Down-regulation of Endogenous Amyloid Precursor Protein Processing due to Cellular Aging

Andreas Kern, Birgit Roempp, Kai Prager, Jochen Walter, and Christian Behl

From the Institute for Physiological Chemistry and Pathobiochemistry, Johannes Gutenberg University Mainz, 55099 Mainz, Germany and Department of Neurology, University of Bonn, 53121 Bonn, Germany

Processing of amyloid precursor protein (APP) is a well acknowledged central pathogenic mechanism in Alzheimer disease. However, influences of age-associated cellular alterations on the biochemistry of APP processing have not been studied in molecular detail so far. Here, we report that processing of endogenous APP is down-regulated during the aging of normal human fibroblasts (IMR-90). The generation of intracellular APP cleavage products C99, C83, and AICD gradually declines with increasing life span and is accompanied by a reduced secretion of soluble APP (sAPP) and sAPPα. Further, the maturation of APP was reduced in senescent cells, which has been shown to be directly mediated by age-associated increased cellular cholesterol levels. Of the APP processing secretases, protein levels of constituents of the γ-secretase complex, presenilin-1 (PS1) and nicastrin, were progressively reduced during aging, resulting in a progressive decrease in γ-secretase enzymatic activity. ADAM10 (a disintegrin and metalloprotease 10) and BACE (β-site APP-cleaving enzyme) protein levels exhibited no age-associated regulation, but interestingly, BACE enzymatic activity was increased in aged cells. PS1 and BACE are located in detergent-resistant membranes (DRMs), well structured membrane microdomains exhibiting high levels of cholesterol, and caveolin-1. Although total levels of both structural components of DRMs were up-regulated in aged cells, their particular DRM association was decreased. This age-dependent membrane modification was associated with an altered distribution of PS1 and BACE between DRM and non-DRM fractions, very likely affecting their APP processing potential. In conclusion, we have found a significant modulation of endogenous APP processing and maturation in human fibroblasts caused by age-associated alterations in cellular biochemistry.

Aging is the most prevailing risk factor of Alzheimer disease, even though the biochemical basis of this association is unknown. A significant pathological feature of Alzheimer disease is the appearance of senile plaques that are composed primarily of amyloid β (Aβ), a 38–42-amino-acid peptide derived from proteolytic processing of the ubiquitously expressed amyloid precursor protein (APP) (1). At least three APP processing secretases are identified. ADAM10 (α-secretase) is involved in non-amyloidogenic processing and cleaves APP within the Aβ domain, whereby release of Aβ is prevented and soluble sAPPα is secreted (2). Amyloidogenic processing is driven by BACE (β-secretase), which cleaves APP at the N-terminal site of the Aβ domain (3). Subsequently, a complex of presenilin, nicastrin, anterior pharynx defective-1 and presenilin enhancer-2 (γ-secretase complex) cleaves the generated C-terminal fragments (CTFs) C83 or C99 at the C-terminal site of the Aβ domain (4–6). This results in the generation of the APP intracellular domain (AICD), and in the amyloidogenic pathway, Aβ is released (7, 8).

BACE and the γ-secretase complex have been well described to be associated to detergent-resistant membranes (DRMs) or lipid rafts (9, 10). These well structured membrane microdomains exhibit high levels of cholesterol, sphingolipids, and structural protein compounds, such as caveolin (11). They function as specialized membrane compartments for channeling and integrating external stimuli into downstream pathways and are also implicated in membrane trafficking (12–14). Modulation of cellular cholesterol levels has been shown to affect amyloidogenic APP processing by altering the constitution of DRMs and thereby the potential of BACE processing activity (15–17). Although APP processing has been extensively studied in cellular models, the influence of cellular aging on the biochemistry of APP processing has not been investigated in molecular detail so far.

Normal human fibroblasts (NHFs) exhibit a well established cellular aging model. In culture, they undergo a limited number of population doubling levels (PDs) before entering a state of irreversible growth arrest (18). At this end-stage of their in vitro mitotic life span, fibroblasts can be maintained and remain metabolically active but cannot be driven into further cell cycling (19). Although the exact molecular mechanisms of this replicative senescence have not been fully understood, multiple age-dependent cellular alterations are described. These include morphological changes with enlarged cell size, dysregulation of protein degradation, as well as post-translational modification of proteins and unresponsiveness to external stimuli (20, 21).

