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The Mammalian Brain rsec6/8 Complex

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Summary

rsec6 and rsec8 are two components of a 17S complex in mammalian brain that is homologous to the yeast 834 kDa Sec6/8/15 complex which is essential for exocytosis. Purification and partial amino acid sequencing of the mammalian rsec6/8 complex reveals that it is composed of eight novel proteins with a combined molecular weight of 743 kDa. The complex is broadly expressed in brain and displays a plasma membrane localization in nerve terminals. Membrane associated rsec6/8 complex coimmunoprecipitates with syntaxin, a plasma membrane protein critical for neurotransmission. These data suggest a role for the mammalian rsec6/8 complex in neurotransmitter release via interactions with the core vesicle docking and fusion apparatus.

Introduction

Neurons communicate with target cells through regulated exocytosis of chemical messengers (reviews: Scheller, 1995; Südhof, 1995). Within the presynaptic terminal, neurotransmitter is packed into synaptic vesicles which are targeted to active zones at the nerve terminal plasma membrane. Action potential–induced elevation of intracellular calcium increases the probability of fusion between synaptic vesicles and the plasma membrane lipid bilayer, resulting in the release of neurotransmitter into the synaptic cleft. Modulation of the probability of neurotransmitter release is an important mechanism of synaptic plasticity; sites of such modulation likely include molecules which mediate synaptic vesicle functioning. Thus, a major endeavor of current neurobiological inquiry is to understand the biochemical events which govern the synaptic vesicle life cycle.

In the past few years, the convergence of three major experimental approaches, yeast genetics, in vitro vesicle trafficking assays and purification and analysis of synaptic vesicle proteins, has brought forth the realization that although neurons have evolved a regulated form of secretion, neurotransmitter release from nerve cells displays similarities to constitutive intracellular vesicle trafficking pathways found in all cells (reviews: Bennett, 1994; Rothman, 1994; Südhof, 1995). Many of the proteins involved in synaptic transmission are conserved from yeast Saccharomyces cerevisiae cells to mammalian neurons. Specifically, SSO1 and SSO2 (Aalto et al., 1993), SNC1 and SNC2 (Gerst et al., 1992), and five of the twelve SEC genes (*SEC1, SEC4, SEC9, SEC17,* and *SEC18*; Novick et al., 1980) which are essential for the later stages of the yeast secretory pathway have well characterized mammalian homologs (reviews: Bennett and Scheller, 1993; Pfeffer, 1994; Ferro-Novick and Jahn, 1994). Homologs of these yeast proteins include cytosolic factors (nsec1, NSF and α-SNAP), vesicle proteins (Rab3A and VAMP/synaptobrevin), and plasma membrane proteins (SNAP-25 and syntaxin). Some of these proteins interact with one another to form the 7S and the 20S complexes, which are thought to mediate vesicle docking, activation and membrane fusion (Söllner et al., 1993b; Söllner et al., 1993a). Seven of the twelve late-acting SEC mutants in yeast, however, still do not have well defined mammalian homologs. Interestingly, the proteins mutated in six of these seven mutants, Sec3, Sec5, Sec6, Sec8, Sec10 and Sec15, have been postulated to be components of a 19.5S complex composed of eight polypeptides (TerBush and Novick, 1995). We recently identified rsec6 and rsec8, putative mammalian homologs of two members of this complex (Ting et al., 1995). Identification of these two mammalian SEC mutant homologs has provided a valuable stepping stone toward the isolation of the mammalian complex.

Here we demonstrate that, in brain, rsec6 and rsec8 are present together in a high molecular weight complex. Purification of this complex from rat brain reveals that it is composed of eight proteins. Biochemical and immunolocalization studies show that the rsec6/8 complex is present in both soluble and membrane-bound states. The presence of the rsec6/8 complex in hippocampal synapses, together with its association with the synaptosomal protein syntaxin, suggests a role for the rsec 6/8 complex in synaptic vesicle trafficking pathway. Isolation of the mammalian rsec6/8 complex more than doubles the previous number of known proteins in the synapse which are similar to the yeast proteins critical for Golgi to plasma membrane vesicle trafficking. It is now possible to further investigate the mechanisms whereby the rsec6/8 complex interacts with the biochemical machinery that governs neurotransmitter release.

Results

rsec6 and rsec8 Are Components of a 17S Complex in Brain

Previous studies demonstrated that rsec8 is a component of a 17S complex in rat brain (Ting et al., 1995). However, the biochemical associations of rsec6 remained unknown. To determine whether rsec6 and

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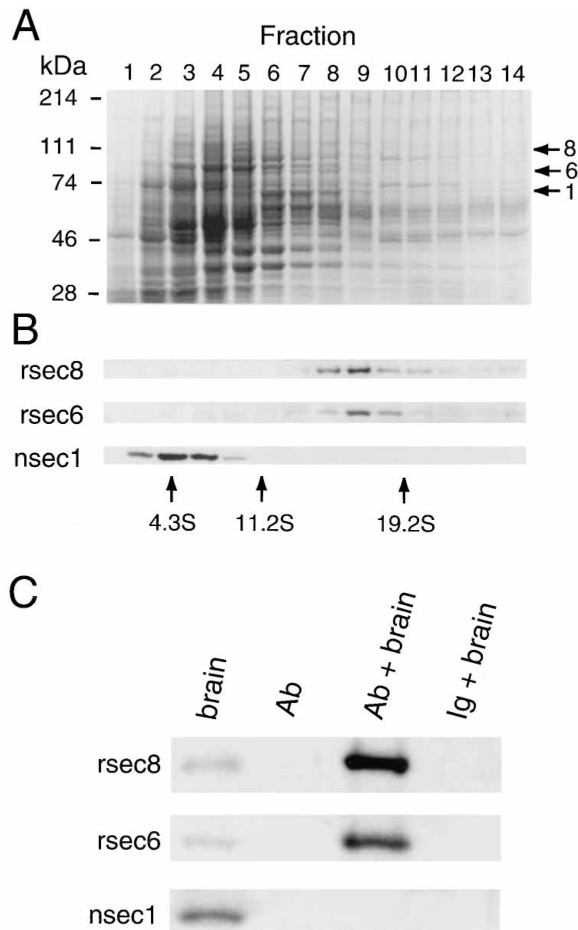

Figure 1. rsec6 and rsec8 Are Components of a 17S Complex

Proteins from rat brain supernatant were separated on a linear glycerol gradient, and gradient fractions were collected and analysed by SDS–PAGE followed by Western blotting.

(A) Coomassie blue–stained protein profile of glycerol gradient fractions. The positions of rsec8, rsec6 and nsec1 on the gel are indicated on the right as 8, 6, and 1, respectively.

(B) Immunodetection of rsec6, rsec8 and nsec1 in gradient fractions using immunoaffinity-purified antibodies. Fraction 1 corresponds to the top of the gradient. Size markers (indicated by the arrows) from left to right are bovine serum albumin (4.3S), β-amylase (11.2S) and thyroglobulin (19.2S).

