Abstract—The sec6/8 (exocyst) complex is implicated in targeting of vesicles for regulated exocytosis in various cell types and is believed to play a role in synaptogenesis and brain development. We show that the subunits sec6 and sec8 are present at significant levels in neurons of adult rat brain, and that immunoreactivity for the two subunits has a differential subcellular distribution. We show that in developing as well as mature neurons sec6 is concentrated at the inside of the presynaptic plasma membrane, while sec8 immunoreactivity shows a diffuse cytoplasmic distribution. Among established, strongly synaptophysin-positive neuronal boutons, sec6 displays highly differential concentrations, indicating a role for the complex independent of the ongoing synaptic-vesicle release activity. Sec6 is transported along neurites on secretogranin II-positive vesicles, while sec8-negative/secretogranin II-positive vesicles stay in the cell body. In PC12 cells, sec6-positive vesicles accumulate at the plasma membrane at sites of cell–cell contact. Neuronal induction of the PC12 cells with nerve growth factor shows that sec8 is not freely soluble, but may possibly interact with cytoskeletal elements. The complex may facilitate the targeting of membrane material to presynaptic sites and may possibly shuttle vesicles from the cytoskeletal transport machinery to presynaptic membrane sites. Thus, we suggest that the exocyst complex serves to modulate exocytic activity, by targeting membrane material to its presynaptic destination. © 2003 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: exocyst complex, sec6/8 complex, tethering, perisynaptic, growth cone, neuron.

In young neurons, axonal elongation and the transformation of growth cones to synaptic terminals are major factors in brain development, underlying the correct wiring of the nervous system. However, even after maturation, synapses maintain a strict regulation of membrane traffic, with regard to the ongoing exo-/endocytotic cycling of synaptic vesicles, as well as to structural, plastic modifications of synapses. Thus, brief periods of strong neuronal activity may induce changes in synaptic size and shape as a morphological correlate to changes in synaptic efficacy and possibly memory and learning ( Weeks et al., 2001 ).

Clearly, both the specific function of synapses and the general mechanisms regarding control of cellular polarization are dependent on Golgi to plasma membrane transport of vesicles. The budding yeast Saccharomyces cerevisiae has proved to be a particularly useful system for the genetic identification of proteins required for vesicle transport (Novick et al., 1980, 1981). Six of the SEC gene products, sec3, -5, -6, -8, -10, -15, have been found in a complex, and mammalian homologues have been identified for these yeast proteins (Ting et al., 1995; Hsu et al., 1996; Kee et al., 1997). In addition two novel gene products, exo70 and exo84, have been identified (Hsu et al., 1996; TerBush et al., 1996; Kee et al., 1997) as part of the complex, now called the sec6/8 or exocyst complex.

The sec6/8 complex is concentrated at sites of active vesicle exocytosis: at the tip of new growing buds in yeast cells (TerBush and Novick, 1995; Drubin and Nelson, 1996; TerBush et al., 1996; Finger and Novick, 1997; Finger et al., 1998) as well as just before cytokinesis at the neck of the budding cells (Mondesert et al., 1997). It has been shown to interact with Ca$^{2+}$-signaling proteins (Shin et al., 2000), and with cytoskeletal elements like actin (Shin et al., 2000), septins (Hsu et al., 1998) and microtubules (Vega and Hsu, 2001), all known to be involved in vesicular trafficking. In polarized Madin-Darby canine kidney (MDCK) epithelial cells, antibodies directed against sec8 block vesicle secretion at the basolateral membrane (Grindstaff et al., 1998).

From these studies a concept of the sec6/8 complex as part of a vesicle targeting mechanism at sites of regulated exocytosis has been implied (reviews: Hsu et al., 1999; Bajjalieh, 1999; Lin and Scheller, 2000). This mechanism would probably contribute with spatial specificity for vesicle transport, in neurons probably during brain development and synaptogenesis (Hazuka et al., 1999; Chin et al., 2000). The sec6/8 complex has recently been shown to interact with Ral GTPase, a member of the Ras superfamily of monomeric 20–30 kDa GTP binding proteins (Bry-
mora et al., 2001), and this interaction has been implicated in the modulation of the readily releasable pool of vesicles (Polzin et al., 2002) and filopodia formation (Sugihara et al., 2002). However, the exact localization and vesicular and plasma membrane interactions of the sec6/8 complex in developing and mature brain remain obscure.

We have undertaken to demonstrate the cellular and subcellular distribution of the sec6/8 complex, primarily through its protein sec6, in developing and mature neurons, using immunocytochemical techniques on rat brain, and PC12 and hippocampal cells in culture.

**EXPERIMENTAL PROCEDURES**

**Animals and antibodies**

Wistar rats were of either sex (250–350 g, Taconic M&B, Ry, Denmark). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and all efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to in vivo techniques, if available. Monoclonal antibodies (mAB) against sec6 (9H5 and 10C3) and sec8 (10C2 and 2E12) were a generous gift from S. C. Hsu. All mABs have been used and characterized previously (Kee et al., 1997; Hsu et al., 1998). The mAB against SV2 was a generous gift from R. H. Scheller. mAB against synaptophysin, SY38, was from Stressgen, Victoria, British Columbia, Canada. Polyclonal antibody (rabbit) against secretogranin II (SgII), 718, was a generous gift from H. H. Victoria, British Columbia, Canada. Polyclonal antibody (rabbit) antibodies (mAB) against sec6 (9H5 and 10C3) and sec8 (10C2 and 2E12) were a generous gift from S. C. Hsu. All mABs have been used and characterized previously (Kee et al., 1997; Hsu et al., 1998).

