Synaptic vesicles (SVs) in the central nervous system upon stimulation undergo rapid calcium-triggered exocytotic cycling within the nerve terminal that at least in part depends on components of the clathrin- and dynamin-dependent endocytosis machinery. How exocytic SV fusion and endocytic retrieval are temporally and spatially coordinated is still an open question. One possibility is that specialized membrane microdomains characterized by their high content in membrane cholesterol may assist in the spatial coordination of synaptic membrane protein recycling. Quantitative proteomics analysis of detergent-resistant membranes (DRMs) isolated from rat brain synapses or cholesterol-depleted control samples by liquid chromatography-tandem mass spectrometry identified a total of 159 proteins. Among these 122 proteins were classified as cholesterol-dependent DRM or DRM-associated proteins, many of which with proven or hypothesized functions in exocytotic vesicle cycling including clathrin, the clathrin adaptor complex AP-2, and a variety of SV proteins. In agreement with this, SV membrane and endocytic proteins displayed a partial resistance to extraction with cold Triton X-100 in cultured rat hippocampal neurons where they co-localized with labeled cholera toxin B, a marker for cholesterol-enriched DRMs. Moreover SV proteins formed cholesterol-dependent complexes in CHAPS-extracted synaptic membrane lysates. Our combined data suggest that lipid microdomains may act as spatial coordinators for synaptosomal membrane lysates. Our combined data suggest that lipid microdomains may act as spatial coordinators for exocytotic vesicle cycling at synapses.

Molecular & Cellular Proteomics 5:2060–2071, 2006.

Synaptic vesicles (SVs) store and release neurotransmitters by calcium-induced regulated exocytosis that at least in part is accompanied by insertion of SV membrane proteins and lipids into the presynaptic plasma membrane from where they are retrieved by temporally and spatially coupled clathrin- (1) and dynamin-mediated endocytosis (2, 3). To accomplish this task SVs comprise a distinct set of proteins including the vesicular SNARE synaptobrevin/VAMP-2, synaptophysin, and the calcium- and phospholipid-binding protein synaptotagmin 1 (4). It is unclear how precisely SVs maintain their compositional identity. Synaptotagmin 1, the presumed calcium sensor for neuroexocytosis (5), interacts with the endocytic adaptor proteins AP-2 (6–8) and stonin 2 (9); this may facilitate its internalization and sorting to the recycling vesicle pool. However, most other SV proteins apparently lack defined interactions with endocytic adaptor or accessory proteins. Although it remains possible that SV proteins are sorted individually during clathrin-mediated recycling an alternative option is that SVs remain clustered at presynaptic sites and are sorted as complete entities. Support for this idea has recently been gained from high resolution stimulated emission-depletion microscopy imaging of actively cycling SVs (10). Such clustering of SV proteins may be aided by membrane lipids, in particular cholesterol, which is highly enriched in SV membranes (i.e. about 40% of the total lipid content) (11). Evidence from neuroendocrine PC12 cells (12, 13) and primary neurons (14) suggests that the membrane cholesterol content is critical for SV protein clustering and endocytic recycling (15). These functional data are paralleled by the observations that synaptophysin and synaptotagmin 1 direct bind to cholesterol in situ (13) and that synaptophysin forms a cholesterol-dependent protein complex with synaptobrevin 2 within the vesicle membrane (16–18). Exocytic SV fusion sites are also dependent on membrane cholesterol, which facilitates clustering of both SNARE proteins (19, 20) and phosphatidylinositol 4,5-bisphosphate (21), an essential factor in coordinating the exo- and endocytic limbs of the vesicle cycle (22, 23). Finally cholesterol may play a role in the organization of the pre- and postsynaptic actin cytoskeleton (2).

The neuronal plasma membrane from which SVs mainly arise is particularly rich in cholesterol. Cholesterol may not only affect the physical properties of the bilayer but could also impact its association with the cytoskeleton and thereby impart a diffusional barrier for the lateral movement of mem-
brane proteins. Consistent with this, cholesterol-dependent microdomains have been implicated in epithelial cell polarization and axonal membrane protein sorting in neurons (24, 25). The size and stability of cholesterol-rich microdomains (26) have been controversially debated (27) and in fact may also vary between cell types. Biochemically cholesterol-rich microdomains can be isolated because of their partial resistance to extraction with non-ionic detergents at low temperature and to float in gradient centrifugations to light density fractions corresponding to the buoyant density of lipids (so-called detergent-resistant membranes (DRMs)) (25, 27).

