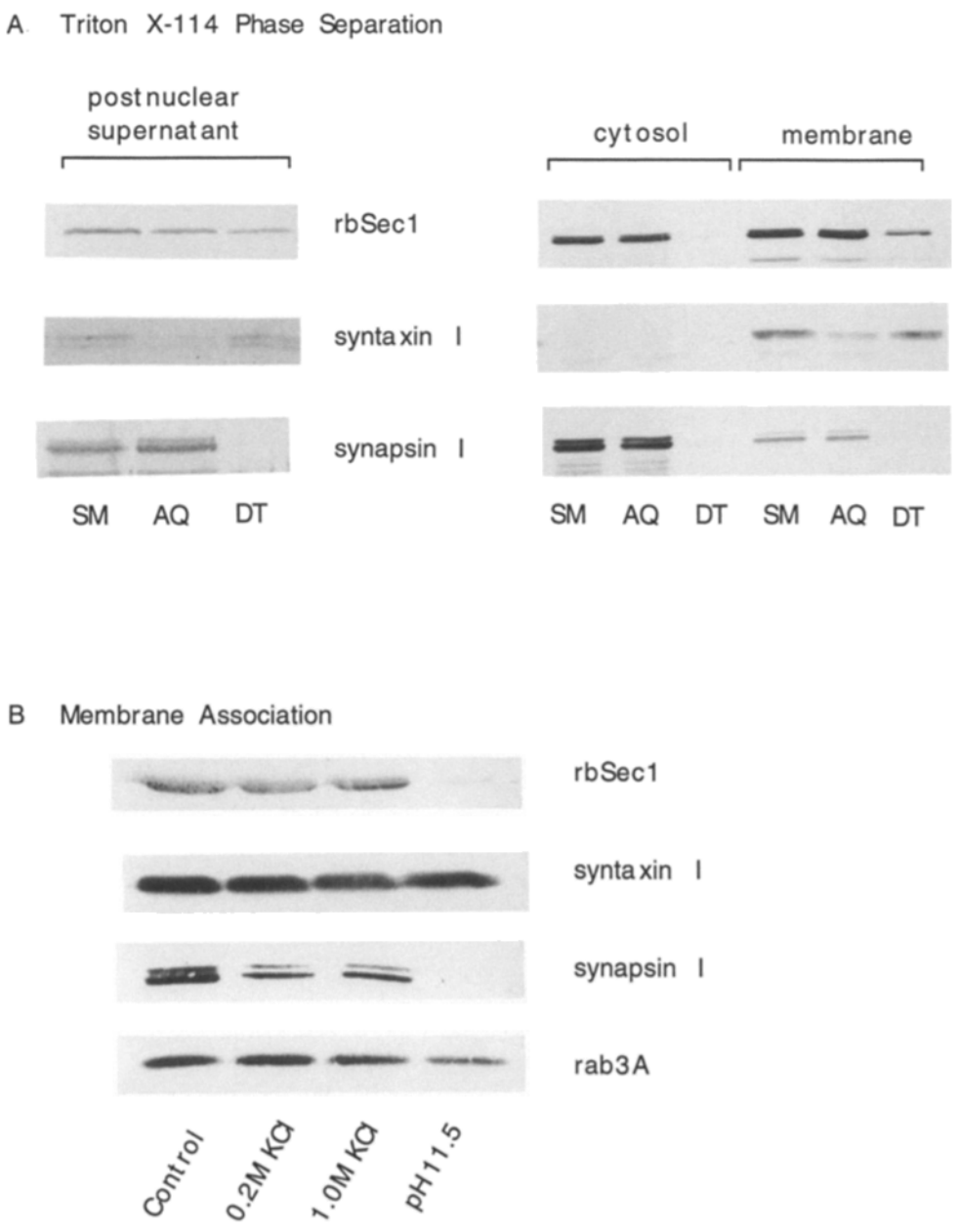

Figure 8. Membrane association of rbSecl. (*A*) Triton X-114 phase separation of rat brain postnuclear supernatant, cytosol, or membrane fraction is shown. Corresponding volumes of each fraction (*SM*, starting material; *AQ*, aqueous phase; *DT*, detergent phase) were separated by SDS-PAGE and analyzed by Western blotting. (*B*) Total particulate fractions of rat brain homogenate resuspended in 10 mM Hepes (*control*) were extracted with the indicated treatments. Nonextracted material was pelleted and analyzed by SDS-PAGE and Western blotting.

