Adaptor protein (AP)-2 and AP-3-dependent mechanisms control the sorting of membrane proteins into synaptic vesicles. Mouse models deficient in AP-3, mocha, develop a neurological phenotype of which the central feature is an alteration of the luminal synaptic vesicle composition. This is caused by a severe reduction of vesicular levels of the zinc transporter 3 (ZnT3). It is presently unknown whether this mocha defect is restricted to ZnT3 or encompasses other synaptic vesicle proteins capable of modifying synaptic vesicle contents, such as transporters or channels. In this study, we identified a chloride channel, CIC-3, whose level in synaptic vesicles and hippocampal mossy fiber terminals was reduced in the context of the mocha AP-3 deficiency. In PC-12 cells, CIC-3 was present in transferrin receptor-positive endosomes, where it was targeted to synaptic-like microvesicles (SLMV) by a mechanism sensitive to brefeldin A, a signature of the AP-3-dependent route of SLMV biogenesis. CIC-3 was packed in SLMV along with the AP-3-targeted synaptic vesicle protein ZnT3. Co-segregation of CIC-3 and ZnT3 to common intracellular compartments was functionally significant as revealed by increased vesicular zinc transport with increased CIC3 expression. Our work has identified a synaptic vesicle protein in which trafficking to synaptic vesicles is regulated by AP-3. In addition, our findings indicate that CIC-3 and ZnT3 reside in a common vesicle population where they functionally interact to determine vesicle luminal composition.

Synaptic vesicles store neurotransmitters and neuromodulators by virtue of specific membrane transporters and channels that pump ions and small molecule mediators into these vesicles (1). Adaptor-dependent biogenesis and sorting mechanisms are critical to deliver these and other synaptic membrane proteins into these secretory vesicles (2, 3). Genetic defects in adaptor-based mechanisms such as AP-2 (4), AP180 (5, 6), or AP-3 (7–9) lead to neurological phenotypes, either because synaptic vesicles are not assembled properly and/or because the sorting of some synaptic vesicle proteins is impaired. The adaptor complex AP-3 is involved in the biogenesis of lysosomes, lysosome-related organelles and synaptic vesicles (10, 11). AP-3-deficient mouse models mocha and mocha2J develop a complex neurological phenotype that at the cellular level is characterized by the lack of intravesicular synaptic vesicle ionic zinc (7, 8). Vesicles are devoid of zinc because the synaptic vesicle-specific zinc transporter 3 (ZnT3) does not reach a specialized population of synaptic vesicles (7–9). The defective sorting of ZnT3 to mocha synaptic vesicles most probably occurs because ZnT3 possesses cytosolic determinants robustly recognized by the adaptor complex AP-3 (9).

So far, ZnT3 is the only known synaptic vesicle protein in which targeting to these vesicles is defective in the absence of functional AP-3 (7, 8). However, the pleiotropic neurological defects observed in mocha alleles are not fully recapitulated by a ZnT3-null mutant mouse (12, 13). This suggests that AP-3 could regulate the targeting of as-yet-unidentified proteins to synaptic vesicles that determine their luminal composition.

To address this question, we performed a search for synaptic vesicle membrane proteins endowed with transport or channel activity in which targeting to synaptic vesicles was impaired in the context of an AP-3 deficiency (mocha). We found that the sorting of a synaptic vesicle channel, CIC-3, was defective in the absence of functional AP-3. CIC-3 is an anion/chloride-permeant channel that provides an anionic shunt for the establishment of a proton gradient generated by the vacuolar ATPase (14–16). Perturbation of chloride membrane permeability impairs the acidification of synaptic vesicles and affects the luminal accumulation of neurotransmitters (17). Biochemical and morphological analyses in wild-type and mocha brain revealed that CIC-3 content in synaptic vesicles was reduced by the presence of the mocha allele. These defects were not restricted to neuronal cells or synaptic vesicles because fibroblasts lacking the adaptor complex accumulated CIC-3 in distinct subcellular compartments. In PC-12 cells, CIC-3 was present in transferrin receptor-positive endosomes, where it was targeted to synaptic vesicles, also known as synaptic-like microvesicles (SLMV), by a brefeldin A-sensitive mechanism. CIC-3 and ZnT3 co-segregated to a common SLMV population. The co-existence of CIC-3 and ZnT3 on the same vesicles was functionally tested in zinc transport assays, which revealed that vesicular zinc content was under control of the vesicular CIC-3 levels. Our work has identified a new synaptic vesicle protein whose trafficking to synaptic vesicles is regulated by AP-3. These results indicate that the functional interplay of chloride and zinc-permeant membrane proteins trafficked by AP-3 determine the intravesicular milieu.
Weinman (University of Texas, Galveston, TX). GFP-tagged form of the short isoform of ClC-3 were gifts from Dr. S. Francisco, CA). Flag-tagged ClC-3 short and long isoforms as well as a taxin 13-GFP was a gift from Dr. R. Scheller (Genentech, South San Anselmo). Affinity purified antibodies directed against the chloride channel 3 were obtained from Synaptic Systems (Göttingen, Germany); anti-transferrin receptor (H68.4; Zyomed Laboratories Inc., South San Francisco, CA); anti-FLAG epitope (generous gift from Dr. A. H. Kowalczyk, Emory University, Atlanta, GA); anti-α-HA (12CA5; a gift from Dr. Y. Altschuler, Hebrew University, Jerusalem); and anti-LAMP 1 and AP-3 delta subunits (from Developmental Studies Hybridoma Bank at the University of Iowa). Affinity purified antibodies directed against the chloride channel 3 were a gift from Dr. D. J. Nelson (University of Chicago, Chicago, IL). Affinity-purified polyclonal antibodies against glutathione S-transferase fusion proteins encompassing ZnT3 residues 1–78 as well as HA- and GFP-tagged ZnT3 DNAs have been described previously (9). Synaptin 13-GFP was a gift from Dr. R. Scheller (Genentech, South San Francisco, CA). Flag-tagged ClC-3 short and long isoforms as well as a GFP-tagged form of the short isoform of CIC-3 were gifts from Dr. S. Weiman (University of Texas, Galveston, TX).

