Autophagosomes cooperate in the degradation of intracellular C-terminal fragments of the amyloid precursor protein via the MVB/lysosomal pathway

Alexis E. González,* Vanessa C. Muñoz,* Viviana A. Cavieres,* Hianara A. Bustamante,* Víctor-Hugo Cornejo,†‡ Yunan C. Januário,§ Ibeth González,* Claudio Hetz,†‡§,† Luis L. daSilva,§ Alejandro Rojas-Fernández,*,*** Ronald T. Hay,§ Gonzalo A. Mardones,*,*** and Patricia V. Burgos*,**,1

*Department of Physiology, School of Medicine, and †Center for Interdisciplinary Studies of the Nervous System (CISNe), Universidad Austral de Chile, Valdivia, Chile; ‡Biomedical Neuroscience Institute, Faculty of Medicine, University of Chile, Santiago, Chile; §Center for Geroscience, Brain Health and Metabolism, Santiago, Chile; ‡Department of Cell and Molecular Biology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil; †Buck Institute for Research on Aging, Novato, California, USA; ‡Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts, USA; and *Centre for Gene Regulation and Expression, College of Life Sciences, University of Dundee, Dundee, United Kingdom

ABSTRACT: Brain regions affected by Alzheimer disease (AD) display well-recognized early neuropathologic features in the endolysosomal and autophagy systems of neurons, including enlargement of endosomal compartments, progressive accumulation of autophagic vacuoles, and lysosomal dysfunction. Although the primary causes of these disturbances are still under investigation, a growing body of evidence suggests that the amyloid precursor protein (APP) intracellular C-terminal fragment β (C99), generated by cleavage of APP by β-site APP cleaving enzyme 1 (BACE-1), is the primary cause of the endosome enlargement in AD and the earliest initiator of synaptic plasticity and long-term memory impairment. The aim of the present study was to evaluate the possible relationship between the endolysosomal degradation pathway and autophagy on the proteolytic processing and turnover of C99. We found that pharmacologic treatments that either inhibit autophagosome formation or block the fusion of autophagosomes to endolysosomal compartments caused an increase in C99 levels. We also found that inhibition of autophagosome formation by depletion of Atg5 led to higher levels of C99 and to its massive accumulation in the lumen of enlarged perinuclear, lysosomal-associated membrane protein 1 (LAMP1)-positive organelles. In contrast, activation of autophagosome formation, either by starvation or by inhibition of the mammalian target of rapamycin, enhanced lysosomal clearance of C99. Altogether, our results indicate that autophagosomes are key organelles to help avoid C99 accumulation preventing its deleterious effects.—González, A. E., Muñoz, V. C., Cavieres, V. A., Bustamante, H. A., Cornejo, V.-H., Januário, Y. C., González, I., Hetz, C., daSilva, L. L., Rojas-Fernández, A., Hay, R. T., Mardones, G. A., Burgos, P. V. Autophagosomes cooperate in the degradation of intracellular C-terminal fragments of the amyloid precursor protein via the MVB/lysosomal pathway. FASEB J. 31, 2446–2459 (2017). www.fasebj.org

KEY WORDS: Alzheimer disease · APP · C99 · multivesicular bodies · amphisome · proteostasis

Abbreviations: AD, Alzheimer disease; AICDγ, β-amyloid precursor protein intracellular domain; APP, β-amyloid precursor protein; Aβ, amyloid β; BACE-1, β-site β-amyloid precursor protein cleaving enzyme 1; BafA1, bafilomycin A1; C83, carboxy-terminal fragment α; C99, carboxy-terminal fragment β; CQ, chloroquine; CTF, C-terminal fragment; DAPT, N-[3(R,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; EBSS, Earle balanced salt solution; ESCRT, endosome-sorting complexes required for transport; GFP, green fluorescent protein; ILV, intraluminal vesicle; LAMP1, lysosomal-associated membrane protein 1; mTOR, mammalian target of rapamycin; MVB, multivesicular body; RNAi, RNA interference; siRNA, short hairpin RNA; SIM, structured illumination microscopy; siNT, nontargeting small interfering RNA; ssiRNA, small interfering RNA

† Correspondence: Laboratory of Cell and Molecular Biology, Department of Physiology, School of Medicine, Universidad Austral de Chile, Valdivia S10566, Chile. E-mail: patricia.burgos@uach.cl
doi: 10.1096/fj.201600713R
This article includes supplemental data. Please visit http://www.fasebj.org to obtain this information.

Alzheimer disease (AD) is the most common form of dementia. However, the identification of cell biologic processes that cause AD remains a challenge. An important feature of brain regions of patients affected by AD is the accumulation of protein aggregates, namely tangles and amyloid β (Aβ) plaques (1, 2). Aβ plaques are formed during the so-called amyloidogenic cleavage of the amyloid precursor protein (APP), which starts with the β-site APP cleaving enzyme 1 (BACE-1) that generates a C-terminal fragment (CTF) named C99 (also called CTβ). In contrast, nonamyloidogenic cleavage of APP by α-secretases generates a shorter CTF called C83 (also known as CTFα). The transmembrane domain of each CTF is further cleaved by γ-secretase, which generates additional fragments: Aβ and APP intracellular domain (AICDγ) for C99, or p3 and AICDγ for C83 (3, 4).
Although enhancement of amyloidogenic proteolytic processing of APP is considered crucial to AD pathogenesis, several reports indicate that defects in autophagy and the endolysosomal pathway of protein degradation are also critical (5–7). Autophagy can proceed via 3 mechanistically distinct pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy (8). Macroautophagy deficiency in mice brain results in neurodegeneration and is characterized by accumulation of ubiquitylated-protein aggregates (9), highlighting the importance of this pathway in neuronal homeostasis. Central to macroautophagy is the existence of transitory organelles called autophagosomes. Formation of autophagosomes occurs constitutively under normal nutrient conditions (9, 10), but it is highly inducible by cellular stress, such as amino acid starvation and inhibition of the key cell regulator mammalian target of rapamycin (mTOR) (11). Autophagosomes sequester cytoplasmic constituents and fuse with lysosomes to form autolysosomes, promoting degradation of sequestered content to recycle macromolecules, a process known as autophagic flux (12). Ultrastructural studies of the brain of postmortem AD patients revealed that autophagic organelles including autophagosomes and autophagolysosomes gradually accumulate in dystrophic neurites, a neuropathologic hallmark of AD, having important implications in Aβ peptide generation and neuronal survival in later stages of AD (13, 14). Although these findings explain the lower rates of protein degradation in AD neurons, it is still under study how to manipulate these pathways to increase protein clearance in neurons (15, 16).