novel sequence including a termination codon and finally by the sequence of the previously cloned rbSecl (referred to henceforth as rbSeclA). We conclude that clone #24 encodes sequences of an alternatively spliced form of rbSecl which we call rbSeclB. The predicted amino acid sequence of each of the rbSecl isoforms and the corresponding nucleotide sequence is shown in Fig. 10, *A* and *B*, respectively. Note that the amino acid sequence of the two COOH termini is very similar, in spite of a substantial difference of the corresponding nucleotide sequence. The isoforms are encoded by two different mRNAs which differ due to the inclusion (rbSeclB) or exclusion (rbSeclA) of a nucleotide sequence of 110 base pairs inserted at position +1702 of the nucleotide sequence of rbSeclA (Fig. 10 *C*). Thus, the nucleotide sequence which encodes the COOH terminus of rbSeclA, is part of the 3′ untraslated sequence (and out of frame) in the rbSeclB mRNA. rbSeclA and B encode proteins of 594 amino acids (67.5 kD) and 603 amino acids (68.4 kD), respectively. The predicted electrophoretic migration of rbSeclA and 1B on SDS-PAGE is consistent with that observed.

As recombinant rbSeclA has been shown to bind syntaxin 1 in vitro (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994*a*; Hodel et al., 1994), we determined whether rbSeclB shared this property. A fusion protein containing GST fused to the entire coding sequences of rbSeclA or rbSeclB was constructed. Recombinant rbSeclA or rbSeclB (100 μg) immobilized on glutathione-Sepharose 4B beads were incubated with Triton X-100–solubilized brain postnuclear supernatant. Proteins specifically bound to the beads were then eluted with glutathione and analyzed by SDS-PAGE and Western blotting. As shown in Fig. 11, both rbSeclA and rbSeclB specifically bound syntaxin 1A and B, but not SNAP-25 or synaptobrevin I/VAMP I as detectable by Western blot analysis.