Immunolocalizations—Cells were washed in PBS with Ca²⁺/Mg²⁺. After washing, cells were fixed for 20 min at 4 °C in 4% paraformaldehyde and processed for immunofluorescence as described previously (20, 21).

Either two wild-type or mocha mouse brains (The Jackson Laboratory, Bar Harbor, ME) were simultaneously fixed by perfusion with 4% paraformaldehyde, sliced coronally, and embedded in paraffin. To assure identical processing, slices containing wild-type and mocha brain hippocampi were included in the same block and sectioned together (8 μm). AP-3 δ, ZnT3, VAMP II, and CIC-3 antigen retrieval was performed by microwave treatment in 0.01 M citrate buffer, pH 6, for 10 min. All stainings were performed in duplicate in at least two independent experiments. Primary antibody incubations were carried out overnight at 4 °C and immunocomplexes detected by species-specific VECTASTAIN avidin-biotinylated enzyme complex kit (Vector Labs, Burlingame, CA) according to manufacturer's instructions.

Co-localization of Two-photon Microscopy—To image zinc-containing compartments, cells were loaded with 25 μM ZnSO₄ in calcium/magnesium-free Hanks’ balanced salt solution containing 10 mM pyruvate, 10 mM glucose, 100 μM/ml penicillin, and 100 μM/ml streptomycin for 1 h at 37 °C. Free zinc was washed at 4 °C and cells were loaded with 25 μM Znquin in 10 mM glucose Dulbecco’s PBS for 30 min at 37 °C (22, 23). Excess dye was extensively washed at 4 °C, and cells were kept at 4 °C until processed. GFP-PC-12 cells were imaged at 37 °C on MatTek dishes warmed with a Bioptechs objective heating system. A single image was taken before the addition of the drug. Time series were taken from each dish with 201.6-s intervals between images. Specimens were viewed using a Zeiss Axiovert 100 μ microscope coupled to a HeNe laser (argon ion and a co-pumped titanium: sapphire laser. Znquin was excited with the Ti:sapphire laser tuned at 750 nm. Images were acquired using the Zeiss LSM 510 mp1 software. Emission filters used for acquisition from live imaging experiments were LP505 and BP465 IR. The emission filters used for immunofluorescence were LP505 and BP500–550 IR. All images were viewed and acquired using a Plan Apochromat 63x/1.4 oil differential interference contrast objective.

Morphometric analysis was performed using Metamorph software (Universal Imaging Corporation, Downingtown, PA). Areas were scored in individual 1-μm confocal optical slices that cross-sectioned the cell nucleus. Identical color thresholds were set for all images, and region measurements were performed after automatic software-driven selection of regions around objects.

Cell Fractionation—PC-12 cells or fibroblasts were homogenized in intracellular buffer (38 mM potassium aspartate, 38 mM potassium glutamate, 38 mM potassium gluconate, 20 mM MOPS-KOH, pH 7.2, 5 mM reduced glutathione, 5 mM sodium carbonate, 2.5 mM magnesium sulfate, and 2 mM EGTA) using a cell cracker with a 12-μm clearance according to Cibb-O’Grady et al. (24). The homogenate was sedimented for 5 min at 1000 × g to obtain an S1 supernatant. Fibroblast S1 supernatants were sedimented in 10–45% velocity sucrose gradients at 116,000 × g for 1 h using a Beckman SW55 rotor (25). PC-12 S1 fractions were sedimented at 27,000 × g for 35 min to generate S2 supernatants. S2 fractions were spun either at 210,000 × g for 1 h in a Beckman TLA-120.2 rotor (FS) or through glycerol velocity gradients (5–25%) prepared in intracellular buffer at 215,000 × g for 75 min in a SW55 rotor (Beckman Coulter). The synaptic vesicle peak was determined by immunoblot. All procedures were performed at 4 °C in the presence of the antiprotease mixture Complete (Roche Molecular Biochemicals).

