Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 9613–9617, October 1995
Neurobiology

rSec6 and rSec8, mammalian homologs of yeast proteins essential for secretion

(neurotransmission/synaptic vesicle/membrane trafficking)

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Communicated by W. Maxwell Cowan, Howard Hughes Medical Institute, Chevy Chase, MD, July 18, 1995

ABSTRACT **Many of the molecules necessary for neurotransmission are homologous to proteins involved in the Golgi-to-plasma membrane stage of the yeast secretory pathway. Of 15 genes known to be essential for the later stages of vesicle trafficking in yeast, 7 have no identified mammalian homologs. These include the yeast *SEC6, SEC8*, and *SEC15* genes, whose products are constituents of a 19.5S particle that interacts with the GTP-binding protein Sec4p. Here we report the sequences of rSec6 and rSec8, rat homologs of Sec6p and Sec8p. The rSec6 cDNA is predicted to encode an 87-kDa protein with 22% amino acid identity to Sec6p, and the rSec8 cDNA is predicted to encode a 110-kDa protein which is 20% identical to Sec8p. Northern blot analysis indicates that rSec6 and rSec8 are expressed in similar tissues. Immunodetection reveals that rSec8 is part of a soluble 17S particle in brain. COS cell cotransfection studies demonstrate that rSec8 colocalizes with the GTP-binding protein Rab3a and syntaxin 1a, two proteins involved in synaptic vesicle docking and fusion at the presynaptic terminal. These data suggest that rSec8 is a component of a high molecular weight complex which may participate in the regulation of vesicle docking and fusion in brain.**

Chemical neurotransmission, a process by which neurons communicate with target cells, is mediated by quantal release of neurotransmitter from the nerve terminal. The cellular organelle containing a quantum of neurotransmitter substance is the synaptic vesicle. Because of its primary importance in the function of neurons, the life cycle of the synaptic vesicle has been intensely studied (for reviews, see refs. 1 and 2). Synaptic vesicles are located in the nerve terminal, where they associate with the cytoskeleton and soluble factors. After synaptic vesicles are loaded with neurotransmitter, they are targeted to the plasma membrane, where they dock at active zones. Upon entry of Ca^{2+}, synaptic vesicles fuse with the plasma membrane, rapidly culminating in the release of neurotransmitter. Though much research has elaborated the life cycle of the synaptic vesicle, progress in understanding the molecular mechanisms underlying these events has only just begun.

Proteins important in regulation of various synaptic vesicle functions have been purified and characterized (3, 4). This approach has led to insights into molecular mechanisms of secretion of neurotransmitter and detailed hypotheses regarding the function of individual proteins (5). For example, vesicles are proposed to recognize target membranes via the association of proteins specific to each membrane. The assembly and disassembly of protein complexes consisting of proteins located on the synaptic vesicle (VAMP and synaptotagmin), on the plasma membrane (syntaxin 1a and SNAP-25), and in cytosol (NSF and αSNAP) are thought to mediate docking and fusion steps in the synaptic vesicle life cycle (6, 7). Targeting of synaptic vesicles to their correct acceptor membranes, a step preceding docking and fusion, may involve another synaptic vesicle protein, the low molecular weight GTPase Rab3a (8, 9). However, insight into the role of Rab3a in the synaptic vesicle life cycle remains elusive.

The number of identified proteins that play important roles in the regulated docking and fusion of synaptic vesicles in the presynaptic terminal is increasing. Biochemical studies demonstrate that many of these proteins interact with one another (10), as shown in Fig. 1 *Right*. Interestingly, these proteins are homologous to yeast proteins which have been shown by genetic studies to be essential for the later stages of yeast constitutive secretion (5, 13, 14). For example, Sec4p, a low molecular weight GTPase crucial for late stages of vesicle secretion in yeast, is homologous to Rab3a. This homology suggests that characterization of Sec4p interactions with other proteins will provide insight into the function of Rab3a in the nerve terminal. However, comparison of proteins involved in the later stages of yeast secretory pathway with those required for mammalian synaptic vesicle docking and fusion reveals a number of yeast secretory proteins which have no known mammalian homologs. These proteins include Sec6p (15) and Sec8p (12), which are components of a 19.5S particle that interacts with Sec4p (12). The absence of mammalian homologs to these proteins suggests that (*i*) only a portion of the yeast secretory mechanism is conserved in mammals or (*ii*) mammalian homologs of these yeast genes have yet to be identified and shown to function in secretion. To further characterize the vesicular trafficking pathway in mammalian cells, we isolated the cDNAs encoding proteins homologous to Sec6p and Sec8p from rat brain libraries and have begun their characterization.§