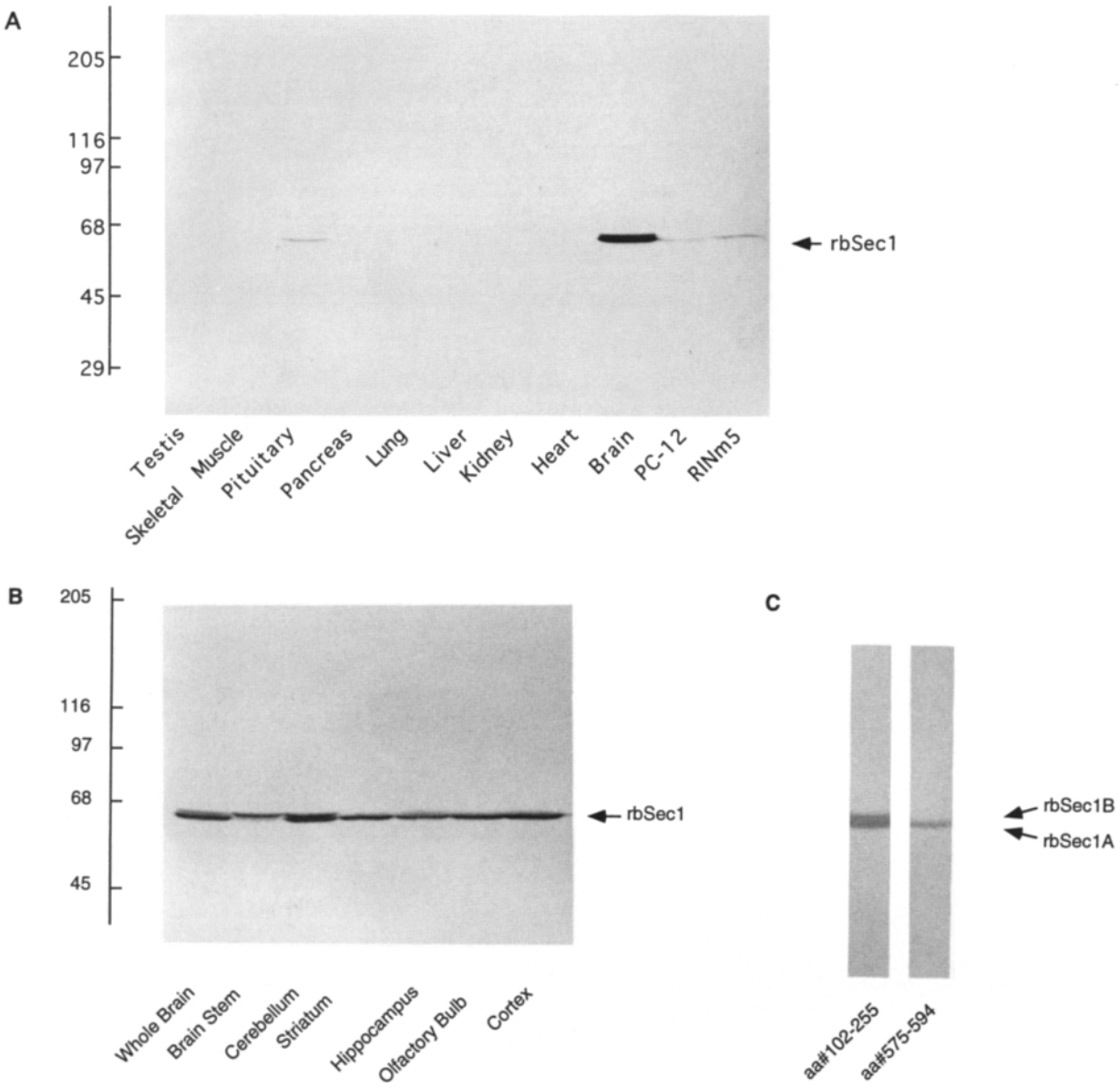

Figure 9. Tissue distribution of rbSecl. Postnuclear supernatants (200 μg) of total brain, brain regions, nonneuronal tissues, and cell lines were analyzed by SDS-PAGE and Western blotting using affinity-purified anti-rbSecl antibody (EGM1; *A*, *B*, and *C*), or anti-rbSecl directed against aa #574-594 (*C*). (*A*) rbSecl is most abundant in brain, but is also present in endocrine tissues and cell lines. (*B*) rbSecl immunoreactivity migrates as a doublet that is differentially expressed in various brain regions. (*C*) Antibodies raised to aa #574-594 of the known rbSecl protein detects only the faster migrating band. Molecular weights (kD) are indicated.

Discussion

Based on genetic and biochemical studies, rbSecl is thought to be involved in synaptic vesicle exocytosis via an interaction with syntaxin 1. We show here that rbSecl and syntaxin 1 are localized in the same regions of the nerve cell, but that the bulk of the two proteins are not associated with each other in situ. We demonstrate that rbSecl has a widespread distribution in the cytoplasm of neurons and endocrine cells. It is present at high concentration throughout the axon and at lower concentration in perikarya and dendrites. The localization of rbSecl throughout the axon parallels the localization of syntaxin 1 and SNAP-25 along the entire axolemma. Consistent with its primary sequence, which does not include any putative transmembrane region, a large fraction of rbSecl is localized in the cytosolic matrix. However, a pool of rbSecl is membrane bound, cofractionates with plasma membrane markers, and can be localized to the plasmalemma by immunoelectron microscopy. Our study does not support the presence of a substantial pool of rbSecl on either synaptic vesicles or secretory granules of chromaffin cells.