(C) Coimmunoprecipitation of rsec6 and rsec8. Immunoprecipitation of polyethylene glycol-fractionated rat brain supernatant was carried out with affinity-purified antibodies against rsec8 or with control rabbit immunoglobulin. The immunoprecipitated sample was analysed for the presence of rsec6, rsec8 and nsec1 by Western blotting. Brain is the starting material; Ab represents proteins bound to the anti-rsec8 antibody in the absence of brain homogenate; Ab + brain represents proteins bound to the anti-rsec8 antibody following incubation with brain homogenate; and Ig + brain represents proteins bound to the control rabbit immunoglobulin following incubation with brain homogenate.

rsec8 function together in a protein complex, soluble brain proteins were fractionated on a linear glycerol gradient and the gradient fractions were analysed by both sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting (Figure 1A). Most of the proteins fractionated between the 4.3S and 11.2S regions of the gradient. The migration of rsec6 and rsec8 in the gradient was monitored by Western blot analysis using affinity-purified anti-rsec6 and anti-rsec8 antibodies. As demonstrated previously, rsec8 migrated to the 17S position. Interestingly, rsec6 also migrated to the 17S position (Figure 1B). In contrast, nsec1, another synaptosomal protein, migrated to the 4.3S position as previously observed (Pevsner et al., 1994b). This experiment indicates that all of the soluble rsec6 and rsec8 protein is in a large complex and that little or no protein is found in monomeric state. Because the Coomassie blue–stained glycerol gradient protein profile (Figure 1A) showed the presence of many proteins in the 17S region, it was necessary to determine whether the cofractionating rsec6 and rsec8 were in the same complex. Figure 1C shows an immunoprecipitation of rsec8 from soluble brain proteins using affinity-purified rabbit polyclonal anti-rsec8 antibodies. rsec6, but not nsec1, coimmunoprecipitated with rsec8. No rsec6, rsec8 or nsec1 were immunoprecipitated by control rabbit immunoglobulin, confirming the specific binding of the rsec6/8 complex to anti-rsec8 antibodies. These data demonstrate that both rsec6 and rsec8 are components of the same high molecular weight complex.

Purification of the Mammalian rsec6/8 Complex

The rsec6/8 complex was purified from rat brain by four sequential chromatographic steps to define other members of the complex (Figure 2A). The enrichment of the rsec6/8 complex following each chromatographic step was monitored by Western blot analysis (Figure 2B). Initially, cytosol prepared from frozen rat brains was applied to and step-eluted from hydroxyapatite. No significant amount of rsec6 and rsec8 was detected in the flow-through from this column. The pooled hydroxyapatite eluant exhibiting an enrichment of rsec6 and rsec8 immunoreactivity was concentrated and further purified using a strongly basic anion exchange resin, TMAE-Fractogel. All rsec6 and rsec8 immunoreactivity was retained by the resin and was eluted by a linear salt gradient. Next, taking advantage of the large size of the complex, the TMAE eluant was further fractionated by HW55-S gel filtration chromatography. The rsec6 and rsec8 immunoreactivity was eluted in a single peak with an apparent native molecular weight of 650–700 kDa. At this stage of purification, eight polypeptides of molecular weights between 70 kDa and 110 kDa were visible on a SDS polyacrylamide gel stained with Coomassie blue (Figure 2A). The last stage of purification involved a second anion exchange chromatography utilizing TMAE-Fractogel. Analysis of salt-eluted fractions from this column by SDS–PAGE revealed that elution of eight distinct proteins with molecular weights ranging from 71 kDa to 110 kDa coincided exactly with the elution of rsec6 and rsec8 immunoreactivity (data not shown). SDS–PAGE analysis of pooled peak rsec6 and rsec8 immunoreactivity fractions indicated that these same eight proteins constitute at least 95% of the total proteins in this fraction (Figure 2A, lane TMAE2; Figure 2C). Fractionation of the purified complex on a glycerol gradient demonstrated that all eight components comigrate at the 17S position (data not shown). In addition, all eight proteins coimmunoprecipitate with rsec8 monoclonal

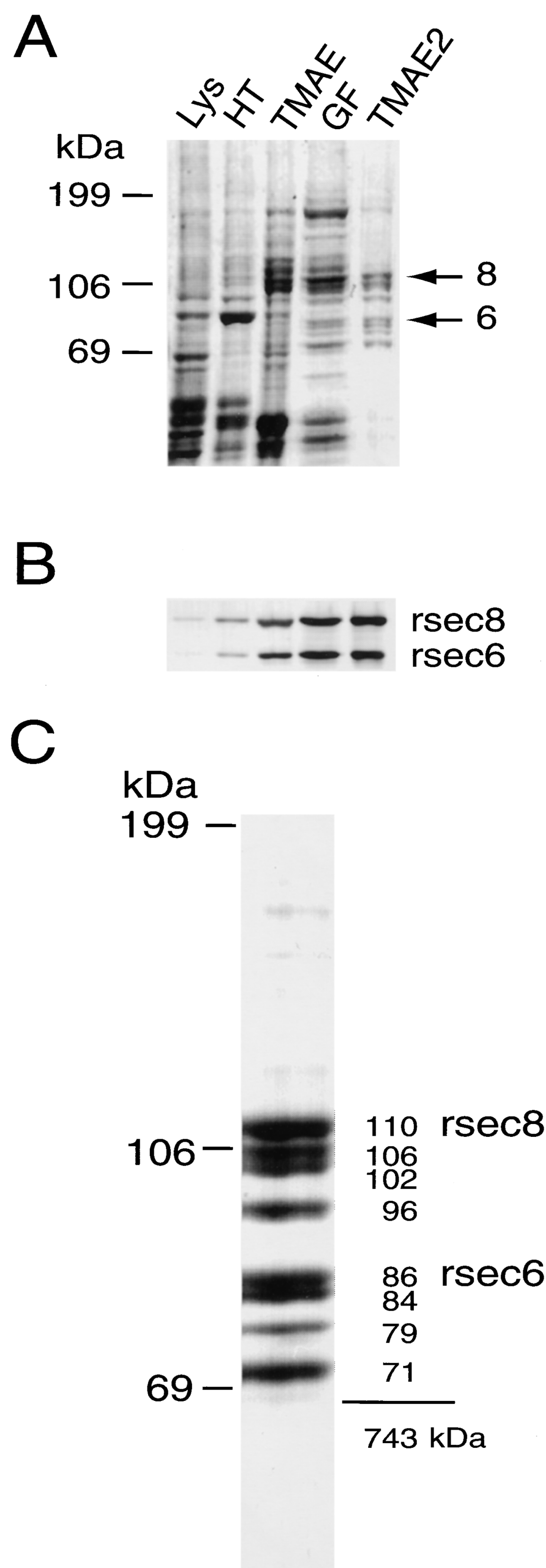

Figure 2. Purification of Rat Brain rsec6/8 Complex

The rsec6/8 complex was purified from frozen rat brains using four sequential column chromatographic steps.

(A) Coomassie blue–stained SDS polyacrylamide gel analysis of pooled column fractions. Lys, starting soluble brain proteins; HT, hydroxyapatite eluant; TMAE, TMAE anion exchanger eluant; GF, HW55S gel filtration eluant; TMAE2, second TMAE anion exchanger eluant. Positions of rsec6 and rsec8 on the gel are indicated on the right as 6 and 8, respectively. Fifteen μg of proteins were loaded in lane Lys, HT and TMAE; eight μg of proteins were loaded in lane GF; and three μg of proteins were loaded in lane TMAE2.

(B) Western blot analysis of the column fractions shown in (A). The enrichment of the rsec6/8 complex following each column chromatography was monitored using mouse polyclonal antibodies against rsec6 and rsec8.

(C) Coomassie blue–stained SDS polyacrylamide gel analysis of purified rsec6/8 complex. Peptide sequences from protein bands 110 kDa and 86 kDa indicate that they are rsec8 and rsec6, respectively.

antibodies (data not shown). Approximately 100–200 μg of rsec6/8 complex was recovered from 50 rat brains after purification. Quantitation of the enrichment of the rsec6/8 complex by Western blot using ^{125}I-labeled secondary antibodies suggest that the complex constitutes approximately 0.1% of the total soluble brain protein. All eight proteins were approximately equally stained by Coomassie blue (although p79 reproducibly appears to stain somewhat less intensely) suggesting a stoichiometry of one copy of each protein within the complex (Figure 2C). This 1:1 stoichiometry is further supported by the apparent molecular weight of 600–700 kDa of the native complex.

