Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling

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Tandem MS has identified 209 proteins of clathrin-coated vesicles (CCVs) isolated from rat brain. An overwhelming abundance of peptides were assigned to the clathrin coat with a 1:1 stoichiometry observed for clathrin heavy and light chains and a 2:1 stoichiometry of clathrin heavy chain with clathrin adaptor protein heterotetramers. Thirty-two proteins representing many of the known components of synaptic vesicles (SVs) were identified, supporting that a main function for brain CCVs is to recapture SVs after exocytosis. A ratio of vesicle-*N*-ethylmaleimide-sensitive factor attachment protein receptors to target-*N*-ethylmaleimide-sensitive factor attachment protein receptors, similar to that previously detected on SVs, supports a single-step model for SV sorting during CCV-mediated recycling of SVs. The uncovering of eight previously undescribed proteins, four of which have to date been linked to clathrin-mediated trafficking, further attests to the value of the current organelle-based proteomics strategy.

The sorting of receptors and other cell-surface proteins from the plasma membrane via clathrin-mediated endocytosis is the basis for a range of essential cellular processes, including the uptake of nutrient and signaling receptors, the control of cell and serum homeostasis through the internalization of plasma membrane pumps, and a contribution to learning and memory through the regulation of surface expression of neurotransmitter receptors (1). Until recently, it was thought that clathrin assembly into progressively curved lattices provided the driving force for the formation of clathrin-coated pits (CCPs) and vesicles (CCVs), and that the adaptor protein 2 (AP-2) complex was solely responsible for recruiting clathrin to the membrane and for binding to endocytic cargo, concentrating the cargo in CCPs (1, 2). However, clathrin assembly may not be sufficient to drive membrane curvature (3), and the previously accepted obligatory role for AP-2 in coat assembly and cargo recruitment has been recently questioned (4–6).

In neuronal tissues, CCVs are postulated to be responsible for the recycling of synaptic vesicles (SVs) during neurotransmission (7). As such, CCVs retrieve SV membranes from the plasma membrane after SV collapse, concomitant with neurotransmitter release. Many of the components of the endocytic machinery are concentrated in the presynaptic compartment (8), and disruption of these proteins affects neurotransmission (9). Moreover, a number of SV proteins have been identified as components of isolated CCVs (10, 11). Synaptic transmission involving intermittent fusion of SVs without complete collapse (12, 13) has also been demonstrated. The prevalence of such a "kiss-and-run" mechanism with the alternative model of full fusion is uncertain (14). Even in the membrane retrieval model via CCVs, it remains unclear whether SVs are generated directly from CCVs (15, 16) or whether they require an additional sorting step through endosomal membranes localized in the presynaptic compartment (7, 17). Here, using a tandem MS (MS/MS) analysis of CCVs isolated from adult rat brain, several of these issues have been addressed.

Experimental Procedures

CCV Purification and Analysis. CCVs were isolated from adult rat brain as described (18). Suspensions of CCVs were deposited on a 0.22-μm nitrocellulose filter and processed for electron microscopy, using a double fixation protocol with osmium and tannic acid (19). For quantification, all vesicles were circled by using PHOTOSHOP (Adobe Systems Canada, Ottawa), filled with white, and the regions outside were darkened artificially to remove all other white spots. The number of white objects was automatically deduced by using the digital measurement system, SIGMA SCAN PRO (SPSS, Inc., Chicago). The diameter of each vesicle was assessed as a function of its surface by SIGMA SCAN PRO. All antibodies used in this study are described in Table 1, which is published as supporting information on the PNAS web site.

After the separation of CCV proteins by 1D SDS/PAGE, the gel lanes were divided horizontally into 60 or 61 slices that were individually processed to yield in-gel trypsin digestion products (18). The resulting peptide mixtures were separated and analyzed in an automated system by nanoscale liquid chromatography quadrupole time-of-flight MS/MS, as described in detail in *Supporting Experimental Procedures*, which is published as supporting information on the PNAS web site. After fragmentation in the MS/MS mode, spectra were analyzed by MASCOT software to identify tryptic peptide sequences matched to the National Center for Biotechnology Information (NCBI) nonredundant protein database with a confidence level of 95% or greater (20). Each MS/MS spectrum was then visually inspected. Spectra with four clearly identified consecutive y ions were chosen as a validated subset of the previous MASCOT assignments. All other MS/MS spectra, amounting to 15% of those assigned by MASCOT, were rejected. For each protein with three or fewer peptide assignments, a further analysis was performed. Peptide sequence tags were generated from the MS/MS spectra as described (21). Only ions at least three times above background were taken into account. The sequence tags were searched against the NCBI nonredundant protein database using the PepFrag search routine (http://129.85.19.192/prowl/pepfragch.html), with parameters of trypsin digest with one missed cleavage, cysteines carboxyamidomethylated, and a tolerance of 1 mass unit for the parent peptide or the fragment ion. PepFrag search results were rejected if different peptide sequences were retrieved for a given sequence tag. Greater than 95% of the MS/MS spectra evaluated were found to be consistently assigned by MASCOT analysis.

