Alkalizing Drugs Induce Accumulation of Amyloid Precursor Protein By-products in Luminal Vesicles of Multivesicular Bodies

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Amyloid precursor protein (APP) metabolism is central to the pathogenesis of Alzheimer disease. We showed recently that the amyloid intracellular domain (AICD), which is released by γ-secretase cleavage of APP C-terminal fragments (CTFs), is strongly increased in cells treated with alkalizing drugs (Vingtdeux, V., Hamdane, M., Bégaré, S., Loyens, A., Delacourte, A., Beauvillain, J.-C., Bueé, L., Marambaud, P., and Sergeant, N. (2007) Neurobiol. Dis. 25, 686–696). Herein, we aimed to determine the cell compartment in which AICD accumulates. We show that APP-CTFs and AICD are present in multivesicular structures. Multivesicular bodies contain intraluminal vesicles (known as exosomes) when released in the extracellular space. We demonstrate that APP, APP-CTFs, and AICD are integrated and secreted within exosomes in differentiated neuroblastoma and primary neuronal culture cells. Together with recent data showing that amyloid-β is also found in exosomes, our data show that multivesicular bodies are essential organelles for APP metabolism and that all APP metabolites can be secreted in the extracellular space.

Amyloid precursor protein (APP) metabolism is central to Alzheimer disease etiopathogenesis. Extracellular amyloid deposits, a neuropathological hallmark of Alzheimer disease, are composed of amyloid-β (Aβ) peptides that derive from APP catabolism. APP is a type I transmembrane glycoprotein processed by an α- or a β-secretase to produce C-terminal fragments (CTFs) (for review, see Ref. 1). γ-Secretase further processes APP-CTFs (2, 3), releasing Aβ from β-CTF and the amyloid intracellular domain (AICD or ε-CTF) from all APP-CTFs (2, 4–8). Several lines of evidence suggest that AICD is a trans-regulating factor of gene expression (nephrilysin, KAI1, APP, and glycogen synthase kinase-3β) (9–12). However, AICD is rapidly degraded and thus seldom detected (13). We showed recently that AICD is strongly increased upon treatment with alkalizing drugs, suggesting that the endosomal/lysosomal pathway regulates AICD degradation (14).

The endosomal/lysosomal pathway is essential for Aβ production and APP catabolism. For instance, BACE-1 (β-site APP-cleaving enzyme 1) resides within endosomes, and endocytosis of BACE-1 and APP is a prerequisite for generating Aβ (15–17). An acidic pH is necessary for optimal BACE-1 protease activity (18), and BACE-1 is degraded in lysosomes (19). The γ-secretase activity has been localized at the endosomal/lysosomal membrane (20–23). Treatment with drugs that prevent endosomal/lysosomal acidification (24–26) or deletion of the APP internalization motif (27, 28) dramatically reduces Aβ secretion.

The endosomal/lysosomal system is likely to be altered in Alzheimer disease (for review, see Ref. 29). Several APP derivatives accumulate in multivesicular bodies (MVBs), in transgenic animal models of amyloidosis (30, 31), in Alzheimer disease (30), and in cell models (32). MVBs belong to the endocytic pathway (33); are at the crossroad of several cellular mechanisms such as membrane receptor recycling and protein degradation; and can release their intraluminal vesicles, known as exosomes (for review, see Refs. 34–36). More recently, exosomes were demonstrated to contain Aβ peptides (37). Taken together, a growing body of evidence suggests that APP processing takes place mainly between the plasma membrane and late endosomal compartments such as multivesicular endosomes. Herein, we studied the localization of APP and its derivatives in SY5Y neuroblastoma cells stably overexpressing human APP and demonstrate that APP, APP-CTFs, and AICD accumulate in the luminal vesicles of multivesicular endosomes and are also found in exosomes.
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EXPERIMENTAL PROCEDURES