For zymosan brains from mocha AP-3 deficient (grigr, mhmlh); and wild-type (grigr) mice were a generous gift of Dr. M. Burmeister (University of Michigan, Ann Arbor, MI). Brains were pulverized to a fine powder using porcelain mortars under a continuous supply of liquid nitrogen. Extracts were thawed at 4 °C in 5 volumes of buffer A (150 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM EDTA, and 0.1 mM MgCl₂) plus Complete antiprotease mixture (26). Homogenates were sedimented at 1000 × g for 10 min to generate S1 supernatants. S1 were further fractionated at 27,000 × g for 45 min to obtain high speed supernatants, which were resolved in glycerol velocity gradients as described above.

Gradient fractions were analyzed by immunoblot and immunoreactivity was revealed by ECL. The synaptic vesicle peak was defined as fractions 7–12 for CIC-3 plots, in which we integrated the immunoreactivity signal only in fractions 8–12 to exclude fraction 7, which overlapped with the larger membranes seen in mocha (fractions 2–7). Immunoreactive bands were quantified using NIH Image 1.62 software (18, 20, 27).

Immunomagnetic Isolations—PC-12 SLMV isolated by glycerol velocity gradient (fractions 7–12) were used in the assays. All the reactions and washes were done at 4 °C in the presence of Complete antiprotease mixture. SV used as input pool were processed in identical conditions as the vesicles bound to beads except that beads and BSA were omitted. Dynabeads M-450 (Dynal, Oslo, Norway) were preincubated with antibodies either against LAMP, HA epitope, or VAMPII. All beads and washes were done in PBS-BSA. To beads was performed for 3 h at 4 °C. Incubations were terminated by washing the beads four times for 10 min each. Unbound membranes were diluted in 1 volume of PBS to dilute BSA and sedimented at 200,000 × g for 1 h in a tabletop Beckman TLA ultracentrifuge. Complexes and unbound membrane pellets were resolved by SDS-PAGE and analyzed by immunoblot. Immune complexes were detected by ECL.

Transferrin Internalization Assays—Transferrin internalization assays were performed with Alexa 568-conjugated human transferrin (Molecular Probes, Eugene, OR) as described previously (18, 20, 27). In brief, PC-12 cells were incubated in the absence or presence of ZnT3 at 37 °C in Dulbecco’s modified Eagle’s medium without fetal bovine serum. Cells were washed at 4 °C, and transferrin (50 μg/ml) binding to the cell surface was carried out in Dulbecco’s modified Eagle’s medium, 0.1% BSA, 20 mM HEPES, pH 7.4, at 4 °C for 2 h. Unbound ligand was washed away at 4 °C, and internalization was resumed at 37 °C. Assays were stopped in ice-cold PBS followed by paraformaldehyde fixation on ice.

Flow Cytometry—PC-12 cells were incubated in the absence or presence of ZnSO₄ and/or Zinquin as described above. Cells were extensively washed at 4 °C, and Zinquin and GFP fluorescence were determined in a MoFlo high performance cell sorter from DAKO Cytomation (Fort Collins, CO). The laser used for the forward and side scatter was a Coherent Innova 9088-nm argon laser and a co-pumped titanium: sapphire laser. Zinquin was excited with the Ti:sapphire laser set to 305 nm. The filters used were a long-pass filter at 440 nm and a 450/65s in front of the photo multiplier tube. Data analysis was performed using FlowJo version 4.3.2. To depict the data in a normalized fashion, we used percentage of maximum, defined as the number of cells in each bin (25%) divided by the number of cells in the bin that contained the largest number of cells.

Other Procedures—Protein assays were performed using the Bio-Rad protein assay dye reagent (Bio-Rad) using BSA as standard. All data are presented as means ± S.E. Statistical analysis was performed using a two-tailed t test.

RESULTS

CIC-3 Targeting to Brain Small Vesicles Is Selectively Modified in AP-3 Deficiencies—It is presently unknown whether other membrane proteins present in synaptic vesicles, in addition to ZnT3 (7, 8), might be targeted to this organelle by an
AP-3-dependent ClC-3 Targeting

To address this question, we analyzed whether a series of known synaptic vesicle proteins were properly targeted to synaptic vesicles of AP-3-deficient neurons (Fig. 1). High-speed supernatants (S2) from wild-type and AP-3-deficient brain homogenates were fractionated in glycerol velocity gradients. This procedure discriminates particles by size, resolving large membranes from those that are the size of synaptic vesicles. These vesicles peak in the middle of the gradient in fractions 7–9 (28–30). Moreover, their synaptic vesicle nature has been established by the redistribution of synaptic vesicle markers to larger membrane fractions in Drosophila melanogaster shibire mutants at restrictive temperature (26).