Peptide Sequencing of the rsec6/8 Complex Components

To further characterize individual components of the mammalian rsec6/8 complex, the purified complex was fractionated on an SDS polyacrylamide gel and each polypeptide band was individually subjected to in-gel proteolysis with trypsin, Lys C and/or Glu C. The digested peptides were eluted from the gel, fractionated by high performance liquid chromatography (HPLC), and in many cases, analysed by mass spectrometry for peptide purity and mass. Figure 3 shows the mass spectrometric analyses of HPLC peptide peaks from four proteins, p102, p96, p84 and p71. A single peptide peak in the mass spectrometry profile suggests the presence of one peptide species in the analysed HPLC peptide peak, as shown in (A), (B), and (C). The multiple peaks shown in (D) are due to the binding of different amounts of salt to the peptide. Amino acid sequence analysis of this peptide peak confirmed the presence of only one peptide species. Preselection of HPLC peaks for single peptide fractions by mass spectrometry greatly increases the clarity and confidence of obtained peptide sequences, as the observed and predicted masses differed by less than 0.1% (See Experimental Procedures and Figure 3 legend).

Figure 4 shows all the peptide sequences obtained from eight predicted members of the rsec6/8 complex. Peptide sequences from p110 and p86 confirmed that these two proteins are rsec8 and rsec6, respectively

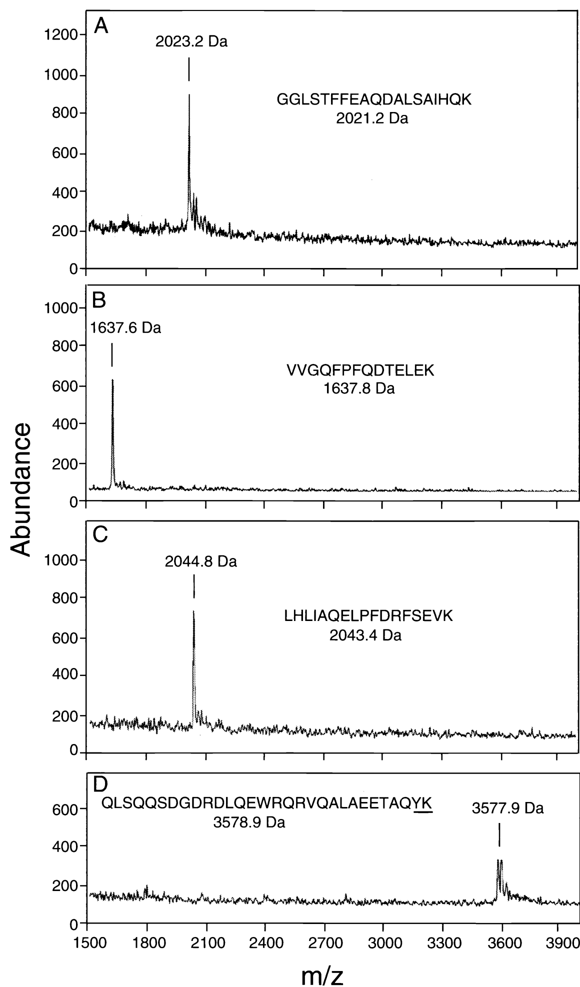

Figure 3. Mass Spectrometric Analysis of rsec6/8 Complex Peptides

Protein components of purified rsec6/8 complex were fractionated on SDS polyacrylamide gels, cut out and digested with Trypsin, Lys C and/or Glu C. The digested peptides were then subjected to HPLC fractionation and some HPLC peptide peaks were analysed by mass spectrometry for their mass and purity. Mass spectrometric analysis of peptides from p102, p96, p71 and p84 are shown in (A) to (D), respectively. The mass of each peptide predicted by mass spectrometry is shown above the peptide peak. The amino acid sequence of each peptide determined by peptide sequencing and its mass (protonated form of peptide) calculated from the determined peptide sequence is shown beside corresponding peptide peak. In (A) to (C) the calculated and observed masses matched with less than 0.1% difference. In (D), mass spectrometry analysis was used to predict the last two amino acids (underlined) of the peptide. Amino acids Y and K were the only combination of amino acids that could be added to the peptide to result in a mass that agrees with the mass spectrometry with less than 0.1% difference. Da = dalton.

(Figure 2C). Furthermore, all of the peptide sequences from these two protein bands match the amino acid sequence predicted by rsec6 and rsec8 cDNAs demonstrating that the bands are free of contaminating proteins and that the rsec6/8 complex is relatively pure. Comparison of remaining peptide sequences to GenBank and a database of human expressed sequence tag (EST) cDNAs generated by the Institute for Genomic Research found matches to two proteins p71 and p79. Overall the amino acid sequences match the sequences predicted from the cDNAs with an accuracy greater than 87%. Taken together, these results suggest that members of the rsec6/8 complex are novel proteins which have not been previously characterized.

The rsec6/8 Complex Is Ubiquitously Expressed

As a first step to determine the site of rsec6/8 complex function in brain, we examined the brain regional distribution of these proteins. Figure 5A shows that rsec8 is expressed in all regions of rat brain examined and there is no significant brain regional variation. A similar expression pattern is also observed for rsec6 (data not shown) suggesting that both proteins are required by all brain cells. To see whether the rsec6/8 complex functions in multiple exocytotic pathways in addition to the regulated pathway underlying neurotransmission, the presence of rsec8 in cell lines derived from kidney, ovary, pituitary and adrenal medulla tissues was investigated (Figure 5B). Western blot analysis using affinity-purified anti-rsec8 rabbit polyclonal antibodies detected rsec8 as a 110 kDa protein in all the cell lines examined. rsec6 was also observed in all cell lines examined (data not shown). Interestingly, the cells with the highest amounts of rsec8 were AtT-20 and PC-12 cells. Both cell lines have regulated secretory pathways and contain numerous secretory vesicles as well as synaptic proteins, suggesting that the expression of the rsec6/8 complex parallels the level of secretory activity in cells.

Subcellular Localization of the rsec6/8 Complex

To understand better the biochemical properties of the rsec6/8 complex, we carried out cell fractionation studies to investigate its solubility properties. Upon homogenization of freshly dissected rat brain, rsec6 and rsec8 were present in both soluble and insoluble fractions. Approximately 25% and 75% of rsec6 and rsec8 were found in the supernatant and the membrane pellet, respectively (Figure 6A). Similarly, in crude synaptosomes, the majority of rsec6 and rsec8 was found associated with synaptosomal membranes. Very little rsec6 and rsec8 were found in the synaptosomal cytosol (Figure 6A). However, in frozen brain more rsec6 and rsec8 (approximately 50%) was found in the soluble fraction (data not shown). The presence of rsec6 and rsec8 in the membrane fraction was not due to the entrapment of the complex in intact cells or organelles in the postnuclear supernatant because the distribution of these two proteins was similar when rat brain was completely homogenized in hypotonic buffer with a Polytron. Likewise, the presence of these two proteins in the supernatant was not due to contaminating membrane fragments because both SV2, a synaptic vesicle marker, and Na/K ATPase, a plasma membrane marker, were detected only in synaptosomal membrane pellet and not in synaptosomal cytosol.