Antibodies—APPcter-C17 is a well characterized rabbit antibody raised against the last 17 amino acids of the human APP sequence (7, 38–40). Anti-HSP70 (heat shock protein of 70 kDa) antibody was purchased from Euromedex (Mundolsheim, France). Anti-flotillin antibody was from Translab (Erembodegem, Belgium); anti-LAMP-2 and anti-Tsg101 antibodies were from Santa Cruz Biotechnology, Inc.; and anti-EEA1 antibody (early endosome antigen 1) was from Upstate (Lake Placid, NY). The mouse monoclonal antibody 8E5 (raised against residues 444–592 of human APP770) was a generous gift from Dr. Peter Seubert (Elan Pharmaceuticals). Anti-β-tubulin antibody and horseradish peroxidase-coupled secondary antibody were purchased from Sigma (Saint-Quentin-Fallavier, France).

Cell Culture of SKNSH-SY5Y Neuroblastoma Cells and Differentiation—The human neuroblastoma cell line SKNSH-SY5Y (referred to as SY5Y) was maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM nonessential amino acids, and 50 units/ml penicillin/streptomycin (Invitrogen) in a 5% CO2 humidified incubator at 37 °C. APP695 cDNA was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen), allowing for G418 (Invitrogen) selection of clones. This APP cDNA was transfected into SY5Y cells using the ethyleneimine polymer ExGen 500 (Euromedex) according to the manufacturer’s instructions. Cells expressing APP were selected by the addition of 200 μg/ml G418 to the cell medium. For differentiation, SY5Y cells were maintained for 15 days in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 supplemented with 2 mM L-glutamine, 50 units/ml penicillin/streptomycin, 7 mg/ml progesterone, 1% insulin/transferrin/selenium (Invitrogen), and 1 mM retinoic acid (Sigma). The medium was replenished every 3 days.

Primary Embryonic Neuronal Culture—Rat primary cortical neurons were prepared from 17–18-day Wistar rat embryos as follows. Briefly, the brain and meninges were removed, and the cortex was carefully dissected out and mechanically dissociated in culture medium by triturating with a polished Pasteur pipette. Once dissociated and after blue trypan counting, cells were plated in 25-cm2 flasks at a density of 900 cells/mm2 in Neurobasal medium supplemented with 10% FBS, 1% B27, 2 mM L-glutamine, and 150 μM pyruvate. The medium was replenished every 3 days and maintenance, we used Neurobasal medium supplemented with 10% FBS, 1% B27, 2 mM L-glutamine, and 150 μM pyruvate.

Drug Treatments—N-[N-(3,5-Difluorophenacyl)-L-ala- nyl]-3-(S)-amino-1-methyl-5-phenyl-1,3-dihydrobenzo[e]- (1,4)-diazepin-2-one (compound E, γ-secr etase inhibitor XVIII; used at a final concentration of 30 nM) and bafilomycin A1 (BafA1; used at a final concentration of 100 nM) were purchased from Calbiochem (Fontenay-sous-Bois, France). Chloroquine (10 μM) was purchased from Sigma. Cells were plated in 6-well plates 24 h before drug exposure. Cells were washed with warmed phosphate-buffered saline (PBS) and scraped with a policeman rubber in 70 μl of Laemmli sample buffer containing protease inhibitors (Complete mini, Roche Applied Science, Meylan, France). The cell homogenate was sonicated and heat-treated for 5 min at 100 °C. The protein concentration was determined using a PlusOne™2D Quant kit (Amersham Biosciences, Orsay, France), and samples were kept at −80 °C until used.