Autophagosomes can also fuse to prelysosomal endocytic organelles, such as late endosomes, also called multivesicular bodies (MVBs). Fusion of autophagosomes to MVBs generates a hybrid organelle called the amphisome, which ultimately also fuses to lysosomes (17, 18). Biogenesis of MVBs involves the formation of intraluminal vesicles (ILVs), which requires the activity of the 4 multisubunit endosome-sorting complexes required for transport (ESCRTs 0 to III), collectively called the ESCRT machinery (19, 20). Macroautophagy is abrogated in cells depleted of ESCRT subunits (21, 22), indicating a close interaction between these 2 pathways (10, 23).

Intracellular trafficking of APP includes its targeting to endolysosomal membranes, either from the trans-Golgi network sorted by the AP-4 adaptor complex (24, 25) or from the plasma membrane by endocytosis (26, 27). Several studies have demonstrated that APP is normally incorporated into the ILVs of MVBs in a process dependent on the ESCRT machinery (26, 28, 29). However, it is still under debate if the MVB pathway prevents amyloidogenic processing of APP (28, 29) or increases Aβ production (26). Moreover, it is not clear how the ESCRT machinery affects both APP processing and CTF levels, and whether its effects are a consequence of ILV formation or via autophagic flux (21, 22, 30). Positive regulators of macroautophagy, such as SMER28 (31) and the transcription factor EB (32), reduce the levels of APP and its CTFs. However, the mechanism that explains how autophagosomes impact the turnover of APP and its CTFs remains unknown.

In the present study, we found that inhibition of either autophagosome formation or the fusion of autophagosomes with endolysosomal compartments caused a dramatic increase in CTF levels. Surprisingly, microscopy revealed that these CTFs accumulated in structures resembling ILVs of MVBs. In agreement with these findings, we found that activation of autophagosome formation, either by starvation or treatment with the mTOR inhibitor Torin-1, enhanced C99 lysosomal clearance. Therefore, we propose that autophagosomes are key organelles that connect with the MVB/lysosomal pathway for efficient turnover of CTFs.

**MATERIALS AND METHODS**

**Chemical reagents and antibodies**

Bafilomycin A1 (BfaA1), chloroquine (CQ), Earle balanced salt solution (EBSS), N-[N-(3,5-difluorophenacyl)-l-alanyl]-5-phenylglycine l-butyl ester (DAFT), puromycin dihydrochloride, thapsigargin, and a cocktail of protease inhibitors were purchased from Sigma-Aldrich (St. Louis, MO, USA). Torin-1 was purchased from Tocris Bioscience (Bristol, UK). The selective Vps34 inhibitor Vps34-IN1 was purchased from MedKoo Biosciences (Chapel Hill, NC, USA). We used the following mouse monoclonal antibodies: clone C4 anti-β-actin, clone 51 anti-TSG101, and clone 3 anti-p62 (BD Biosciences, San Jose, CA, USA); clone WO2 anti-C99 fragment (Merck Millipore, Billerica, MA, USA); clone HA3 anti–lysosomal-associated membrane protein 1 (LAMP1), clone H4B4 anti-LAMP2, and clone H5C6 anti-CD63 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA); and anti–green fluorescent protein (GFP; Roche Applied Sciences, Indianapolis, IN, USA). We used the following polyclonal antibodies: human anti-EEA1 (the gift of A. Gonzalez, Pontificia Universidad Católica, Chile); rabbit anti–cathepsin D (R&D Systems, Minneapolis, MN, USA); rabbit anti-LC3 and rabbit anti-Atg5 (Cell Signaling Technology, Danvers, MA, USA); and rabbit anti-APP (Thermo Fisher Scientific, Waltham, MA, USA). Horseradish peroxidase–conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA), and DAPI and fluorophore-conjugated secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

**Cell culture, plasmids, and generation of stable cell lines**

H4 human neuroglioma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). H4-derived cell lines were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin (G418. Cells were grown to subconfluence, then treated with drug or lentiviral particles containing specific short hairpin RNAs (shRNAs) for further Western blot and immunofluorescence analyses. Nutrient starvation assays were performed in the presence of EBSS.
RNA interference with small interfering RNA

A small interfering RNA (siRNA) designed to target human TSC101 (5′-CCUCCAGUCUUCUCUGUC-3′) was purchased from GE Dharmacon (Lafayette, CO, USA). siRNA transfections were carried out using the oligofectamine reagent from Thermo Fisher Scientific using similar conditions as previously described (25).

RNAi with shRNA

We generated stable H4 neuroglioma cell lines with reduced Atg5 levels by introducing shRNA-containing lentiviral particles. shRNA sequences were cloned in the pLKO.1 vector, and a shRNA against the luciferase gene was used as a control. Lentiviral particles were generated by cotransfection of HEK293 cells with pLKO.1-shRNA constructs (1 μg), VSV-g (1 μg), and pA8.9 (1 μg). Transfection was performed with Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer’s instructions. Forty-eight hours after transfection, the medium containing lentiviral particles was transferred to H4 cells at a 1:2 dilution in the presence of 8 μg/ml polybrein. After 24 h, cells were selected with 3 μg/ml of puromycin. pLKO.1 vectors were generated by the Broad Institute (Boston, MA, USA; http://www.broad.mit.edu/genome_bio/rna.html). Stable H4 cells with reduced Atg5 levels were maintained in medium containing 3 μg/ml puromycin. We used a shRNA sequence against Atg5 that we selected and described previously (34).