In the present study we undertook a non-biased quantitative characterization of DRMs isolated from highly purified synaptic membranes using nano-LC-MS/MS in combination with stable isotope labeling. We identified a variety of SV and endocytic proteins and provide evidence for their association with cholesterol-rich membrane domains in situ.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Sources for antibodies were as follows: monoclonal antibodies against flotillin 1, AP-2γ, and phosphatidylinositol 4-phosphate 5-kinase type Iy (PIPK Iγ); BD Transduction Laboratories; mouse anti-human transferrin receptor antibody; Zymed Laboratories Inc.; monoclonal antibodies against synaptobrevin/VAMP-2 or synaptophysin: Synaptic Systems (Göttingen, Germany); monoclonal antibodies against synaptotagmin 1 (41.1) and clathrin heavy chain (TD1): gifts from Reinhard Jahn (Göttingen, Germany) and Pietro De Camilli (Yale University School of Medicine), respectively; and monoclonal antibody against α-adaptin (AC1M1): Affinity Bioreagents. H$_{18}$O was purchased from Euriso-top GmbH, Saarbrücken, Germany (95% 18O). Amplex Red cholesterol assay kit was from Molecular Probes. All other reagents, if not mentioned specially, were from Sigma.

Isolation of DRMs from Highly Purified Synaptosomes—Rat brain synaptosomes were prepared according to Ref. 28. Synaptosomal pellets were resuspended in homogenization buffer followed by resolation from a 0.8 M/1.2 M sucrose step gradient. Pellets were resuspended in TNE buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM DTT). Gradient-purified synaptosomes were strongly enriched in synaptic marker proteins such as synaptophysin, synaptotagmin 1, etc., and de-enriched in glial contaminations including myelin basic protein in agreement with previous findings (29). For cholesterol depletion these highly purified synaptosomes were extracted twice with 10 mM methyl-β-cyclodextrin (MβCD) in TNE buffer. 450 μg of control or MβCD-extracted synaptosomes were solubilized with 2% Triton X-100 (or 2% CHAPS for the experiment shown in Fig. 3C) in a total volume of 1.5 ml on ice for 10 min followed by mixing on a rotating wheel at 4 °C for 20 min. Samples were adjusted to a final volume of 4 ml in 30% Optiprep and loaded to the bottom of an ultracentrifuge tube. The material was overlaid with 7 ml of 25% Optiprep followed by 1 ml of 5% Optiprep in TNE with 2% Triton X-100. Gradients were centrifuged at 36,100 rpm in an SW41.Ti rotor overnight. Fractions of 1.1 ml were taken from top to bottom and subjected to precipitation with TCA. Western blots were performed to verify the localization of DRM markers and other proteins.

Crude Synaptic Vesicle (LP$_3$) Preparation and Immunoprecipitation—LP$_3$ was prepared according to Ref. 28. 250 μl of LP$_3$ (2 mg/ml total protein concentration) were solubilized by adding equal volumes of 4% CHAPS in HKA buffer (10 mM HEPES-KOH, pH 7.4, 140 mM potassium acetate, 1 mM MgCl$_2$, 0.1 mM EGTA) on ice for 30 min (2% CHAPS final concentration). Insoluble material was removed by centrifugation at 18,500 × g for 15 min. The supernatant was added to 30 μl of protein A/G-Sepharose beads precooled with antibodies against transferrin receptor, synaptotagmin 1, or γ-glutamyl acid decarboxylase and incubated by end-over-end rotation at 4 °C for 4 h. Beads were washed several times with HKA buffer, extracted by SDS sample buffer, and analyzed by quantitative immunoblotting using 125I-protein A for detection followed by phosphorimaging analysis.

Cholesterol Determination—Equal amounts of protein from mock-treated or MβCD-extracted synaptosomes (or LP$_3$ membranes) were subjected to total lipid extraction. Free cholesterol was determined by using the Amplex Red cholesterol assay kit (Molecular Probes). Fluorescence was measured at 610 nm.