MATERIALS AND METHODS

Cloning and Sequence Analysis of rSec6 and rSec8 cDNAs. Two oligonucleotides corresponding to nt 1–25 (5′-GAAATTATAAGGAAGTACGTCCTGG-3′) and 293–314 (5′-ATGTACGTGTCAAGCAGCTCAG-3′) of a human infant brain expressed sequence tag (EST) cDNA (Genbank accession no. Z46204) were synthesized for rSec6 screening. Two oligonucleotides corresponding to nt 1–20 (5′-TGCCATTGTGGCTAATGTGG-3′) and 301–320 (5′-CTGTGTTGTAAAGCATCTCG-3′) of the human fetal brain EST 56101 cDNA were generated for rSec8 screening. By using a human brain cDNA library (Clontech) and the EST 56101 cDNA as templates,

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Abbreviation: EST, expressed sequence tag.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U32498 and U32575).

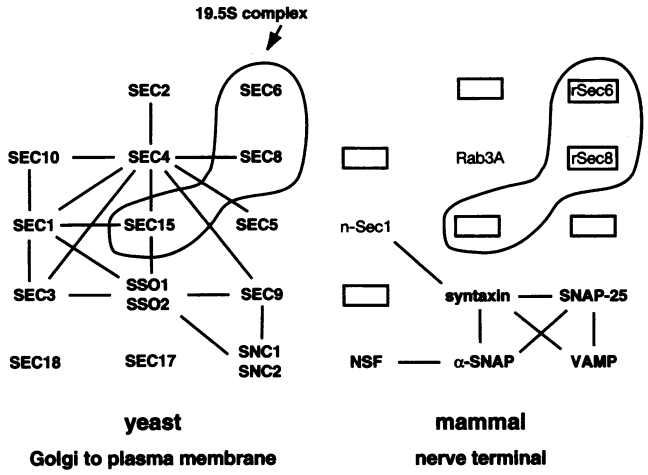

FIG. 1. Comparison of proteins required for yeast constitutive secretion and mammalian regulated neural secretion. Genes essential for the later stages of the yeast secretory pathway (*Left*) and their corresponding mammalian homologs (*Right*) are depicted. The lines between yeast genes indicate genetic interactions identified in suppression studies (11). The lines between mammalian proteins indicate interactions identified in biochemical studies (10). Genes (*SEC6, SEC8*, and *SEC15*) encoding components of a soluble 19.5S particle isolated in yeast are circled (12).

these two sets of primers were used to produce either a 314-bp or a 320-bp DNA fragment by PCR as described (16) and used as probes to screen several rat brain cDNA libraries (Clontech and Stratagene) for rSec6 and rSec8, respectively. The consensus sequence for rSec6 has an open reading frame of 2265 nt that predicts a 755-aa hydrophilic protein of 87 kDa and a pI of 6.1. The rSec8 cDNA has an open reading frame of 2925 nt that predicts a 975-aa hydrophilic protein of 110 kDa and a pI of 6.5

Northern Blot Analysis of rSec6 and rSec8 mRNA Expression. A 1221-bp rSec6 cDNA fragment (nt 1049–2269) and an 844-bp rSec8 cDNA fragment (nt 17–860) were radiolabeled by random hexamer priming and were used to probe RNA blots containing 2 μg of poly(A)$^+$ RNA from various rat tissues (Clontech).