Preparation of protein extracts and Western blot analysis

Cells were washed in cold PBS and lysed at 4°C in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with a cocktail of protease inhibitors (Sigma-Aldrich). Lysates were cleared by centrifugation at 16,000 g for 20 min at 4°C, and protein concentration was determined with a protein assay solution (Bio-Rad, Hercules, CA, USA). Samples with equivalent amount of protein were boiled for 5 min with Laemmli SDS-PAGE sample buffer, and then analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electroblotted onto nitrocellulose membranes and incubated sequentially with primary and secondary antibodies for 1 h at room temperature, or overnight at 4°C. Chemiluminescence protein detection was performed using Pierce Western blotting substrate (Thermo Fisher Scientific). β-Actin was used as an internal loading control.

Fluorescence microscopy

Cells grown on glass coverslips were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. After fixation, cells were washed with PBS and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. Cells were incubated with the indicated primary antibodies diluted in immunofluorescence buffer (PBS containing 10% fetal bovine serum and 0.1% [w/v] saponin) for 45 min at 37°C. Coverslips were washed with PBS and incubated with the corresponding fluorochrome-conjugated secondary antibody diluted in immunofluorescence buffer for 30 min at 37°C. For nuclei staining, cells were washed with PBS and incubated for 10 min at room temperature with 0.1 μg/ml DAPI. After the final wash, coverslips were mounted onto glass slides with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Images of fixed cells were acquired with an AxioObserver.D1 microscope equipped with a PlanApo ×63 oil-immersion objective (NA 1.4) and an AxioCam MRm digital camera (Carl Zeiss GmbH, Jena, Germany), using similar settings described previously (34), or with an Olympus Fluoview FV1000 scanning unit fitted on an inverted Olympus IX81 microscope equipped with a PlanApo ×60 oil-immersion objective (NA 1.4; Olympus, Tokyo, Japan), using similar settings as described previously (35). Alternatively, images of fixed cells were acquired by a DeltaVision OMX system (GE Healthcare Life Sciences, Issaquah, WA, USA) for superresolution by structured illumination microscopy (SIM), using similar settings as described previously (36, 37). For fluorescent signal quantification, 12- or 16-bit images (± stack, with 0.2-μm intervals) were acquired under identical settings avoiding signal saturation, and each image was corrected for the background. The corrected fluorescent signal in each cell of each image was used to determine the total integrated pixel intensity per cell area by ImageJ 1.44o software [Image Processing and Analysis in Java; National Institutes of Health (NIH), Bethesda, MD, USA; http://imagej.nih.gov/]. Colocalization analyses and estimation of the Pearson’s correlation coefficient (38) were performed as described in Wong and Holzbaumer (35).

Densitometric quantification and statistical analysis

Quantification of immunoblot signals was performed using ImageJ 1.44o software (NIH). For each condition, protein bands were quantified from at least 3 independent experiments. Data analysis was performed by Microsoft Excel 2011 (Microsoft, Redmond, WA, USA) or GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Results were represented in graphs depicting the means ± SD. Statistical significance was determined by a 2-tailed, paired Student’s t test. Values of P > 0.05 or P ≤ 0.05 were regarded as not statistically significant or statistically significant, respectively.

RESULTS

Impaired lysosomal activity increases APP full-length and C99 levels

Previous reports have demonstrated that the perturbation of lysosomal pH causes an increase in the levels of APP and CTFs (33, 39). However, it is not well defined if all CTFs are equally perturbed by lysosomal dysfunction. Here, we used a H4 human neuroglioma cell line stably expressing a GFP-tagged amyloidogenic version of APP (APP-GFP), which we previously used to facilitate the detection of C99 in the form of C99-GFP (33, 40). We tested the effect of CQ and BfaA1 on the levels of APP-GFP, C99-GFP, and C83-GFP (Fig. 1A). CQ and BfaA1 are 2 drugs that raise organelle pH in the late secretory pathway, resulting in perturbation of both endosomal maturation and lysosomal function (41). Western blot analysis showed that treatment with CQ or BfaA1 led to a significant ~2.4- or ~2.1-fold increase in APP levels, respectively, compared to nontreated cells [Fig. 1B (lanes 1, 3, and 5)]. To detect C99-GFP and C83-GFP, we treated cells with DAPT, a specific γ-secretase inhibitor (42). As expected, Western blot analysis using the WO2 antibody (which distinguishes between C99 and C83; Fig. 1A) (43) showed that incubation with DAPT alone caused a steady-state accumulation of C99-GFP (Fig. 1B, lanes 1 and 2). We observed that in the presence of DAPT, the treatment with
CQ caused a significant ~1.8-fold increase in C99-GFP levels compared to cells treated with DAPT alone [Fig. 1B (lanes 2 and 4), C]. Likewise, treatment with BfaA1 in the presence of DAPT caused a significant ~3.0-fold increase in C99-GFP levels [Fig. 1B (lanes 2 and 6), C]. In contrast, we found that in the presence of DAPT, the treatment with CQ or BfaA1 led to a significant decrease of C83-GFP to ~55% or ~45%, respectively, of the levels found in control cells [Fig. 1B (lanes 2, 4, and 6), C]. In addition, we found that a combination of BfaA1, E64, and leupeptin, a cocktail that strongly perturbs lysosomal function (44), increased the levels of endogenous C99 (Supplemental Fig. 1). Together, these results indicate that impaired lysosomal function causes a differential effect on the CTFs, suggesting that C99 could be a better substrate of lysosomes.