We used ZnT3 as a bona fide AP-3-interacting membrane protein in which synaptic vesicle content is AP-3-dependent. ZnT3-containing synaptic vesicles peaked at fractions 7–9 (Fig. 1, A and E), comigrating with the synaptic vesicle proteins synaptophysin and VAMP II (Fig. 1B). As described previously, ZnT3 content was reduced to 20% of control values, in both synaptic vesicles and in the P1 and P2 pellets from mocha brain (Fig. 1J) (9). However, synaptophysin and VAMP II levels remained constant in mocha brain membranes and synaptic vesicles (Fig. 1, B and H). In contrast, the velocity sedimentation of membranes containing the synaptic vesicle chloride channel 3 (CIC-3) was affected by AP-3 deficiency (Fig. 1, C, F, and H). Rather than a generalized reduction of CIC-3, as seen with ZnT3, we observed that in the mocha background the majority of CIC-3 was redistributed from the synaptic vesicle peak to a region of the gradient containing larger membranes (fractons 2–7, compare plots in E and F). CIC-3 distribution in P1 and P2 pellet was not affected by the mocha allele (Fig. 1J), showing that the changes in CIC-3 distribution in AP-3-deficient brain were restricted to a limited set of small vesicles.

To assess the CIC-3 content in the synaptic vesicle-containing fractions, we integrated the immunoreactivity signal in fractions 8–12 to exclude fraction 7, which overlapped with the larger membranes seen in mocha (fractions 2–7). Thus, we estimated that in mocha brain, the CIC-3 content in vesicles the size of synaptic vesicles was reduced by at least half (55.4 ± 10%, n = 3) (Fig. 1H). P1, P2, and synaptic vesicle levels of SV2 (p > 0.08), and the vacuolar ATPase (V-ATPase, p > 0.5) were not significantly different between wild-type and mocha brain membranes (Fig. 1H), indicating that the effects of the mocha allele upon CIC-3 and ZnT3 were selective.

CIC-3 is targeted to synaptic vesicles in brain (17), yet it is present in late endosome-lysosomal compartments in non-neuronal cells (31). To explore whether synaptic vesicle-positive fractions co sedimented with membranes containing lysosomal antigens on glycerol gradients, we tested for the presence of the AP-3-sorted lysosomal protein LAMPI on them. LAMPI was detected in fractions 7–9 comigrating with the synaptic vesicle proteins synaptophysin, VAMP II, and ZnT3 (Fig. 1, compare D with A–C and open circles in G with E and F). In the absence of AP-3, LAMPI immunoreactivity along the glycerol gradient was generally reduced (Fig. 1D), whereas...
AP-3-dependent ClC-3 Targeting

Tomoko Kuriyama, Mami Suzuki, Takakazu Kato, Masahiro Maruyama, and Kiyosuke Takahashi

Fig. 2. ClC-3 content decreases in mocha hippocampal mossy fiber nerve terminals. Wild-type (A, C, E, G, I, and K) and mocha (B, D, F, H, J, and L) hippocampal mocha brains sections were stained with antibodies against the AP-3 delta subunit (A and B), ZnT3 (C and D), and ClC-3 (E–L). Immunocomplexes were detected with the ABC peroxidase reagent and DAB deposition. A–H show representative images of the hilus at low (A–F, 20×) and high magnification (G–H, 63×), whereas I–L correspond to the CA3 region of the hippocampus acquired at low (I and J) or high power (K and L). Note the reduction of ClC-3 immunoreactivity in the hilus mossy fibers and CA3 mossy fiber synaptic terminals. Images are representative of sections obtained from two wild-type and mocha brains simultaneously stained in two independent experiments. DG, dentate gyrus; HF, hippocampal fissure.

its content in P2 membranes was increased (Fig. 1I). Nonetheless, like ClC-3, the remaining LAMPI present in these gradients appeared in faster sedimenting membranes in the absence of AP-3 (fractions 2–7), whereas the LAMPI content in fractions 8–12 was reduced to 42.9 ± 10.4 (Fig. 1H, n = 2). These results suggest that at least part of the ClC-3 protein present in brain is bound to late endosome/lysosomes in vesicles that possess physical properties identical to those of synaptic vesicles.

In summary, these results indicate that AP-3 deficiency selectively affects the targeting of ClC-3 and LAMPI to a vesicle population with physical properties similar to those of synaptic vesicles. Moreover, they suggest that ClC-3 targeting to synaptic vesicles may be impaired in mocha nerve terminals.

ClC-3 Content Decreases in Nerve Terminals from AP-3-deficient Brain—We analyzed the distribution of ClC-3 in hippocampal mossy fiber synaptic terminals from wild-type and mocha brain sections to define whether the ClC-3 targeting defects observed in mocha small membranes reflected alterations in targeting to synaptic vesicles. We focused in mossy fiber nerve terminals because they can be easily identified at the light microscopic level (32, 33) and because the ZnT3 synaptic vesicle mocha phenotypes can be unambiguously determined in these synaptic terminals. In addition, ZnT3 and ClC-3 transcript levels are high in the hippocampus (17, 34).

Wild-type and mocha hippocampus sections were immunolabeled with AP-3 delta, ZnT3 and ClC-3 antibodies. Two hallmarks of the mocha phenotype were readily identified in stained hippocampal brain sections. First, AP-3 immunoreactivity was reduced in the mocha phenotype were readily identified in stained hippocampal brain sections. Second, AP-3 immunoreactivity was reduced in the mocha phenotype were readily identified in stained hippocampal brain sections. Second, AP-3 immunoreactivity was reduced in the mocha phenotype were readily identified in stained hippocampal brain sections. In the contrary, cell body ClC-3 immunoreactivity was reduced in mocha brain sections, although the extent of ClC-3 immunoreactivity reduction was different in hilus and CA3. Other brain regions were not assessed because ClC-3 levels were too low.