Western Blotting and Mass Spectrometry—An equal quantity of total proteins (20 μg/lane) was loaded onto a 16.5% Tris-Tricine- or 8–16% Tris/glycine-polyacrylamide gel. Tris-Tricine/SDS-PAGE was performed following the procedure of Schägger and von Jagow (41) with a PROTEAN II xi cell (Bio-Rad, Marnes-la-Coquette, France). Following the final sucrose density gradient (see below), proteins were loaded onto Criterion XT/MES gels (Bio-Rad) as described previously (14). Proteins were transferred to nitrocellulose membrane (Hybond, Amersham Biosciences) at 2.5 mA/cm2/gel using a semidyrid NovaBolt transfer system (Amersham Biosciences) according to the manufacturer’s instructions. Proteins were reversibly stained with Ponceau red to check the quality of protein transfer. Membranes were blocked in 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% (w/v) Tween 20, and 5% (w/v) skim milk for 30 min. Membranes were then incubated overnight at 4 °C with the appropriate dilutions of the primary antibody and incubated for 1 h at room temperature with the secondary antibody. The immunoreactive complexes were revealed using an ECL™Western blotting kit and Hyperfilm (Amersham Biosciences). For mass spectrometry analyses, proteins were resolved with Criterion XT gels (Bio-Rad) and stained with Coomassie Brilliant Blue G-250 (Sigma). In-gel trypsin digestion and mass spectrometry analyses were performed as described previously (42).

Exosome Isolation and Sacrose Density Gradients—The protocol used was derived from that of Thery et al. (43). The cell culture media were centrifuged for 10 min at 1200 × g, and the supernatant was filtered on a 0.22-μm filter (Milllex® GP, hydrophilic membrane of polyethersulfone; Millipore, Molsheim, France) and centrifuged for 30 min at 12,000 × g before ultracentrifugation at 100,000 × g for 1 h at 4 °C. The pellet was added once with a large volume of PBS and further centrifuged at 100,000 × g for 1 h. For purification of exosomes on a sucrose density gradient, the 100,000 × g pellet was resuspended in 0.25 M sucrose in HEPES/NaOH buffer (pH 7.4). A step gradient of sucrose at 2.25, 2.00, 1.75, 1.5, 1.25, 1.00, 0.75, 0.5, and 0.25 M was layered with 0.25 M exosome-containing solution. The gradient was spun at 200,000 × g for 1 h using an SW 41 Ti rotor (Beckman Coulter). Fractions of 1 ml were diluted with 3 ml of HEPES/NaOH buffer (pH 7.4) and spun at 200,000 × g for 1 h using an SW 60 Ti rotor. Pellets were used for Western blotting or electron microscopy.

Immunofluorescence—SY5Y cells expressing wild-type APP (APPWT; referred to as SY5Y-APPWT cells) were differentiated on poly-L-lysine-coated glass coverslips. After drug treatment, cells were fixed in 0.1 M PBS containing 4% paraformaldehyde for 30 min at room temperature and further permeabilized with 0.25% (v/v) Triton X-100 in PBS. After blocking in 1% (w/v) bovine serum albumin, the fixed materials were incubated for 2 h at room temperature with the primary antibody in PBS containing 1% (w/v) bovine serum albumin and 0.25% Triton X-100. After washing, the secondary antibody (Alexa Fluor
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For APP internalization analysis, cells were incubated with the 8E5 antibody for 1 h at 4 °C in ice-cold Dulbecco’s modified Eagle’s medium supplemented with 1% (w/v) bovine serum albumin and then washed and incubated at 37 °C for the times indicated. Cells were fixed as described above, permeabilized by incubation in 0.3% (v/v) Triton X-100 in PBS, and incubated with Alexa Fluor 568-conjugated anti-mouse IgG antibody.

Electron Microscopy and Gold Immunolabeling—For conventional observations, differentiated SY5Y cells pretreated or not with 10% fetal calf serum for 2 min were fixed in 2% glutaraldehyde in 0.1 M PBS (pH 7.2) for 10 min. After washing, they were post-fixed with 1% osmic acid in the same buffer. The cells were then embedded in Araldite after dehydration in ethanol. Sections were collected on Parlodion-coated nickel grids and counterstained with uranyl acetate and lead citrate before observation on a Zeiss 9025 electron microscope.

