Amyloidogenic Processing of Amyloid Precursor Protein: Evidence of a Pivotal Role of Glutaminyl Cyclase in Generation of Pyroglutamate-Modified Amyloid-β[†]

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ABSTRACT: Compelling evidence suggests that N-terminally truncated and pyroglutamyl-modified amyloid-β (Aβ) peptides play a major role in the development of Alzheimer's disease. Posttranslational formation of pyroglutamic acid (pGlu) at position 3 or 11 of Aβ implies cyclization of an N-terminal glutamate residue rendering the modified peptide degradation resistant, more hydrophobic, and prone to aggregation. Previous studies using artificial peptide substrates suggested the potential involvement of the enzyme glutaminyl cyclase in generation of pGlu-Aβ. Here we show that glutaminyl cyclase (QC) catalyzes the formation of A$\beta_{3(pE)-40/42}$ after amyloidogenic processing of APP in two different cell lines, applying specific ELISAs and Western blotting based on urea−PAGE. Inhibition of QC by the imidazole derivative PBD150 led to a blockage of A$\beta_{3(pE)-42}$ formation. Apparently, the QC-catalyzed formation of N-terminal pGlu is favored in the acidic environment of secretory compartments, which is also supported by double-immunofluorescence labeling of QC and APP revealing partial colocalization. Finally, initial investigations focusing on the molecular pathway leading to the generation of truncated Aβ peptides imply an important role of the amino acid sequence near the β-secretase cleavage site. Introduction of a single-point mutation, resulting in an amino acid substitution, APP(E599Q), i.e., at position 3 of Aβ, resulted in significant formation of A$\beta_{3(pE)-40/42}$. Introduction of the APP KM595/596NL "Swedish" mutation causing overproduction of Aβ, however, surprisingly diminished the concentration of A$\beta_{3(pE)-40/42}$. The study provides new cell-based assays for the profiling of small molecule inhibitors of QC and points to conspicuous differences in processing of APP depending on sequence at the β-secretase cleavage site.

Alzheimer's disease (AD)[1] has emerged as the major cause of dementia in the developed world, affecting approximately 20−25 million patients (*1*). Neurofibrillary tangles and senile plaques, which are found *post mortem* in cortical and hippocampal brain sections of AD patients, represent the major histopathological hallmarks of the disease. According to the amyloid cascade hypothesis, amyloid-β (Aβ) peptides, the primary components of senile plaques, are key for the development of the disease (*1, 2*). These A$\beta_{40/42}$ peptides are liberated sequentially by proteolysis of the amyloid precursor protein (APP) by β- and γ-secretases (*3, 4*). Aβ undergoes a high level of turnover within the brain, and its impaired degradation is presumed to be the primary cause of Aβ deposition (*1, 5, 6*).

It has been shown that N-terminal variants of Aβ are abundant in human amyloid deposits and soluble Aβ. Modifications affect the aspartic acids at positions 1 and 7, which are isomerized or racemized (*7–10*), or the glutamic acid residues at position 3 and 11, which are found to be cyclized into pyroglutamic acid (pGlu) after liberation by peptidases. In this regard, the most prominent N-terminal variant has been identified as A$\beta_{3(pE)-42}$ (*7, 11–15*). The A$\beta_{3/11(pE)-40/42}$ peptides are suggested to play a crucial role in the development of AD, since deposition occurs early in AD and A$\beta_{3(pE)-40/42}$ exhibited pronounced toxicity in neuronal and glial cell cultures (*14, 16*). In addition, pGlu-modified Aβ species exhibit an up to 250-fold accelerated initial rate of aggregation compared to that of unmodified Aβ, suggesting these peptides as potential seeding species for neurotoxic aggregate formation in vivo (*17, 18*). Importantly, in healthy and pathologically aged brains, profound plaque pathology without signs of dementia has been observed, which is accompanied by A$\beta_{3(pE)-42}$ at low levels (*19*). Finally, the occurrence of intracellular N-truncated Aβ species correlates with hippocampal neuron loss in a mouse model (*20*), supporting a decisive role of pGlu-Aβ in the development of AD.