In this study, we examined age-associated alterations in the processing of endogenous APP by analyzing intracellular and secreted APP cleavage products throughout the life span of NHFs by immunoblotting as well as pulse-chase analysis. The impact of cholesterol on APP maturation was determined by depletion of intracellular cholesterol levels. We further focused on age-dependent changes in secretase levels, enzymatic activity of BACE, and the γ-secretase complex, as well as the impact of age-related alterations in DRM integrity on proteins involved in APP processing.

EXPERIMENTAL PROCEDURES

Cell Line and Culture Conditions—IMR-90 NHFs (Coriell Institute for Medical Research, Camden, NJ) were grown in phenol red-free Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO2.

This is an Open Access article under the CC BY license.

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
becco’s modified Eagle’s medium (Invitrogen) supplemented with antibiotics (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 10% charcoal-dextran-treated fetal calf serum (HyClone). These deficient culture conditions accelerate aging without influencing the resulting general aged phenotype, as described previously (22). At subconfluence, cells were passaged by trypsinization. PDLs were calculated as \((\log C_n - \log C_i) / \log (2)\), where \(C_n\) and \(C_i\) are defined as the cell number harvested and seeded, respectively. The aged phenotype was identified by use of a senesence-associated \(\beta\)-galactosidase staining kit (Cell Signaling), following the manufacturer’s instructions.

**Immunoblotting—**Adherent fibroblasts were scraped off the plates in lysis buffer (60 mM Tris-HCl, 2% SDS, 10% sucrose) sonicated briefly, and boiled for 5 min. Equal amounts of protein were separated on a SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane using a wet blot chamber (Bio-Rad). Commercial 4–12% NuPage BisTris gels (Invitrogen) were used to simultaneously separate high and low molecular weight proteins. For analysis of secreted APP, the medium was collected after 72 h, and the cells were scraped off the plates, as described above. According to the cell lysate protein concentration, the medium was loaded on an 8% SDS-polyacrylamide gel, separated, and transferred onto a nitrocellulose membrane. The blots were incubated overnight with the primary antibody at 4 °C. Proteins were detected using the ECL kit (Amersham Biosciences) after incubation for 2 h at room temperature with the appropriate secondary antibody. For detection of BACE, a biotinylated secondary antibody was used. Primary antibodies used were APP C-terminal antibody A8717 (Sigma), APP cytoplasmic domain polyclonal antibody CT-15 (kind gift of Dr. C. U. Pietrzik), APP mid-region polyclonal antisera 863 (kind gift of Dr. S. Weggen), human APP monoclonal antibody (clone 6E10) (Biocat), actin (Santa Cruz Biotechnology), ADAM10 (Chemicon), BACE (Ab-2) (Oncogene), caveolin-1 (Transduction Laboratories), nicastrin (Sigma), Notch (Santa Cruz Biotechnology), PS1 N-terminal (3110), which was raised against a fusion protein of the maltose-binding protein and amino acids 2–80 of human PS1.

**Quantitative Real-time Reverse Transcription-PCR—**Total RNA from cells grown to subconfluence was prepared using the Absolutely RNA reverse transcription-PCR miniprep Kit (Stratagene) according to the manufacturer’s instructions. Reverse transcription was performed on 500 ng of total RNA with the Omniscript reverse transcriptase kit (Qiagen) according to the supplier’s instructions. The quantitative real-time PCR was carried out in triplicates containing 1 µl of cDNA, 100 pmol sense and antisense primer (sense, 5′-GGAGCTCCTTCCGGTGAATGG-3′; antisense, 5′-CGTAGCCGTTCTGCTGCATC-3′) and 12.5 µl of 2× SYBR Green Supermix (Bio-Rad) in a final volume of 25 µl. PCR was performed using the iCycler (Bio-Rad). PCR conditions were as follows: initial denaturation at 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 59 °C for 20 s, 72 °C for 30 s, and final elongation at 72 °C for 5 min. The generation of specific PCR products was confirmed by melting curve analysis. The PCR cycle number that generated the first fluorescence signal above threshold (threshold cycle, \(C_T\)) was determined. Glyceraldehyde-3-phosphate dehydrogenase served for normalizations and calculation of \(\Delta C_T\).