For immunoelectron microscopy observations, the same cells (treated or not) were fixed in 0.2% glutaraldehyde and 4% paraformaldehyde in PBS for 15 min and directly embedded in LR White after dehydration. Polymerization occurred under UV light for 48 h. Sections were collected on coated nickel grids and treated for gold immunolabeling. Briefly, the sections were successively incubated for 30 min in buffer A (0.1 M Tris, 0.15 M NaCl, 1% bovine serum albumin, and 4% goat serum; antibody dilution in buffer A (for 24 h), and colloidal gold (18 nm)-labeled goat anti-rabbit Ig in buffer A (1.5 h). After washing, the sections were counterstained in 4% uranyl acetate in H2O. For observation of isolated exosomes, 400 mesh carbon grids were put for 5 min on a drop of exosome pellet diluted 1:50 in PBS. After blotting, the grids were successively put in fixative solution (0.2% glutaraldehyde and 2% paraformaldehyde in PBS), glycine solution (0.2 M in PBS), APPCter-C17 antiserum (12 h), and colloidal gold-labeled goat anti-rabbit Ig (1.5 h). Counterstaining was performed in uranyl acetate.

RESULTS

Accumulation of APP in Late Endosomes after Alkalizing Drug Treatments—Alkalizing drugs induce an accumulation of APP-CTFs and AICD as determined by biochemical analyses (14). Herein, we investigated the cellular compartments in which this accumulation may arise. We investigated the cellular localization of APP in chloroquine- and BafA1-treated SY5Y-APPWT cells by immunofluorescence using 8E5 and APPCter-C17 antisera (Fig. 1). After drug treatment, APP accumulated in numerous engulfed vesicles (Fig. 1). These large engulfed vesicles were not seen under control conditions and could result from defective endocytosis or routing of APP.

BafA1 has been demonstrated to reduce slightly the rate of internalization, recycling, and intracellular sorting of transmembrane protein and to completely block the transport from late endosomes to lysosomes (44, 45). We assessed the internalization of APP in control and BafA1-treated cells. SY5Y-APPWT cells were incubated with the 8E5 antibody at 4 °C, and the kinetics of endocytosis of 8E5-APP complexes was followed by indirect immunofluorescence (Fig. 2A). Following incubation of the 8E5 antibody at 4 °C in both control and BafA1-treated cells, we observed that the membrane was delineated by immunocomplexes, demonstrating that endocytosis was repressed at low temperatures. After 5 min at 37 °C, APP was rapidly internalized in small vesicles under both conditions. Noteworthy, in control cells, the plasma membrane was not visualized, whereas in BafA1-treated cells, the plasma membrane was detected. Although indirect immunofluorescence is not quantitative, we observed that APP staining strongly disappeared under control conditions after 60 min at 37 °C. In contrast, APP staining remained intense in vesicular structures in BafA1-treated cells, and the signal at the plasma membrane was diminished. BafA1 induced the accumulation of the 8E5 antibody in vesicles resembling those observed in Fig. 1, suggesting that these vesicles belong to the endosomal/lysosomal pathway. To further characterize these vesicles, we used antibodies against markers of the early and late endosomal compartments (Fig. 2B). In control and BafA1-treated cells, antibody APPCter-C17 labeling partially overlapped EEA1 early endosome marker labeling. However, EEA1 staining did not correspond to large engulfed vesicles. Tsg101 is a marker of late endosomal compartments and particularly of multivesicular endosomes (46). In control SY5Y-APPWT cells, few vesicles were stained by both anti-Tsg101 and APPCter-C17 antibodies. In sharp contrast, all large vesicles were stained by anti-Tsg101 and APPCter-C17 antibodies in BafA1-treated cells. We showed previously that these large vesicles are also partially positive for the LAMP-2 lysosome marker (14). We suggest that BafA1 treatment results in the accumulation of APP and its derivatives in late endosomal compartments.