**Western blot analysis**

Rats were deeply anesthetized with sodium pentobarbital (50 mg/kg) and decapitated. The brains were cleansed, and rinsed in hypotonic homogenization solution (0.32-M sucrose, 10-mM HEPES-KOH pH 7.5, 1-mM EGTA, 0.1-mM EDTA, 0.3-mM PMSF in DMSO, all Sigma, St. Louis, MO, USA). The brains were then harvested in lysis buffer by pipetting 10–12 times in 300 μl buffer, supernatant was carried out at 1000×g for 10 min. The supernatant was then spun at 100,000×g for 1 h. The resulting pellet was resuspended in H-buffer, aliquotted and stored at −20 °C.

PC12 cells in confluent 100-mm dishes were washed in PBS and harvested in lysis buffer by pipetting 10–12 times in 300 μl buffer (25-mM Tris–Ac, pH 7.8, 2-mM dithiothreitol, 1-mM EDTA, 10% glycerol, 1% Triton X-100, 0.5-mM PMSF, all Sigma). Samples were processed further by centrifugation at 6000×g for 6 min, to obtain supernatant samples and pellet samples.

Aliquots were resolved on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes for Western blot analysis. The nitrocellulose membranes were blocked by incubation with 5% non-fat dry milk in phosphate-buffered saline containing 0.05% Tween (PBST). Primary mAB 9H5 (1:10) and 10C2 (1:50) were used. After incubation, the membranes were washed three times with PBST and incubated with goat anti-mouse HRP-conjugated secondary antibodies (NA 931, 1:1000, Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 h. Membranes were visualized by enhanced chemiluminescence.

**Light microscopic immunocytochemistry of Vibratome sections**

Rats were deeply anesthetized with sodium pentobarbital (50 mg/kg) and subjected to transcardiac perfusion with fixative (4% formaldehyde), after a brief flush of 2% dextran (MW 70,000) in sodium phosphate buffer (pH 7.4) (PB), all solutions 4 °C. The fixative was bicarbonate-buffered, initially at pH 6.0, then at pH 10.5 (‘pH shift protocol’; 0.2% picric acid was added to both solutions). The brain was left in situ overnight, before dissection and sectioning on a Vibratome at 50 μm. To facilitate diffusion of antibodies, some sections were pretreated with 0.5% Triton X-100. Immunocytochemistry was performed as described previously (Tsujimoto et al., 1999). Primary antibodies were used in the following dilutions: 9H5 1:300, 10C2 1:100, SY38 1:10 and SV2 1:800.

**Electron microscopy**

From rat-brain Vibratome sections, the hippocampus and cerebellar folia were dissected free. The tissue samples were incubated sequentially in the following solutions: (1) TBST (Tris-buffered saline with Triton X-100) with 2% human serum albumin (HSA); (2) primary antibody diluted in TBST w/HSA for 48 h at 4 °C; (3) TBST w/HSA; (4) secondary gold-coupled antibody 1:40 in TBST w/HSA (Nanogold 2004; Nanoprobes, Inc., Stony Brook, NY, USA); (5) TBST w/HSA; (6) PB; (7) 2.0% glutaraldehyde in PB; (8) PB; (9) H2O silver enhancement kit in dark room with safelight (HQ silver; Nanoprobes, Inc.); (10) double-distilled water; (11) PB; (12) 0.5% osmium tetroxide in PB and (13) PB. For immunoperoxidase reactions, step 4 was changed to secondary antibody 1:100 in buffer (biotinylated sheep anti-mouse Ig; Amersham, Arlington Heights, IL, USA), step 7 was changed to horseradish peroxidase complex 1:100 in buffer (streptavidin biotinylated; Amersham). The tissue was then dehydrated using increasing concentrations of ethanol. At 70%, uranyl acetate was added to the ethanol. Subsequently, the tissue was treated with propyleneoxide, before embedding in resin (Durecupan; Sigma). Ultrathin sections were cut on a Leica Reichert Ultracut S ultramicrotome and mounted on nickel mesh grids. Sections were counterstained in 1% uranyl acetate and 0.3% lead citrate. The specimens were examined and photographed on a Phillips EM 300 electron microscope.

**Fluorescence immunocytochemistry of cryosections**

After fixation, the cerebellum was dissected out, rinsed in PB and incubated in cryoprotectant, at 4 °C overnight. The tissue was quickly frozen in CO2 gas, and sectioned on a cryostat (Cryocut 1800, Reichert-Jung). Sections were collected on gelatin-coated microscope glass slides. The staining protocol was used as described by Veruki and Wassef (1996). The sections were treated with a mixture of 9H5 and polyclonal anti-synaptophysin antibody (B24-1) diluted 1:300 each in PB overnight at 4 °C. Secondary antibodies were at 1:300 each (Cy3 [red] conjugated donkey anti-rabbit IgG and FITC-conjugated donkey anti-mouse IgG, both Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Digital images were obtained with a Leica DM RXA confocal laser scanning microscope using FITC/TRITC filter set and the Leica TCS NT software.