**Metabolic Labeling—**IMR-90 cultures were incubated with methionine-free Dulbecco’s modified Eagle’s medium supplemented with 150 µCi/ml [35S]methionine/cysteine for 15 min (pulse). Cells were lysed immediately or chased for the indicated time periods. APP and APP-CTFs were immunoprecipitated using the APP-CT antibody 140. This antibody was raised against the C-terminal 20 amino acids of human APP, which were conjugated to keyhole limpet hemocyanin before inoculation in rabbits. Immunoprecipitates were separated by SDS-PAGE. Radiolabeled proteins were detected and quantified by phosphorimaging.

**Determination of Free Cholesterol—**Free unesterified cholesterol species were analyzed using the Amplex Red cholesterol assay kit (Molecular Probes) following the manufacturer’s instructions. For determination of the cholesterol content in fractions of sucrose gradients for DRM isolation, 50 µl of each fraction were analyzed.

**Isolation of Detergent-resistant Membranes—**Cells were scraped off the plates in phosphate-buffered saline and lysed in 300 µl of lysis buffer (20 mM CHAPS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). The cell lysate was mixed with the same volume, 80% sucrose (in lysis buffer without CHAPS). The resulting 40% sucrose solution was overlaid by a step gradient of 1.3 ml of 30% and 0.25 ml of 5% sucrose. By centrifugation at 200,000 × g for 2 h at 4 °C, DRMs floated at the border of 5 and 30% sucrose. Fractions were collected, and 30 µl of each were analyzed by immunoblotting.

**Determination of \(\beta\)- and \(\gamma\)-Secretase Activity—**Enzymatic activities of \(\beta\)- and \(\gamma\)-secretase were analyzed using the \(\beta\)-Secretase Activity assay kit (BioVision) or the \(\gamma\)-Secretase Activity assay kit (R & D Systems), respectively, following the manufacturer’s instructions. For analysis of total cellular activity, the same amounts of cellular protein were used. Activity measurements in fractions of sucrose gradients for DRM isolation were done with 50 µl of each fraction.

**Statistical Analysis—**Optical band densities from immunoblotting were measured with Aida Image Analyzer version 3.28 software (Raytest). Mean values of data from at least three independent experiments were subsequently calculated and plotted as percentage change compared with young cells (PDL 20/23), which have been set to 100%. Standard deviations were calculated by analysis of variance using Sigma Stat software (SPSS Science). Significance was analyzed using the post hoc Tukey test.

**RESULTS**

**Levels of Mature APP and APP Cleavage Products Decrease during Cellular Aging—**IMR-90 NHFs were sequentially passaged until growth arrest of the cells at PDL46. The aged phenotype was determined using a senesence-associated \(\beta\)-galactosidase staining, which identified almost 100% of stained cells at PDL 46 (data not shown).

Processing of endogenous APP throughout the in vitro life span of the NHF culture was analyzed by monitoring the protein levels of full-length APP, the intracellular cleavage products C99, C83, and AICD, as well as the secreted cleavage products sAPP and sAPPα at increasing PDLs by immunoblotting (Fig. 1). We observed ~50% reduction in the ratio of mature to immature APP in senescent cells (Fig. 1A). Real-time PCR analysis did not show any decrease in APP gene expression (data not shown), and additionally, no significant alterations in immature APP protein levels were detected. Levels of the intracellular APP cleavage products C99 and C83, deriving from either \(\beta\)- or \(\alpha\)-secretory cleavage, respectively, significantly decreased from young to old PDLs, resulting in reduced ratios of these C-terminal fragments (CTFs) to total or mature APP. C99 and C83 are further processed by the \(\gamma\)-secretase complex, leading to the generation of AICD. This ~6-kDa APP-CTF is known to be rapidly degraded and has not been shown at endogenous cellular levels previously. To verify the AICD identity, we treated young fibroblasts (PDL 25) with the \(\gamma\)-secretase inhibitor H-5106 (Sigma), after which the ~6-kDa band was not detectable anymore (Fig. 1B). In addition, a further APP C-terminal antibody was used, which is well described to be specific for AICD (23). This antibody also recognized the 6-kDa band, emphasizing the identity of the fragment (supplemental Fig. 1). Thus, we suggest that this APP-derived fragment indeed
age-associated decrease in levels of mature APP and APP cleavage products. Full-length APP and APP cleavage products were analyzed by immunoblotting. A, total cell lysates of different PDLs were separated on a 4–12% NuPage BisTris gel. Proteins were detected using the CT-15 antibody. Full-length APP and CTFs were identified on the same membrane but at different exposure times, allowing the demonstration of C83, C99, and AICD. Actin was used for control of equal loading. Graphical representations were calculated using optical band densities and are indicated as the percent of change to PDL 23 (#, PDL 23–40, *, PDL 23–46; p < 0.05; Tukey test, n = 4). B, for identification of endogenous AICD, cells at PDL 25 were treated overnight with 10 μM H-3106 or Me2SO (DMSO), respectively. AICD levels were analyzed using the CT-15 antibody. C, culture media of different PDLs were collected, separated on an 8% polyacrylamide gel, and analyzed using the monoclonal 6E10 antibody (sAPPα) or polyclonal antiserum 863 (sAPP). Graphical representations were calculated using optical band densities. *, PDL 23–46; p < 0.05; Tukey test, n = 4.