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Abbreviations: CCV, clathrin-coated vesicle; cpCCV, cushion-pellet CCV; SV, synaptic vesicle; MS/MS, tandem MS; CHC, clathrin heavy chain; CLC, clathrin light chain; TGN, transGolgi network; EM, electron microscopy; ER, endoplasmic reticulum.

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Peptide assignments were based on the number of peptides found specifically in their cognate protein (defined as specific peptides) and those found in two or more different proteins (shared peptides) within a given band. The identifications presented in Table 2, which is published as supporting information on the PNAS web site, are the minimal set of proteins to explain the peptides assigned by MASCOT on a band-to-band basis. Proteins are identified as a single entry with the band-to-band distribution shown (Table 2). For Figs. 2 and 4, the peptide numbers for each protein were a sum of all peptides counted as specific and a proportion of those peptides shared with other proteins in the gel slice with proportionality a consequence of the number of specific peptides. For example, β1- and β2-adaptin had 64 and 208 specific peptides, respectively. The 90 peptides shared between the two proteins were thus apportioned by the ratio of specific peptides (i.e., 21 to β1, 69 to β2). As a final criterion, only those proteins identified in at least two of the three CCV preparations analyzed were retained. Therefore, the protein assignments were a consequence of 9,239, 9,904, and 12,091 fragmented ions from the three CCV preparations. From these, 1,560, 2,225, and 2,348 MS/MS profiles were assigned.

Results

Isolation of CCVs. Brain CCVs were isolated by differential centrifugation, Ficoll density gradient centrifugation, and pelleting through a D_2O/sucrose cushion (10). These cushion-pellet CCVs (cpCCVs) were further enriched by centrifugation on linear 20–50% sucrose gradients (18). A clear enrichment for polypeptides at the mobility of clathrin heavy chain (CHC), clathrin light chains (CLCs), and the α-, β-, γ-, and μ-adaptin subunits of the AP-2 complex, which functions at the plasma membrane, and the AP-1 complex, which functions at the transGolgi network (TGN), was evident in the CCV fractions (Fig. 1*A*). Western blots confirmed enrichment for CHC, CLCs, and α-adaptin (Fig. 1*B*). Using a filtration apparatus to ensure random sampling (20), transmission electron microscopy (EM) revealed a preparation highly enriched in CCVs of uniform shape and size with a diameter of 53 ± 1 nm (based on morphometric analysis of 269 CCVs) (Fig. 1*C*). Quantitation over three separate preparations revealed 73% of all structures as coated, whereas 65% of the structures were coated in cpCCVs (Fig. 1*D*). Uncoated structures were heterogeneous in shape and size and varied from 13 to >200 nm in diameter (based on the analysis of 1,033 noncoated structures).

Protein Characterization by Liquid Chromatography–MS/MS. In three independent preparations, a total of 209 proteins were reproducibly identified with up to 17 different proteins found in one gel slice (Fig. 2*A*, from one of three preparations). Notably, in gel slices 9–11, where little Coomassie blue staining was observed, 23 proteins were identified, albeit with low peptide abundance (33 assigned peptides in total, Fig. 2 *A* and *B*). In contrast, gel slice 49 contained only one protein, CHC, with 55 assigned peptides (Fig. 2 *A* and *B*). In fact, the distribution of the total number of assigned peptides revealed peaks corresponding to peptide peaks for CHC, CLCs, and μ-adaptins (Fig. 2*C*). An average of 10,430 (±1,492 SD) MS/MS profiles were generated per CCV preparation, from which 19.6% (±2.8% SD) were assigned to proteins by using described criteria (*Experimental Procedures*). The lack of assignment of profiles can be for many reasons, including low-quality spectra, posttranslational modifications, or limitations in the rat database. However, the percentage of MS/MS profiles assigned here is greater than in a study of the human spliceosome (22).