**Preparation of cell cultures and transient transfection**

PC12 cell cultures were prepared and maintained as described by Greene et al. (1991). PC12 cells were seeded at a cell density of 5×104 or 1×105/well in 24-well plates for immunocytochemistry analysis and transfection experiments respectively. Transfection of PC12 cells was performed using lipofectAMINE 2000 reagent (Life Technologies Inc., Rockville, MD, USA) according to standard procedures, with nerve growth factor (NGF) (50 ng/ml) to differentiate cells and enhance gene expression.

Primary hippocampal CA1/CA3 cultures were obtained and maintained as described by Malgaroli and Tsien (1992).
Plasmid construction

A 3’ green fluorescent protein (GFP)-tagged sec6 eukaryotic expression plasmid was constructed by a two-step strategy. First, a PCR-amplified fragment of full-length sec6 was cloned into the pcDNA3.1/Myc-His A vector (Invitrogen), using the unique introduced HindIII (5’-end) and XhoI (3’-end) sites. The 3’-end primer was designed to contain a unique AseI site upstream of the XhoI site. Second, a GFP PCR-amplified fragment, using a humanized GFP expression vector as template (pCMX-SAH/Y145F; Ogawa et al., 1995) was cloned into the previously generated plasmid by employing AseI (5’-end) and XhoI (3’-end) sites. Restriction enzyme analysis and sequencing was performed to verify inserts. For PC12 cell transfection, DNA was prepared using CONCERT maxi-prep kit (Life Technologies Inc.).

Fluorescence immunocytochemistry of cell cultures

Cultures were sequentially subjected to (1) quenching solution of 0.1-M glycine in PB; (2) permeabilization solution (PS) of 0.4% saponin, 1% bovine serum albumine and 2% normal goat serum in PB; (3) primary antibody (9H5 1:1, B24-1 1:300 or SgII 1:200) in PS; (4) PS; (5) a mixture of fluorescent secondary antibodies at 1:100 each in PS (rhodamine-Cy3 and FITC-conjugated antibodies); (6) PS; (7) PB. Cultures were examined on a Leica TCS NT scanning confocal microscope.

Quantification of immunofluorescence intensity

Quantification of immunofluorescence intensity in confocal sections through boutons was performed with Leica TCS NT software. The data were further processed and graphically presented in Microsoft Excel. The correlation coefficient with 95% confidence interval was calculated using the correlation and Fisher z transformation functions in Microsoft Excel.

RESULTS

The sec6/8 complex is present in neuron-rich areas of the adult brain

The sec6/8 complex has been shown to be present in high concentrations in developing brain, and is believed to play a role in synaptogenesis (Hazuka et al., 1999; Hsu et al., 1999; Lin and Scheller, 2000). To study the expression and localization of the complex in adult rat brain, we characterized the mAB 9H5 toward sec6 and 10C2 toward sec8 with Western blots of homogenates of PC12 cells and adult rat brain. For sec8, a band of similar molecular size was present in all PC12 and brain samples (Fig. 1), corresponding to the previously reported size of the protein (110 kDa; Kee et al., 1997). The sec6 protein was found in PC12 and brain samples, with a molecular size corresponding to previous reports (86 kDa; Kee et al., 1997). In order to assert the regional and cellular distribution of sec6 and sec8 in adult rat brain, sagittal and coronal sections of formaldehyde-fixed rat brain were treated with mAB against the two proteins (9H5 and 10C3 toward sec6 and 10C2 and 2E12 toward sec8) (Fig. 2). (Micrographs labeled with 9H5 and 10C2 are shown.) Parallel sections were treated with an anti-synaptophysin, or anti-SV2, antibody (Fig. 2E, F, respectively). Synaptophysin and SV2 are well-characterized synaptic vesicle membrane protein proteins, present in the axon terminals of neurons. At low magnifications, all of the antibodies generally produced similar and distinct labeling of neuron-rich areas in the brain sections. Specifically, the cerebral cortex, striatum, hippocampus, and the cerebellar cortex were heavily labeled by the antibodies. Many areas of the thalamus were also densely labeled by the anti-sec6 and synaptophysin antibodies, while showing moderate immunoreactivity for sec8. The brain stem was moderately labeled by the antibodies. White matter, e.g. the corpus callosum, cerebellar white matter and corticobulbar fiber tracts, were only weakly to moderately labeled with the sec6 and sec8 antibodies, while being distinctly negative for synaptophysin and SV2. This widespread staining pattern indicates that the sec6/8 complex has a universal role in neuronal function in the mature, adult brain.