Protein Digestion and Isotope Labeling—DRM fractions obtained from flotation gradients derived from untreated or cholesterol-depleted synaptosomes were analyzed by SDS-PAGE side-by-side. Gel lanes were cut into 16 slices of equal size. Excised gel slices were washed with 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate, shrunk by dehydration in acetonitrile, and dried in a vacuum centrifuge. The dried gel pieces were incubated with 120 ng of trypsin (sequencing grade, Roche Diagnostics) in 50 μl of 5 mM ammonium bicarbonate. The enzymatic protein in-gel digestions were performed in the presence of H$_{18}$O (Euriso-top GmbH; 95% 18O) and H$_{16}$O for mock-treated and MβCD-extracted DRM proteins, respectively. After 17 h of incubation at 37 °C, 50 μl of 0.3% TFA in acetonitrile was added, the samples were sonicated for 5 min, and the separated supernatant was dried under vacuum. Samples were reconstituted in 6 μl of 0.1% (v/v) TFA, 6% (v/v) acetonitrile in water. Samples from paired gel slices (16O and 18O samples of adjoining slices) were combined immediately before nano-LC-mass spectrometry.

Nano-LC-MALDI-MS/MS and Identification of Proteins—An Ultimate HPLC system ( Dionex, Idstein, Germany) was coupled off line to MALDI-MS using a Probit Micro fraction collector (Dionex) deposition interface. For desalting and concentrating, the samples were loaded onto a precolumn ( PepMap C$_{18}$, 5 μm, 1 mm inner diameter, Dionex) using a Famos autosampler and a Switchos II HPLC system. Peptides were eluted onto an analytical column ( PepMap C$_{18}$, 3 μm, 100 Å, 150 mm × 75-μm inner diameter, Dionex), and separations were performed at an eluent flow rate of 200 nl/min. Mobile phase A was 0.1% TFA in acetonitrile/water (5:95, v/v), and B was 0.085% TFA in acetonitrile/water (8:2, v/v). Runs were performed using a gradient of 20–65% B in 40 min. The eluent was directly injected into a matrix solution (2 mg of α-cyano-4-hydroxycinnamic acid in 1 ml of 0.1% TFA in acetonitrile/water, 7:3) at a 1:4 flow rate ratio and spotted onto blank target plates ( Applied Biosystems, Framingham, MA). A total of 312 spots per run were deposited in a 24 × 26 array in which each spot consisted of 10 s of chromatographic time.

MS/MS experiments was performed on a MALDI-TOF/TOF instrument (4700 Proteomics Analyzer, Applied Biosystems) equipped with an Nd:YAG (neodymium-doped yttrium aluminium garnet) laser (355 nm) operating at a frequency of 200 Hz. MS spectra were acquired in positive ion reflector mode by accumulation of 3000 consecutive laser shots. After completion of the acquisition of MS spectra the precursor ions for MS/MS analysis were selected automatically according to the selection criteria (a maximum of five peaks per spot, signal-to-noise ratio > 35, and 60 ppm fragment-to-fragment precursor mass tolerance). Both MS and MS/MS spectra were acquired using the instrument default calibration that was updated directly before the run. Fragmentation spectra were acquired with a minimum of 4000 and a maximum of 8000 laser shots (signal-to-noise of fragment ion-dependent stop condition was used). The precursor mass window was set to 80 (full width at half-maximum), the collision energy was 1 keV, and air was used as the collision gas. The GPS Explorer (Version 3.5, Applied
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Biosystems) was used for processing and to submit the data to the MASCOT server (Version 2.0, Matrix Science Ltd., London, UK) for in-house search against the National Center for Biotechnology Information non-redundant protein database (NCBI, June 5, 2005). The maximum of two missed cleavages was allowed, and the mass tolerance of precursor and sequence ions was set to 100 ppm and 0.15 Da, respectively. The search included variable modifications of cysteine with acrylamide, methionine oxidation, and the C-terminal 16O/18O exchange. A protein was accepted as identified if the total MASCOT score was greater than the significance threshold and at least two peptides appeared the first time in the report and were the first ranking peptides. The entire experiment including preparation of synaptic DRMs from rat brain, SDS-PAGE, cutting the gel into 16 slices of equal size, tryptic in-gel digestion, LC-MS analysis, database search, and protein identification was performed twice.