Antiserum Production and Western Blot Analysis. A 3′ Myc-tagged rSec8 was constructed by replacing the 3′ end of the full-length rSec8 cDNA in a pBluescript II SK vector (Stratagene) with a 390-bp DNA *Nco* I–*Cla* I fragment encoding the Myc epitope QKLISEEDL. The 390-bp fragment was generated by PCR using oligonucleotides that correspond to nt 2219–2236 and 2907–2925 of rSec8 (5′-CTGCTCAAGAGAGCCACG-3′ and 5′-GGATCGATCAGGTCCTCTTCGCTGATCAGTTTCTGCTCCACAGTGGTTATTTTCTTGTCC-3′) and was digested with *Nco* I and *Cla* I. The Myc-tagged rSec8 construct was then subcloned into the histidine-tag expression vector pQE9 (Pharmacia) at *Hin*dIII (blunt-ended) and *Bam*HI sites to generate a 5′ histidine-tagged and 3′ Myc-tagged rSec8 construct. The histidine-tagged rSec8 fusion protein was expressed in the M15 strain of *Escherichia coli* (Pharmacia). Polyacrylamide gel slices containing rSec8 fusion protein were injected into rabbits to generate polyclonal antisera. Antibodies against rSec8 were affinity purified by incubating the crude sera with histidine-tagged rSec8 fusion protein electrophoresed onto nitrocellulose paper and eluting the bound antibodies with 0.1 M glycine (pH 2.5). For regional Western blot analysis, a rat was decapitated and the tissues were homogenized in 3 volumes (vol/wt) of 20 mM Hepes, pH 7.4/0.32 M sucrose/1 mM EDTA containing pepstatin, leupeptin, aprotinin, and phenylmethanesulfonyl fluoride each at 1 μg/ml. The homogenates were centrifuged at 1000 × *g* to obtain postnuclear supernatants.

Glycerol Gradient Centrifugation Analysis of Brain rSec8 Complex. Rat brain supernatant was prepared by homogenization in 6 ml of 40 mM Hepes, pH 7.4/150 mM NaCl/10% (vol/vol) glycerol/1 mM EDTA/1 mM dithiothreitol containing leupeptin, aprotinin, pepstatin, and phenylmethanesulfonyl fluoride each at 1 μg/ml. The homogenate was centrifuged at 1000 × *g* for 5 min and the supernatant was centrifuged at 100,000 × *g* for 20 min. Eighty microliters (40 μg of protein) of the resulting supernatant was layered onto a 1.2-ml ten-step 22.5–36% (vol/vol) glycerol gradient in 40 mM Hepes, pH 7.4/150 mM NaCl/1 mM dithiothreitol. The gradient was centrifuged at 91,000 × *g* for 16 hr at 4°C. Fractions (95 μl) were collected and analyzed by SDS/PAGE and Western blotting. Migration of brain rSec8 and n-Sec1 was monitored by Western blotting using affinity-purified antibodies against rSec8 and n-Sec1 (16). Na^+,K^+-ATPase, a plasma membrane marker, and SV2, a synaptic vesicle membrane protein, were detected in the 5S region and the pellet fractions of the glycerol gradients, respectively. These data suggest that rSec8 present in the 17S region was not associated with these membranes.

Microscopic Localization of rSec8. A *Bam*HI–*Cla* I fragment of Myc-rSec8 DNA was subcloned into the expression vector pCMV and used to study the localization of rSec8 in COS cells (17). A Rab3a pCMV construct was generated by subcloning a *Hin*dIII–*Sma* I (blunt-ended) restriction fragment from a Rab3a pBluescript II KS construct into pCMV.

RESULTS

Cloning and Sequence Analysis of Mammalian Homologs of *SEC6* and *SEC8*. The yeast Sec6p and Sec8p sequences were compared with GenBank and a data base of human EST cDNAs generated by the Institute for Genomic Research (18, 19). One human infant brain EST cDNA was found to have 24% identity over 104 aa to Sec6p and a second human fetal brain EST cDNA was discovered that had 24% identity over 129 aa to the C terminus of Sec8p. Based on the sequence of these clones, DNA probes were generated to screen rat brain cDNA libraries. The cDNAs identified as rat brain homologs of Sec6p (rSec6) and Sec8p (rSec8) encode hydrophilic proteins of 87 and 110 kDa, respectively (Fig. 2). Comparisons of the predicted amino acid sequences of full-length rSec6 and rSec8 to their respective yeast homologs revealed 22% and 20% identity and probability values of 5.3×10^{-12} and 1.8×10^{-17}, respectively, as determined by BLASTP analysis of GenBank. These identities occurred over the entire length of both proteins (Fig. 2). When randomized sequences of the rSec6 and rSec8 proteins were compared with the yeast proteins of Sec6p and Sec8p by BESTFIT analysis, the quality scores of the nonrandomized rSec6 and rSec8 protein sequences were 8.9 and 13.7 standard deviations above the average quality score of randomized sequences. Thus, the identities between rat and yeast sequences are significant and indicate an ancestral relationship between these proteins. Further, the homology between the mammalian and yeast forms of these proteins is consistent with previously identified mammalian homologs to Sec proteins. For example, n-Sec1 is 26% identical to yeast Sec1p over its entire length (16). This identity is attained with the incorporation of gaps similar in number to the amount of gaps present in rSec6 and rSec8 comparisons to their respective yeast homologs. The rSec6 protein also has 24% identity to a protein encoded by the mouse primary response gene B94, which has been proposed to be involved in fusion of sperm acrosomal and plasma membranes (22, 23).