FIGURE 1. Age-associated decrease in levels of mature APP and APP cleavage products. Full-length APP and APP cleavage products were analyzed by immunoblotting. A, total cell lysates of different PDLs were separated on a 4–12% NuPage BisTris gel. Proteins were detected using the CT-15 antibody. Full-length APP and CTFs were identified on the same membrane but at different exposure times, allowing the demonstration of C83, C99, and AICD. Actin was used for control of equal loading. Graphical representations were calculated using optical band densities and are indicated as the percent of change to PDL 23 (#, PDL 23–40, *, PDL 23–46; p < 0.05; Tukey test, n = 4). B, for identification of endogenous AICD, cells at PDL 25 were treated overnight with 10 μM H-3106 or Me2SO (DMSO), respectively. AICD levels were analyzed using the CT-15 antibody. C, culture media of different PDLs were collected, separated on an 8% polyacrylamide gel, and analyzed using the monoclonal 6E10 antibody (sAPPα) or polyclonal antiserum 863 (sAPP). Graphical representations were calculated using optical band densities. *, PDL 23–46; p < 0.05; Tukey test, n = 4.

resembles endogenous AICD. During aging, detectable levels of AICD decreased rapidly, reflecting the progressive decline in C99 as well as C83 generation. Therefore, we conclude that APP processing is gradually reduced throughout the life span of NHFs, resulting in lowered intracellular APP-CTF levels. To further confirm the age-related decline in endogenous APP processing, we analyzed the secreted cleavage products of APP (Fig. 1C). Although secretion of total sAPP and sAPPα decreased, no significant age-associated alteration in the ratio of sAPPα to sAPP was detected.

Maturation of APP Is Reduced during Cellular Aging—Interestingly, we observed an age-associated reduction in levels of mature APP as well as APP-CTFs. We next examined the kinetics of APP metabolism by pulse-chase experiments. Analysis of the time course of APP maturation demonstrated a significant delay in generation of mature APP in senescent NHFs (Fig. 2A). Maturation of 50% APP (based on immature APP levels at t = 0 min) was reached after ~15 min in young cells (PDL 23), whereas ~24 min were needed in cells at PDL 46. As shown by immunoblotting (Fig. 1A), total levels of mature APP were significantly reduced in aged cells. The half-lives of mature as well as immature APP (~60 min) did not show an age-associated alteration, suggesting that degradation rates of APP were unchanged during aging. Thus, the reduced levels of mature APP observed in cells at PDL 46, were caused by an age-associated down-regulation in the efficiency of APP maturation, rather than by an enhanced degradation or processing. We also found that APP-CTFs were generated earlier and reached higher levels in young cells as compared with aged cells (Fig. 2B). Together, these data demonstrate that maturation and proteolytic processing of APP is down-regulated during cellular aging.