**Quantification of Proteins—Relative quantitation of proteins was performed using an algorithm described previously (30). Briefly relative protein amounts were calculated from relative amounts of 18O-labeled peptides that were identified by MS/MS with a score above the MASCOT homology threshold. Using signal intensities of tryptic peptides containing no, one, and two 18O at m/z, (m + 2)/z, and at (m + 4)/z, respectively, the method considers that either one or both oxygen atoms of the carbonyl group could be exchanged during in-gel digestion of the protein. The contribution of naturally occurring isotopes at (m + 2)/z and at (m + 4)/z as well as the isotopic purity of 18O water (95% 18O) were considered. Quantification of all proteins was based on calculations of isotope intensity ratios of at least two different tryptic peptides. For quantitation, two independent experiments starting from preparation of synaptic DRMs from rat brain and including LC-MS analysis were performed. If the resulting protein ratio deviated by more than 30%, a third independent repeat of the experiment was carried out.**

**Triton X-100 Extraction of Primary Hippocampal Neurons—** Mixed hippocampal neuron-glia co-cultures (14–20 DIV) grown on glass coverslips were washed with PBS and extracted with 500 µl of 120 mM sodium phosphate, pH 7.4, with 0.5% Triton X-100 either on ice or at 37 °C for 10 min. Neurons were carefully washed twice with PBS, fixed, and processed for immunostaining. Images were acquired on a Zeiss Axiovert 200M microscope equipped with the Stallion system (3i Inc.) and analyzed by SlideBook™ software using nearest neighbor deconvolution.

**Cholera Toxin Endocytosis—** Hippocampal neurons prepared from E18 rat embryos (14 DIV) were briefly washed with Krebs-Ringer-HEPES (KRH) solution containing 128 mM NaCl, 25 mM HEPES, 4.8 mM KCl, 1.3 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, and 1.2 mM KH2/K2HPO4, and a corresponding reduction in Na+ to 37 °C for 5 min. Samples were thoroughly washed with KRH buffer, fixed, and processed for indirect immunofluorescence microscopy using monoclonal antibodies against flotillin 1, transferrin receptor, or synaptotagmin 1.

**RESULTS**

**Quantitative Proteomics Analysis of DRMs Isolated from Nerve Terminal Membranes—** To get insights into the protein composition of putative synaptic DRMs we took a proteomics approach. To this aim highly purified rat brain synaptosomes isolated from sucrose step gradients were either mock-treated or pre-extracted twice with 10 mM MgCD to remove cholesterol. This procedure efficiently removed about 95% of the total cholesterol as determined by fluorescence measurements using the Amplex Red assay (data not shown). Synaptosomal membranes were then solubilized with 2% Triton X-100 at 4 °C, thus ensuring a larger excess of detergent over membrane lipids (i.e. detergent-to-lipid ratio ~90; detergent-to-protein ratio ~5,000) and subjected to flotation density gradient centrifugation in the continued presence of detergent. Under these stringent solubilization conditions we recover only a minor part of the DRM marker protein flotillin accumulated within the lighter DRM fractions of the gradient (i.e. fractions 2 and 3). The conditions used are thus designed to ensure a relatively conservative assignment of DRM proteins. Flotillin recovery within DRMs was completely dependent on the presence of cholesterol (Fig. 1). By contrast, transferrin receptor, a non-DRM membrane protein enriched in the cell soma and thus not very abundant in synaptosomes, was undetectable in these fractions irrespective of prior treatment of the synaptosomal starting material with MgCD (data not shown).

To analyze the protein content of synaptic DRMs by quantitative high resolution MS/MS, DRM fractions (corresponding to fraction 2 in Fig. 2) derived from mock- or MgCD-treated (3-fold excess to ensure unequivocal later identification by MS/MS) samples were resolved side-by-side using one-dimensional SDS-PAGE followed by staining with Coomassie Blue (Fig. 2A). Horizontally taken gel slices from each sample were then subjected to in-gel tryptic digestion in the presence of H216O (MgCD-treated sample) or H218O (control sample), and samples were mixed and finally analyzed by nano-LC-MALDI-MS/MS. The prior isotope encoding allows assignment of specific quantitative changes of only the cholesterol-dependent proteins in the DRM fraction (31). Comparison of the isotopic distribution patterns of peptides obtained from the vacuolar proton pump, a DRM protein (Fig. 2C, a and b) (16) and the non-DRM protein α-ketoglutarate carrier protein (Fig. 2C, c and d) indeed displayed dramatically different 18O/16O ratios (15.03 and 15.75 for the V-ATPase versus 1.14 and 1.23 for 2-oxoglutarate carrier protein; isotope ratios normalized for protein content). These data confirm that isotope encoding is a valuable method to quantitatively detect DRM or DRM-associated proteins from synaptic membranes.