Depletion of TSG101 increases levels of APP and CTFs, and number of autophagosomes

Several studies have proposed that APP is normally sorted into ILVs of MVBs, and that this process is dependent on the ESCRT machinery (26, 28, 29). Although these studies demonstrated that depletion of the ESCRT-0 subunit HRS, or of the ESCRT-I subunit TSG101, block APP sorting into ILVs, it is still a matter of discussion if these disturbances promote amyloidogenic processing (28, 29) or reduce Aβ production (26). Therefore, we evaluated whether the formation of MVBS was important for lysosomal degradation of C99. For this, we investigated the effect of depleting TSG101 by RNA interference (RNAi) in H4 cells expressing the amyloidogenic version of GFP-tagged APP were left untreated (lane 1) or treated either with 1 μM DAPT for 16 h (lane 2), 200 μM CQ for 4 h (lane 3), 1 μM DAPT for 12 h followed by combination of 1 μM DAPT and 200 μM CQ for 4 h (lane 4), 100 nM BfaA1 for 4 h (lane 5), or 1 μM DAPT for 12 h followed by combination of 1 μM DAPT and 200 μM CQ for 4 h (lane 6). Detergent-soluble protein extracts were analyzed by Western blot either with anti-GFP (to detect GFP-tagged APP, C83, and AICDγ), monoclonal antibody WO2 (used to show detection of only C99-GFP), or anti-β-actin used as loading control. Position of molecular mass markers is indicated on left. C) Densitometric quantification of Western blot signal of GFP-tagged APP (APP-GFP), C99 (C99-GFP), and C83 (C83-GFP) from cells treated with DAPT alone (control), or in combination with either CQ or BfaA1, as shown in A. Bars represent mean ± SD of Western blot signal normalized with β-actin signal (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001.

To study whether the depletion of TSG101 affected APP-GFP or CTF localization, we performed a confocal microscopy analysis. In different cell types, including H4 cells, a large proportion of the steady-state distribution of endogenous APP is in early endosomes (26, 45). Similarly, in H4 cells, the majority of APP-GFP localizes in early endosomes (25). Consistent with this notion, we found that in cells transfected with siNT, little APP-GFP colocalized with LAMP1, a late endosome/MVB/lysosome protein.
Figure 2. TSG101 depletion increases levels of APP species generated from amyloidogenic version of APP-GFP. A) H4 cells stably expressing amyloidogenic version of GFP-tagged APP were transfected with either control siRNA (siNT) or siRNA targeting TSG101 (siTSG101), and further left untreated (lanes 1 and 3), or treated with 1 μM DAPT for 16 h (lanes 2 and 4). Detergent-soluble protein extracts were analyzed by Western blot either with anti-GFP (to detect GFP-tagged APP, C99, C83, and AICDy), monoclonal antibody WO2 (used to show detection of only C99-GFP; C99-GFP*), or anti-TSG101. Western blot with anti-β-actin was used as loading control. Position of molecular mass markers is indicated on left. Bars represent means ± sd of Western blot signal normalized with β-actin signal (n = 3). **p < 0.01, ***p < 0.001.

(Supplemental Fig. S2A–C, G–H). In contrast, depletion of TSG101 resulted in a significant redistribution of APP to enlarged, LAMP1-containing endolysosomal compartments (Supplemental Fig. S2D–H). We obtained similar results with RNAi-mediated depletion of HRS (data not shown). Together, these findings suggested that impaired MVB formation caused accumulation of APP and CTFs in altered endolysosomal compartments.

Because MVBs eventually fuse with autophagosomes (17), we hypothesized that increased levels of APP and CTFs in TSG101-depleted cells could be the result of autophagosome accumulation. Therefore, we analyzed by confocal fluorescence microscopy the presence of puncta containing the protein p62. During autophagy, these puncta recruit specific ubiquitylated cargos by binding to the ubiquitin-associated domain of p62 (46), puncta that accumulate when the fusion of autophagosomes with endolysosomal compartments is blocked (47). We found that H4 cells depleted of TSG101 showed a significant increase in the number of puncta containing p62 compared to cells treated with siNT (Supplemental Fig. S3A, D, G), in agreement with the effect of a similar treatment in HeLa cells (22). A classic marker of autophagosomes is the protein LC3 (47), and accordingly, we found a significant colocalization of p62-containing puncta with LC3 (Supplemental Fig. S3D–F, N) compared to the negligible colocalization of p62-containing puncta with the lysosomal protein cathepsin D (Supplemental Fig. S3K–N). These results indicate that TSG101 depletion precludes autophagic flux, and hence this effect could be the cause of APP-GFP and CTF accumulation.

To further analyze whether the accumulation of APP-GFP and CTFs is produced when autophagosomes are accumulated, we studied the effect of thapsigargin, a drug that inhibits the fusion of autophagosomes to lysosomes (48). As expected, we found that cells treated with thapsigargin also accumulated p62-containing puncta (Supplemental Fig. S4A, E), similar to the effect reported for MEFs cells (48). Moreover, we observed a significant higher colocalization of the p62-containing puncta with LC3 compared to that with cathepsin D (Supplemental Fig. S4A–J), indicating that thapsigargin is also a potent inhibitor of autophagic flux in H4 cells. Western blot analysis showed that thapsigargin caused a significant increase in the levels of both APP-GFP and C99-GFP, but with no apparent effect in the levels of C83-GFP (Supplemental Fig. S4K, L), suggesting that the disruption of autophagic flux by thapsigargin affects differentially the turnover of APP species. Nevertheless, because the treatment with thapsigargin can also result in stress of the endoplasmic reticulum (49), we also analyzed the effect of the treatment with Vps34-IN1, which is a selective inhibitor of Vps34, a class III phosphatidylinositol 3-kinase that is required for autophagosome formation (50, 51). As expected, immunofluorescence analysis showed both that nutrient starvation by incubating cells in EBSS resulted in accumulation of puncta that contained LC3 and p62 (Fig. 3A–F), and that the treatment with Vps34-IN1 of cells incubated in complete medium did not accumulate puncta containing LC3 or p62 (Fig. 3G–I). In contrast, the treatment with Vps34-IN1 of cells incubated in EBSS significantly reduced the accumulation of puncta containing LC3 and p62 (Fig. 3F–I), confirming that Vps34-IN1 is a potent blocker of autophagosome biogenesis (52). Additional confirmation of the effect of Vps34-IN1 was obtained by Western blot analysis that showed a time-dependent increase in the levels of LC3-I, and a corresponding decrease in the levels of LC3-II, which is the lipidated form of LC3 that decorates autophagosomes (53) (Fig. 3N). Interestingly, the treatment with Vps34-IN1 caused a time-dependent increase in the levels of endogenous C99, although with no apparent effect on the levels of endogenous APP or C83 (Fig. 3N). Altogether, our results indicate that different treatments that disrupt autophagy lead to C99 accumulation.
**Depletion of Atg5 accumulates endogenous CTFs in the lumen of enlarged MVBs**