Our data argue against a binding of rbSecl to the plasma membrane mediated by syntaxin 1. First, membrane-bound rbSecl could not be solubilized by 1 M KCl, a treatment which is known to disrupt the binding of rbSecl to syntaxin 1 (our unpublished observations). Second, we did not find any evidence for the existence of a significant pool of rbSecl bound to syntaxin 1 in situ. Triton X-100 solubilized mem-

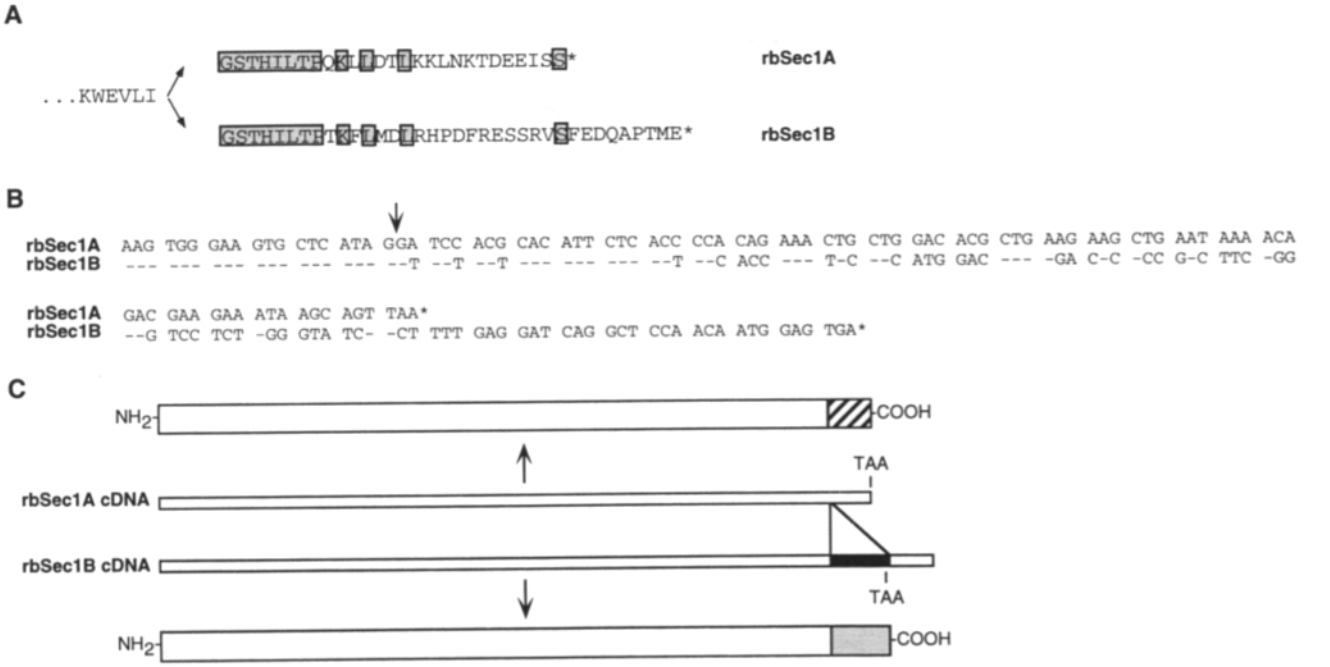

Figure 10. rbSecl is alternatively spliced. (*A*) A comparison of the deduced amino acid sequences of the COOH termini of rbSeclA and rbSeclB are shown starting from aa +561 of rbSecl (Garcia et al., 1994). Shaded boxes indicate amino acids identical in the two different COOH termini. (*B*) cDNA nucleotide sequence of rbSeclA and 1B starting from nucleotide +1684 (which corresponds to aa +561 in rbSeclA). The putative splice junction is indicated by an arrow (+1702). These sequence data are available from EMBL/GenBank/DDBJ under accession number U06069 for rbSeclA, and V21116 for rbSeclB. (*C*) Schematic drawing depicting the structure of the rbSeclA and rbSeclB cDNAs and of the corresponding proteins. rbSeclA cDNA results from the removal of a 110-nucleotide exon from the rbSeclB cDNA.

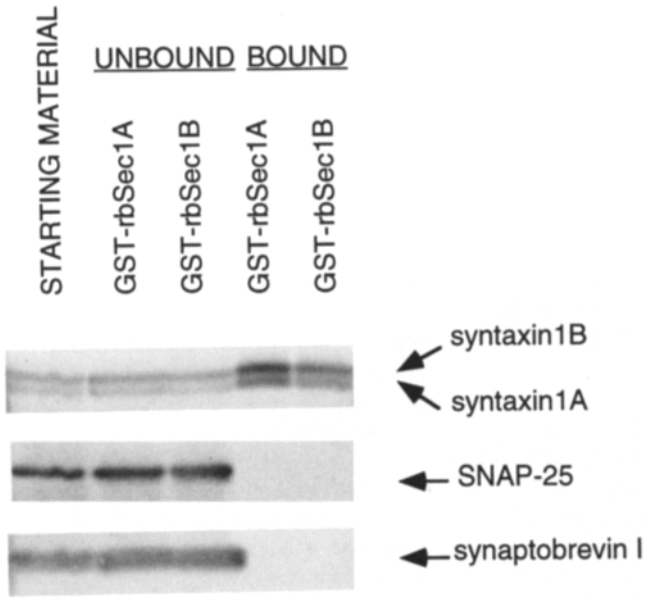

Figure 11. Both rbSeclA and 1B bind syntaxin 1 but not SNAP-25 or synaptobrevin I. Recombinant rbSeclA or rbSeclB fused to GST (100 μg) were bound to glutathione-agarose beads. Triton X-100 solubilized rat brain extracts (500 μg) were mixed with the beads and washed extensively. Bound proteins were eluted from the beads with 5 mM glutathione and analyzed by SDS-PAGE and Western blotting.

brane-bound rbSecl migrated identically to soluble rbSecl with the molecular mass expected for the monomer. This finding also rules out the possibility that rbSecl may be bound in situ to a syntaxin isoform distinct from syntaxin 1, the major neuronal isoform (Bennett et al., 1993).

Additionally, using a battery of antibodies directed against different regions of rbSecl and syntaxin 1, we did not observe substantial coprecipitation of the two proteins from membrane extracts prepared in Triton X-100, CHAPS, or *n*-octylglucoside. These negative results cannot be explained by a sensitivity of the rbSecl-syntaxin 1 interaction to the detergent/salt conditions used for immunoprecipitation as the two proteins bind to each other in vitro under similar conditions. Pevsner et al. (1994*b*) reported that anti-rbSecl (n-Secl) antibodies coprecipitate syntaxin 1 from solubilized brain LP_2 fractions. Using the same starting material and conditions, we coprecipitated only small amounts of two proteins using a variety of anti-syntaxin 1 and anti-rbSecl antibodies. It is possible that even in the experiments reported by Pevsner et al. (1994*b*) coprecipitation may involve only a small fraction of the two proteins, since the relative proportion of the two proteins in the precipitate was not reported.