Proteins Involved in Cycling of Presynaptic Vesicles Are
Found Together with Scaffolding Molecules in Synaptic DRMs—Quantitative nano-LC-MALDI-MS/MS analysis of synaptic DRMs identified a total of 159 proteins, which were grouped according to their isotopic distribution patterns into three main categories. 101 proteins were identified based on peptides with $^{18}\text{O}/^{16}\text{O}$ ratios greater than 6 and were thus classified as DRM proteins (see also Ref. 31). 21 proteins displaying peptide $^{18}\text{O}/^{16}\text{O}$ isotope ratios between 3 and 6 were deemed DRM-associated. Finally $^{18}\text{O}/^{16}\text{O}$ ratios smaller than 3 indicate that the remaining 37 proteins were not dras-
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| Protein name                          | Molecular mass | Peptides (MS/MS) | Peptides used for quantification | Normalized \(^{18}\text{O}/^{16}\text{O} \) ratio |
|---------------------------------------|---------------|-----------------|----------------------------------|---------------------------------------------|
| Rab3a                                 | 24,954        | 3               | YADDSTPAVSTVGIDFK                | >6                                          |
| Synaptic vesicle glycoprotein 2a (SV2a) | 82,608        | 4               | QVHDTNMR                        | >6                                          |
| Synaptobrevin 2 (VAMP 2)              | 12,683        | 2               | LQQTQAVDEVDIMR                  | >6                                          |
| Synaptophysin                         | 33,289        | 3               | MATDPENIIK                      | 4.26                                        |
| Synapsin I                            | 73,943        | 10              | QTTAAATFSEQVGGSGGAGR             | >6                                          |
| Synaptophysin                         | 63,417        | 5               | SGSLTAFSFSESSFR                 | >6                                          |
| Synaptotagmin I                       | 47,441        | 2               | VFGYNSTGAELR                    | >6                                          |
| Thy-1 antigen                         | 18,160        | 3               | VNLFSDR                         | >6                                          |
| V-ATPase, V0 subunit A, isoform 1     | 96,265        | 6               | SVFIIFQGDQLK                    | >6                                          |
| V-ATPase, V1 subunit C, isoform 1     | 43,873        | 2               | TEFWISAPGK                      | >6                                          |
| V-ATPase, V0 subunit D, isoform 1     | 51,010        | 9               | NVADYYPEYK                      | >6                                          |
| V-ATPase, V1 subunit A, isoform 1     | 68,222        | 16              | ADYAOQLEDMONAFR                 | >6                                          |
| V-ATPase subunit E1                   | 26,112        | 3               | IMEYYEK                         | >6                                          |
| Exo- and endocytotic proteins         |               |                 |                                  |                                             |
| Adaptor protein complex, AP-2, α subunit | 103,979       | 3               | YGGTFQNVSVK                     | >6                                          |
| Adaptor protein complex, AP-2, β 2 subunit | 105,691      | 2               | ALQHMDFIAQFNK                   | >6                                          |
| Adaptor protein complex, AP-3, β 3B subunit | 119,096      | 3               | ATGYQELPDWPEEAPDPSVR            | >6                                          |
| Clathrin, heavy chain                 | 191,477       | 7               | SVNESLNLITEEQYQAER              | >6                                          |
| Clathrin, light chain                 | 26,964        | 3               | VADEAFYK                        | >6                                          |
| V-ATPase subunit E1                   | 95,867        | 11              | TGLFTPDLAFEVTK                  | >6                                          |
| Hsc70                                 | 70,884        | 18              | IINEPTAAIAAYGLDDK               | >6                                          |
| Munc18-1                              | 67,568        | 3               | REPLPSLEAVYLITPSEK              | >6                                          |
| N-Ethylmaleimide-sensitive fusion protein (NSF) | 82,600      | 8               | VVNGPEILKNYVGSEANIR             | >6                                          |
| SNAP-25a                              | 20,545        | 5               | AWGNQDGWASQPAR                  | >6                                          |
| SNAP25-interacting protein            | 129,665       | 9               | HTQAOPQGLADQAAK                 | >6                                          |