Age-associated Increased Cholesterol Levels Inhibit APP Maturation—We observed a significant reduction in APP maturation efficiency in cells at PDL 46, which resulted in decreased mature APP protein levels. Previously, it was shown that APP maturation is affected by increased cholesterol levels (24, 25) and analysis of intracellular cholesterol in the NHF aging model detected an age-associated increase by 37% (±4, p = 0.006, n = 3) (data not shown), consistent with a previous study (26). To determine whether age-related increased levels of cellular cholesterol affect APP maturation, we depleted cholesterol by treatment with methyl-β-cyclodextrin (MβCD), exactly as shown previously (27). After cholesterol depletion, levels of mature APP were selectively increased in aged cells as compared with untreated cells, without any effect on immature APP levels (Fig. 3). This resulted in an increased ratio of mature to immature APP, almost to the level of young cells. Thus, APP maturation was recovered after depletion of cellular cholesterol, demonstrating that the age-associated decreased maturation efficiency of APP was directly affected by increased levels of intracellular cholesterol. Notably, MβCD treatment of young cells did not significantly affect APP maturation. Further, depletion of cholesterol is well described to affect the cleavage of APP, resulting in enhanced non-amyloidogenic processing (27). However, we observed no significant influence on C83 levels after MβCD treatment. The rather short time of treatment, necessary to avoid the toxic potential of MβCD as well as the slow turnover of APP-CTFs, very likely did not permit resolution of these changes at levels of endogenous APP processing. In addition, analysis of secreted APP cleavage products did not detect differences in APP processing in comparison to untreated cells (data not shown).

Age-associated Changes for Constituents of the γ-Secretase Complex—To analyze age-associated alterations in levels of enzymes involved in APP processing, we examined protein levels of ADAM10, BACE, PS1, and nicastrin throughout the life span of NHFs. Immunoblotting detected a selective down-regulation of PS1 and nicastrin, whereas ADAM10 and BACE protein levels were not altered (Fig. 4). Presenilin undergoes endoproteolysis, which results in a C- and N-terminal subunit (28). Protein levels of the PS1 N-terminal subunit as well as of the full-length precursor protein were significantly reduced with increasing PDLs, without any change in the ratio of the PS1 N-terminal subunit to full-length protein. Therefore, cellular aging was not affecting PS1 maturation. Additionally, protein levels of
mature nicastrin, another constituent of the γ-secretase complex, were
down-regulated throughout the life span of the NHF culture, confirming
that total γ-secretase complex levels were reduced during aging. Protein
levels of ADAM10 exhibited no age-associated decrease. The inactive pro-
form is cleaved by secretory proprotein convertases generating the active
protease (29). This maturation process was not exhibiting any changes with
increasing PDLs. Also for BACE, no age-dependent alterations in total pro-
tein levels and maturation efficiency, mediated by a furin-like convertase
(30), were detected (see supplemental Fig. 2 for graphical representations
of maturation efficiencies).

Age-associated decrease in DRM integrity affects the distribution of
proteins involved in APP processing—To analyze age-associated alter-
ations in membrane constitution and its influence on proteins involved
in APP processing, we isolated DRMs, indicating lipid rafts, by sucrose
gradient ultracentrifugation, as previously described (16, 17). Thereby,
due to their buoyant density, DRMs accumulate between 5 and 30%
sucrose (Fig. 5, A and C, fraction 2). Analyzing cholesterol levels within
fractions of DRM isolations, we found decreased levels within DRM
fractions of aged cells (Fig. 5, A), even though total intracellular choles-
terol levels were increased, consistent with a previous report (26). Fur-
thermore, total caveolin-1 protein levels were progressively up-regu-
lated during cellular aging and, interestingly, caveolin-1 was also
observed to significantly migrate into the non-DRM fraction of senes-
cent cells (Fig. 5, B and C, upper panels). To investigate whether this

FIGURE 2. Cellular aging decreases maturation and proteolytic processing of APP but does not affect degradation of APP and APP-CTFs. APP maturation efficiency as well as the turnover of APP and APP-CTFs were analyzed by pulse-chase analysis. A, cells at PDL 23 and 46, respectively, were labeled with [35S]methionine for 15 min. At the indicated chase
times, the cells were lysed and APP was immunoprecipitated using the APP-CT antibody 140. Immunoprecipitates were separated on a 7% SDS-polyacrylamide gel, and APP variants
were detected and quantified by phosphorimaging. B, turnover of APP-CTFs was analyzed accordingly.
age-dependent disintegration of DRMs or reduction in total DRM levels influenced the localization of APP and its processing secretases, we analyzed their distribution between DRM and non-DRM fractions by immunoblotting (Fig. 5C). In agreement with previous reports, significant levels of BACE, PS1, and also APP were DRM-associated (9, 10, 16), and as expected, their immature precursors were almost completely excluded from DRMs of young cells. Senescent fibroblasts exhibited an altered pattern of distribution. Increased levels of immature APP accumulated within DRMs and significant levels of PS1 migrated into the non-DRM fraction, reflecting the age-associated reduction in DRM integrity or total levels. DRM-associated levels of total BACE were not decreased in senescent cells, but interestingly, mature BACE was almost