Sec6 is associated with the plasma membrane of boutons in mature CNS neurons

At high magnification the labeling visualized using the anti-sec6 mAB 9H5 and 10C3 and anti-sec8 mAB 10C2 and 2E12 showed differential labeling. Strong 10C2 immunoreactivity was observed throughout major parts of the neuronal cytoplasm, i.e. in the cell body and the large

Abbreviations used in the figures

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>Cx</td>
<td>cerebral cortex</td>
</tr>
<tr>
<td>Cb</td>
<td>cerebellar cortex</td>
</tr>
<tr>
<td>Hc</td>
<td>hippocampus</td>
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<tr>
<td>Th</td>
<td>thalamus</td>
</tr>
<tr>
<td>SV2</td>
<td>synaptic vesicle protein #2</td>
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<tr>
<td>SP</td>
<td>stratum pyramidale</td>
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Fig. 2.
dendrites (Fig. 2H, K). Intense immunoreactivity for 9H5 was typically found as punctate labeling (Fig. 2G, J). These immunoreactive puncta were clearly outlining neuronal dendrites and cell bodies. However, specific but weaker immunoreactivity for 9H5 (sec6) was also found throughout the neuronal cytoplasm. The immunoreactive pattern for synaptophysin was almost identical to the punctate labeling of 9H5 (Fig. 2I, L). A similar difference in labeling using different mAB against the sec6/8 complex has been published by Yeaman et al. (2001). They showed that different epitopes may be accessible for the antibodies when the complex is associated with different subcellular compartments. Thus our findings suggest that the 10C2 mAB recognizes the sec8 subunit of the sec6/8 complex in a cytoplasmatic distribution, while the 9H5 anti-sec6 mAB more readily binds to the complex in synaptic boutons.

However, the sec6/8 proteins are not exclusive to neurons. To varying degrees, glia cells were also labeled by the respective antibodies. 10C2-positive Bergman glia fibers, e.g. may be seen in the cerebellar cortex. At high magnifications, it is also evident that the weak to moderate labeling seen for both sec6 and sec8 in white matter (e.g. the corpus callosum) is due to astrocyte labeling (data not shown).

The sec6 labeling pattern strongly indicated a presynaptic localization for this protein. In order to determine this unequivocally, three additional methods were applied. First, adult rat-brain cryosections were subjected to double immunofluorescence against sec6 and synaptophysin. Also with this procedure the antibodies against sec6 (green in Fig. 3A, C) as well as synaptophysin (red in Fig. 3B, C) gave punctate labeling in the neuropil. Overlay pictures with fluorescent probes of different wavelengths showed that there was a high degree of punctate colocalization for sec6 and synaptophysin (Fig. 3C), showing that sec6 indeed localized to vesicle-filled terminals. However, the relative strength of the two immunoreactivities seemed to vary between the different terminals. In some puncta only synaptophysin immunofluorescence was seen, or there was a relative abundance of synaptophysin toward sec6 (red or orange fluorescence), in others the opposite was observed (green), while many terminals were about equal in immunofluorescence for the two proteins (yellow). Though this gives no measure of the absolute concentration of the two proteins, it does indicate that the concentration of sec6 may not be strongly dependent of the concentration of synaptophysin within a bouton.

Second, the presence of sec6 in terminals was visualized at the ultrastructural level with preembedding immuno-}

nostaining of sections from adult rat hippocampus. A dominating labeling of presynaptic terminals (Fig. 3D) is visible, with the diaminobenzidine (DAB) chromogen filling the terminal and the space between the vesicles. This clearly shows that sec6 is concentrated within terminal boutons, but due to the local diffusion of the DAB reaction product, it is not possible from these experiments to establish the exact intraterminal localization of the protein.

Third, to finally determine the localization of sec6 within mature, established terminals, preembedding immunogold labeling was performed on vibratome sections of adult rat hippocampus. With this method it is possible to determine the exact ultrastructural localization of sec6 to the inside of the plasma membrane in established, mature terminals in the brain (Fig. 3E). Although there was labeling both in the cytoplasm and in close vicinity of cellular organelles, there was a higher level of sec6 labeling at the inside of the boutonal membrane. Labeling along the perisynaptic membrane was evenly dispersed. However, the active zone typically contained less sec6 labeling than the rest of the presynaptic membrane.

**Mature boutons show a wide distribution range of sec6 immunoreactivity**

Hazuka et al. (1999) showed that the exocyst complex in the developing brain is found in highest concentrations at sites of synaptogenesis, preceding synapsin I labeling. Our results with immunofluorescence of intact rat brain indicate that though the overall majority of synaptic boutons in adult rat brain contain significant concentrations of sec6, there is a range of different concentration levels among these boutons. However, due to the dense packing of boutons in the neuropil of brain sections, it was not feasible to determine the relative levels of sec6 and synaptophysin in any given terminal. Therefore, we chose to investigate this further in dissociated hippocampal cultures. Synaptophysin immunoreactivity was used as a marker for vesicle-containing boutons. As in the intact brain, most of these boutons colocalized sec6 (Fig. 3F–I). Though it has been shown that the portion of boutons colocalizing sec6 and synapsin I is highest in younger cultures (Hazuka et al., 1999), we consistently found high numbers of double-labeled boutons (sec6 and synaptophysin) in all ages of cultures investigated, even up to 36 days 

in vitro. Indeed, the degree of colocalization was often strongest in cultures with a dense meshwork of fibers and boutons. However, overlay pictures showed that the relative strengths of sec6 and synaptophysin immunoreactivity displayed high levels of
Fig. 3.
variability, i.e. sec6 intensity was strong in some of the boutons, synaptophysin was strong in other boutons, while in many they were fairly equal in intensity.