Proteins including synaptobrevin 2, synaptotagmin 1, synaptophysin, synapsins, SV2a, Rab3A, and the V-ATPase, some of which were identified based on as many as 36 peptides in total (Table I, top). The glycosylphosphatidylinositol-linked “raft” marker protein Thy-1 identified here as a component of...
synaptic DRMs has also been demonstrated to be a common component of SVs (32). In addition, we found factors involved in exocytic membrane fusion such as SNAP-25a, Munc18-1, or NSF and in SV recycling including clathrin (both heavy and light chains), the α and β2 subunits of AP-2, neuronal AP-3B (2, 3), uncoating ATPase (Hsc70) (33), and dynamin 1. Many of these factors have previously been shown to associate with cholesterol-rich membrane sites (i.e. SNAP-25, clathrin, and synaptobrevin 2) (19, 20), to directly bind to cholesterol (i.e. synaptotagmin 1, synaptophysin) (13), or to form cholesterol-dependent protein complexes (i.e. synaptophysin-synaptobrevin 2) (16, 17). As expected, a variety of signaling proteins (31) such as Gαq, Gαz, the Gαi family, Gβγ, cGMP and cAMP phosphodiesterases, protein kinase C (β and γ), cAMP-dependent protein kinase, calcium-calmodulin-dependent protein kinase II, the α and δ1 subunits of voltage-gated calcium channels, and the NRI subunit of N-methyl-o-aspartate receptors (supplemental table) were specifically enriched in synaptic DRMs. Finally we identified synaptic scaffolding molecules including PSD-95 (34), PSD-93, densin-180, ProSAP/Shank 2, the homer family protein Vesl-1L, SynGAP-a (a PSD-95-binding Ras-GAP), 14-3-3 (γ and δ), GAP-43, and septins 5 and 7 as well as the lipid-anchored proteins neurotrimin and paralemmin within DRMs (supplemental figure and supplemental table). Surprisingly several mitochondrial membrane proteins (i.e. F0F1-ATPase) were also present in the DRM fraction (supplemental table). These may either correspond to a pool of mitochondrial enzymes located in cholesterol-rich plasma membrane sites as previously shown by surface biotinylation, immunofluorescence, and subcellular fractionation experiments (35, 36) or be nonspecific contaminants in the preparation.

To confirm the localization of SV cycling and endocytic proteins to synaptic DRMs we analyzed their distribution within flotation density gradients prepared from mock-treated or cholesterol-depleted synaptosomal membrane lysates. In agreement with our proteomics analysis the SV proteins synaptotagmin 1, synaptobrevin 2, and synaptophysin were all present in DRM fractions isolated from Triton X-100-lysed synaptosomes, and this was dependent on the cholesterol content (Fig. 3A). Similar results were seen for the endocytic proteins clathrin and AP-2. Moreover we could detect small amounts of PIPK Iγ (23) in synaptic DRMs (Fig. 3B), suggesting that phosphatidylinositol 4,5-bisphosphate synthesis may at least in part occur within cholesterol-rich membrane sites (21). PIPK Iγ escaped detection in our proteomics analysis presumably due to its relatively low abundance. The presence of SV and endocytic adaptor proteins in synaptic DRM fractions was not an artifact caused by the use of Triton X-100 as a detergent because we could detect synaptobrevin 2, syn-
The SV Proteins Synaptotagmin 1, Synaptophysin, and Synaptobrevin 2 Form a Cholesterol-dependent Protein Complex—Based on the presence of SV proteins in the DRM fraction, the ability of some of them to directly bind to cholesterol (13), and the unusually high cholesterol content of SV membranes (11) we speculated that SV proteins might form cholesterol-dependent protein complexes. To this aim crude SVs were solubilized with CHAPS and subjected to immunoprecipitation using monoclonal antibodies against synaptotagmin 1 or γ-glutamic acid decarboxylase (GAD6) or the transferrin receptor (8). Following MJCD-mediated depletion of cholesterol by 80% (mean ± S.D.; n = 3) the amounts of synaptophysin and synaptobrevin 2 associated with synaptotagmin 1 were reduced by about 80 and 50%, respectively. Quantitative immunoblots were developed with 125I-protein A and analyzed by phosphorimage analysis. C, quantitative analysis of the amount of synaptophysin and synaptobrevin 2 associated with synaptotagmin 1. Data represent normalized mean (±S.E.; n = 3). 20% Std, ¼ of the total material loaded at the bottom of the gradient; IP, immunoprecipitate; CoIP, co-immunoprecipitate; TR, transferrin receptor; GAD6, glutamic acid decarboxylase; MβCD, 2,3-dimethylbutane; GAD6, γ-glutamic acid decarboxylase; GAD6, glutamic acid decarboxylase.