To determine if the pool of the rsec6/8 complex that pelleted with crude synaptosomal membranes at 100,000 $\times$ g was due to its association with synaptosomal membranes or with large proteinaceous aggregates, we subjected crude synaptosomal membranes

Protein	Peptides
rsec8	1. XAPEGPLIDVXNI 2. EFAAFFAK 3. XLGVQRPLLQSTSIXE
p106	1. XXYGEIAXK 2. XDYGVIAND 3. ELPEFNLHFF 4. XLQDVDLASXR 5. XNRXNEPAVNVL 6. XQLXNIVEPEXIY
p102	1. YLSGLQAPGXPASQSIGA 2. GGLSTFFEAQDALSAIHQK 3. ASNTADT 4. ENLGRLFENY 5. XDYDVVINDYE
p96	1. VVGQFPFQDTELEK 2. XVYEIF 3. DFLESIR 4. XDQDLQLADYD 5. STNLLLTRTL
rsec6	1. DFRQSINTIEXL 2. QGPSQASPNYXP 3. AAIQSQLDGVRTGLSQ
p84	1. QLSQQSDGDRDLQEWRQRVQALAEETAQYK 2. XIPLALLPAA 3. DAVXQNSTQAAETEN 4. DYRNDEA 5. ENNPEEDDP 6. XLSQQSDXG 7. AAALRAPPXVTS 8. XKREPLE
p79	1. XTDYIAE 2. ETYGAFLSR 3. XXQEEETLMFIR 4. NLPVFQ 5. AVEYFQDKFPD 6. YRVEQVGDMIDRLFDTS 7. VYEDPALSAIFLHNNYNY 8. XXYGAFLHRYSSVPF
p71	1. XNQVAFQHFQELDEHI 2. VCHLXDQLEXVN 3. LHLIAQELPFDRFSEVK

Figure 4. Peptide Sequences from the rsec 6/8 Complex

Protein components of purified rsec6/8 complex were digested with a combination Trypsin (cuts after a lysine or an arginine residue), Lys C (cuts after a lysine residue) and/or Glu C (cuts after a glutamic acid) as described in the Experimental Procedures. p110 or rsec8 peptides were obtained from a combination of Lys C and Trypsin digestion; p106 peptides 1–4 were obtained from a combination of Lys C and Trypsin digestion and p106 peptides 5–6 were obtained from Lys C digestion; p102 peptides 1–3 were obtained from a combination of Lys C and trypsin digestion and p102 peptides 4–5 were obtained from Lys C digestion; p96 peptides 1–3 were obtained from Lys C digestion and p96 peptides 4–5 were obtained from a combination of Lys C and Glu C digestion; p86 or rsec6 peptide 1 was obtained from Lys C digestion and p86 or rsec6 peptides 2–3 were obtained from a combination of Lys C and Glu C digestion; p84 peptide 1 was obtained from Lys C digestion and p84 peptides 2–8 were obtained from a combination of Lys C and Glu C digestion; p79 peptides 1–4 were obtained from Lys C digestion and p79 peptides 5–8 were obtained from a combination of Lys C and Glu C digestion; p71 peptides were obtained from Lys C digestion.

to sucrose gradient flotation (Figure 6B). Both rsec6 and rsec8 floated up into the sucrose gradient, as observed with both Na/K ATPase, and synaptophysin (a synaptic vesicle marker). Very little rsec6 and rsec8 were detected at the bottom of the gradient as protein aggregates. These data suggest that the insoluble pool of rsec6/8 complex is associated with synaptosomal membranes.

To investigate the nature of rsec6/8 complex association with membranes, brain membranes were extracted with 20 mM Tris buffer, 1.5 M NaCl, 4% Triton X-100, or 4 M urea (Figure 6C). The extracted membranes were centrifuged and the resulting supernatants and membrane pellets were subjected to Western blot analysis. The amount of complex extracted under these conditions was examined by probing the Western blots with antibodies against rsec8 and quantitating by phosphorimaging using ^{125}I-labeled secondary antibodies (See Experimental Procedures). Approximately 4%, 46%, 60% and 88% of the rsec6/8 complex were extracted by Tris buffer, 1.5 M NaCl, 4% Triton X-100 and 4 M urea, respectively. The incomplete extraction of the rsec6/8 complex in the presence of a high concentration of salt suggests that this complex may bind very tightly to the membranes. Interestingly, a fraction of the native rsec6/8 complex is found in the Triton X-100 insoluble pellet following membrane solubilization, contrary to the observation that purified soluble rsec6/8 complex is completely soluble in Triton X-100, suggesting that the membrane-bound complex may be associated with cytoskeletal elements. Taken together, these observations suggest that the insoluble pool of rsec6/8 complex may be associated with cytoskeletal elements which, in turn, bind to membranes. Further studies will be required to understand these issues.

Since rsec6 and rsec8 floated to the same region of the sucrose gradient as synaptic vesicles and plasma membranes, we investigated the possibility of an association between the rsec6/8 complex and synaptic vesicles. Synaptic vesicles were immunoprecipitated using a monoclonal antibody against SV2, a synaptic vesicle–specific integral membrane protein. Figure 6D shows the Western blot analysis of immunoprecipitated synaptic vesicles. While the synaptic vesicle–specific protein synaptotagmin was present in anti-SV2 antibody immunoprecipitated vesicles, no rsec8 was detected even upon longer exposure. Neither protein was immunoprecipitated by control mouse immunoglobulin indicating a specific association of synaptic vesicles with anti-SV2 antibody. These results suggest that rsec6/8 complex is not associated strongly or stably with synaptic vesicles leaving the plasma membrane as the likely site of its membrane localization.

Localization of rsec8 in Cultured Hippocampal Neurons

To confirm the presence of the rsec6/8 complex in synapses, its localization in primary hippocampal cultures was studied by immunofluorescence microscopy. Synaptic terminals in these cultures were identified by the presence of the synaptic vesicle marker, synaptotagmin. Figure 7A shows that rsec8 labeling was detected throughout hippocampal neurons, including the cell bodies, dendrites, axons and nerve terminals. Brighter rsec8 staining at synapses suggests an accumulation of the rsec6/8 complex in nerve terminals (Figures 7A and 7B). Although both synaptotagmin and rsec8 were detected in synaptic terminals, staining of rsec8 was more concentrated near the plasma membrane than that of synaptotagmin (Figures 7B, 7C, and 7D). Many terminals displayed rsec8 labeling in peripheral areas where it did not colocalize with the more centrally positioned synaptotagmin staining. Glial and other non-neural cells in the hippocampal neuron cultures were also stained, although typically more weakly than the neurons (data not shown). As controls, preimmune immunoglobulins of same protein concentrations as the anti-rsec8 antibodies were used as the primary antibodies. At the same time exposures, no staining was observed in these neurons using identical labeling procedures. Taken together, these biochemical and histochemical data suggest that the rsec6/8 complex is localized to the plasma membrane.