However, the total population of varicosities in a culture will comprise both immature and mature boutons, as well as boutons with and without synaptic vesicles. Hazuka et al. (1999) showed that the exocyst complex might be recruited to boutons in an early phase of their development, before they are set up with significant numbers of synaptopophsin-containing synaptic vesicles. Conversely, we wanted to focus on boutons that are already established with significant levels of synaptophysin. Such boutons should be mature terminals with densely packed synaptic vesicles and high synaptic activity. Boutons were selected visually, and the average fluorescent intensities for both sec6 and synaptophysin in a cross-section through the boutons were measured. As such, these observations would not give us the information on which of the proteins that were present at the strongest concentration. Rather, our aim was to establish whether the concentrations of sec6 and synaptophysin were dependent or independent of each other within boutons. We found that most of the boutons contained significant concentrations of sec6, and that there is very little or no correlation (n=110, r=−0.054, 95% confidence interval: −0.239−0.134) between sec6 and synaptophysin fluorescence levels (Fig. 3I). This implies that the recruitment of sec6 to an individual bouton is virtually independent of the established number or density of vesicles.

In growing neurons, sec6 is localized to Sgll-positive vesicles in growth cones and neurites and to the plasma membrane in boutons

Having visualized the labeling of sec6 in mature rat brain, we wanted to investigate its distribution during cellular growth and development. Thus, we investigated the localization of sec6 immunoreactivity in growing hippocampal cultures with high-magnification confocal immunofluorescence.

Generally, we observed a high degree of labeling of boutons, as described above. However, with high magnification, it was evident that many boutons harbor small, granular structures immunoreactive for sec6 (Fig. 4A). Similar structures are present in a densely aggregated pattern within many growth cones, and singular granules were observed along neurites between varicosities. In large growth cones, a combination of granular and plasma membrane labeling was evident.

Sgll is a peptide of the granin family that is present in a subset of secretory granules in neurons and neuroendocrine cells (Rosa et al., 1985; Tooze et al., 1991). While synaptophysin is present on small synaptic vesicles, Sgll is a part of large dense-core vesicles in neurons. Double labeling hippocampal cell cultures with anti-Sgll antibodies in addition to the anti-sec6 antibodies (Fig. 4B) showed that the sec6-positive granules in the processes and boutons also harbor this granin peptide. Almost all granular labeling for sec6 colocalized with Sgll-positive puncta, but the opposite was not the case. While Sgll-positive, sec6-negative granules were diffusely spread within cell bodies, granules labeling positive for both fluorophores were concentrated in neurites. Sec6-positive granules were typically somewhat larger than Sgll-positive granules that did not harbor sec6.

In our hippocampal cultural preparations, many terminals were of significantly large size, up to about 5 μm in diameter. In these terminals, sec6 immunofluorescence was usually observed as a circle in the periphery of the terminal (Fig. 4C). Double labeling with synaptophysin would either fill the entire terminal, or exist in an area central to the sec6 immunofluorescence. This is in concordance with previous reports (Hsu et al., 1996; Hazuka et al., 1999), as well as with our own electron microscopical observations. These data confirm that sec6 is associated with peripheral areas in established boutons, while synaptophysin (associated with small synaptic vesicles) occupies the more central domains.

Sec6-containing vesicles relocate during different stages of PC12 development

The targeting process of sec6 and synaptophysin to their different locations was investigated in PC12 cells. This cell line is originally developed from a rat neuroendocrine cell tumor, and has the ability to differentiate into a neuronal growth pattern after stimulation with NGF. PC12 cell cytoplasm is abundant in synaptic-like microvesicles, equivalent to synaptic vesicles in neurons, as well as secretory granules, equivalent to large dense-core vesicles in neurons. To establish the vesicular association of sec6 also in neuroendocrine cells, immunolabeling with markers of small synaptic-like vesicles (synaptophysin) and secretory granules (Sgll) was performed on PC12 cell cultures.

Fig. 3. Sec6 is present in presynaptic terminals in adult rat brain, and has a differential immunoreactivity in synaptophysin-positive terminals. (A–C) Immunofluorescence of cerebellar cortex. Sec6-9H5 (A), synaptophysin (B), overlay (C). Molecular layer with a Purkinje cell dendrite (small arrow) and an interneuron cell body (asterisk). Abundant colocalization of the two proteins is seen, although some of the puncta labeled with sec6 seem to be larger than the equivalent labeling of synaptophysin. Also, it is evident that some puncta are predominantly green (high in sec6), some are predominantly red (high in synaptophysin), while some are yellow (indicating more equal immunofluorescence intensities). (D) Sec6-9H5-immunoreactive presynaptic terminal in the cerebellar cortex (molecular layer) in an electron microscopic preembedding preparation. Due to the possible diffusion of the reaction product (DAB) it is not possible to determine which membranes are more strongly labeled. (E) Electron microscopy of preembedding immunogold labeling. Sec6-9H5 immunoreactivity represented by silver-intensified gold particles is present on the inside of the plasma membrane of a presynaptic terminal. (F–H) Immunofluorescence of cultured hippocampal neurons. Sec6-9H5 (F), synaptophysin (G), overlay (H). Synaptophysin is visible as smaller puncta enwrapped by sec6 labeling. The majority of varicosities colocalize sec6 and synaptophysin, but with a wide range of ratios between the two immunolabeling intensities. Yellow designates fairly equal labeling. (I) Scatter diagram showing sec6 immunofluorescence in 110 boutons selected for synaptophysin immunoreactivity. There is no obvious correlation (r=−0.054, 95% confidence interval: −0.239−0.134) between the two immunoreactive intensities. Scale bars=10 μm (A–C), 500 nm (D, E) and 20 μm (F–H).
The anti-synaptophysin antibody gave densely packed small-vesicle labeling throughout the cytoplasm, while the anti-SgII antiserum produced staining of singular, large cytoplasmic granules. While a high level of colocalization was obvious for sec6 and SgII (Fig. 5E), colocalization of sec6 and synaptophysin was not evident (Fig. 5C, D). As in neurons, all granular labeling for sec6 largely colocalized with SgII positive puncta, but the opposite was not the case. The 10C2 labeling showed sec8 immunoreactivity present in a diffuse manner throughout the cells (Fig. 5A, B). However, in many cells a perinuclear enrichment of sec8 was evident.