Accounting for all fragmented peptides matched to their cognate proteins revealed those assigned specifically to individual proteins and those shared among different proteins within a given band (Table 2). Approximately 50% of the assigned peptides (3,069 of the 6,133 total) were assigned to the 18 identified coat proteins, which represent most known components of clathrin coats (Fig. 2*D* and Table 2). As expected, Western blots for proteins selected from this group [CHC, CLC, α-adaptin, γ-adaptin, and HIP12 (huntingtin-interacting protein 12)] revealed a high degree of enrichment in CCVs compared to brain homogenate (Fig. 3). The proteins were generally enriched in CCVs compared to cpCCVs (Fig. 3), consistent with the enrichment of coated profiles between these two fractions observed by EM (Fig. 1*D*).

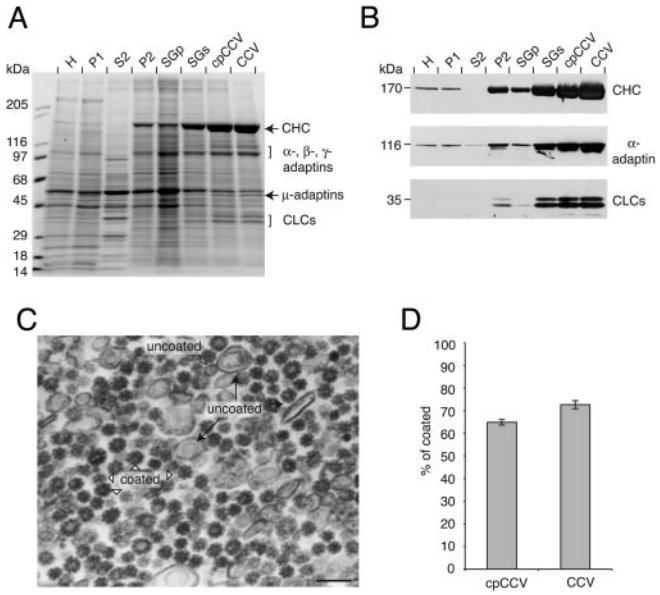

Fig. 1. Isolation of CCVs. (*A*) Coomassie blue staining of proteins separated by SDS/PAGE from each step of the enrichment protocol for CCVs. Aliquots of 40 μg of protein were loaded on each lane. H, P1, S2, P2, SGp, SGs, cpCCV, and CCV represent homogenate, first pellet, second supernatant, second pellet, sucrose/Ficoll gradient pellet, sucrose/Ficoll gradient supernatant, D_2O cushion pellet, and CCVs, respectively. The predicted migratory positions of CHC, CLCs, and α-, β-, γ- and μ-adaptins are indicated. (*B*) Immunoblots detected the presence CHC, CLCs, and α-adaptin as indicated. (*C*) CCV fractions were evaluated by random sampling EM. Examples of CCVs are indicated by arrowheads, noncoated vesicles with comparable diameter to CCVs are indicated by short arrows, and noncoated profiles of diameter larger than CCVs are indicated by long arrows. (Bar = 200 nm.) (*D*) The proportion of coated profiles in the cpCCV and CCV preparations was evaluated from 64 and 66 independently and randomly sampled EM images, respectively. In total, 9,974 (cpCCV) and 10,457 (CCV) profiles were counted. The mean and SD from three independent experiments are 64.9 ± 1.2% and 72.7 ± 1.7% for cpCCV and CCV, respectively.

Peptide Accounting Reveals Endocytic Protein Complexes. Assignment of peptides to proteins was reproducible among the three CCV preparations (note the SD bars in Fig. 4*A*). When such peptide accounting was normalized for the size of the protein, the expected 1:1 relationship was found for CHC and CLCs (Fig. 4*A*). A comparable stoichiometry was evident for AP-2 adaptin subunits and for the subunits of AP-1, with the exception of γ-adaptin. Normalization of peptides for the various adaptin subunits reveals that AP-1 and -2 complexes combined are less abundant than predicted if all CHCs are occupied by AP complexes (Fig. 4*B*). Moreover, AP-2 was ≈3-fold more abundant than AP-1, suggesting a 3:1 ratio in whole brain for CCVs arising from the plasma membrane vs. the TGN. In contrast, AP-1 complexes are significantly more abundant than AP-2 complexes in CCVs isolated from rat liver (M.G., unpublished data).