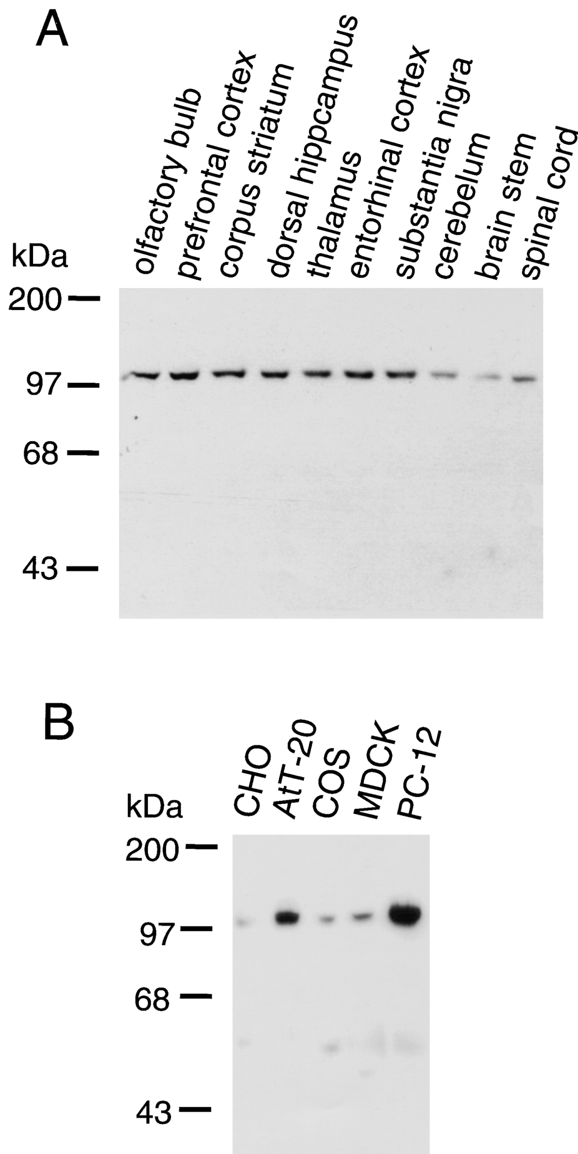

Figure 5. The rsec6/8 Complex Is Broadly Distributed

(A) Western blot analysis of brain regional expression of the rsec 6/8 complex. Ten μg of brain proteins were loaded per lane.

(B) Distribution of the rsec6/8 complex in cultured cell lines. Ten μg of proteins were loaded per lane.

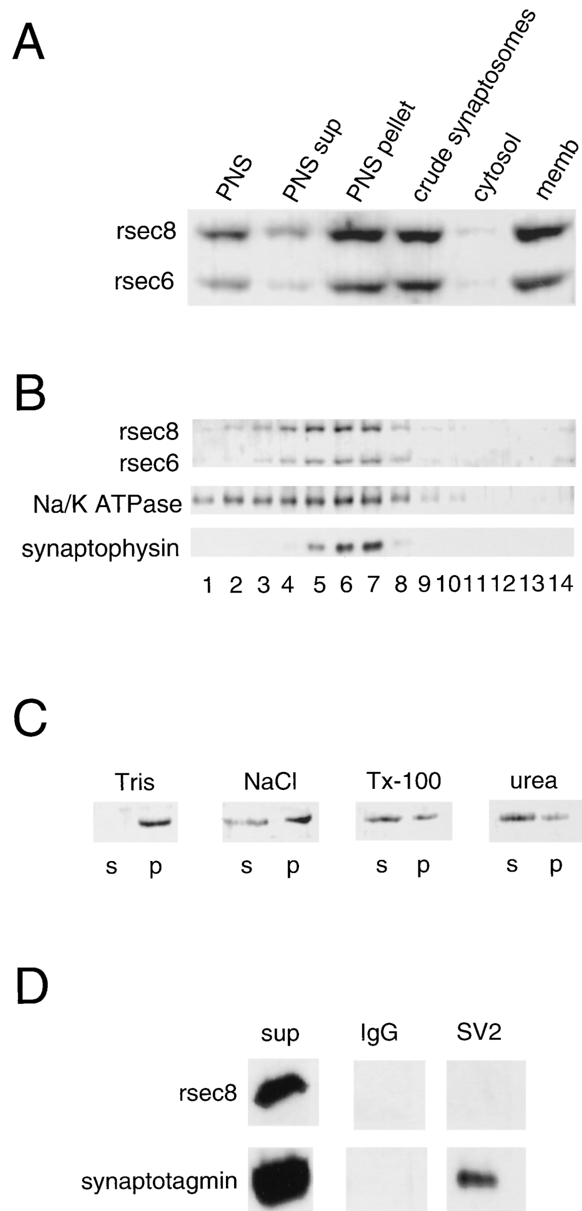

Figure 6. Subcellular Distribution of the rsec6/8 Complex

(A) Western blot analysis of subcellular fractionation of the rsec6/8 complex. Rat brain homogenate was subjected to a 1,000 × g spin to yield postnuclear supernatant (PNS).. The PNS was then subjected to either a 100,000 × g spin to yield soluble (PNS sup) and membrane protein (PNS pellet) fractions or a 25,000 × g spin to yield a supernatant (not shown) and a crude synaptosomal pellet (crude synaptosomes). The synaptosomal pellet was lysed in 2 mM Hepes, pH 7.4 and homogenized with a Polytron. The homogenate was then subjected to a 100,000 × g spin to yield both soluble (cytosol) and membrane (memb) fractions. The membrane pellets were resuspended in 20 mM Hepes, pH 7.4 to volumes equal to that of the corresponding supernatants. Five μl of each protein sample was loaded per lane. The presence of rsec6 and rsec8 in these fractions were analysed by Western blotting.

(B) The rsec6/rsec8 complex is associated with brain membranes. Crude synaptosomal membranes were placed at the bottom of a sucrose gradient and allowed to float up into the gradient. The flotation of the rsec6/8 complex into the gradient was monitored by Western blot analysis using mouse polyclonal antibodies. The migration of plasma membrane and synaptic vesicles was also mon-

Coimmunoprecipitation of rsec8 Complex with Syntaxin

To better study the properties of the rsec6/8 complex, including its membrane association in brain, we generated monoclonal antibodies against rsec8. These antibodies, with the exception of mAb15E12, recognize a single band of 110 kDa in brain postnuclear supernatant (Figure 8A). Monoclonal antibody 15E12 detected a major band of 110 kDa in addition to two minor bands of 65 kDa and 90 kDa. These two lower molecular weight bands are likely to be degradation products of rsec8. Of the seven monoclonal antibodies generated, only mAbs 2E9 and 17A10 immunoprecipitate the rsec6/8 complex.

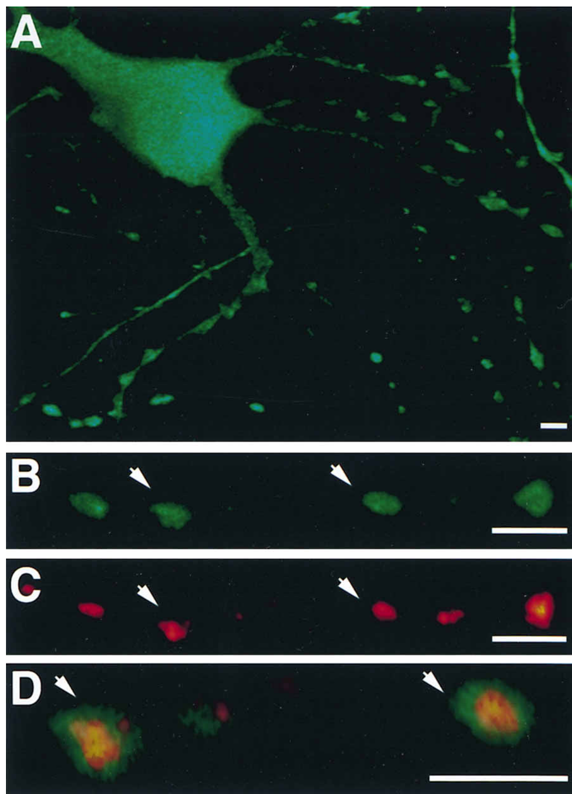

Figure 7. The rsec6/8 Complex Is Present in Nerve Terminals of Cultured Primary Hippocampal Neurons

15- to 20-day-old cultures of primary hippocampal neurons were fixed and immunostained with antibodies against rsec8 (A and B) and synaptotagmin (C). (D) shows a larger magnification of rsec8 staining overlaid with that of synaptotagmin at two synaptic terminals indicated by arrows. Bar = 7 μm.