APP and Its Derivatives Are Found in MVBS in BafA1-treated Cells—We next observed cellular organelles at the ultrastructural level in control and BafA1-treated cells. Numerous large...
vesicles containing smaller intraluminal vesicles were observed in BafA1-treated SY5Y-APPWT cells, but not in control cells (Fig. 3A and supplemental Fig. 1, A and D). Immunogold labeling with our APPCter-C17 antibody showed that few gold particles were localized in vesicular structures and MVBs in control cells (supplemental Fig. 1C). SY5Y-APPWT cells were not stained by the secondary antibody alone (supplemental Fig. 1, A and D). In BafA1-treated cells, intraluminal vesicles were stained by our APPCter-C17 antibody. We hypothesized that these large vesicles correspond to engulfed MVBs.

MVBs can fuse with the plasma membrane to release their luminal content, including the small vesicles termed exosomes (33, 36). The latter originate from the budding of the endosomal membrane to form intraluminal vesicles. If MVBs are the cell compartments in which APP metabolites accumulate, then these products should be associated with exosomes.

We first investigated the presence or absence of exosome vesicles in the medium of SY5Y-APPWT cells (Fig. 4). SY5Y-APPWT cells were differentiated with retinoic acid for 15 days in a medium that did not contain fetal calf serum, which is a source of exogenous exosomes (43). To ascertain the presence of exosome-like vesicles in differentiated SY5Y-APPWT cells, the medium was collected after 24, 48, or 72 h of culture and processed as described for the isolation of secreted vesicles (43). Flotillin and HSP70, which are often found in exosome vesicles, were used as exosome markers (36, 43, 47). We also used electron microscopy to determine the morphology of the secreted vesicles. Following two low speed centrifugations, filtration, and ultracentrifugation, the pellet was analyzed for the presence of flotillin and HSP70. We observed both flotillin and HSP70 by Western blotting (Fig. 4B). Electron microscopy analysis of the same pellet revealed the presence of numerous membrane vesicles with a mean size of 50–90 nm and a typical “cup shape” (Fig. 4B), hence corresponding to the morphological characteristics of exosomes (35). We excluded the possibility that the release of such vesicles is a characteristic of our cell model, as secreted exosome vesicles were isolated in mock SY5Y cells as well as in primary neuronal culture cells (Fig. 4, C and D).

To further demonstrate that these vesicles correspond to exosomes, we analyzed the protein in a 100,000 × g pellet. SY5Y-APPWT cells were differentiated with retinoic acid for 15 days, and the medium was collected from the last 72 h. Exosome vesicles were isolated following the established procedure, and the final pellet was homogenized in SDS lysis buffer. Proteins were resolved by SDS-PAGE and stained with Coomassie Blue (Fig. 5A). The identities of the proteins were determined by peptide mapping after in-gel trypsin digestion and mass spectrometry analyses (42). A low parts/million error (inferior to 30 ppm) was applied to match a protein with a minimum of four matching peptides and a high probability score (Fig. 5A, table). The proteome of exosomes isolated from multiple biological sources has been described (43, 48, 49). The proteins that were identified herein, such as the clathrin heavy chain, translation initiation factor-3, HSP90, pyruvate kinase, tubulin, actin, elongation factor-1, and Ras-
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APP, APP-CTFs, and AICD Are Secreted in Exosome Vesicles—We next addressed the question of the presence of APP metabolic products in SY5Y-APPWT exosomes (Fig. 6A). Immunogold labeling with our APPCter-C17 antibody was performed to localize APP metabolic products. Numerous vesicles of 50–90 nm were stained with gold particles 18 nm in diameter (Fig. 6A). The gold particles localized inside the vesicles. Using the same antibody, we next characterized the APP metabolic products by Western blotting (Fig. 6A). This analysis was performed on exosome vesicles obtained from differentiated SY5Y-APPWT cells maintained in the culture medium for 24, 48, or 72 h. An increase in HSP70 staining was observed (Fig. 6A), suggesting that exosomes accumulated in the cell medium with time. The APPCter-C17 antibody stained full-length APP, APP-CTFs, and AICD (Fig. 6A). Whereas full-length APP staining only slightly increased under the three conditions, APP-CTF staining strongly increased at 48 and 72 h. AICD was observed mainly after 72 h. To further demonstrate that APP, APP-CTFs, and AICD are associated with exosomes, the 100,000 × g pellet was loaded on the top of a 0.25–2.5 M sucrose density gradient. After centrifugation at 200,000 × g for 18 h, 1-ml fractions were collected and analyzed by Western blotting for the presence of EEA1, APP, APP-CTFs, AICD, HSP70, and flotillin (Fig. 6B). Exosomes were found principally between 1.0 and 1.25 M sucrose, corresponding to a density of 1.13–1.16 g/ml, as described previously (50, 51). APP, APP-CTFs, and AICD were strongly detected in the 1.0–1.25 M fractions; HSP70 and flotillin were strongly detected as well (Fig. 6B). Moreover, we verified the presence of exosomes in the 1.0 M sucrose fraction by electron microscopy (Fig. 6B) and showed also that EEA1 was not detected in this fraction, but was slightly