This general labeling pattern showed some interesting variants in different stages of cell differentiation. In PC12 cells fixed shortly after (1 h) being plated out, many of the cells have not yet adhered properly to the glass coverslip. These cells are small, circular or ovoid, with only a small area of plasma membrane contacting the supporting glass coating. In these cells, most of the sec6-positive granules were typically located in the periphery, close to the plasma membrane. Sec8 immunoreactivity (10C2), however, while present throughout most of the cytoplasm, was not observed on the most peripheral synaptophysin-positive granules, the latter often being seen protruding from the outline of the cell (data not shown).

After stable attachment of the cells to the coverslip, the sec6-immunopositive granules relocated to the interior of the cells, being more or less evenly spaced out in the cytoplasm (Fig. 5C). If the cultures are densely grown, granules concentrate close to the plasma membrane at cell–cell contacts (Fig. 5F, G). Granules thus demarcate the face of contact.

After neuronal induction of the cells with NGF, the sec6-immunopositive granules increased in number and relocated once more, now being typically found within the enlarged endings of the neurite-like extensions (Fig. 5D). Some sec6-immunopositive vesicles were also seen along the plasma membrane of the cell bodies. When the cells were treated with NGF, only those SgII-positive granules that contained sec6 were enriched in the growth cone of the neurites (Fig. 5E). Sec8 immunoreactivity, being still diffusely spread out within the cytoplasm of the stably attached cells, largely relocated to the shafts of the neurite-like extensions after differentiation with NGF (Fig. 5B). Sec8 immunoreactivity was now comparatively weaker in the cell bodies and in the tip of the growth cones than in the processes. Quantification of the labeling intensities accordingly showed a mean of 51 (arbitrary units) over cell-body cytoplasm, while the corresponding figure for dendrite cytoplasm was 190 ($N=8$, $P=0.0002$).

We transfected PC12 cells with a GFP–sec6 vector in an attempt to follow the transport of the sec6-positive vesicles. We found GFP labeling diffusely spread throughout the cytoplasm, similar to what Matern et al. (2001) showed for N- and C-terminal GFP constructs of sec3, -5, -8 and -10. Transfected cells were also immunolabeled with sec6 (9H5), using Cy3-coupled secondary antibody (Fig. 5F, G). As expected, red immunofluorescence was seen diffusely throughout the cytoplasm recognizing recombinant as well as native sec6. However, sec6 protein associated with vesicles were immunolabeled (red),
Fig. 5. (Caption overleaf).
though they did not contain recombinant sec6 (lacking green label). Thus, the C-terminal GFP tag seems to inhibit the association of sec6 with functional vesicles. As was also observed with non-transfected cells, sec6-immunopositive vesicles seemed to associate with the plasma membrane at sites of cell–cell contact.

**DISCUSSION**

We show that the sec6/8 complex is present in significant concentrations in adult rat brain. Mature synaptic boutons contain significant concentrations of sec6, and the concentration of sec6 is here virtually independent of the presence of the synaptic vesicle-marker synaptophysin. The sec6 protein is attached to the cytoplasmic side of the neuronal terminal plasma membrane, though it is not concentrated at the active zone. Finally, we demonstrate that sec6 is transported, in neurons and PC12 cells, with SgII-positive secretory granules, which concentrates at areas of active membrane addition.

**mAB against sec6 and sec8 show differential immunolabeling**

A striking observation throughout the present study is the different intracellular immunolabeling patterns of sec6 and sec8. This would not be expected, due to the fact that the two proteins are subunits of the same complex. Despite this, sec8 immunoreactivity was predominantly seen diffusely throughout the cytoplasm in adult brain neurons, in cultures hippocampal neurons, and in PC12 cells. Much less sec8 immunoreactivity may be seen in synaptic terminals. In the same cellular preparations, sec6 immunoreactivity is clearly strongest in synaptic boutons and on vesicles (different from synaptophysin positive small synaptic vesicles), though some immunoreactivity is also present throughout the cytoplasm. This pattern was evident with two different anti-sec6 mAB (9H5 and 10C3) and with two different anti-sec8 mAB (10C2 and 2E12), respectively. All four antibodies have been published and characterized previously (Hsu et al., 1996; Kee et al., 1997; Grindstaff et al., 1998; Hazuka et al., 1999; Vega and Hsu, 2001 and Yeaman et al., 2001).