SV Recycling. The abundance of plasma membrane-derived CCVs suggests that the brain is specialized for endocytosis relative to

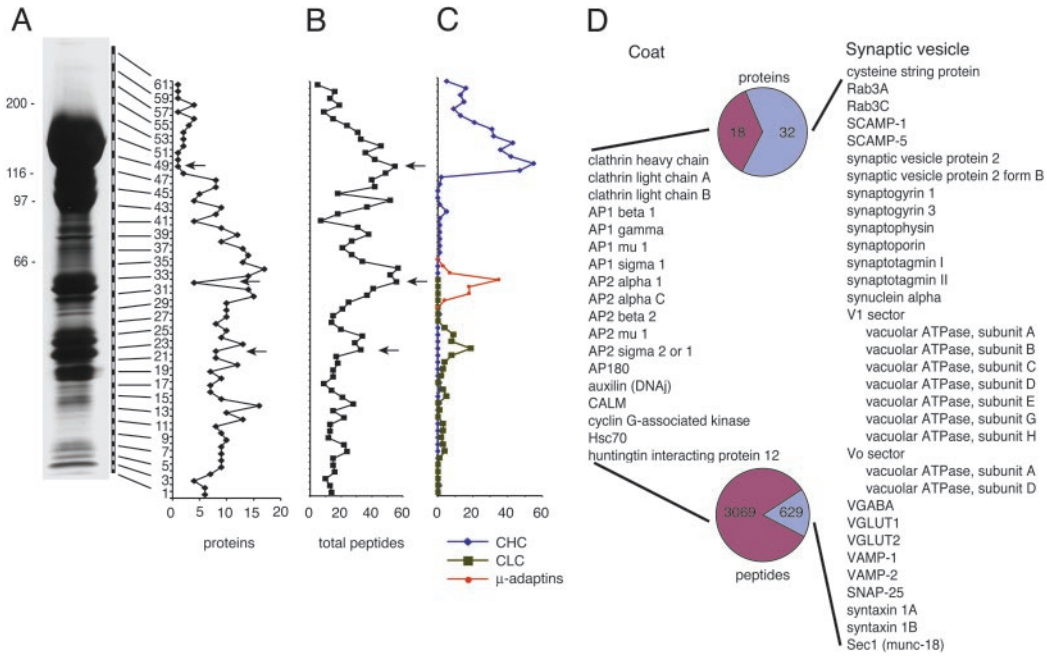

Fig. 2. Distribution of proteins and peptides in CCVs. Proteins from one of the three independent CCV preparations were separated on SDS/PAGE, and 61 gel slices were cut as illustrated and processed for MS/MS. Molecular weight markers are indicated on the left. (*A*) Number of proteins identified in each gel slice. (*B*) Number of fragmented peptides assigned to proteins in each gel slice. (*C*) Numbers of peptides assigned to CHC (blue triangles, total assigned 398), CLC (black squares, total assigned 28), and μ-adaptin subunits of AP-1 and -2 (red circles, total assigned 84) are indicated. In slice 49 (arrow), 100% of the peptides are assigned to the CHC. In slice 32 (arrow), 63% of the total peptides are accounted for by the μ-subunits of AP-1 and -2, and in slice 21 (arrow), 39% of the total peptides are accounted for by CLC. (*D*) Known components of clathrin coats (coat) and SVs identified on CCVs by MS/MS are listed. The total number of proteins and peptides assigned to each category are presented in the pie graphs as a percentage of the total for the two categories.

clathrin trafficking at the TGN. Remarkably, the single largest category of proteins found consisted of the 32 that define many known components of SVs (23) (Fig. 2*D*). With few exceptions, these proteins were identified with multiple peptides that together represent ≈20% of the total number of peptides assigned to clathrin coat proteins (Fig. 2*D*). Western blots for synaptophysin and synaptotagmin reveal their enrichment in CCVs and their comigration with CHC and α-adaptin on sucrose gradients (Fig. 3). Interestingly, we did not detect the abundant synapsin proteins, which are known to dissociate from SVs after nerve stimulation (24).