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related nuclear protein, were characterized previously in exosomes (43, 48, 49). Our data indicate that the isolated vesicles are SY5Y-derived exosomes. These exosomes are most likely not contaminated by proteins of other unrelated organelles, as the vast majority of the proteins we have characterized were shown previously (43, 48, 49) in exosomes from other sources. Moreover, we performed staining with anti-EEA1 antibody. EEA1 was detected in the SY5Y-APPWT lysate, but not in exosomes obtained from 10 ml of culture medium (Fig. 5B).

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FIGURE 4. Secretion of exosome-like vesicles by SY5Y-APPWT cells. SY5Y-APPWT cells were differentiated for 15 days with retinoic acid, and the medium was collected after the last 24, 48 or 72 h. Shown is a summary of the protocol for the presence of EEA1, APP, APP-CTFs, and AICD; HSP70 and flotillin were strongly detected as well (Fig. 6B). Shown are the results from morphological analysis by electron microscopy of the 100,000 × g pellet obtained from the 72-h cell culture supernatant. The pellet was composed of 30–100-nm vesicles. Scale bar = 100 nm. The 100,000 × g pellet obtained after differential centrifugation from the crude control (Ctrl) medium (B, first lane) or from the supernatant after 24, 48, and 72 h of cell culture (second through fourth lanes) and the cell lysate (fifth lane) were analyzed by Western blotting with anti-flotillin and anti-HSP70 antibodies. Shown are the results from electron microscopy of the 100,000 × g pellet obtained from the 72-h cell culture medium of differentiated SY5Y cells (C) or rat primary neuronal culture cells (D). Scale bars = 100 nm. Note the characteristic cup shape of the 50–90-nm vesicles (insets in D).

FIGURE 3. MVB localization of APP in SYSY-APPWT cells. A, ultrastructural analysis of BaF2-treated SYSY-APPWT cells; B, immunogold labeling of APP in SYSY-APPWT cells treated with BaF2. 18-nm gold particles were localized in the small intraluminal vesicles of a larger vesicle. Note that the shape of these vesicles is very similar to that of multivesicular structures observed in the same cells under the same conditions by ultrastructural electron microscopy (A). Nu, nucleus. Scale bars = 1 μm.

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detected in fractions below 2.0 M sucrose when the blot was overexposed (Fig. 6B). Our data show that SY5Y-APPWT cells produced exosomes in which APP and APP metabolites were co-sedimented with exosome markers. Finally, to demonstrate that the AICD we observed in exosomes was released by /H9253-secretase and not by an unrelated proteolytic cleavage, we used a specific /H9253-secretase inhibitor. SY5Y-APPWT cells were treated for 24 h with either compound E or chloroquine, and we analyzed the exosome content after treatment (Fig. 7A).