**FIGURE 3.** Age-associated increased cholesterol levels interfere with APP maturation. Subconfluent IMR-90 cells of the indicated PDLs were treated with 10 mM MβCD for 30 min. After washing, the cells were incubated for 4 h in serum-free Dulbecco’s modified Eagle’s medium and thereafter lysed. Total cell lysates were separated on a 4–12% NuPage BisTris gel. APP and C83 were detected using the CT-15 antibody. Actin was used for the control of equal loading. C indicates control cells. Graphical representations were calculated using optical band densities. *, p < 0.05; Tukey test, n = 6.

**FIGURE 4.** Protein levels of secretases throughout the life span of NHFs. Protein levels of secretases were analyzed by immunoblotting. Total cell lysates of different PDLs were separated on a 12% SDS-polyacrylamide gel, and proteins were detected using specific antibodies. Actin was used for the control of equal loading. Graphical representations were calculated using optical band densities. #, PDL 23–40; *, PDL 23–46; p < 0.05; Tukey test, n = 4.
completely replaced by the immature form, resulting in only marginal levels of DRM-associated mature protease.

**β-Secretase Enzymatic Activity Is Increased in Aged Cells and Migrates into the Non-DRM Fraction, whereas γ-Secretase Activity Progressively Decreases**—Because we observed an age-related down-regulation in the generation of C99 and AICD, we analyzed alterations in enzymatic activity of β- and γ-secretase during cellular aging. β-secretase enzymatic activity was analyzed in total cell lysates and in fractions of DRM isolations. Interestingly, senescent fibroblasts exhibited a significant increase in total BACE activity by 44% in comparison to young and middle-aged cells (Fig. 6A). Within fractions of DRM isolations, β-secretase enzymatic activity was prominently present within DRMs of young cells (PDL 20) but migrated into the non-DRM fraction of cells at PDL 46 (Fig. 6B). Thus, we observed a permanent co-localization of enzymatic activity with the mature form of the enzyme. Analyzing changes in γ-secretase enzymatic activity during aging detected a progressive down-regulation in total enzymatic activity, which reflected the decrease in PS1 N-terminal subunit and nicastrin protein levels, and, thus, total γ-secretase levels (Fig. 6C). To analyze whether the age-related reduction in γ-secretase activity exclusively affected APP processing or also additional substrates of the complex, we investigated the cleavage of the Notch extracellular truncation, which results in genera-
tion of the Notch intracellular domain (31). Immunoblotting demonstrated a progressive reduction in Notch intracellular domain generation with increasing PDLs, even though total Notch extracellular truncation protein levels were decreased during cellular aging (supplemental Fig. 3). Therefore, the age-related down-regulation of total γ-secretase activity resulted in a reduced processing of APP as well as Notch and, thus, of at least two independent substrates.

DISCUSSION

In this study, we observed a gradual down-regulation of endogenous APP processing during in vitro aging of NHFs. This was associated by a reduced APP maturation efficiency in aged cells, which was directly affected by age-related increased cellular cholesterol levels. Of the APP processing secretases, only constituents of the γ-secretase complex were decreased with increasing life span, which resulted in an age-dependent down-regulation of total γ-secretase enzymatic activity. The aging phenotype was associated by a reduction in DRM integrity or total DRM levels, resulting in decreased levels of the PS1 N-terminal domain and mature BACE in these membrane subdomains. In aged cells, mature BACE was almost completely replaced by the immature form. Although enzymatic activity of the β-secretase was increased in aged cells, it co-localized with mature BACE outside of DRMs, which very likely reduced its APP processing potential.