Because the ClC-3 protein levels were not globally reduced in whole-brain extracts (Fig. 1I), these data suggest that in the absence of AP-3, a limited set of small vesicles containing ClC-3 is affected or that the ClC-3 targeting defects are prominent only in the hippocampus. The reduction in mossy fiber ClC-3 and ZnT3 content was not caused by a disappearance of the mossy fibers, in that VAMP II staining was identical in wild-type and mocha fibers.7

To exclude the possibility that part of the ClC-3 immunoreactivity observed in brain sections could be caused by its expression in glial cells, we compared the ClC-3 protein expression levels in primary cultures enriched either astrocytes (Fig. 3, odd lanes) or hippocampal neurons (Fig. 3, even lanes). ClC-3, transferrin receptor, and cell type-specific markers were assessed by immunoblot. Although transferrin receptor was similarly expressed in both glial cells and neurons, most of ClC-3 was detected in neuronal cells. Thus, these results suggest...
Fig. 4. CIC-3 intracellular distribution is altered in mocha fibroblasts. A, immortalized mocha fibroblasts transduced either with a retrovirus carrying the AP-3 δ subunit or with an empty retrovirus were transiently transfected with plasmids containing Flag-tagged versions of the short and long isoforms of CIC-3. Mocha cells rescued by the δ-subunit were used as controls. Both CIC-3 isoforms were targeted to small vesicles in rescued fibroblast; in contrast, the channel was targeted to big vesicles in the absence of functional AP-3. B, depicts frequency histograms of the vesicle size determined by Metamorph software. Data represent the area of vesicles acquired from 19 and 27 wild-type and 19 and 17 mocha cells transfected with either the short or long CIC-3 isoform respectively. Each histogram was built with at least 450 vesicle measurements. C, wild-type (open circles) and mocha fibroblast (closed circles) were transiently transfected with the GFP-tagged short isoform of CIC-3. Equal amounts of postnuclear supernatant were resolved in non-equilibrium 5–45% sucrose gradients and fractions analyzed by immunoblot with CIC-3 antibodies. Wild-type and mocha cells' CIC-3-positive membranes sediment differently (*, p < 0.05, n = 3).

gest that most of the CIC-3 immunoreactivity in brain is present in neuronal cells.

In summary, our immunocytochemistry and biochemical data indicate that the targeting of ZnT3 and CIC-3 to synaptic vesicles is impaired in the absence of functional AP-3.

CIC-3 Subcellular Distribution Is Affected in mocha-derived Fibroblasts—To further explore whether CIC-3 targeting was dependent on AP-3 in non-neuronal cells, we examined by confocal microscopy and subcellular fractionation whether AP-3-deficient fibroblasts possessed an altered distribution of CIC-3. Although CIC-3 is a ubiquitously expressed protein (17, 35), endogenous levels in fibroblasts were below the immunofluorescence detection level. To overcome this limitation, we transfected Flag- and GFP-tagged CIC-3 constructs into immortalized mocha fibroblasts rescued or not with the AP-3 δ subunit. Neither tag affects the functional properties of CIC-3 (31, 36). In AP-3-deficient cells, CIC-3 was distributed in large vesicles. In contrast, in cells bearing the δ subunit, the CIC-3 positive vesicles were smaller (Fig. 4A). We substantiated this observation with morphometric analysis of the CIC-3 positive vesicles using Metamorph software. Irrespective of whether the long or short isoforms of CIC-3 were expressed, we observed that the CIC-3-positive organelles present in AP-3-deficient cells were consistently bigger than in wild-type cells. We next sought to independently test whether CIC-3 subcellular distribution was affected in AP-3-deficient fibroblasts. Thus, we analyzed the migration of CIC-3 in postnuclear supernatants from CIC-3 transfected wild-type and mocha cells resolved by non-equilibrium sucrose sedimentation. In wild-type cells, 57 and 24% of the CIC-3 protein contained in the gradient sedimented around 34% (fraction 5) and 24% sucrose peaks (fraction 12), respectively. Instead, 33 and 40% of CIC-3 were present in the same sucrose peaks in AP-3-deficient fibroblast (n = 3). These results indicate that the adaptor AP-3 regulates CIC-3 targeting in non-neuronal cells.