In the presence of the /-secretase inhibitor compound E, AICD was detected neither in the cell lysate nor in the exosomes. Conversely, the amount of APP-CTFs was strongly increased in SY5Y-APPWT cells and exosomes (Fig. 7B), demonstrating that AICD in exosomes is produced by /-secretase cleavage of APP-CTFs. Moreover, we showed that, in chloroquine-treated cells, AICD was increased in the SY5Y-APPWT lysate and in exosomes, suggesting further that chloroquine might repress AICD degradation in MVBS. Altogether, our data demonstrate that APP, APP-CTFs, and AICD are found in intraluminal vesicles of MVBS, where they are likely degraded, as are other type I transmembrane proteins.

**DISCUSSION**

In this study, we have shown that differentiated SY5Y cells stably expressing APP or not produced and secreted small vesicles of 50–90 nm in diameter, consistent with morphological and biochemical properties of exosomes. We have also shown that proteins often found in exosomes, e.g. HSP70 and flotillin (36), were detected in these small vesicles secreted by SY5Y cells. In contrast, a marker of early endosomes (EEA1) was not found associated with exosomes. Further similarities were deduced from characterization of the proteome SY5Y-derived vesicles. Fifty percent of the proteins characterized were identified previously in exosomes from other biological sources (43, 48). Similar vesicles were also isolated from primary neuronal culture cells, demonstrating that the production of exosomes is not a specificity of SY5Y neuroblastoma cells, but is possibly a physiological process of neuronal cells (51). More important, our data demonstrate that APP derivatives, including full-length APP, APP-CTFs, and more surprisingly, AICD, are found in exosomes secreted by differentiated SY5Y cells. These APP fragments are also co-sedimented with markers of exosomes at a sucrose density corresponding to that described for exosomes (50, 51). The presence of soluble full-length APP in the medium was demonstrated previously in cells transfected with APP, but remained unexplained (52, 53). Secretion of APP within exosomes is con-
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FIGURE 6. APP, APP-CTFs, and AICD are secreted and co-sedimented with exosome markers. **A**, shown are the results from electron microscopy of the 100,000 × g pellet obtained from 2 ml of the 72-h cell culture medium immunogold-labeled with the APPCter-C17 antibody. Scale bars = 0.1 μm. Exosomes were also analyzed by Western blotting with anti-flotillin, anti-HSP70, and anti-APPCter-C17 antibodies. An equal volume (3 ml) of the native culture medium (control (Ctrl)) was processed for exosome isolation and loaded in the first lane. The last lane corresponds to the cell lysate. Ten micrograms of protein was loaded. **B**, exosomes were prepared from 65 ml of medium obtained after 72 h of culture of differentiated SY5Y-APPWT cells. The 100,000 × g pellet was recovered in 0.25M sucrose and layered over a sucrose density gradient. Ten fractions of 1 ml were recovered after centrifugation at 200,000 × g for 18 h and concentrated. The concentrations of the sucrose fractions are indicated. The whole proteins were loaded onto SDS-polyacrylamide gel. Full-length APP, APP-CTFs, AICD, EEA1, HSP70, and flotillin were detected. EEA1 staining was performed after APP labeling. The residual staining of APP is indicated by the asterisk because a very long exposure time was necessary to visualize EEA1 staining. AICD was observed after a longer exposure (10 min (mn)) and is indicated by the arrow. Electron microscopy was performed to verify the presence of exosomes in the 1 m sucrose fraction. Scale bar = 100 nm.

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use of a γ-secretase inhibitor abrogated the release AICD. Moreover, AICD was not produced during the purification of exosomes. First, we failed to detect presenilin in exosomes; and second, when exosomes were incubated at 37°C for 24 h, APP-CTFs were not processed further to produce AICD or other catabolites (data not shown). However, our negative findings do not exclude the possibility that γ-secretase cleavage occurs prior to exosome secretion.