In classic studies, Heuser and Reese (7) found that loss of SVs

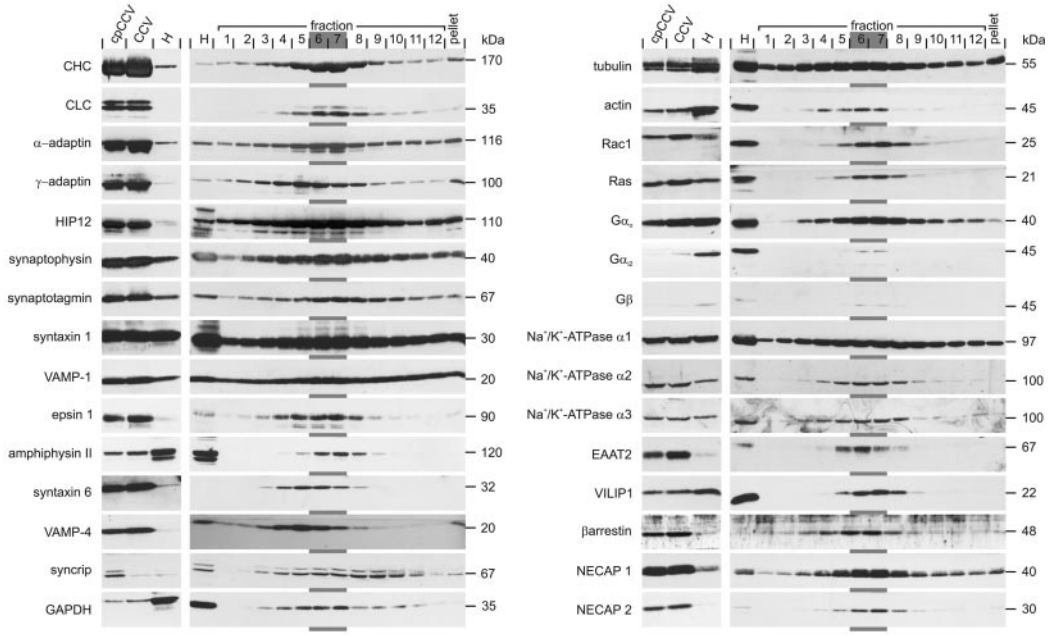

Fig. 3. Western blot of selected proteins found in CCVs. Equal protein aliquots (40 μg) for cpCCV, CCV, and homogenate (H) fractions were prepared for Western blots with the indicated antibodies. For sucrose gradient fractions, an equal volume (30 μl) was applied to each lane.

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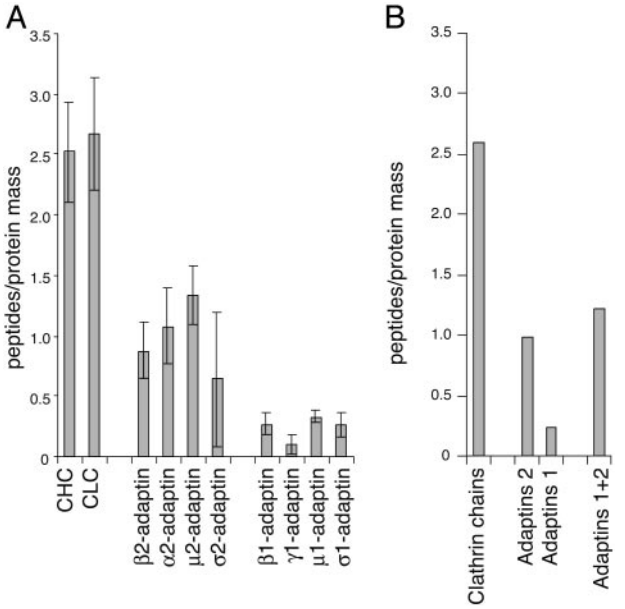

Fig. 4. Clathrin coat. (*A*) Normalization of the total number of peptides found in each preparation for each protein by dividing by the calculated mass of the protein (in kilodaltons) reveals a 1:1 relationship for CHC and CLCs as well as AP-2 and -1 subunits. The error bars represent the SD among the three preparations. (*B*) Peptide to protein mass of CHC and CLCs combined (clathrin) compared to the subunits of AP-2 combined, AP-1 combined, or the subunits of AP-2 and -1 combined.

after nerve stimulation at 10 Hz was balanced by membrane retrieval from the plasmalemma, coincidental with tracer entry into neuronal CCVs. They concluded that sorting would occur by clathrin coats "gathering components of vesicle membrane and excluding components of the plasma membrane as it pinched off vesicles." An alternative model to CCV-mediated SV recycling is the kiss-and-run model, in which SVs fuse transiently with the plasma membrane with direct retrieval of the SV membrane on neurotransmitter release. Recent studies have suggested that kiss-and-run is the predominant mode of SV retrieval, at least in specific neuronal populations (12, 13). Although our data do not dispute that kiss-and-run is used in neurons, the finding of a near-complete inventory of the known SV proteins in CCVs from whole brain (Fig. 2*D*), with a lack of abundant presynaptic plasma membrane proteins, supports an important role for CCVs in SV recycling. Moreover, the abundance of CCVs engaged in recycling SVs in brain may explain why factors that participate in "housekeeping" forms of clathrin-mediated endocytosis, such as transferrin receptor or low-density lipoprotein receptor-related protein, were not detected in our analysis, even though they are readily detected by MS/MS in CCVs from liver (M.G., unpublished data).