The rSec6 and rSec8 protein sequences contain regions with a high probability of forming coiled-coiled domains (Fig. 2). This structural motif is frequently observed in vesicle trafficking proteins and is required for their interactions with other proteins (24). Since the coiled-coil motif has been shown to participate in many heterotypic and homotypic interactions

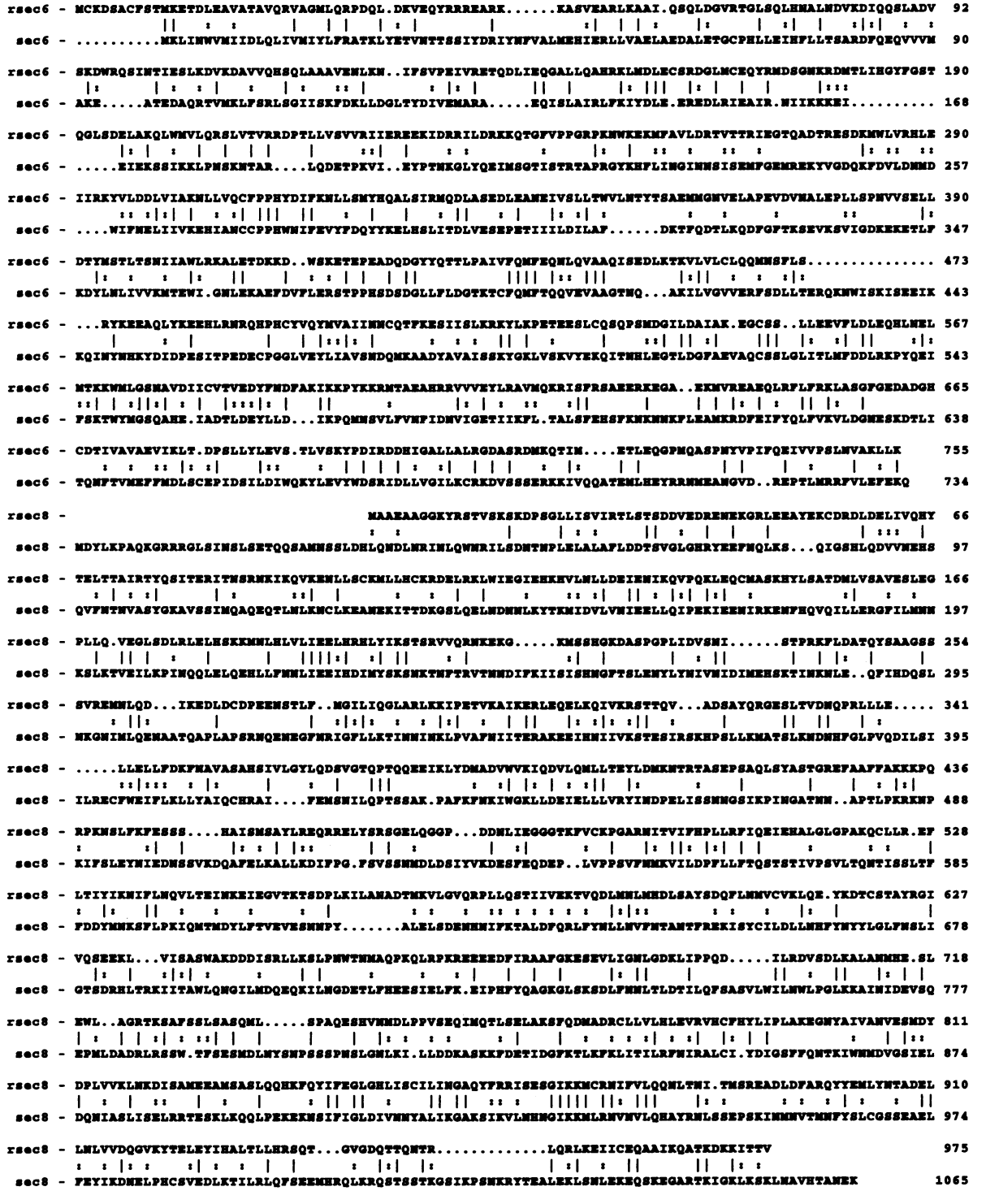

FIG. 2. Comparison of rSec6 and rSec8 protein sequences with their yeast homologs. Solid lines indicate identical amino acids and dashed lines indicate conservative amino acid substitutions (L, I, V, M, F, and W; S and T; K and R; Q and N; D and E). Analysis of the rSec6 and rSec8 protein sequences with the program NEWCOILS (21) reveals that both proteins have a possible coiled-coil region at the N terminus aa 97–171 for rSec6 and aa 34–99 (with a break in the middle due to a tyrosine residue at aa 66) for rSec8. For the yeast protein Sec8p, there is also a predicted coiled-coil region at the N terminus; aa 117–159.

(25, 26), conservation of this motif between rSec6 and rSec8 and their yeast homologs further suggests that these regions are essential for important protein–protein interactions. In addition, yeast Sec8p has a 202-aa leucine-rich region near its N terminus that is 25% identical to the leucine-rich *RAS*-responsive domain of yeast adenylate cyclase (12). This region has been postulated to interact with the GTP-binding protein Sec4p. Correspondingly, rSec8 also contains a leucine-rich region near its N terminus, aa 23–200. This region may interact with a vesicle-associated GTP-binding protein such as Rab3a.

Regional Distribution of rSec6 and rSec8 mRNAs and rSec8 Protein. The proteins involved in synaptic vesicle trafficking vary in their tissue distribution. Several are brain-specific, whereas others are expressed in all tissues (1). Northern blot analyses showed rSec6 and rSec8 message sizes of 2.9 and 5.4 kb, respectively (Fig. 3). Both messages were present in all the

tissues examined, with higher amounts found in brain, lung, muscle, kidney, and testis. This similarity in tissue distribution suggests that rSec6 and rSec8 function in a variety of secretory pathways and may also be in association with one another.