At least two different interpretations may account for these observations. First, the sec6/8 complex may possibly dynamically assemble and disassemble at different functional states. Thus, sec6 and sec8 could at some points in the trafficking pathway belong to separate subcomplexes, associated with different organelles or compartments, at other points or states fully assemble to a complete complex. In line with this, several studies have shown that a portion of the complex exists in a partially assembled state with some of the protein being found dissociated from sec6 (Grindstaff et al., 1998; Shin et al., 2000). Also, studies by Finger et al. (1998) and Guo et al. (1999) show that the localization of sec8 to the plasma membrane at sites of polarized exocytosis is dependent on accumulation of all other proteins of the complex. Sec8 may thus be recruited to sites where sec6 and other components of the complex are waiting. Possibly, the potential physiological binding between sec6 and sec8 may facilitate the interaction between their respective attached organelles or molecules.

A second interpretation is that the antigenic epitope of at least one of our proteins may be structurally hidden from immunolabeling at different physiological states or interactions of the protein, e.g. before or after binding to the rest of the complex or to other proteins, before or after phosphorylation, etc. Thus, it is possible that our immunocyto-chemical images underestimate the relative degree of cellular colocalization. In line with this interpretation, Yeaman et al. (2001) show that immunoreactivity with different mAB against the same protein (sec6) may localize to different compartments of NRK cells, i.e. to the trans Golgi network or to the plasma membrane.

However, it is not the aim of the present study to determine the molecular interactions of the different sec6/8 complex proteins at distinct stages of the exocytic trafficking pathway. We have primarily focused on the vesicle and plasma membrane interaction of sec6 in neurons and neuron-like cells. We acknowledge that sec6 in these interactions may form part of the whole complex, part of a subcomplex, or possibly at some stage exist unassociated with other parts of the complex.

**The sec6/8 complex may contribute to the regulation of synaptic activity through presynaptic mechanisms**

Hazuka et al. (1999) showed that the sec6/8 complex in the developing brain is found in highest concentrations at sites of synaptogenesis, preceding synapsin1 labeling, while being down-regulated in adult brain. These and other findings have led to the interpretation that the complex works primarily during the establishment of synapses and cell polarity, with other mechanisms or proteins being responsible for vesicle targeting once synapses have matured (Hsu et al., 1999; Lin and Scheller, 2000). However, this interpretation may prove to be premature. First, several studies have shown biochemically that the exocyst complex is enriched and broadly expressed in

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**Fig. 5.** Sec6 localizes to SgII-positive granules in PC12 cells and redistributes on neuronal differentiation. Immunofluorescence of PC12 cells. (A–E) Overlay pictures; sec8-10C2 (green, A, B), sec6-9H5 (green, C–E), synaptophysin (red, A–D) and SgII (red, E). (A, C) Native, before NGF stimulation. (B, D, E) After NGF stimulation. (A) Before NGF stimulation, sec8 has a diffuse cytoplasmic staining pattern. (B) When NGF treated, sec8 has an enhanced labeling along the neurite-like extensions, but little colocalization with synaptophysin in the distal part of the growth cone. (C) After adhesion, sec6 immunoreactivity shows a granular pattern within the cytoplasm, not colocalizing with synaptophysin. (D, E) After stimulation with NGF, sec8-positive organelles accumulate in the distal parts of the neurite-like extensions. Sec6 colocalizes with a subset of SgII-positive granules (note yellow color), but not with synaptophysin. (F, G) Transiently transfected PC12 cells expressing GFP–sec6 (green), labeled with anti-sec6 mAB (red), G showing a high magnification of the area shown in F. GFP–sec6 is present throughout the cytoplasm. mAB-tagged, non GFP–sec6-containing vesicles are present along the cell–cell contact membrane. Scale bar=15 μm.
adult rat brain (Ting et al., 1995; Hsu et al., 1996; Kee et al., 1997). Second, the present immunocytochemical results clearly show the distinct and widespread existence of the exocyst proteins sec6 and sec8 in adult rat brain, the former predominantly localized to presynaptic terminals. It is then reasonable to assume that the function of the exocyst complex is required also in the mature brain, indicating a role beyond synaptogenesis.

Recently published results from several laboratories show that the sec6/8 complex interacts with active Ral GTPase (Brymora et al., 2001; Sugihara et al., 2002; Moskalenko et al., 2002). Polzin et al. (2002) suggests that this interaction takes part in modulation of the readily releasable pool of synaptic vesicles. Using Ral knockout mice they show that the Ral protein is necessary for refilling of the readily releasable pool of synaptic vesicles, and that in the mutants several synaptosomal proteins are present at reduced levels. They also suggest a role for the Ral-sec6/8 complex in regulating synaptic strength.

In concordance with these reports, our results show that the synaptic level of the sec6/8 complex is not correlated with the concentration of synaptophysin in individual boutons. The level of synaptophysin immunoreactivity has been shown by others to correlate well with the vesicle-turnover activity in individual terminals (Staple et al., 1997). Thus, the exocyst complex, while probably serving a role in the targeting and addition of membrane material to the presynaptic terminal, may not be involved in the ongoing exo-/endocytotic cycle of small synaptic vesicles.