Atg5 conjugated to Atg12 is part of a protein complex that also contains Atg16L, a complex that controls an essential step in autophagosome formation (54). Therefore, to further study the contribution of autophagosomes in the clearance of APP/CTFs, we analyzed by Western blot the steady-state levels of endogenous APP and CTFs in H4 cells stably depleted of Atg5 by shRNA-mediated RNAi. We found that in cells stably expressing a shRNA targeting Atg5 (shAtg5), the levels of APP were significantly decreased to ~50% the levels found in cells expressing a control shRNA [shLuc; Fig. 4A (lanes 1 and 3), B]. We observed a similar result in cells treated with DAPT [Fig. 4A (lanes 2 and 4), C], indicating that decreased APP levels were not the result of increased γ-secretase activity. Moreover, we found that in contrast to control cells, the levels of APP in Atg5-depleted cells were not rescued with the combination of BfaA1, E64, and leupeptin (Supplemental Fig. S5), indicating that the decreased level of APP in Atg5-depleted cells was not the result of an increase in APP degradation. On the other hand, we observed a significant ~3.9-fold increase in C99 levels in Atg5-depleted cells (Fig. 4A (lanes 1 and 3), D). We also observed a significant ~23.1-fold increase in C83 levels in Atg5-depleted cells (Fig. 4A (lanes 1 and 3), E). These observations strongly indicate that perturbation of Atg5-dependent autophagy results in augmented proteolytic processing of APP, with generation of CTFs that ultimately accumulate.
To evaluate the possible effect of the γ-secretase activity on the levels of CTFs in Atg5-depleted cells, we treated cells with DAPT. Treatment of control cells with DAPT resulted in increased levels of both C99 and C83 (Fig. 4A, lanes 1 and 2), indicative of the expected γ-secretase activity on the CTFs. In Atg5-depleted cells, treatment with DAPT also resulted in a significant increase in the levels of both CTFs (Fig. 4A, lanes 3 and 4). However, when comparing the treatment with DAPT between control and Atg5-depleted cells, we observed that the levels of C99 underwent the highest increase: 7.9-fold increase of C99 vs. 2.0-fold increase of C83 (Fig. 4A, lanes 2 and 4). These results are consistent with the notion that impaired autophagosome formation caused increased amyloidogenic processing of APP.

To determine the effect that Atg5 depletion could have on the intracellular localization of APP and CTFs, we performed immunofluorescence analysis using an antibody targeting the C-terminal, cytosolic domain of APP (anti-Tail; Fig. 1A), and we examined cells by confocal microscopy. Consistent with the localization of APP-GFP, we found that in control H4 cells, little endogenous APP/CTFs localized in MVBs/lysosomes decorated with antibody to LAMP1, and that a higher proportion localized instead in early endosomes decorated with antibody to EEA1 (Fig. 5A–D, I–L). In contrast, Atg5 depletion led to a significant ~3.0-fold increase in the colocalization of APP/CTFs with LAMP1, and a corresponding significant decrease in the colocalization with EEA1 to ~45% the levels found in control cells (Fig. 5E–H, I–L). Similarly, we observed that depletion of Atg5 led to a significant ~2.6-fold increase in APP/CTF colocalization with cathepsin D, while the expected colocalization of cathepsin D with LAMP1 remained largely unaffected (Supplemental Fig. S6B, C, F, G, K, M). On the other hand, quantification of fluorescence intensity showed a significant ~4.6-fold increase in the levels of APP/CTFs in Atg5-depleted cells (Fig. 5M). This last result correlates with the Western blot analysis (Fig. 4A) and further supports the conclusion that Atg5 depletion causes a massive increase in APP/CTF levels in organelles positive to LAMP1 and cathepsin D. Because the anti-Tail antibody recognizes the cytosolic domain of APP and both CTFs, the APP species that accounts for the increase in fluorescence intensity cannot be discriminated. Nevertheless, the Western blot analysis suggests that the majority of the fluorescence signal corresponded to CTFs. We also observed that in cells depleted of Atg5, the organelles decorated with antibodies to
LAMP1 or cathepsin D were enlarged, and were mainly localized at the perinuclear region (Fig. 5B, F, and Supplemental Fig. S6B, C, F, G), in agreement with a previous study performed in mouse embryonic fibroblasts (55), suggesting that depletion of Atg5 caused a disruption of MVBs/lysosomes. Accordingly, these cathepsin D– and LAMP1–positive large organelles were also positive to CD63 (Supplemental Fig. S7A–C, F, G), both proteins that have been found localize mainly in MVBs/lysosomes (56, 57), and, similar to the colocalization of cathepsin D with LAMP1, the colocalization of cathepsin D with CD63 or LAMP2 was unaffected in cells depleted of Atg5 (Supplemental Fig. S7M, N). All these results strongly suggest that Atg5 depletion perturbs the homeostasis of MVBs/lysosomes, a condition that enhances the accumulation of intracellular CTFs.