Other SV proteins identified include vesicle-associated membrane protein (VAMP)-1 and –2, which are v-soluble-*N*-ethylmaleimide-sensitive factor attachment protein receptors (v-SNARES) (51 matched peptides), and syntaxin 1A/B and synaptosomal associated protein of 25 kDa (SNAP-25), which are t-SNAREs (38 matched peptides) (Fig. 2*D* and Table 2). These proteins form the core complex responsible for SV fusion (25). Syntaxin 1 and VAMP-1 are enriched on CCVs (Fig. 3). Given that the averaged molecular mass of SNAP-25 and syntaxin 1A/1B is ≈1.7 times that of VAMP-1 and -2, there appears to be a near 2.4-fold excess of v-SNAREs over t-SNARES. Walch-Solimena *et al.* (26) previously reported a similar ratio of v-SNAREs to t-SNAREs on SVs, supporting the hypothesis that CCVs directly recycle SVs without a need to sort out plasma membrane-derived t-SNAREs via endosomes (15, 16). This mechanism of SV sorting is also supported by the detection of 68 peptides for the SV proteins synaptotagmins 1 and 2 with none detected for synaptotagmins 3 or 7, abundant proteins of the synaptic plasma membrane (28).

Additional Identified Proteins

Endocytic Accessory Proteins. We found one endocytic accessory protein, epsin 1, whereas others such as amphiphysin I and II, dynamin, and synaptojanin were not detected (Table 2). This is not surprising, because the components of the endocytic machinery transiently associate with CCVs (8). MS/MS results were confirmed by Western blots, which revealed that epsin 1 was enriched on CCVs and comigrated with CHC and α-adaptin after fractionation of cpCCVs on sucrose gradients (Fig. 3). Amphiphysin II also comigrates with CHC and α-adaptin but is present on CCVs at much lower levels than in an equal protein aliquot of brain homogenate (Fig. 3). Therefore, the MS/MS methods used here were limited in their ability to comprehensively detect CCV proteins.

Vesicle Trafficking. Twenty-nine proteins were identified that function in vesicle trafficking, in addition to those associated with SVs (Table 2). Included were syntaxin 6 and VAMP-4, which form a SNARE complex on TGN-derived CCVs (28) and are enriched on CCVs (Fig. 3). Strikingly, these proteins cosediment on sucrose gradients with CCVs containing γ-adaptin and run one fraction denser than the SV SNARE complex proteins syntaxin 1 and VAMP-1, which comigrate with the plasma membrane-derived CCV coat proteins α-adaptin and HIP12 (Fig. 3). These results suggest a distinction in density between TGN- and plasma membrane-derived CCVs in the brain. We also detected abundant levels of α-SNAP and *N*-ethylmaleimide-sensitive factor, which are necessary to uncouple v-/t-SNARE cis pairs on CCVs, freeing the v-SNAREs for another round of fusion after clathrin uncoating and neurotransmitter replenishment (25).

In addition to SV-associated Rab3A/3C, we were surprised to detect Rabs that are thought to localize predominantly to endosomal membranes (Rab4A/B, -5A-C, -7, and -11), endoplasmic reticulum (ER)/Golgi (Rab1, -2, -6, and -10), and postGolgi (Rab8) (29). The CCV preparations appear to be relatively free of contaminating membranes, because we identified no mitochondrial proteins, no nuclear proteins such as histones and nuclear lamins, and no Golgi contaminants (GM130, etc.). Moreover, we identified only one protein that may be considered a resident ER component (calreticulin, two peptides), and we did not detect other ER residents such as protein disulfide isomerase, GRP 94, or calnexin. These results make it difficult to suggest that non-SV Rabs were detected due to membrane contamination.

Myelin and RNA Granule. We identified two myelin proteins that may be present due to contamination, because myelin is very abundant in the adult brain. Moreover, we obtained 25 ribosomal and RNA-binding proteins. These proteins appear to be components of an RNA granule, which cofractionates with CCVs due to a coincidental size and density (S.W., unpublished data). However, we were able to partially separate RNA granules from CCVs using the 20–50% linear sucrose gradients as indicated by Western blot against syncrip (Fig. 3), an RNA granule component (S.W., unpublished data). Given that the RNA granule represents a minor contaminant of CCVs, it is not surprising that many of its components were detected with limited numbers of peptides (Table 2).