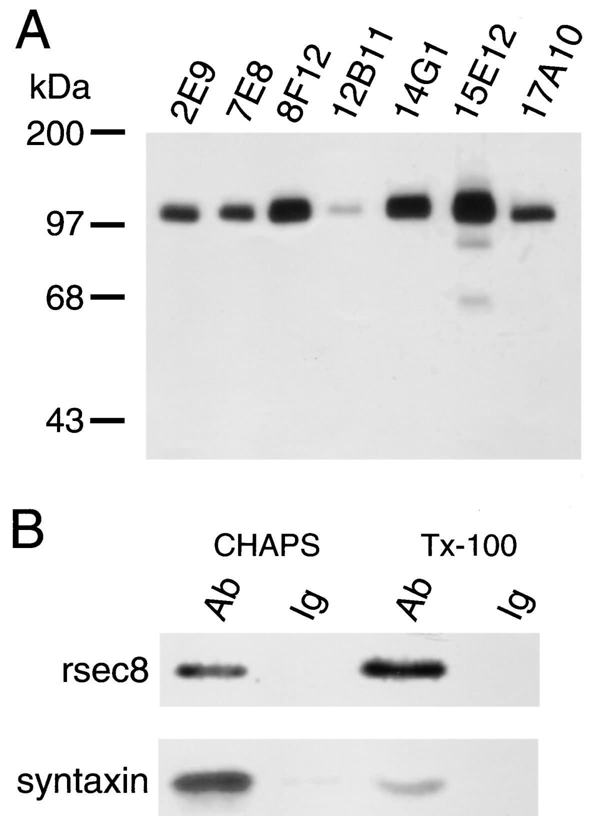

Figure 8. Syntaxin Associates with the rsec6/8 Complex

(A) The specificity of seven monoclonal antibodies generated against rsec8 was investigated by Western blot analysis. Ten μg of brain postnuclear supernatant were loaded per lane.

(B) Coimmunoprecipitation of syntaxin with rsec8. Immunoprecipitation of CHAPS or Triton X-100 solubilized rat brain membranes was carried out either with monoclonal antibodies 2E9 and 17A10 or with control mouse immunoglobulins. The immunoprecipitated sample was analysed for the presence of rsec8 and syntaxin by Western blotting. Ab represents proteins bound to the anti-rsec8 monoclonal antibodies. Ig represents proteins bound to control mouse immunoglobulins.

Figure 8B shows the immunoprecipitation of the rsec6/8 complex from 1% CHAPS or 1% Triton X-100 solubilized rat brain membranes. Interestingly, syntaxin coimmunoprecipitates with rsec8 in the presence of both detergents (the association appears more stable in the presence of CHAPS). Under these conditions rsec8 is present in the 743 kDa complex (data not shown) suggesting that the entire native complex is associated with syntaxin. While more than 90% of rsec6/8 complex protein was immunoprecipitated from solubilized brain membranes, no significant depletion of syntaxin was observed (data not shown). This suggests that only a very small subset of syntaxin, possibly those syntaxin molecules which are in a specific conformation or engaged in protein complexes, interacts with the rsec6/8 complex. Prolonged incubation of brain membranes in detergents or solubilization of brain membranes at higher detergent concentrations appears to weaken the syntaxin association with the rsec6/8 complex. In the same immunoprecipitation studies, no n-sec1, Rab3A or N-ethylmaleimide-sensitive factor (NSF) coimmunoprecipitated with the rsec6/8 complex confirming the specificity of the immunoprecipitation. In addition, no rsec8 or syntaxin were precipitated by control mouse immunoglobulin. Together, these data indicate the specific coimmunoprecipitation of syntaxin with the rsec 6/8 complex by the anti-rsec8 antibodies. Thus, syntaxin or one of the previously described syntaxin complexes may serve as a plasma membrane receptor for the rsec 6/8 complex.

itored using rabbit polyclonal antibodies against Na/K ATPase and a monoclonal antibody against synaptophysin, respectively. Fraction 1 corresponds to the top of the gradient.

(C) Nature of rsec8 association with brain membranes. Lysed brain membranes were extracted with either 20 mM Tris, pH 8.0, 1.5 M NaCl, 4% Triton X-100 or 4 M urea. Following extraction, the membranes were centrifuged to yield soluble (s) and membrane (p) fractions. The presence of rsec8 in these fractions was detected and quantitated by phosphorimaging using anti-rsec8 primary and ^{125}I-labeled secondary antibodies (see Experimental Procedures).

(D) Immunoprecipitation of synaptic vesicles from rat brain supernatant with anti-SV2 antibody or control mouse immunoglobulin coupled to Dynabeads M-500. The immunoprecipitated vesicles were examined for the presence of synaptotagmin and rsec8 by Western blotting.

Discussion

We previously identified two mammalian proteins that are homologs of yeast Sec6p and Sec8p based on sequence identity (Ting et al., 1995). In this report, glycerol gradient and immunoprecipitation analyses demonstrate that rsec6 and rsec8 are components of a 17S protein complex. Purification of this mammalian rsec 6/8 complex by column chromatography revealed that it is composed of eight distinct proteins with a combined molecular weight of 743 kDa. Although the sequence homology of Sec6 and Sec8 proteins between yeast and rat is only in the 20%–25% range, the purified mammalian rsec6/8 complex exhibits several striking physical and biochemical similarities to its yeast counterpart. First, both mammalian and yeast complexes are relatively large in size. The predicted molecular weights for the mammalian and yeast sec6/8 complexes are 743 kDa and 834 kDa, respectively (TerBush and Novick, 1995). Second, both complexes are composed of eight polypeptides with similar molecular weights ranging from 70 kDa to 140 kDa. Third, both complexes are unusually stable. Specifically, both Sec6 and Sec8 in yeast and rsec6 and rsec8 in rat brain are always found as components of a large complex on glycerol gradients; monomeric forms of these proteins are never observed under nondenaturing conditions. Unlike the 20S particle composed of syntaxin, VAMP, SNAP-25, α-SNAP and NSF, the 17S rsec6/8 complex does not dissociate in response to ATP hydrolysis. In fact, this complex does not dissociate in the presence of ATP, GTP, ATPγS, GTPγS, EGTA and/or Ca^{2+} (data not shown). Fourth, the mammalian rsec6/8 complex displays solubility properties similar to its yeast counterpart. In both organisms, the complex is found in soluble and membrane-bound states. In yeast the Sec6/8/15 complex is found in association with plasma membrane (Bowser et al, 1992), while in brain, the rsec6/8 complex has been observed to bind to synaptosomal membranes other than synaptic vesicles. Peripheral rsec8 staining in synaptic terminals suggests that the rsec6/8 complex is associated with the synaptic plasma membrane. The evolutionary conservation of an entire complex composed of eight distinct proteins between two distantly related organisms is most likely due to a critical function of this complex in the exocytotic pathway of yeast and mammals.