To analyze in more detail the organelles that accumulate CTFs in Atg5-depleted cells, we used superresolution SIM. As shown by standard confocal microscopy (Fig. 5A–C), most of the APP/CTF fluorescent signal in control cells was detected in cytoplasmic structures that were different than MVBs/lysosomes decorated with antibody to LAMP1 (Fig. 6A–C). However, the APP/CTF fluorescent signal in Atg5-depleted cells was detected in structures resembling ILVs of enlarged LAMP1-containing MVBs (Fig. 6D–G). Moreover, fluorescence intensity analysis of SIM images showed that the APP/CTF signal is restricted to the lumen of enlarged LAMP1-containing organelles and therefore excluded from their limiting membrane.}

**Figure 5.** Atg5 depletion increases amount of endogenous APP and CTFs localized in enlarged LAMP1-containing endolysosomal compartment. A–H) H4 cells stably expressing either control shRNA (shLuc, A–D) or shRNA targeting Atg5 (shAtg5, E–H) were fixed, permeabilized, and incubated with antibody targeting cytosolic tail of APP (to detect APP, C99, C83, and AICDγ; APP/CTFs; E), with anti-LAMP1 (B, F), and with anti-EEA1 (C, G), followed by Alexa Fluor 488–conjugated donkey anti-rabbit IgG (green channels), Alexa Fluor 594–conjugated donkey anti-mouse IgG (red channels), and Alexa Fluor 647–conjugated goat anti-human IgG (blue channels). Images were acquired by laser scanning confocal microscopy. Merging green, red and blue channels generated fourth image in each row; yellow indicates overlapping of green and red channels, cyan indicates overlapping of green and blue channels, magenta indicates overlapping of red and blue channels, and white indicates overlapping of all channels. Insets: ×3 magnification, with arrows indicating colocalization of APP/CTFs and EEA1 in shLuc cells or colocalization of APP/CTFs and LAMP1 in shAtg5 cells. Scale bar, 10 μm. I) Quantification of fluorescence signal of APP/CTFs that colocalized with fluorescence signal of LAMP1, from images of control cells (shLuc; A, B), or Atg5-depleted cells (E, F). J) Quantiﬁcation of fluorescence signal of APP/CTFs that colocalized with fluorescence signal of EEA1 of control cells (shLuc) (A, C), or of Atg5–depleted cells (E, G). Bars represent means ± so (n = 12). K, L) Scatter-plot graphs with Pearson’s correlation coefficients obtained from colocalization analysis (I, J). M) Quantification of fluorescence intensity of APP species (A, E). Bars represent means ± so of fluorescent signal per cell area (n = 12). ***P < 0.001.
membrane, suggesting that APP/CTFs were efficiently sorted into ILVs (Fig. 6G, H). These results indicate that accumulation of APP/CTFs in Atg5-depleted cells was not due to impaired incorporation of APP/CTFs into ILVs. Together, this suggests that APP/CTFs are sorted into ILVs of MVBs en route to lysosomes for degradation.

**Activation of autophagy promotes intracellular clearance of C99**

Our results showed that autophagosomes are necessary for the clearance of intracellular CTFs via the endosomal pathway. To directly examine the active role of autophagy in C99 clearance, we subjected H4 cells stably expressing C99-GFP to nutrient starvation by incubation in EBSS, an established treatment that activates autophagy (16). Western blot analysis showed that nutrient starvation led to a significant, time-dependent reduction in C99-GFP levels as well as of AICDγ-GFP (Fig. 7A, B). Likewise, treatment with Torin-1, a potent and selective inhibitor of mTORC1/2 that also activates macroautophagy (58), similarly reduced C99-GFP and AICDγ-GFP levels in a time-dependent manner (Fig. 7C, D). Treatment with either EBSS or Torin-1 in the presence of DAPT did not prevent the reduction of both C99-GFP and AICDγ-GFP levels (Fig. 7E, F), indicating that the reduction in the levels of C99-GFP was not the result of γ-secretase activity. These last results strongly indicate that autophagy can participate in the clearance of amyloidogenic APP fragments. All the results shown in the present report are consistent with a model of a concerted function of autophagosomes and the MVB pathway for the clearance of amyloidogenic C99.

**DISCUSSION**

Several lines of evidence indicate that defects in long-term potentiation and cognitive impairment correlate better with increased intracellular Aβ-like immunoreactivity than with the presence of extracellular Aβ aggregates (59–61). The analysis of a murine model of AD (3xTgAD mice) showed that intracellular accumulation of early and age-dependent Aβ-like species corresponds to C99 rather than full-length APP or the Aβ peptide (59). Thus, C99 seems to be the earliest initiator of synaptic plasticity and long-term memory impairment in AD (62), presumably as a result of its early accumulation at the hippocampus (59, 61).
Although the cause of C99 accumulation remains unknown, several early studies point out abnormalities of endolysosomal compartments within neurons located in affected brain regions of patients with AD (7, 14). The abnormalities include an increased number of endolysosomal compartments, particularly in the cell soma of pyramidal neurons (7). Of note, the dependence of neuronal function on the endolysosomal system is well documented because of the existence of many lysosomal primary disorders, in which defects in lysosomal function cause severe neurodegeneration phenotypes (63), including pathologic features of AD (64).

Early accumulation of C99 in enlarged endolysosomal neuronal compartments of 3xTgAD mice suggests an impaired clearance of C99 by the endolysosomal system (59). In fact, it has been proposed that depletion of some ESCRT subunits inhibits APP sorting into ILVs of MVBs (26, 28, 29).