It has been shown that rbSecl binds to syntaxin 1 in a manner exclusive of the other SNAREs. (Garcia et al., 1994; Pevsner et al., 1994*a*). It is possible that a rbSecl/syntaxin 1 complex present in situ might be disrupted after detergent solubilization by competition for the rbSecl binding site on syntaxin 1. This is not the case for synaptobrevin as rbSecl

did not coprecipitate with syntaxin 1 even in immunoprecipitations carried out on extracts of white matter, i.e., a brain tissue which contains axon tracts only, and therefore extremely low levels of synaptobrevin. Furthermore, the far greater affinity of syntaxin 1 for rbSec1 (Pevsner et al., 1994*b*) than for synaptobrevin (Calakos et al., 1994) speaks against the possibility that synaptobrevin may displace rbSec1 from syntaxin 1 after detergent solubilization.

A large pool of SNAP-25 coprecipitates with syntaxin 1 from extracts of both gray and white matter, it is unlikely that this reflects an interaction which occurs after solubilization. The two proteins are highly colocalized in situ. In addition, studies in yeast have shown that substantial fractions of the yeast homologues of SNAP-25 and syntaxin 1 (Sec9 and the Sso proteins, respectively), can be cross-linked in situ before homogenization in a complex which does not include the Snc proteins (the yeast homologues of synaptobrevin) (Brennwald et al., 1994). Finally, immunoprecipitation experiments from both the gray and the white matter demonstrate the presence of a pool of syntaxin 1 not bound to SNAP-25.

Given the very high affinity of rbSec1 for syntaxin 1 (Pevsner et al., 1994*b*), the failure to demonstrate a rbSec1/syntaxin 1 complex after detergent solubilization is striking and suggests that this interaction is highly regulated in vivo. Our observation that particulate rbSec1 cannot be solubilized by 1 M KCl, and that a fraction of particulate rbSec1 partitions in the detergent phase after Triton X114 detergent phase separation are consistent with an association of rbSec1 with the plasmalemma mediated at least in part by a hydrophobic modification.

Sögaard et al. (1994) reported that Sly1 can be coprecipitated by anti-Sed5 antibodies from yeast extracts. Sly1 and Sed5 are the yeast members of the Sec1 and syntaxin families, respectively, which participate in vesicular transport from the ER to the Golgi complex. The difference between Sly1 and rbSec1 may be related to the involvement of Sly1 in a constitutive transport step and of rbSec1 in a highly regulated vesicle fusion reaction.

rbSec1 Is Alternatively Spliced

We report here that rbSec1 as previously described (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994*a*) represents only one of two isoforms of rbSec1 which are produced by alternative splicing of a single gene. The two isoforms which we refer to as rbSec1A and B, differ at their COOH termini. A peculiar feature of the two COOH termini is their similar amino acid sequence in spite of substantial divergence at the nucleotide level. This observation suggests that the two COOH termini originate from exon duplication and that there has been strong evolutionary pressure to conserve some of the amino acids. rbSec1A and 1B behave similarly in all biochemical assays performed in this study but have different cellular distributions. rbSec1B is expressed at higher levels relative to rbSec1A in regions that are evolutionarily older. Additionally, rbSec1A is also expressed in peptide-secreting endocrine cells (Hodel et al., 1994, and this study). Immunofluorescent staining of rat brain sections performed with antibodies which recognize both rbSec1 isoforms (EGM1) and antibodies specific for rbSec1A (directed to the COOH terminus of rbSec1A) (Pevsner et al., 1994*a*) confirmed the different cellular localization of the two proteins but suggested a similar intracellular distribution (data not shown). Upon subcellular fractionation, rbSec1B appeared to be more enriched in the soluble fraction of synaptosomes (LS_2 fraction) than rbSec1A. The possible significance of these observations is being further investigated.