Culture and serial passaging of NHFs resulted in post-mitotic senescent cells exhibiting typical age-associated features, such as increased senescence-associated β-galactosidase staining and caveolin-1 protein levels. Age-associated changes in cellular biochemistry have been described previously and may directly or indirectly affect the cleavage of APP and result in the observed overall decrease in this complex processing event. Besides alterations in post-translational protein modification and in membrane constitution, such as demonstrated for APP or DRMs, respectively, these changes may further include intracellular protein trafficking mechanisms, which resemble well characterized age-associated cellular alterations (32, 33).

The progressive decrease in APP processing was associated with a reduced APP maturation, whereas immature protein levels and APP gene expression were unchanged. APP is subjected to post-translational modification, such as glycosylation, sulfation, and phosphorylation during transit through the intracellular protein secretory pathway (34–37). N-glycosylated immature APP is missing O-glycosylations and tyrosyl-sulfations and is described as not being transferred to the plasma membrane but remaining within the endoplasmic reticulum or cis-Golgi (38). Interestingly, depletion of intracellular cholesterol levels resulted in recovery of the reduced maturation efficiency in aged cells, directly linking age-associated alterations in cholesterol metabolism with APP maturation. Previously, for neuronal and glial cultures, increased cholesterol levels have been shown to interfere with APP maturation by inhibiting APP glycosylation (24). Thus, the age-associated increase in intracellular cholesterol levels very likely also affects APP glycosylation mechanisms, resulting in a significant down-regulation of APP maturation efficiency in cells at PDL 46. We recently found that glycosphingolipids also regulate maturation of APP in the Golgi compartment, indicating that APP forward transport is modulated by the membrane lipid composition (39). Interestingly, we observed a reduction in maturation exclusively for APP, whereas the secretases did not show any age-associated decrease.

The progressive decline in APP processing was accompanied by a redistribution of the PS1 N-terminal subunit into non-DRM fractions of aged cells, reflecting the reduction in DRM integrity or in total DRM levels. Further, total PS1 as well as nicastrin protein levels were decreased during aging, which emphasized that total γ-secretase complex levels were progressively reduced. This was associated by a decreased enzymatic activity of the complex, resulting in a reduced processing of APP as well as Notch. Because the progressive reduction in γ-secretase enzymatic activity reflected the decline in levels of PS1 N-terminal domain as well as nicastrin, the actual enzymatic activity very likely was unaffected by cellular aging, but correlating with decreased levels of the secretase, the total activity was reduced.

Beyond their protease activity, presenilins are also described to control subcellular trafficking of a number of proteins, such as APP, Trk receptor, nicastrin, and Pen-2 (40–42). Recently it was shown that caveolin-1 transport is also regulated by presenilins, and PS deficiency leads to a severe loss in caveolae (43). A possible link between the demonstrated age-associated reduction in total PS1 protein levels and caveolin-1 trafficking, which may lead to the decreased DRM association, needs to be further analyzed.

Protein levels of ADAM10 and BACE were not altered during cellular aging. However, an enhanced β-secretase enzymatic activity was detected, consistent with what has previously been described for the brains of aged humans, mice, and monkeys (44). Intracellular regulative factors of β-secretase activity, such as sorting in acidic endosomal or lysosomal compartments, were disrupted by the type of activity assay carried out here. Thus, the observed enhanced enzymatic activity very likely was because of altered post-translational modification or allos-

FIGURE 6. Age-associated alterations in β- or γ-secretase enzymatic activity. A, analysis of BACE enzymatic activity in total cell lysates is presented as percentage of activity to PDL 20 based on relative fluorescence units/μg of protein. *, p < 0.05; Tukey test, n = 3. B, analysis of activity in sucrose fractions for DRM isolations were carried out with 50 μl of each fraction and presented as percentage of activity to total. The ratio of raft to non-raft activity was calculated with fractions 1–3 as DRM and 7–9 as non-DRM fractions. *, p < 0.05; Tukey test, n = 3. C, γ-secretase enzymatic activity was analyzed in total cell lysates and is presented as percentage of activity to PDL 20 based on relative fluorescence units/μg of protein. #, PDL 20–34; *, PDL 20–46; p < 0.05; Tukey test, n = 3.
Cellular Aging Down-regulates APP Processing

teric modulation of the protease. Such modulations are supported by the recent finding of a copper-binding ability of the C-terminal domain of BACE, which might suggest a copper-dependent alteration in BACE enzymatic activity (45).