In agreement with this, we found that TSG101 depletion in H4 cells caused an increase in APP-GFP levels detected by Western blot analysis, which correlated with accumulation of APP-GFP in enlarged endolysosomal compartments decorated with antibodies to LAMP1. Moreover, we found that TSG101 depletion increased CTF levels, suggesting that CTFs are also sorted into ILVs. We also observed a robust increase in the levels of C99-GFP and endogenous C99 in response to treatments that perturb lysosomal function, supporting the conclusion that lysosomes are implicated in C99 clearance, as well as in APP turnover (26, 33, 61). Moreover, perturbed lysosomal function led to a significant decrease in C83-GFP levels. These results indicate that proper lysosomal function favor the nonamyloidogenic processing of APP, preventing the accumulation of C99. Interestingly, TSG101 depletion results in accumulation of APP/CTFs in an endolysosomal compartment that appears to contain proteins of both early endosomes and MVBs/lysosomes, such as EEA1 and LAMP1, respectively (26, 28, present study), emphasizing the need of normal maturation of early endosomes into MVBs to avoid accumulation of amyloidogenic proteins.
In addition to the evidence showing that APP is sorted to the endolysosomal pathway for delivery to lysosomes (25, 26, 28, 33), other findings indicate that the cellular levels of both APP and C99 are also regulated by autophagy (31, 32, 34). However, the contribution of both pathways in the total clearance of these amyloidogenic membrane proteins remains unknown. In this regard, different reports indicate that the endolysosomal system is functionally connected to autophagy (10, 17, 18, 23). In fact, autophagosomes can fuse to MVBS before lysosome delivery (18). Moreover, depletion of some ESCRT subunits accumulates autophagosomes by precluding their fusion to MVBS or lysosomes (22, 30, 65). In the present study, we show that, similar to HeLa cells (22), TSG101 depletion in H4 cells also inhibited autophagic flux. We found that TSG101 depletion resulted in accumulation of APP species in endolysosomal organelles containing LAMP1. To distinguish between autophagic flux inhibition and impaired sorting into ILVs of MVBS as the cause of APP and CTF accumulation in TSG101-depleted cells, we investigated the effect of treatment with thapsigargin. Thapsigargin is a classic stressor of the endoplasmic reticulum (66) that also blocks the fusion of autophagosomes to endolysosomal compartments, but by hampering recruitment of Rab7 to autophagosomes, a critical step during macroautophagy (48). Treatment with thapsigargin resulted in impaired autophagic flux and increased APP and C99 levels, which is consistent with the notion that autophagy is critical for APP/CTF clearance. In addition, we found increased levels of endogenous C99 upon selective inhibition of Vps34, which is a kinase that produces phosphatidylinositol-3-phosphate (PI3P), a crucial lipid for autophagosome nucleation and maturation (50, 51). This result is in agreement with a previous report showing that silencing of Vps34 enhances the production of Aβ in primary mouse cortical neurons (29). Because inhibition of Vps34 also resulted in inhibition of both basal autophagy and autophagy induced by nutrient starvation, the higher levels of Aβ peptide in cells depleted of Vps34 could be consequence of impairment in the MVB/lysosomal pathway due to perturbed autophagosome biogenesis. Together, these results suggest that autophagosomes are necessary for the degradation of C99 in the MVB/lysosomal pathway. Thus, increased CTF levels in LAMP1-containing organelles during impaired, basal Atg5-dependent autophagy provide additional support to the notion that autophagic flux is necessary for C99 clearance. Moreover, our SIM analysis in Atg5-depleted cells strongly suggests that accumulation occurred in ILVs of enlarged MVBS.

Because the fusion between an autophagosome and an MVB is the convergence point between these 2 pathways (17, 18), our findings indicate that upon Atg5-dependent autophagy impairment the MVB/lysosomal pathway could be dramatically affected, and hence the degradation of cargos such as APP/CTFs. In this regard, we showed that Atg5 depletion caused an apparent accumulation of CTFs in perinuclear, enlarged organelles positive to LAMP1, as well as positive to CD63, LAMP2, and cathepsin D, suggesting that a dramatic effect on the maturation of MVBS affected the degradation of CTFs. In contrast, recent studies in murine models of Down syndrome and AD show that C99 accumulates in enlarged early endosomes that contain overactivated Rab5 (67–69), suggesting the intriguing possibility that in those cases a similar mechanism of inter organelle fusion is impaired, although it is not well known whether autophagosomes can fuse to early endosomes (70, 71). Nevertheless, our immunofluorescence analysis to EEA1 showed no apparent differences in the morphology of early endosomes in control and Atg5-depleted cells. In addition, we observed a reduction in the colocalization of APP/CTFs and EEA1 upon Atg5 depletion, suggesting that perturbations at different levels of the endolysosomal system could lead to a similar outcome of C99 accumulation.

Likewise, it will be important to determine if an initial accumulation of C99 triggers the enlargement of endolysosomal organelles affecting their functions. In any case, our results provide strong evidence that autophagosomes cooperate in maintaining reduced levels of C99 either indirectly, by avoiding C99 production in MVBS, or directly, by accelerating C99 degradation in autolysosomes.

Our findings suggested that Atg5 depletion also elicited processing of APP into both C99 and C83. A likely explanation is that APP being accumulated in enlarged MVBS was subsequently processed by α-secretases and/or BACE-1. This is in agreement with the popular idea that amyloidogenic processing of APP occurs mainly in endosomes (72). A different scenario is that APP could have been processed in a preendosomal compartment, such as the endoplasmic reticulum or the Golgi apparatus, and that the resulting products C83 and C99 accumulated in MVBS. Although less popular, the possibility of amyloidogenic processing in a preendosomal compartment is supported by several reports (25, 73). However, the finding that Atg5-depleted cells presented a disproportionate increase of C83 indicates that macroautophagy also plays an important but unexpected role in the nonamyloidogenic processing of APP.