Functional Considerations

What are the implications of these data towards the elucidation of the function of Sec1? A role of Sec1 and its homologous proteins in exocytosis is strongly suggested by genetic studies in yeast, *C. elegans* and *Drosophila* (Novick et al., 1981; Aalto et al., 1993; Genyo-Ando et al., 1993; Harrison et al., 1994). The direct interaction of rbSec1 with syntaxin 1 demonstrated by binding assays using recombinant proteins (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994*a*; Hodel et al., 1994) suggests that Sec1 is involved in the function of the SNARE complex. However, rbSec1 is not part of the synaptic 7-S SNARE complex or of the syntaxin 1/SNAP-25 complex which we show to be present along the entire axonal plasmalemma. In fact, the interaction of rbSec1 with syntaxin 1 is mutually exclusive of the interaction of syntaxin 1 with SNAP-25 and synaptobrevin (Garcia et al., 1994; Pevsner et al., 1994*a*,*b*). While this finding would be consistent with a possible role of rbSec1 after fusion, the genetic interaction between proteins of the Rab and Sec1 families (Ossig et al., 1991; Dascher et al., 1991) suggest rbSec1 acts before docking and fusion. Rab proteins are postulated to promote and facilitate the formation of the fusion complex or make vesicular carriers competent for fusion with target membranes (Novick and Brennwald, 1993; Nuoffer and Balch, 1994; Geppert et al., 1994; Sögaard et al., 1994).

The mutually exclusive interaction of rbSec1 and other SNAREs with syntaxin 1, raises the possibility that rbSec1 may act to negatively regulate the formation of the synaptic SNARE fusion complex through a stable association with syntaxin 1. For example, rbSec1 binding to syntaxin 1 at nonsynaptic regions of the plasmalemma may prevent fusion of synaptic vesicles away from active zones. Our study, which demonstrates the lack of substantial pools of syntaxin 1/rbSec1 complex in situ, speaks against such a hypothesis. This conclusion is also in agreement with the demonstration that overexpression of yeast syntaxin (the Sso proteins) suppresses the secretory defect of a temperature sensitive *sec1-1* mutant, but not the *sec1* null phenotype (Aalto et al., 1993).

Our data are consistent with a scenario in which rbSec1 transiently interacts with syntaxin 1 to produce a change in this molecule which then allows it to interact with downstream effectors. One finding which suggests a highly regulated interaction of rbSec1 with syntaxin 1 was reported by Hodel et al. (1994). These investigators found that in homogenates from bovine adrenal medulla only the rbSec1 pool present in a secretory granule fraction was competent to bind to immobilized recombinant syntaxin 1.

The presence of high concentrations of rbSec1, SNAP-25, syntaxin 1, and SNAP-25/syntaxin 1 complexes throughout the axon raises the question as to whether the main function of these three proteins is to participate in synaptic vesicle exocytosis, a process which occurs selectively in nerve terminals. For many neurons, the area of the axonal plasmalemma involved in synaptic vesicle exocytosis represents

an extremely small fraction of the entire plasmalemma. rbSec1, syntaxin 1, and SNAP-25 are also present in axonal segments surrounded by myelin. These areas are devoid of any type of vesicle (Pappas and Waxman, 1972) suggesting the absence of exocytotic events. In yeast, the Sso and Sec9 proteins are localized along the entire plasmalemma and not only at the tips of the bud, where the bulk of exocytosis occurs (Brennwald et al., 1994). The localization of t-SNARE's throughout the plasmalemma and of at least some of their accessory proteins throughout the cell may allow rapid plastic changes when vesicle exocytosis must be redirected from one site to another of the cell surface. One example involves axon sprouting which takes place proximal to an axon cut. t-SNAREs may have a general distribution in a given membrane in order to define its identity, i.e., they serve as landmarks to guide membrane-vesicle interactions. This identity is crucial to maintaining the fundamental structural organization of an eukaryotic cell. Additional mechanisms, superimposed on this basic membrane recognition code, may define where fusion must occur and help to establish the specific identity of a highly differentiated cell. One cannot exclude the possibility, however, that the localization of rbSec1, syntaxin 1, and SNAP-25 throughout the axon may reflect other functions of these molecules, in addition to their participation in the formation of SNARE fusion complexes.

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