Synaptic plasticity has been shown to involve also structural changes like increasing the size of the presynaptic terminal (Pierce and Lewin, 1994; Weeks et al., 2001). Thus, the up- or down-regulation of synaptic size would be expected to be a dynamic membrane trafficking process in many areas of the brain. We speculate that the sec6/8 complex may serve as a marker or link for the accumulation of vesicle membrane to growing boutons, irrespective of their developmental age. A strong immunocytochemical signal for sec6 in a synaptic terminal would then indicate a bouton that is increasing in membrane size (positive modulation), a low concentration of sec6 would indicate a bouton of possibly stable, or decreasing size.

**Sec6 is targeted to presynaptic terminals in developing and mature neurons**

This report is the first presentation of the specific localization of the sec6/8 complex in mature brain neurons. We show sec6 in the conformation detected by the 9H5 antibody, at the ultrastructural level, to be localized to the inner surface of the plasma membrane in presynaptic terminals. This corresponds well with studies of the exocyst complex in yeast, where sec3 localizes to sites of polarized exocytosis (Finger et al., 1998), and in polarized MDCK cells, where sec6 labeling was restricted to probable baso-lateral exocytotic release sites (Grindstaff et al., 1998). In the presynaptic bouton synaptic vesicles are targeted specifically to the active zone. While the sec6/8 complex is not absent from presynaptic active zones, the whole perisynaptic membrane was more densely labeled. Thus, our findings do not support the exocyst as a direct and single determinant for targeting or tethering to the active zone, because of a distribution mostly lateral to, rather than within, this membrane area. Other proteins would still be necessary to ensure docking specifically and exclusively at the active zone.

**Sec6 is transported to sites of regulated exocytosis on SgII-positive vesicles before insertion in the presynaptic plasma membrane**

The sec6/8 complex, or a subcomplex containing at least sec6, is present on a type of vesicle in neurons and PC12 cells. Although all or most sec6-positive vesicles seem to contain SgII, not all SgII-positive vesicles contain sec6. This is strikingly demonstrated by the fact that only granules colocalizing both sec6 and SgII are seen in neurites, while sec6-negative/SgII-positive vesicles dominate in the cell bodies of neurons and NGF-treated PC12 cells. Taken together, our results show that the large sec6-labeled vesicles represent a functional subpopulation of secretory granules, possibly at a certain maturational stage. The fact that the sec6-containing vesicles are summoned in growth cones, and at PC12 cell–cell contacts as well as presynaptic terminals, implies a function for the complex in the polarizing growth of, and in the establishment and maintenance of communicative contact between cells.

Although we were not able to visualize the transport of sec6 on vesicles using GFP-tagged sec6, it seems highly likely from our results that the sec6/8 complex is transported on SgII-containing vesicles prior to associating with the plasma membrane. In accordance with this, Yeaman et al. (2001) conclude that exocytosis is required to develop plasma membrane association of the exocyst complex in NRK cells. Also, our finding of large growth cones containing both granular and plasma membrane sec6 labeling is indicative of a vesicular mode of transport and insertion.

Hatada et al. (1999) have shown that the formation of varicosities involves transient cessation of growth-cone advancement, and the varicosity develops as a distinct swelling in the central region of the growth cone. The large growth cones in our hippocampal cell cultures may thus be in the process of generating varicosities. As neuronal varicosities may develop without preexisting postsynaptic contacts, the signal to incorporate sec6 in the membrane at developing varicosities may be independent of a postsynaptic substrate.

In conclusion, we suggest two alternative functions for sec6 and the complex in neurons, depending on whether sec6 exerts its main function before or after associating with the plasma membrane: First, the sec6-containing part of the complex, associated with the perisynaptic membrane, is perfectly localized to facilitate receipt and tethering of vesicles that have been transported along e.g. microtubules to neuronal boutons or terminals. One may thus interpret the sec6- (and SgII-) positive granules as large transport vesicles for the protein, en route to its plasma membrane destination in developing boutons during synaptogenesis, or in mature, but growing, terminals in adult rat brain. Sec6 would serve a functional role as a tethering
factor after transport and relocation to the perisynaptic plasma membrane.

Alternatively, the targeting of SgII-containing granules may be a specific task of the sec6/8 complex in neurons. Its predominant localization lateral to the active zone is just the place where exocytosis of neuronal large, peptide-containing vesicles takes place (Thureson-Klein and Klein, 1990). Thus, the SgII-positive granules may use sec6 and the sec6/8 complex for correct targeting to their destination membrane. Sec6 immunoreactivity on the presynaptic plasma membrane would then be a residue after the exocytosis of SgII-positive granules. An interaction between two components, one on the vesicle membrane, the other on the plasma membrane in presynaptic terminals, would ensure the spatial specificity of SgII-vesicle targeting, distinct from similar targeting systems for small synaptic, or other, vesicles. The component on the vesicle membrane comprises sec6, as part of a subcomplex or the whole sec6/8 complex. The counterpart on the target membrane could be a matching sec6/8 subcomplex, or a totally different but unknown sec6/8 interacting factor.

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