Presynaptic Vesicle Membrane Proteins as Well as Clathrin and AP-2 Display Partial Resistance to Detergent Extraction—To investigate whether SV proteins are localized to cholesterol-rich membranes in situ we examined their resistance to extraction with Triton X-100 in the cold in primary neurons in culture. Following treatment of hippocampal neurons with 0.5% Triton X-100 at 4 °C the SV proteins synaptophysin, synaptobrevin 2, and synaptotagmin 1 remained concentrated at presynaptic sites, similar to what is seen in the absence of detergent (Fig. 5A). The transferrin receptor, which mainly localizes to recycling endosomes within the somatodendritic compartment, was solubilized completely under these conditions (Fig. 5B), consistent with its absence from cholesterol-enriched membranes in vitro. Labeling for all of the AP-2 complex in DRMs derived from synaptosomal lysates that had been extracted with CHAPS instead of Triton X-100 (Fig. 3C).
these proteins was lost if detergent extractions were carried out at 37 °C, conditions under which cholesterol-rich membrane microdomains are solubilized. Because SV proteins undergo rapid, activity-dependent recycling (2–4) we also analyzed the distribution of clathrin and the AP-2 adaptor complex. Similar to what was observed for bona fide SV membrane proteins both clathrin (Fig. 6, A and B) and AP-2 (Fig. 6, C and D) were partially resistant to Triton X-100 extraction and remained co-localized with synaptobrevin 2. Presynaptic Cycling Vesicles Co-localize with FITC-labeled Cholera Toxin B in Stimulated Primary Neuron—Because treatment of hippocampal neurons with detergent is a relatively crude method to assign localization of proteins to cholesterol-enriched microdomains, we decided to tag living neurons actively undergoing SV cycling with a DRM marker. To this aim primary neurons stimulated by elevated concentrations of K⁺/H₁₁₀₀₁ and calcium were incubated with FITC-CTB, which selectively associates with GM1-containing cholesterol-rich membrane domains (25, 27). FITC-CTB displayed a punctate distribution that largely overlapped with that of the DRM marker protein flotillin 1 (also termed reggie-2) and the SV membrane protein synaptotagmin 1 but not with the transferrin receptor (Fig. 7). Our combined data thus suggest that presynaptic recycling vesicles display properties characteristic for cholesterol-rich membrane microdomains both in vitro and in living primary neurons.

**DISCUSSION**

There are only a few proteomics studies on DRMs from brain, but these have either focused on individual proteins (34, 39, 40) or not been quantitative (41). Thus, to our knowledge we present here the first quantitative study on the composition of DRMs isolated from synapses using nano-LC-MALDI-MS/MS in combination with isotope labeling. In agreement with previous studies in labeled HeLa cells (31) we identified known components of DRMs including the glycosylphosphatidylinositol-linked marker protein Thy-1, flotillin (40), several α and β subunits of heterotrimeric G proteins, prion protein (42), and the Na⁺/K⁺-ATPase along with a variety of other signaling and synaptic scaffolding proteins such as septins, PSD-95, PSD-93, densin-180, spectrins, SynGAP-a, and Vesl-1L.