CIC-3 Isoforms are Targeted to PC-12 Cell SLVM from Endosomes—The neuroendocrine cell line PC-12 gives rise to vesicles that bear all the biochemical and physical properties of brain synaptic vesicles (2, 11, 29). These vesicles are assembled from transferrin receptor-positive endosomes by an AP-3- and ARF-1-dependent and brefeldin A-sensitive mechanism (11, 20, 37–39). This pathway can be tracked by assessing the targeting to SLMV of a VAMP II mutant carrying the N49A mutation, which efficiently recruits AP-3 to membranes (40). Alternatively, these vesicles can be labeled with the AP-3-interacting membrane protein ZnT3. A reasonable prediction is that if CIC-3 is trafficked by AP-3, then in PC-12 cells this chloride channel should follow the same route to SLMV as ZnT3. To test this hypothesis, we used a three-pronged approach asking whether: 1) CIC-3 was present in brefeldin A-sensitive early endosomes, 2) CIC-3 targeting to SLMV was impaired by brefeldin A, and 3) CIC-3 and ZnT3 cosegregated to the same SLMV.

CIC-3 is endogenously expressed in PC-12 SLMV, although at low levels (Fig. 6D). To surmount this limitation, we transfected cells with Flag- or GFP-tagged versions of the channel. We first defined which of the two endocytically sorted CIC-3 splicing variants (CIC-3 short or CIC-3 long) was targeted to PC-12 SLMV. Both Flag-tagged CIC-3 isoforms were indistinguishable in their subcellular distribution as assessed by differential sedimentation (Fig. 5A). Moreover, both channel isoforms were concentrated in P3 pellets, a membrane fraction enriched in PC-12 SLMV (41) that contains negligible amounts of early endosome, endoplasmic reticulum, and Golgi markers (9). To further assess whether both channel isoforms were targeted to SLMV, we sedimented the membranes contained in the P3 pellets in glycerol velocity gradients. PC-12 SLMV were identified using the synaptic vesicle marker synaptophysin. Both CIC-3 isoforms were present in vesicles that cosedimented with synaptophysin (Fig. 5B). The presence of a GFP tag did not alter the SLMV targeting of the short isoform compared with its Flag-tagged counterpart (Fig. 5B).

Donor endosomes that give rise to SLMV in PC-12 cells can be pulse-labeled from the cell surface after short incubation with labeled transferrin (25) (Fig. 6A). PC-12 cells expressing CIC-3-GFP were surface labeled at 0 °C with Alexa-conjugated
transferrin. The ligand excess was washed and endocytosis was resumed at 37 °C. At 0 °C, labeled transferrin remained at the cell surface with little colocalization with the perinuclear GFP signal. However, after resuming internalization at 37 °C, the transferrin signal moved from the cell surface to the perinuclear region, where it showed extensive colocalization with CIC-3-GFP. Transferrin and GFP signals colocalized even after 5 min, showing that CIC-3-GFP is present in early endosomes.

Time-lapse confocal microscopy in PC-12 cells carrying CIC-3-GFP indicated that these perinuclear endosomes were modulated by brefeldin A (Fig. 6B). Syntaxin 13-GFP was used as a bona fide early endosome marker (42) and ZnT3-GFP as an AP-3-dependent cargo routed to SLMV via early endosomes (9). After drug treatment, syntaxin 13, ZnT3 and CIC-3 positive endosomes became congregated in the perinuclear region, indicating that, at this subcellular location, the chloride channel dynamic is controlled by a brefeldin A-sensitive mechanism.

We tested the effects of brefeldin A upon CIC-3 targeting to PC-12 SLMV using ZnT3 and synaptophysin as controls (Fig. 7). After drug treatment, ZnT3 levels were drastically reduced in PC-12-derived SLMV, whereas synaptophysin levels were not modified (Fig. 7B), thus mimicking the effects of the mocha allele on ZnT3 and synaptophysin synaptic vesicle protein sorting (Fig. 7A). SLMV CIC-3 content was also decreased after brefeldin A treatment of PC-12 cells (Fig. 7C), although to a lesser extent than ZnT3. The response of CIC-3 and ZnT3 to brefeldin A indicates that part of the CIC-3 protein follows the endosomal pathway of SLMV formation. Moreover, these results suggest that the brefeldin A-sensitive ZnT3 and CIC-3 pools probably exit endosomes together in a common vesicle carrier.

CIC-3 and ZnT3 Coexist in the Same Vesicles and Functionally Interact—To address whether ZnT3 and CIC-3 are both present in the same SLMV, we isolated SLMV from a PC-12 cell line expressing ZnT3 carrying a HA tag in its carboxyterminal cytosolic tail (clone 4). SLMV from peak glycerol gradient fractions were immunoisolated with antibodies against the HA tag present in ZnT3 or the synaptic vesicle protein VAMPII. Attempts to generate PC-12 lines carrying both ZnT3-HA and CIC-3-GFP failed.3 Thus, we determined by immunoblot the presence of the endogenous channel in the bead-isolated vesicles (Fig. 7D).