An affinity-purified polyclonal antibody generated against rSec8 recognized an immunoreactive band of ≈110 kDa in brain tissue, the molecular size of rSec8 predicted from its cDNA sequence (Fig. 4*A*). Analysis of several rat tissues revealed rSec8 protein in all the tissues examined, with higher amounts observed in brain, kidney, testis, and pancreas (Fig. 4*B*). This broad tissue distribution of rSec8 was consistent with the results of the mRNA analysis.

rSec8 Is a Component of a High Molecular Weight Complex. As a first step to determining the function of rSec8, we investigated the intracellular distribution of rSec8 and its interactions with cellular proteins and membranes. The intracellular distribution of rSec8 was analyzed both by cell fractionation studies and by microscopic examination of COS cells transfected with Myc-tagged rSec8. The transfected rSec8 was present both in a membrane-associated fraction containing the plasma membrane marker Na^+,K^+-ATPase and in the supernatant after centrifugation at 10,000 × *g*. To determine whether rSec8 was present as a high molecular weight complex in brain, soluble brain extracts were separated on a linear glycerol gradient and rSec8 was detected by immunoreactivity. rSec8 peaked around 17 S, indicating that endogenous rSec8 is part of a high molecular weight complex in brain. In contrast, the neural-specific protein n-Sec1 did not migrate as a high molecular weight complex under similar gradient conditions (Fig. 4*C*).

Immunolocalization of rSec8 in COS Cells. In agreement with the cell fractionation studies, immunofluorescence studies of the rSec8-transfected COS cells revealed rSec8 both in the cytoplasm and associated with cellular membranes, including the plasma membrane (Fig. 5 *A* and *C*). Cotransfection of COS cells with rSec8 and Rab3a or syntaxin 1a revealed that rSec8 colocalized with Rab3a or syntaxin 1a in many regions of the cell (Fig. 5). In particular, the colocalization was notably visible at patches along the plasma membrane (Fig. 5, arrowheads). These patches may represent plasma membrane ruffles, regions of increased membrane activity. In contrast, the lectin concanavalin A stained the plasma membrane evenly and did not accumulate at plasma membrane ruffles. This implies that the similar staining patterns between rSec8 and Rab3a or syntaxin 1a may represent specific associations, thus further suggesting a role for rSec8 in the synaptic vesicle trafficking pathway.