Exosomes derive from the inward budding of the membrane of endosomes en route for multivesicular endosomes (54). Several proteins are markers of this cellular compartment such as flotillin, HSP70, and Tsg101 (36, 43). Interestingly, flotillin was shown recently to associate with AICD (55). We have shown here that APP and its derivatives were found in exosomes and multivesicular endosomes and accumulated in engulfed vesicles following treatments with alkalizing drugs. Together with our recent data, we suggest that APP and APP-CTFs that are not processed by γ-secretase are directed to this late endosomal compartment for degradation by fusion of MVBs with lysosomes. Aβ and AICD, which result from the γ-secretase cleavage of APP-CTFs, are also directed to multivesicular endosomes and are integrated in the luminal vesicles, namely exosomes. The presence of AICD in exosomes is consistent with γ-secretase cleavage of APP-CTFs that would occur during the budding of the late endosomal membrane. However, the contribution of flotillin to this mechanism remains to be established. The process of APP following endocytosis is illustrated in Fig. 8.

Recent data suggest that multivesicular endosomes are important cellular compartments in neurodegenerative disorders. Exosomes that are produced in these compartments contain prion protein and have been suggested to mediate the diffusion of the scrapie form of the protein (56). More recently, Aβ has been shown to be a component of the exosomal membrane (37). Protease
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A

FIGURE 7. Inhibition of $\gamma$-secretase or treatment with chloroquine abolishes the production or enhances the quantity of AICD in exosomes, respectively. A, SY5Y-APPWT cells were differentiated for 14 days and treated the last 24 h with compound E (CompE) or chloroquine (Chloro); the medium (3 ml/condition) was collected, and exosomes were isolated. APP, APP-CTFs, AICD, flotillin, and HSP70 were detected by Western blotting in exosomes and cell lysates. Molecular masses are indicated to the right. Ctrl, control. B, the ratio of APP-CTFs detected in exosomes to those detected in cells is expressed in arbitrary units.

B

FIGURE 8. Schematic representation of the sorting of APP and its catabolic derivatives in MVBs and exosomes. APP or APP-CTFs are internalized and directed to early endosomes. Budding from the limiting membrane of endosomes into their lumen forms internal vesicles characteristic of MVBs. En route for degradation, MVBs containing APP, APP-CTFs, and AICD fuse with lysosomes. Alternatively, MVBs can fuse with the plasma membrane, in which case the internal vesicles of the MVBs are released into the extracellular space, the so-called exosomes containing APP and its catabolic derivatives.

implicated in APP metabolism and other proteins showing similarities to APP metabolism such as L1 and CD44 are found in exosomes (57). Exosomes in the immune system are known to contribute to antigen presentation. Neurons also produce exosomes, but their potential roles remain unclear (51). Herein, we have shown that several metabolites of APP, including AICD, are found in exosomes.

MVBs and exosomes are of growing interest in neurodegenerative diseases. We do not know whether the “alkalizing” conditions used herein reflect an Alzheimer disease-related situation, although we have shown that alkalizing drugs induced an intracellular accumulation of APP catabolites. However, an altered routing of APP to late endosomal compartments could be associated with an increase risk of developing Alzheimer disease. A recent work by Rogaeva et al. (58) demonstrates that genetic polymorphisms of SORL1 are at risk for late onset Alzheimer disease. SORL1 acts as a sorting receptor for full-length APP and favors its recycling. A loss of function of SORL1 results in increased production of Aβ in late endosomal compartments. Further work will be needed to study markers of exosomes in Alzheimer disease and the relationship, if any, to a potential defect of late endosomal compartments such MVBs.

The release of exosomes is most likely regulated as demonstrated by Faure et al. (51); thus, depolarization stimulates the exosomes released. The release of full-length APP was also demonstrated to be stimulated by cholinergic agonists (52). These data, together with the data from this study, show that APP and its catabolites can be released associated with exosomes and that this process is likely modulated by several parameters. However, further work will be needed to investigate these hypotheses.

In conclusion, we have demonstrated a novel secretion pathway of APP metabolites (including APP, APP-CTFs, and AICD) that is mediated by exosome vesicles. Our work provides new research perspectives toward the understanding of APP biological function. A more complete characterization of the proteome of neuron-derived exosomes could also be helpful in discovering new biomarkers useful for diagnostic purposes and in developing therapeutic drugs for Alzheimer disease.

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