Metabolic Enzymes. We identified 17 metabolic enzymes, 12 of which cover 10 of the 11 enzymatic steps necessary to convert glucose to lactate (glycolysis). Western blots confirm that a pool of glyceraldehyde 3-phosphate dehydrogenase, a key glycolytic enzyme, is associated with CCVs (Fig. 3). Both aldolase and

phosphofructokinase have been demonstrated to interact with subunits of the vacuolar ATPase (proton pump) (30, 31), which is a component of CCVs and was readily detected in our preparations. In fact, previous studies suggest that the ATPase and glycolytic components form a "metabolon" to maximize energy provision for the proton pump (ref. 31 and references therein). Such a complex may thus assemble on CCVs for the acidification of the CCV lumen.

Cytoskeleton. We detected 11 proteins implicated in cytoskeletal function. The vast majority of peptides in this category assigned to α- and β-tubulin, consistent with the previous identification of tubulin as a major component of CCVs (32) and with more recent evidence of a role for the tubulin cytoskeleton in CCV trafficking (33, 34). Western blots for tubulin, actin, and Rac1 reveal that only Rac1 is enriched on CCVs, although a pool of each protein codistributed with CCVs on sucrose gradients (Fig. 3).

Signaling GTPases. We found eight GTPases including Ras, a constitutive CCV component (35) that is enriched on CCVs (Fig. 3). The heterotrimeric G-protein G_{o2} has been found constitutively associated with SVs where the α-subunit regulates the activity of vesicular monoamine transporters (36). It has also been demonstrated that functional G-protein heterotrimers control glutamate uptake into SVs (37). We have confirmed that $G_{\alpha o}$, along with two other subunits identified in our analysis, $G_{\alpha i2}$ and G_{β}, are found on CCVs (Fig. 3), suggesting that heterotrimeric G-proteins remain associated with SVs during their recycling. βarrestin associates with G-protein-coupled receptors for their internalization by CCVs. This protein is a known constituent of CCVs and was readily detected by Western blot (Fig. 3) yet was undetectable by MS/MS.

Integral Plasma Membrane. The 18 integral components of the plasma membrane identified, along with SVs, appear to represent the major constitutive cargo for recycling brain CCVs. Of interest, we detected several classes of plasma membrane transporters, including Na^+/K^+-ATPase subunits and Na^+-dependent transporters, responsible for neurotransmitter uptake (Table 2). Western blots for Na^+/K^+-ATPase α1, α2, and α3 revealed their enrichment in CCVs (Fig. 3). Our study indicates that in brain, Na^+/K^+-ATPases are constitutively recycling, suggesting a mechanism to rapidly regulate the cell surface expression of these transporters, as was already shown in renal epithelial cells (38). We detected three isoforms of glutamate transporters (EAAT1, -2, and -4) and two GABA transporters (GAT-1 and -3). The presence of synaptic glutamate stimulates the rapid accumulation of glutamate transporters into the plasma membrane at the expense of intracellular pools (39, 40). We have demonstrated that EAAT2, the key regulator of synaptic glutamate levels, is highly enriched in CCVs (Fig. 3). Thus, it appears that EAAT2 and perhaps other identified transporters are undergoing constitutive recycling via clathrin-mediated endocytosis, suggesting a novel locus for regulation of their activity.

Calcium and Phosphorylation. We identified 23 proteins that regulate phosphorylation and Ca^{2+} handling. The dephosphorylation of endocytic proteins by the Ca^{2+}/calmodulin-sensitive phosphatase calcineurin is required for formation of protein complexes that control endocytosis and drugs that inhibit calcineurin function, cyclosporin A and FK506, impair endocytosis (41, 42). Interestingly, we identified protein phosphatase 2B (calcineurin A), calcineurin B-like protein p22, and the cyclosporin-binding protein peptidylprolyl isomerase (Table 2). Thus, it appears that pools of these proteins are constitutively associated with CCVs, perhaps to regulate dephosphorylation events important for endocytic function. The poorly characterized Ca^{2+}-binding proteins, VILIP1 and -3, were also identified. We confirm that VILIP1 is present on CCVs and comigrates with α-adaptin (Fig. 3), providing previously undescribed evidence for VILIP protein function in endocytosis.

Noncategorized Proteins. We identified 18 proteins that could not be readily categorized. However, some of these proteins could be placed in the established groups. For example, both acidic nuclear phosphoprotein 32 and Crmp-2 bind components of the microtubule cytoskeleton and could thus be placed in the cytoskeleton category (43, 44). Further work will indicate which, if any, of these proteins are bona fide constituents of CCVs.

Previously Undescribed Proteins. In addition to known proteins, we identified eight previously undescribed proteins that at the time of the original analysis were described only as ORFs. Several of these proteins, including enthoprotin and AAK1, have been subsequently linked to clathrin-mediated membrane budding (18, 45). Two of the proteins, named NECAP1 and -2, are found on CCVs (Fig. 3) and interact with AP-2 (46). These proteins have no modular domains and display no homology to known proteins, making them difficult to identify through a bioinformatics approach. Thus, subcellular proteomics can provide a functional framework for studies on previously uncharacterized proteins.