Beads coated with antibodies directed against a human lysosomal luminal antigen (LAMP; Fig. 7D, lane 2) did not bind vesicles, thus excluding spurious binding of membranes to the magnetic beads. Conversely, beads coated with antibodies

3 G. Salazar and V. Faundez, unpublished observations.
against a cytosolic epitope present in VAMP II retained SLMV containing both zinc transporter and chloride channel 3 (Fig. 7D, lane 4). HA antibody-coated beads were less efficient in binding SLMV (Fig. 7D, compare lanes 3–4 and 6–7), yet a population of CIC-3 and ZnT3-positive SLMV was identified because membranes isolated with HA epitope antibodies also contained endogenous CIC-3 (Fig. 7D, lane 3). As seen in the mocha-derived synaptic vesicles, CIC-3 was decreased although to a lesser extent compared with ZnT3 (n = 3). Fraction 1 corresponds to the bottom in all gradients. D, ZnT3 and CIC-3 are targeted to the same vesicle carrier. SLMV isolated from ZnT3-HA clone 4 were immuno-magnetically isolated with antibodies against LAMP II, VAMP II, or the HA epitope. LAMP antibodies did not retrieve vesicle antigens (lane 2). Anti-HA antibodies retrieved vesicles that contain HA-ZnT3 and endogenous CIC-3 (lane 3) and VAMP II antibodies bind vesicles that contain CIC-3 as well as ZnT3 (lane 4). Input represents 10% of the material loaded onto the immunomagnetic column. Unbound represents the membrane effluent from the column (lanes 5–7). Arrowheads show two nonspecific bands that cosedimented with SLMV. CIC-3 appears as a more diffuse band between the arrowheads.

Fig. 7. Brefeldin A (BFA) mimics the effects of the mocha allele in the targeting of synaptic vesicle antigens to PC-12 synaptic vesicles. Homogenates were resolved by differential centrifugation and high-speed supernatants (S2) were sedimented in glycerol velocity gradients. Synaptic vesicles migrated in the middle of the gradient as revealed by the antigens synaptophysin (Sphysin), ZnT3, and CIC-3. A depicts a quantification of the synaptophysin (Sphysin) and ZnT3 levels in wild-type (+/+) and AP-3-deficient mocha synaptic vesicles. Only ZnT3 levels are reduced in the absence of AP-3. B–D, PC-12 cells were treated for 2 h either in the absence (closed symbols) or presence of brefeldin A (open symbols). B, synaptophysin levels were not affected by brefeldin A; in contrast, ZnT3 levels were dramatically reduced. C, as seen in the mocha-derived synaptic vesicles, CIC-3 was decreased although to a lesser extent compared with ZnT3 (n = 3). Fraction 1 corresponds to the bottom in all gradients. D, ZnT3 and CIC-3 are targeted to the same vesicle carrier. SLMV isolated from ZnT3-HA clone 4 were immuno-magnetically isolated with antibodies against LAMP II, VAMP II, or the HA epitope. LAMP antibodies did not retrieve vesicle antigens (lane 2). Anti-HA antibodies retrieved vesicles that contain HA-ZnT3 and endogenous CIC-3 (lane 3) and VAMP II antibodies bind vesicles that contain CIC-3 as well as ZnT3 (lane 4). Input represents 10% of the material loaded onto the immunomagnetic column. Unbound represents the membrane effluent from the column (lanes 5–7). Arrowheads show two nonspecific bands that cosedimented with SLMV. CIC-3 appears as a more diffuse band between the arrowheads.

In summary, these results indicate that CIC-3 and ZnT3 functionally interact and that they reside in a common vesicle population. Furthermore, they suggest that a reduction of chloride transport into the vesicle lumen could further contribute to the lack of zinc observed in synaptic vesicles from AP-3-deficient brains (7, 8) and fibroblasts (43).
untransfected cells processed in parallel. Results for clone 10 are from six independent experiments. All the other determinations were done at least twice. The ClC-3 protein is present in neurons. Furthermore, our immunocytochemical studies in hippocampal mossy fiber synaptic terminals indicated that at least part of ClC-3 is targeted to synaptic vesicles by an AP-3-dependent mechanism. However, in addition to the ClC-3 protein targeted to synaptic vesicles, we found that the same fractions that contain synaptic vesicles also include vesicles that carry the AP-3-dependent lysosomal cargo LAMP I. Similar to ClC-3, in the absence of functional AP-3, LAMP I was redistributed into faster glycerol sedimenting vesicles. These results have two important implications; first, the fact that LAMP I, an AP-3 interacting cargo, and ClC-3 containing vesicles are similarly redistributed to faster sedimenting membranes in the mocha allele provides additional support to the hypothesis that ClC-3 is targeted by AP-3. On the other hand, these results suggest that at least part of the ClC-3 protein present in brain is bound to late endosome/lysosomes in vesicles that possess physical properties identical to those of synaptic vesicles. We believe that in neuronal cells, the ClC-3 protein present in LAMP I-positive vesicles is likely to be just a part of the total ClC-3. Our immunocytochemical results indicate that ClC-3 is concentrated in CA3 mossy fiber nerve terminals. These results are consistent with those reported by Stobrawa in retina or transfected neurons, where ClC-3 immunoreactivity is almost exclusively present in synaptic areas (17). How can ClC-3 reach two apparently dissimilar organelles? An attractive hypothesis to explain these results is that some of the membrane proteins sorted by AP-3 can be recognized by both the neuronal and ubiquitous versions of the AP-3 complex, yet the neuronal form of AP-3 could preferentially target other membrane proteins, like the VAMP II N49A mutant (37). This mechanism could provide a way to target the same protein, like ClC-3, either to lysosomes or synaptic vesicles. A prediction from this hypothesis is that some synaptic vesicle proteins initially identified in non-neuronal cells. In fact, the synaptic vesicle protein VAT1 has been recently identified as a constituent of melanosomes (44), a lysosome-related organelle in which bio-