Electron microscopy studies have long shown that some synaptic vesicles are closely apposed to plasma membrane at active zones of nerve terminals (Heuser and Reese, 1973). A possible molecular mechanism of the specific docking and fusion of synaptic vesicles with the presynaptic plasma membrane may be the formation of a 7S complex comprised of two synaptic vesicle proteins, VAMP/synaptobrevin and synaptotagmin, and two presynaptic plasma membrane proteins, syntaxin 1A and SNAP-25 (Bennett et al., 1992b; Söllner et al., 1993a; Söllner et al., 1993b). A role for the rsec6/8 complex at this step is suggested by studies of yeast secretory mutants which have revealed genetic interactions between Sec8/Sec15 and the yeast homologs of syntaxin and SNAP25, SSO1 and Sec9. Specifically, overexpression of SSO1 can suppress mutations in the SEC15 gene (Aalto et al., 1993), while overexpression of Sec9 can suppress mutations in both the SEC8 and SEC15 genes (Brennwald and Novick, 1993). These observations in yeast can be extended to mammals with the demonstration of an in vivo interaction between rsec8 and syntaxin as revealed by the immunoprecipitation of syntaxin with anti-rsec8 antibodies. Additionally, staining of the Sec6/8/15 complex in yeast appears enhanced at the site of vesicle docking and fusion in the tip of the bud. This observation is echoed in neurons stained with antibodies to rsec8. The area near the plasma membrane of the nerve terminals, the site of exocytosis, are enriched in anti-rsec8 labeling. Thus, the yeast studies, along with the data presented here regarding mammalian brain, support the proposal that the rsec6/8 complex, directly or indirectly interacts with syntaxin and is a critical component of the vesicle docking and/or fusion machinery. The data are consistent with a role for the rsec6/8 complex in synaptic vesicle exocytosis upstream of the previously proposed 7S and 20S synaptic vesicle docking/fusion machinery. However, at this early stage of analysis we cannot rule out a role for the complex in later stages of exocytosis as well.

In addition, a genetic interaction has also been observed between Sec8/Sec15 and Sec4, a yeast homolog of Rab3A (Brennwald and Novick, 1993). In synaptic terminals, Rab3A, a GTPase, is found associated with synaptic vesicles (Farnsworth et al., 1991; Fischer von Mollard et al., 1990). Thus the data presented here are suggestive of a role for the rsec6/8 complex in a Rab mediated event at the plasma membrane, possibly intervening between the Rab and the 7S complex (Bowser et al., 1992). It is possible that one of the mechanisms of action of the rsec6/8 complex includes a transient interaction with Rab3A on synaptic vesicles. Characterization of the protein–protein interactions of the rsec 6/8 complex may help elucidate the precise role of Rab3A in neurotransmission.

Expression of the rsec6/8 complex is not as high in neurons and endocrine cells as some of the isoforms of vesicle trafficking proteins involved in the synaptic vesicle docking and fusion pathway. Northern and Western blot analyses as well as fluorescence microscopy studies detect its expression in all tissues and cultured cell lines examined. This ubiquitous distribution of the rsec6/8 complex suggests that it is important in both constitutive and regulated membrane trafficking and that its function is fundamental to the exocytotic process. Identification of the members of the rsec6/8 complex in mammalian cells, including neurons, enables future experimentation aimed at establishing the function of these proteins and raises the possibility that modulation of the activity of these molecules in brain may be important in regulating synaptic plasticity.

Experimental Procedures

Glycerol Gradient Analysis

Rat brain supernatant was fractionated on a linear glycerol gradient and analyzed by Western blotting as described (Ting et al., 1995).

Purification of the Rat Brain rsec6/8 Complex

All procedures were carried out at 4°C unless otherwise noted.

Bio-Gel HT Hydroxyapatite Chromatography

Fifty frozen rat brains (Harlan, IN) were homogenized in 400 ml of homogenization buffer (20 mM Hepes, pH 7.4, 200 mM NaCl, 1 mM

dithiothreitol (DTT), 0.1 mM EDTA and 0.3 mM PMSF) by eight up-and-down strokes with a Teflon/glass homogenizer. A postnuclear supernatant was obtained by centrifuging the homogenate at 5000 rpm in a JA-10 rotor (Beckman, CA) for 15 min. Soluble brain protein supernatant was obtained by centrifuging the postnuclear supernatant at 36,000 rpm in a Ti45 rotor (Beckman, CA) for 1 hr. Following the centrifugation, a final concentration of 110 mM sodium phosphate, pH 7.4 was added to the supernatant before it was applied to a 25 ml hydroxyapatite (BioRad, CA) column equilibrated with the homogenization buffer containing 110 mM sodium phosphate at a flow rate of 20 ml/hr at 18°C. The column was washed with 75 ml of 0.15 M sodium phosphate in buffer A (0.15 M NaCl, and 1 mM DTT, pH 7.4) and eluted stepwise with 5 ml aliquots of 0.2M to 0.65 M phosphate in buffer A, with 50 mM phosphate increment per step. Fractions containing rsec6 and rsec8 as determined by Western blot analyses were pooled and dialyzed twice against 2 liters of 20 mM Tris, pH 8.0, 100 mM NaCl, 0.2 mM EDTA, and 0.5 mM DTT overnight.

Fractogel EMD TMAE-650 (S) Chromatography

The dialyzed hydroxyapatite eluant was diluted with an equal volume of 20 mM Tris, pH 8.0 and loaded onto a 3 ml column of Fractogel TMAE anion exchange resin (EM Separations Technology, NJ) equilibrated with 20 mM Tris, pH 8.0, 50 mM NaCl, and 1 mM DTT at a flow rate of 15 ml/hr. The column was washed with 20 mM Tris, pH 8.0, 50 mM NaCl, 1 mM DTT and eluted with a linear gradient of 0 to 350 mM NaCl in 20 mM Tris, pH 8.0 and 1 mM DTT.

HW-55S Gel Filtration Chromatography

TMAE eluant fractions enriched in rsec6 and rsec8 were pooled and fractionated over a 180 ml HW-55S gel filtration column (TosoHaas, PA) equilibrated in 20 mM Hepes, pH 7.4, 200 mM NaCl, and 1 mM DTT at a flow rate of 5 ml/hr.

Second Fractogel EMD TMAE-650 (S) Chromatography

rsec6 and rsec8-enriched fractions from HW-55S gel filtration column chromatography were pooled and diluted to a final buffer concentration of 20 mM Tris, pH 8.0 and 50 mM NaCl. The diluted sample was applied to and eluted from a 0.5 ml Fractogel TMAE-650 column as described above. Fractions containing purified rsec 6/8 complex were pooled and used for SDS–PAGE analyses and peptide sequencing.

Mass Spectrometry and Peptide Sequence Analysis

Purified rsec6/8 complex was fractionated on an 8% SDS polyacrylamide gel. Individual protein bands were cut out and subjected to in-gel proteolysis at the Stanford Pan facility by Lys C, trypsin and/or Glu C using the method of Hwang et al. (1996). The digested peptides were fractionated by HPLC (Applied Biosystems, CA). Selected peptide peaks were then subjected to amino acid sequencing by Edman degradation method (Applied Biosystems, CA). Following digestion, some peptides from p106, p96 and p79 were also fractionated on a 1090 HPLC (Hewlett-Packard, CA). Selected HPLC fractions were subjected to MALDI-TOF mass analysis (Hewlett-Packard, CA). Following mass spectrometric analysis, selected peptide fractions were subjected to amino acid sequencing (Hewlett Packard, CA).

Brain Regional and Tissue Culture Cell Western Blot Analysis

For brain regional Western blot analysis, various rat brain regions were dissected from fresh brain and sonicated in 10 mM Hepes, pH 7.4, 2.5 mM KOAc, 1 mM $MgCl_2$, 0.1 mM EGTA, and 0.3 mM PMSF. The homogenates were centrifuged at 10,000 $\times$ g for 5 min and the postnuclear supernatants were collected.

For Western blot analysis of the rsec8 distribution in various cultured cells, tissue culture cells were grown to confluency in 10 cm petri dishes, scraped, and dounce homogenized in 20 mM Hepes, pH 7.4. The homogenates were centrifuged at 5000 $\times$ g for 5 min and the supernatants were collected for Western blot analysis.