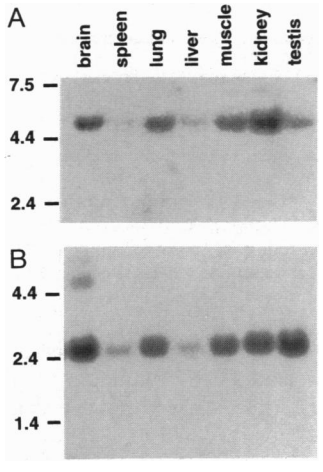

FIG. 3. Northern blot analysis of rSec6 and rSec8 mRNA expression. Lanes contained 2 μg of poly(A)$^+$ RNA from the indicated tissues. Molecular size markers are indicated in kilobases.

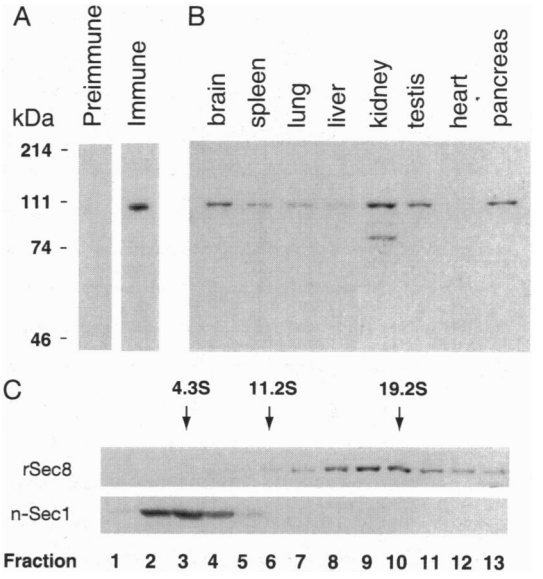

FIG. 4. Western blot and glycerol gradient analysis of brain rSec8. (*A*) Western blot detection of rSec8 in brain postnuclear supernatant. (*B*) Regional Western blot analysis of rSec8. Samples (20 μg) of postnuclear supernatant proteins from the indicated tissues were loaded per lane. (*C*) Glycerol gradient analysis of brain rSec8 complex. Fraction 1 (left) corresponds to the top of the gradient. Migration of rSec8 and n-Sec1 was monitored by Western blot analysis. Size markers (shown by arrows) from left to right were bovine serum albumin (4.3S), β-amylase (11.2S), and thyroglobulin (19.2S).

DISCUSSION

Here we identify mammalian homologs of the yeast proteins Sec6p and Sec8p may be involved in the docking and fusion of vesicles with the plasma membrane. Sequence analysis of these mammalian homologs, rSec6 and rSec8, revealed that both proteins have significant identities to their yeast counterparts and that rSec6 may have additional mammalian homologs that may be involved in secretion. Several pieces of evidence suggest that rSec6 and rSec8 are homologs of the yeast proteins Sec6p and Sec8p, respectively. (*i*) The sequence homologies extend over the entire length of both proteins and are highly significant by BESTFIT analysis. (*ii*) Both proteins are similar in size to their yeast homologs. (*iii*) Structural motifs are conserved between rat and yeast for both proteins, including the coiled-coil domains present in both rSec6 and rSec8 and a leucine-rich region that occurs in rSec8.

The similar tissue distribution of rSec6 and rSec8 mRNAs suggests that the two proteins may interact with one another, as observed with their yeast counterparts. The presence of rSec6 and rSec8 in brain also implies that they may be involved in secretion at the nerve terminal. Additionally, the broad tissue distribution of rSec6 and rSec8 suggests that, like NSF and αSNAP, they may also be involved in a number of different trafficking pathways and possibly in different steps of the secretory pathway.

In yeast, Sec6p and Sec8p are part of a 19.5S complex that includes Sec15p and that also associates with the GTP-binding protein Sec4p (12). Thus, a first priority is to determine whether the mammalian homologs form an analogous complex. Indeed, analysis of brain protein extracts demonstrated that rSec8 was present in a 17S complex. While the composition of this complex other than rSec8 is unknown, one candidate protein is the yet unidentified mammalian homolog of Sec15p. Furthermore, Western blot analysis of both transfected COS cells and brain homogenates revealed rSec8 in both cytoplasmic and plasma membrane fractions. These results are in agreement with yeast biochemical data (12) and