Discussion

The methodology outlined here illustrates the usefulness of MS/MS in the elucidation of protein components of an organelle. The accounting of fragmented peptides successfully assigned to protein databases enabled an estimate of relative proportions of proteins in the CCV preparations. This was most evident for abundant proteins such as the CHC and CLCs, which were detected in a 1:1 molar ratio. Although more variable, the subunits of the AP-2 complex were comparable in normalized peptide abundance, as were the subunits of the AP-1 complex with the exception of γ-adaptin. γ-Adaptin has a clustered distribution of arginines and lysines and may yield uneven sized peptides on tryptic cleavage, possibly accounting for the fewer peptides detected. This observation highlights one limitation of the quantitative approach outlined here. A second limitation involves the abundance of a protein relative to other proteins within a gel band. Specifically, the number of peptides detected for any given protein may underrepresent the level of that protein if the gel band in which it is found has a complex protein composition or contains a second, highly abundant protein. Thus, the stoichiometric analysis is most successfully applied to abundant proteins with similar arginine/lysine distributions.

Interestingly, a 2:1 molar ratio was observed for CHC to AP-1 and -2 complexes combined. Because the functional clathrin unit is the triskelia, composed of three CHCs, there are $\approx$1.5 APs for each triskelia. This is in good agreement with previous data demonstrating that clathrin cages assembled *in vitro* contain 1 AP-2 complex per triskelia (47). Thus, the CHC is found nonstoichiometrically with APs. As such, assembled clathrin triskelia could interact with APs simultaneously with proteins such as HIP12, epsin, enthoprotin, AP180, and CALM, even though these proteins use similar clathrin-binding motifs as the APs. These data support a model in which each of these proteins, which were detected in our preparation, bridge clathrin to membranes (48) and cooperate with APs in clathrin membrane recruitment.

A major concern of organelle proteomics is the extent of contamination by other organelles, which may vitiate the conclusions drawn. The random-sampling EM approach used here enabled an unbiased morphological assessment of our starting material with 73% of all structures identified as CCVs. The uncoated profiles were variable in size and shape and may thus derive from multiple sources. As such, protein components from any given membrane are likely to fall below the level of detection of the methods used. Based on MS/MS, contamination by ER, Golgi apparatus, endo-

somes, peroxisomes, mitochondria, and lysosomes was considered negligible. The assignment of a percentage of the identified proteins to CCVs was further validated by organelle sedimentation through a sucrose gradient. We thus conclude that each of the categories identified here, with the exception of myelin, ER, and RNA granule, represent bona fide CCV constituents.

Wu *et al.* (49) have identified >1,600 proteins from a crude rat brain pellet with multidimensional protein identification technology, which is two to three times more sensitive than liquid chromatography–MS/MS (50). Interestingly, 74 proteins in the enriched CCVs were not detected in the Wu *et al.* study, and they detected only one of the eight previously undescribed proteins found in CCVs. Moreover, none of the abundant Na^+, K^+, and Ca^{2+} channels identified by Wu *et al.* (49) were found in CCVs. These observations further validate the ability of the organelle-based proteomics approach to discriminate proteins associated with specific subcellular compartments from abundant components of the tissue of origin.

From the 6,133 peptides conclusively assigned, 209 proteins were identified. Of these, 92 had been previously associated with CCVs (noted with references in Table 2), and 25 appear to be contaminants (RNA granule, ER, and myelin) (Fig. 5, which is published as supporting information on the PNAS web site). We have thus identified 92 potentially new CCV-associated proteins, including four in the previously undescribed category that were subsequently assigned a CCV function. These 92 proteins are primarily CCV cargo, but several are likely to play regulatory roles in CCV formation and function. Included as a newly discovered CCV cargo is EAAT2, which is enriched on CCVs comparably to coat components. This provides evidence of CCV-mediated retrieval of the protein from the cell surface. Additionally, our studies demonstrate a mechanism for peptide accounting that can provide a measure of the relative ratios of proteins within a subcellular fraction. These data have been used to demonstrate a nonstoichiometric relationship between CHC and APs and to support that SVs recycle directly from CCVs. Finally, our data strongly support that a major function for CCVs in brain is the recycling of SVs, suggesting that the full fusion form of SV exocytosis is used robustly in the brain. Hence proteomics of the highly enriched and well characterized rat brain CCVs provide insight into the major functions of the organelle.

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