Subcellular Localization of rsec6/8 Complex

For brain subcellular fractionation studies, postnuclear supernatant and crude synaptosomal membrane fractions were prepared as described (Bennett et al., 1992a). Soluble brain proteins and brain membranes were obtained by centrifuging the postnuclear supernatant or lysed crude synaptosomal fraction at 100,000 $\times$ g for 20 min in a TLS 55 rotor (Beckman, CA) at 4°C. All protein samples were subjected to SDS–PAGE and Western blot analysis.

For brain membrane extraction studies, lysed brain membranes resuspended in 20 mM Tris, pH 8.0, 1 mM DTT and 10% sorbitol (2 mg/ml) were incubated with equal volumes of either 20 mM Tris, pH 8.0, 3 M NaCl in 20 mM Tris, pH 8.0, 8% Triton X-100 in 20 mM Tris, pH 8.0, or 8 M urea in 20 mM Tris, pH 8.0. The incubations were carried out at 4°C for 1 hr. Following the incubation, the membranes were pelleted at 50,000 $\times$ g. The resulting supernatants and membrane pellets were analysed for their rsec8 content by Western blot. The amount of rsec8 extracted from membranes were quantitated by phosphorimaging using ^{125}I-labeled secondary antibodies. The pixel values for 20 mM Tris extraction were 6713 for supernatant and 173171 for pellet; for 1.5 M NaCl extraction were 39189 for supernatant and 45414 for pellet; for Triton X-100 extraction were 56302 for supernatant and 37254 for pellet; and for urea extraction were 71419 for supernatant and 9999 for pellet.

For sucrose gradient flotation analysis of rsec6/8 complex, lysed crude synaptosomal membranes were resuspended in 55% sucrose in gradient buffer (20 mM Hepes, pH 7.4, 150 mM NaCl and 1 mM DTT) at a protein concentration of 4 mg/ml. The resuspended membranes were placed at the bottom of a linear 25%–52.5% sucrose gradient. After centrifugation at 42,000 rpm for 16 hrs in a TLS 55 rotor (Beckman, CA), the gradient was fractionated and analysed by Western blot for rsec6, rsec8, Na/K ATPase (a plasma membrane marker) and synaptophysin (a synaptic vesicle marker).

Immunoprecipitation Studies

Immunoprecipitation of the rsec6/8 Complex

Immunoaffinity purified anti-rsec8 antibodies (Ting et al., 1995) or purified rabbit immunoglobulin were coupled to protein A beads at a final concentration of 0.5 mg/ml as previously described (Pevsner et al., 1994a; Pevsner et al., 1994b). Three ml of rat brain supernatant (Ting et al., 1995) was precipitated with 5% polyethylene glycol 3000 at 4°C and centrifuged at 20,000 $\times$ g for 10 min. The pellet was dissolved in 1 ml 20 mM Tris, pH 8.0 and 50 mM NaCl and precleared by incubation with 200 μl protein A beads for 4 hrs at 4°C. Following incubation, 500 μl of precleared brain supernatant was incubated with 30 μl of either anti-rsec8 antibody or rabbit immunoglobulin coupled to protein A beads overnight at 4°C. The beads were then washed three times with 200 μl 20 mM Tris, pH 8.0, 150 mM NaCl and 0.05% Tween 20. Proteins bound to the beads were solubilized in protein sample buffer, and subjected to Western blot analysis.

Immunoprecipitation of Synaptic Vesicles

Four frozen rat brains were powderized in liquid nitrogen using a Waring blender. The powderized brain was then homogenized in 20 ml of 0.3 M sucrose and 10 mM Hepes, pH 7.5 with a Teflon-glass homogenizer. The homogenate was centrifuged at 100,000 $\times$ g for 1 hr and the supernatant was incubated for 2 hrs at 4°C with 50 μl of anti-SV2 monoclonal antibody or purified mouse immunoglobulin coupled Dynabeads M-500 (Dynal, Oslo; 16 μg antibody/50 μl beads) in 0.3 M sucrose, 10 mM Hepes, pH 7.5 and 0.15% bovine serum albumin. Following incubation, the beads were washed four times with 0.3 M sucrose, 10 mM Hepes, pH 7.5 and solubilized in protein sample buffer. Protein samples were subjected to SDS–PAGE and analysed by Western blotting.

Coimmunoprecipitation of Syntaxin and the rsec6/8 Complex

Anti-rsec8 monoclonal antibodies 2E9 and 17A10 or mouse immunoglobulin were coupled to protein G beads at a final concentration of 2 mg/ml using the cross-linker dimethylpimelimidate as previously described (Pevsner et al., 1994a). Rat brain membranes prepared as described above were resuspended in 20 mM Tris, pH 8.0, 150 mM NaCl and solubilized with either 1% CHAPS or Triton X-100 at a final protein concentration of 2 mg/ml. The solubilized sample was incubated at 4°C for 30 min before centrifuging at 20, 000 $\times$ g for 20 min. Following centrifugation, the solubilized brain membranes were precleared by incubation with protein G beads (1 ml solubilized membranes per 200 μl protein G beads) for 30 min at 4°C. The precleared supernatant was then incubated with 20 μl protein G beads with either immobilized anti-rsec8 monoclonal antibodies 2E9/17A10 or immobilized control mouse Ig for 4 hrs at 4°C. The

beads were then washed three times with 300 μl 20 mM Tris, pH 8.0, 150 mM NaCl containing either 0.7% CHAPS or Triton X-100. Proteins bound to the beads were analyzed by Western blotting.

Monoclonal and Polyclonal Antibodies

Mouse polyclonal antibodies against rsec6 were generated against a His-tagged rsec6 fusion protein. The mouse serum recognizes a single band of 86 kDa in brain homogenate. This band is not present when preimmune serum was used as the primary antibody (data not shown). Mouse polyclonal antibodies against rsec8 were generated against a His-tagged rsec8 fusion protein. The immunized but not preimmune mouse serum recognizes one single band at 110 kDa in brain homogenate. Monoclonal antibodies against rsec8 were obtained from the corresponding hybridoma cell lines generated by fusion of NS-1 mouse myeloma cells with spleen cells from BALB/c mice immunized with His-tagged rsec8 fusion protein (Lane et al., 1986). Rabbit polyclonal antibodies against rsec8 were generated and affinity-purified as described (Ting et al., 1995). Polyclonal antibodies against nsec1 were affinity purified as described (Pevsner et al., 1994b). Rabbit polyclonal antibodies against Na/K ATPase (Mays et al., 1995) was a generous gift of Dr. W. James Nelson. Monoclonal antibody against SV2 (Buckley and Kelly, 1985) was a generous gift of Dr. Kathy M. Buckley, and monoclonal antibody against synaptophysin was purchased from Boehringer Mannheim (IN).

Hippocampal Cell Culture and Immunocytochemistry

Primary hippocampal CA3/CA1 cultures were obtained and maintained as described by Malgaroli and Tsien, 1992. Immunocytochemistry was carried out as previously described (Ting et al., 1995) using affinity-purified anti-rsec8 mouse polyclonal antibodies at 0.01 μg/ml and affinity-purified rabbit anti-synaptotagmin rabbit polyclonal antibodies at 1:1000 dilution as primary antibodies. The labeling was visualized using fluorescein-labeled donkey anti-mouse and Cy3-labeled donkey anti-rabbit antibodies. In control experiments, identical staining procedures were used except that the cells were labeled using preimmune sera or purified immunoglobulin of equal protein concentration as the primary antibody instead.

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