Moreover we identified a number of SV proteins including the vesicular SNARE synaptobrevin 2, synaptophysin, SV2a, V-ATPase, and the calcium-sensing protein synaptotagmin 1 as DRM or DRM-associated proteins. These are likely to be specific components of synaptic cholesterol-rich membranes based on the following criteria. First, the SV proteins men-
tioned above are specifically enriched within synaptic DRMs because cholesterol depletion reduced their amount in light density floating fractions by a factor of 4 (for synaptophysin) to more than 6 as quantified by differential $^{18}$O/$^{16}$O labeling. Second, immunoblot analysis of density gradients confirmed the cholesterol-dependent partitioning of SV proteins into the floating fractions. Solubilization of synaposomes with CHAPS instead of Triton X-100 yielded similar results, indicating that DRM association is not caused by the nature of the detergent. Third, the localization of SV proteins to DRMs was paralleled by their partial resistance to extraction with Triton X-100 at 4 °C in primary neurons and the formation of a cholesterol-dependent complex between synaptotagmin 1, synaptophysin, and synaptobrevin 2. Finally recycling synaptotagmin 1 co-localized with labeled cholera toxin B, a marker for cholesterol-enriched DRMs (25, 43), in hippocampal neurons. Our data thus support and extend previous studies that have indicated functions for cholesterol in toxin internalization into neurons (43, 44), synapse formation (14), and SV protein clustering and in the biogenesis of SVs from recycling plasmalemmal pools in neuroendocrine cells (13) in agreement with the unusually high cholesterol content of SV membranes (11). High resolution stimulated emission-depletion microscopy imaging of actively cycling SVs suggests that SV proteins indeed may remain clustered following exocytic insertion into the plasma membrane (10), a process that may be aided by the membrane lipid content, in particular cholesterol. Moreover at least in neuroendocrine cells cholesterol is important for the organization of plasmalemmal SNAREs (19, 20) and phosphatidylinositol 4,5-bisphosphate into microdomains (20, 21) and may thus serve as a spatial organizer of neurosecretory vesicle membrane traffic in agreement with our observation that the “raftophile” cholera toxin B co-localized with recycling vesicles in hippocampal neurons. The finding that the endocytic proteins clathrin, AP-2, and dynamin 1, which form preassembled pools at pre- and postsynaptic sites, were also specifically enriched in synaptic DRMs along with scaffolding proteins is also in line with this hypothesis. In addition, the AP-2-related neuronal AP-3 complex implicated in an alternative SV biogenesis pathway from endosomes (45) was present within synaptic DRMs. Whether DRM association is a specific property of clathrin and AP-2 at synapses or reflects a general phenomenon in all cell types is unclear at present, but we note that clathrin has been identified as a DRM protein in HeLa cells (31) and B-lymphocytes (46). Consistent with this, it has been reported that cholesterol depletion leads to a partial inhibition of clathrin-dependent endocytosis and the accumulation of flat plasmalemmal coated pits (47, 48).

Unlike proposed “lipid rafts” within the plasma membrane, which may be heterogenous in size and composition (42), SVs constitute distinct membrane-bounded entities with a specific protein and lipid content (4), and this molecular identity is maintained during repetitive cycles of exo- and endocytosis.
Although the question to which extent DRMs are a true correlate of “rafts” within native cell membranes (26, 27) cannot be precisely answered at this time, we believe that our combined data obtained in vitro and in hippocampal neurons in culture are likely to be of physiological relevance and may serve as a starting point for the further analysis of the role of cholesterol in axon guidance (50), synapse formation (14), maintenance, and synaptic vesicle cycling (12, 15) in vivo.

Acknowledgments—We gratefully acknowledge Inge Walther for help with the preparation of neurons and Drs. Reinhard Jahn (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany) and Pietro De Camilli (Yale University School of Medicine, New Haven, CT) for the kind gift of antibodies.

Fig. 7. Internalized FITC-labeled cholera toxin colocalizes with flotillin 1 and synaptotagmin 1 at synapses. Hippocampal neurons isolated from E18 rats (14 DIV) were washed briefly with KRH solution and incubated with 10 μg/ml FITC-CTB in KRH/high K⁺ at 37 °C for 5 min. Samples were thoroughly washed (three times) with cold KRH solution on ice, fixed, and analyzed by indirect immunofluorescence deconvolution microscopy using antibodies against flotillin 1, transferrin receptor (TfR), or synaptotagmin 1 (Syt 1). Boxed areas are magnified in the upper right corner of the merged images. Scale bar, 20 μm.

The image contains fluorescent microscopy images of hippocampal neurons showing colocalization of FITC-CTB with flotillin 1 and synaptotagmin 1 at synapses.
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