Our data allow us to propose that autophagosomes have an important function in APP and CTF proteostasis. Our proposal is consistent with the observation that autophagosomes gradually accumulate in dystrophic neurites, a well-documented neuropathologic hallmark of AD (14). Autophagy has been proposed as an essential activity for neuronal survival, acting as a neuroprotective process activated to alleviate cellular stress (9). In the nervous system, a major negative regulator of autophagy is the serine/threonine kinase mTOR; therefore, it is plausible that its inhibition would reduce the burden caused by deleterious accumulation of intracellular materials. Supporting this notion is the observation that reduced mTOR signaling extends the life span of model organisms and protects them against age-related pathologies, including AD (74, 75). Our finding that autophagy activation by 2 conditions that inhibit mTOR signaling—nutrient starvation or treatment with Torin-1—reduced the levels of C99 highlights this pathway’s potential as a pharmacologic target.

Collectively, our data support a model in which autophagosomes cooperate in the turnover of C99, as well as of APP and C83, forming an amphisome-like compartment by fusing with MVBS before fusion with lysosomes (Fig. 8).
Our model predicts that conditions that compromise the fusion of autophagosomes to endolysosomal compartments accumulate C99 and thus promote neuronal dysfunction. Further studies will help to clarify whether fusion of autophagosomes with MVBs is a frequent process or a specific event, taking place with a distinct pool of MVBs, and for the clearance of certain cargos, such as APP and CTFs. As a corollary, failures in macroautophagy could impact the balance of proteins that are incorporated into ILVs of MVBs, and therefore, we propose that the fate of autophagosomes constitutes an inherent part of the membrane flux that exists in the endolysosomal pathway of protein clearance. Hence, the pharmacologic booster of autophagy could promote the degradation of C99 and likely of other substrates destined to the endolysosomal pathway, emerging as an attractive approach for therapeutic intervention of AD, and eventually of other neurodegenerative disorders related to protein accumulation.

Figure 8. Model of mechanism underlying turnover of APP and CTFs via Atg5-dependent macroautophagy. 1, 2) APP, C99, and C83 can reach early endosome either via trans-Golgi network (TGN) (1), or via cell surface (2). 3) Early endosome matures to late endosome, also called MVB. 4) APP, C99, and C83 are incorporated into ILVs of MVB in process facilitated by ESCRT machinery (ESCRT-0 to ESCRT-III), in which TSG101 is part of ESCRT-I. 5) In parallel, Atg5-Atg12-Atg16L complex facilitates LC3 lipidation (LC3-I) with phosphatidylethanolamine. 6) Lipidated LC3 (LC3-II) associates to preautophagosomal membrane called phagophore, process that promotes recruitment of autophagy cargos and adaptor proteins, such as p62. 7) Phagophore matures to form autophagosome, double-membrane-bound organelle. 8) Autophagosome can fuse with endolysosomal compartments, such as MVB. Fusion of autophagosome with MVB results in formation of hybrid organelle called amphisome. 9) Amphisome can fuse with lysosome, promoting final degradation of APP, C99, and C83.

ACKNOWLEDGMENTS

This work was funded by the National Fund for Scientific and Technological Development (FONDECYT; Grants 1130929 to P.V.B., 1130710 to G.A.M., and 1140549 to C.H.); International Cooperation Program (PCI) of the National Commission for Scientific and Technological Research (CONICYT; Grants ECOS/CONICYT C14B03 to P.V.B., Fondo Newton-Picarte CONICYT DPI20140068 to P.V.B., and Fondo CONICYT Chile-Brazil 441921/2016-7 to C.H.); Fondo de Financiamiento de Centros de Investigación en Áreas Prioritarias (FONDAP; Program 15150012 to C.H.); Millennium Institute (Grant P09-015-F to C.H.); Office of Naval Research-Global (ONR-G; N62909-16-1-2003 to C.H.); São Paulo Research Foundation (FAPESP; 2014/25812-0 to L.L.D.); Dirección de Investigación y Desarrollo—Universidad Austral de Chile (DID-UACH; Grant D-2013-07 to A.E.G. and D-2015-02 to H.A.B.); and Programa de Mejoramiento de la Calidad y la Equidad de la Educación (MECESUP; AUS 1205). A.E.G., V.A.C., and H.A.B. were supported by CONICYT fellowships 201110746, 21151194, and 21130315, respectively. Y.C.J. was supported by a Coordination for the Improvement of Higher Education Personnel (CAPES) fellowship from the Brazilian Ministry of Education. The use of the OMX microscope was funded by a Medical Research Council (MRC) Next Generation Optical Microscopy award (MR/K015869/1).

AUTOPHAGOSOMES COOPERATE IN DEGRADATION

AUTHOR CONTRIBUTIONS

A. E. González, V. A. Cavieres, H. A. Bustamante, and P. V. Burgos conceived and designed the experiments; A. E.
REFERENCES


Multiple proteases generate and degrade potentially amyloidogenic fragments. *J. Biol. Chem.* **268**, 16602–16609


Autophagosomes cooperate in the degradation of intracellular C-terminal fragments of the amyloid precursor protein via the MVB/lysosomal pathway


FASEB J 2017 31: 2446-2459 originally published online March 2, 2017
Access the most recent version at doi:10.1096/fj.201600713R

Supplemental Material
http://www.fasebj.org/content/suppl/2017/03/02/fj.201600713R.DC1

References
This article cites 73 articles, 37 of which can be accessed free at:
http://www.fasebj.org/content/31/6/2446.full.html#ref-list-1

Subscriptions
Information about subscribing to The FASEB Journal is online at
http://www.faseb.org/The-FASEB-Journal/Librarian-s-Resources.aspx

Permissions
Submit copyright permission requests at:
http://www.fasebj.org/site/misc/copyright.xhtml

Email Alerts
Receive free email alerts when new an article cites this article - sign up at
http://www.fasebj.org/cgi/alerts

© FASEB

Dive into lipidomics with
SPLASH™ Lipidomix® Mass Spec Standard*
*Includes all major lipid classes in ratios relative to human plasma