BACE is synthesized as a precursor protein containing a N-terminal propeptide, which is cleaved within the Golgi apparatus. However, already immature BACE has been described to be enzymatically active (46). Interestingly, analysis of β-secretase enzymatic activity within fractions of DRM isolations detected a significant migration of activity into the non-DRM fraction of aged cells. Therefore, we observed a permanent co-localization of activity with the mature form of the enzyme, which suggests that at endogenous levels immature BACE is enzymatically inactive and complete maturation is a prerequisite for enzymatic activity.

Surprisingly, despite the increased BACE enzymatic activity, which was exclusively detected in cells at PDL 46, generation of C99 was progressively reduced throughout the NHF life span, suggesting that the gradual down-regulation of APP processing is independent of the actual enzymatic activity of the β-secretase itself. One regulatory factor of amyloidogenic APP processing is resembled by DRMs, which are modulated by cellular cholesterol levels. Treatment of cells with statins, inhibitors of intracellular cholesterol synthesis, directs APP processing into the non-amyloidogenic pathway (27, 47). Therefore, DRMs are a well-characterized prerequisite for amyloidogenic APP processing, with DRM-associated APP being the substrate pool for β-secretase processing (16, 48, 49). Consequently, the localization of mature BACE and β-secretase activity in the non-DRM fraction of aged cells very likely reduces the potential of β-secretase-mediated cleavage of APP, which results in a decreased generation of C99. Nevertheless, different effects are possible, because recently it was shown that disintegration of DRMs by moderate cholesterol depletion enhances AB production, emphasizing a cholesterol concentration dependence of amyloidogenic APP processing (17). There in the analyzed cell lines and primary neuronal cultures, endogenous APP was described to be located in the non-DRM fraction, leading to a spatial separation of BACE from its substrate by a DRM-mediated boundary, which was abolished by moderate cholesterol reduction. Analysis of APP processing at endogenous levels does not permit resolution of changes in AB generation because of the extremely low levels of this peptide. However, because we observed significant levels of endogenous APP within DRMs, which is in line with rafts being the location of amyloidogenesis, we suggest that DRMs indeed resemble membrane subdomains, necessary for amyloidogenic APP processing. Thus, the age-associated migration of mature BACE into the non-DRM fraction of aged cells very likely results in a decreased APP processing potential.

In our study, replicative senescent NHFs served as a cellular aging model to investigate age-associated changes in the biochemistry of APP processing. The complex cleavage process is a key event in age-related Alzheimer disease; however, the impact that cellular aging exerts on this process has not been well characterized. Interestingly, age-related changes described for senescent NHFs have also been observed in aged or Alzheimer diseased brains, such as up-regulated caveolin-1 protein levels (50), altered lipid raft constitution (51), age-related increased cholesterol levels (52), and increased BACE enzymatic activity, without changes in BACE expression levels (44). Because processing of APP resembles a constitutive mechanism, whose cleavage products are detected in normal human cerebrospinal fluid as well as in cultured cell lines, they are not abnormal cellular products. Thus, the increased AB levels found in aged brains (53–56) may be the result of a pathological imbalance in APP biochemistry, very likely not occurring during normal aging.

We found that endogenous APP processing is gradually declining with increasing cellular age and is accompanied by a reduced APP maturation efficiency in aged cells caused by increased cellular cholesterol levels. Analysis of age-associated alterations in γ- as well as β-secretase, demonstrated a down-regulation in γ-secretase complex levels as well as enzymatic activity, which resulted in a reduced APP processing. BACE exhibited an increased enzymatic activity in aged cells, which was located outside of DRMs, reflecting the disintegration of these membrane subdomains. This very likely is associated with an age-related down-regulation of amyloidogenic APP processing independent of the increased BACE enzymatic activity. Because the pathological cleavage of APP is well acknowledged as one of the central pathogenic mechanisms for familial as well as for the large group of age-associated sporadic Alzheimer disease, insights into the age-related changes in biochemistry will add important information for future approaches toward an Alzheimer disease therapy.

Acknowledgments—We thank Drs. C. U. Pietrzik and S. Weggen for providing antibodies and for helpful advice. We also thank Drs. A. B. Clement and D. Manthey for help in DRM isolations and real-time PCR analysis, respectively.

REFERENCES

Cellular Aging Down-regulates APP Processing