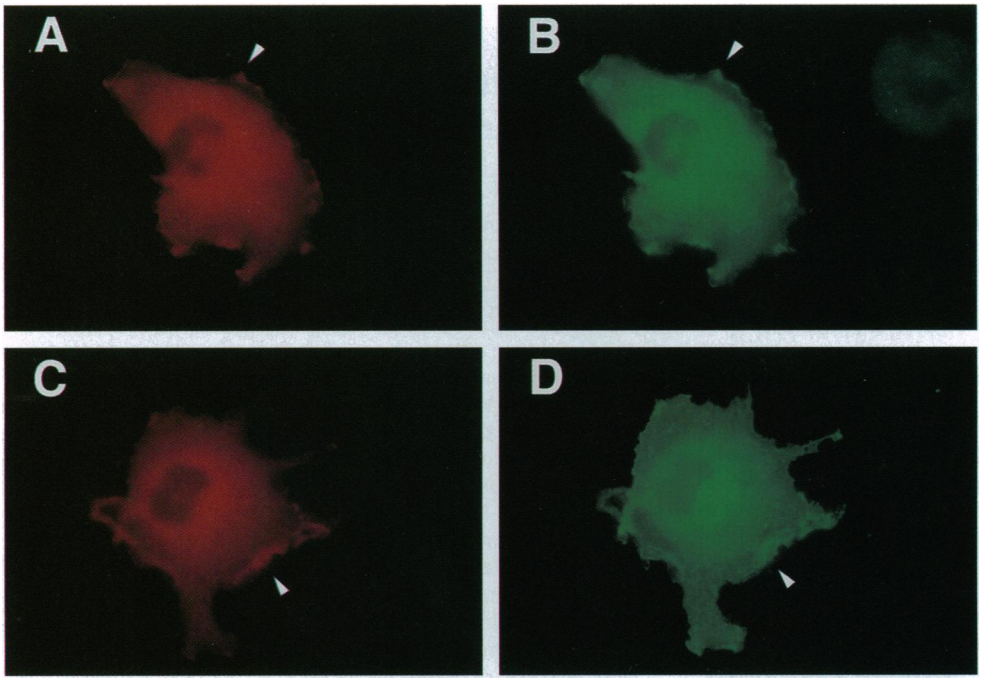

FIG. 5. rSec8 colocalizes with Rab3a and syntaxin 1a in transfected COS cells. COS cells were cotransfected with Rab3a and Myc-tagged rSec8 (*A* and *B*) or syntaxin 1a (17) and Myc-tagged rSec8 (*C* and *D*). For immunofluorescence detection, cells were incubated with primary antibodies against Myc epitope (*A* and *C*), Rab3a (*B*), or syntaxin 1a (*D*) and then with rhodamine (*A* and *C*) or fluorescein (*B* and *D*)-conjugated secondary antibodies.

provide further evidence that rSec8 is a homolog of Sec8p. Finally, these data suggest that the rSec8-containing complex can associate with the plasma membrane, possibly through a specific receptor.

Recently two protein complexes, the 7S (containing VAMP, synaptotagmin, syntaxin, and SNAP-25) and the 20S (containing VAMP, syntaxin, SNAP-25, α-SNAP, and NSF) particles (6, 7), have been identified as putative synaptic vesicle docking/fusion particles in the presynaptic terminal. Genetic studies indicate that the yeast homologs of protein components in these complexes such as syntaxin, VAMP, and SNAP-25 function downstream of Sec8p, Sec15p, and Sec4p (11, 20, 27, 28), suggesting that the formation of these complexes may be regulated by homologs of Sec8p, Sec15p, and Sec4p. Hence, it is possible that the interaction of soluble proteins such as Sec8p and Sec6p with Rab3a located on the synaptic vesicle mediates the delivery and docking of synaptic vesicles to the plasma membrane. Upon docking, two vesicle proteins, VAMP and synaptotagmin, interact with two plasma membrane proteins, syntaxin and SNAP-25, to form the 7S complex. Subsequent formation and dissociation of the 20S complex then precedes Ca^{2+}-stimulated vesicle fusion. We speculate that the isolation and characterization of rSec6- and rSec8-containing complexes will lead to an increased understanding of the processes that occur prior to and during vesicle docking and fusion. Elucidation of the functions of rSec6 and rSec8 is likely to provide insight into the molecular and cellular mechanisms of synaptic transmission.

We thank Dr. Jonathan Pevsner for helpful advice and Karen Peterson for excellent technical assistance. This work is supported by a Conte Center grant from the National Institute for Mental Health. C.D.H. is supported by a National Science Foundation predoctoral fellowship.

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