![Fig. 8. ClC-3 modulates the vesicular zinc stores. Wild-type PC-12 cells (A–C), PC-12 cells ZnT3-HA clone 4 (C and F), or PC-12 cells ClC-3-GFP clone 10 (D–F) were incubated either in the absence (A–B) or presence of zinc (B–D). Cells were subsequently labeled with the zinc fluoroprobe Zinquin (A–D). Fluorescent signal intensity for Zinquin (Log scale abscissa; A–C, and F) and GFP (abscissa, E) was measured by fluoroctometry in the cell population (y-axis, normalized cell count). Increasing levels of ZnT3 by transfection do not modify the zinc uptake by PC-12 cells (C and F). In contrast, endogenous levels of ZnT3 are enough to modulate the zinc uptake by expression of ClC-3 (D). ClC-3 induced increase happened irrespective of the clone analyzed (F). F, median values for the Zinquin signal expressed as percentage increase compared with untransfected cells processed in parallel. Results for clone 10 are from six independent experiments. All the other determinations were done at least twice.](http://www.jbc.org/content/full/25437/Fig8.png)
genesis is perturbed in ubiquitous AP-3-deficient mice mutants (43) but not in a neuronal AP-3–/– mouse model (45).

An unexpected finding is that in the absence of AP-3, the fates of ZnT3, CIC-3, and LAMP 1 are not identical. In the absence of AP-3, ZnT3 levels drop dramatically in all brain membranes, whereas CIC-3 is only redistributed along glycerol gradients. Instead, LAMP 1 is redistributed both in glycerol resolved as well as P2 membranes. CIC-3 and ZnT3 are endowed with several putative tyrosine and di-leucine sorting motifs, yet LAMP 1 possesses only one tyrosine motif (46). If a hierarchy of sorting signals/adaptor interactions controls the traffic of a particular membrane protein, then it is likely that in the absence of a particular “dominant” adaptor, other complexes might command the targeting of the membrane protein, thus rerouting the protein to a different subcellular location. In a proteomic analysis of AP-3-derived SLMV,2 we have identified new membrane proteins in which targeting in mocha brain resembles the behavior of either LAMP I or CIC-3, whereas other proteins have a fate with elements that partially recapitulate the ZnT3 and CIC-3 mocha phenotypes. The identification of the compartments where these proteins are destined in the absence of AP-3 and the sorting signals involved will help us to further understand the mocha neurological phenotype.

Is AP-3 the only mechanism to target CIC-3 to synaptic vesicles? Our results provide clues about this question. Synapt vesicle chloride channel levels are partially reduced in mocha brain vesicles and mossy fiber synaptic terminals as well as in PC-12 SLMV from cells incubated in the presence of brefeldin A. This decrease contrasts with the marked inhibition that brefeldin A or the mocha allele exerts upon ZnT3 targeting (Fig. 6) (9). On the other extreme, none of these conditions significantly affects synaptophysin. Synaptophysin targeting to SLMV is sensitive to plasma membrane cholesterol depletion, yet the sorting of ZnT3 is completely insensitive (9). Interestingly CIC-3 targeting to SLMV is partially sensitive to both cholesterol depletion and brefeldin A, suggesting that two different pathways contribute to the sorting of this protein (9). ZnT3 and synaptophysin would represent examples of membrane proteins that preferentially partition in one or the other pathway, whereas CIC-3 would be equally targeted to both routes. A prediction from this hypothesis is that part of the channel routed to synaptic vesicles should be trafficked along with ZnT3. Our immunomagnetic isolation experiments (Fig. 7D) and the functional zinc uptake assays (Fig. 8) strongly support this notion. Increasing the levels of CIC-3 in PC-12 cells increases the vesicular zinc transport. Our data also suggest a more complex picture to explain the zinc-deficient phenotype observed in mocha and mocha2J nerve terminals (7, 8). Even, in the presence of ZnT3, changes in the anion channel levels could determine the presence or absence of zinc from nerve terminals. Anion-permeant synaptic vesicle proteins whose vesicle distribution is not affected by the lack of AP-3 could supply an anionic shunt mechanism alternative to CIC-3.

Vesicular chloride content controls ionic zinc uptake (Fig. 8) and glutamate uptake (17, 47–49) suggesting that the absence of vesicular chloride could trigger complex glutamatergic phenotypes. Ionic zinc is a powerful inhibitory modulator of NMDA-mediated responses in the hippocampus (50–52). Thus, a reduction in the vesicular chloride permeability could reduce the vesicular content of glutamate but still have a normal or enhanced post-synaptic NMDA response because of the absence of zinc. In fact, miniature post-synaptic currents in CIC-3–/– hippocampus are slightly enhanced despite a reduction in

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