Human glutaminyl cyclase (QC) was recently shown to convert N-terminal glutamate residues into pyroglutamic acid in vitro (*21, 22*). In the study presented here, the glutam(in)yl cyclase-mediated pGlu formation was analyzed in detail in cultures of human cell lines HEK293 and LNZ308. The aim of the work was to investigate the generation of A$\beta_{3(pE)-42}$ in
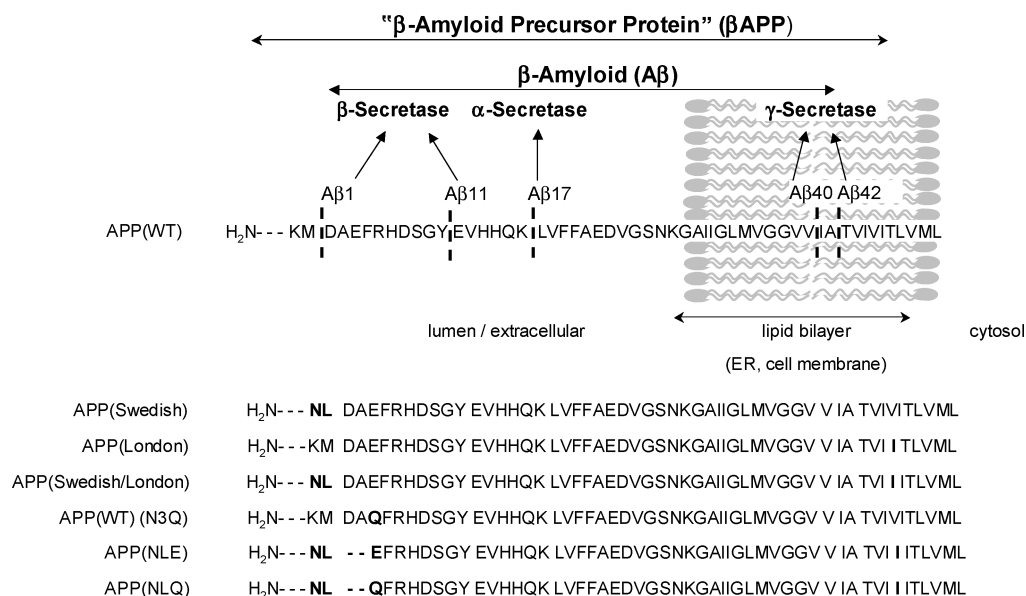
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[1] Abbreviations: Aβ, β-amyloid peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; ELISA, enzyme-linked immunosorbent assay; FBD, familial British dementia; FDD, familial Danish dementia; H-Gln-βNA, glutamine β-naphthylamine; PAGE, polyacrylamide gel electrophoresis; pGlu, pyroglutamic acid; QC, glutaminyl cyclase; TMB, tetramethylbenzidine; WT, wild type.

Scheme 1: APP695 constructs used in this study, including APP(WT), APP(Swedish) (KM595/596NL), APP(London) (V642I), and APP(Swedish/London) (KM595/596NL, V642I)[a]

APP(Swedish)	H₂N- - - **NL** DAEFRHDSGY EVHHQK LVFFAEDVGSNKGAIIGLMVGGV V IA TVIVITLVML
APP(London)	H₂N- - -KM DAEFRHDSGY EVHHQK LVFFAEDVGSNKGAIIGLMVGGV V IA TVI I TLVML
APP(Swedish/London)	H₂N- - - **NL** DAEFRHDSGY EVHHQK LVFFAEDVGSNKGAIIGLMVGGV V IA TVI I ITLVML
APP(WT) (N3Q)	H₂N- - -KM DA**Q**FRHDSGY EVHHQK LVFFAEDVGSNKGAIIGLMVGGV V IA TVIVITLVML
APP(NLE)	H₂N- - - **NL** - - **E**FRHDSGY EVHHQK LVFFAEDVGSNKGAIIGLMVGGV V IA TVI I ITLVML
APP(NLQ)	H₂N- - - **NL** - - **Q**FRHDSGY EVHHQK LVFFAEDVGSNKGAIIGLMVGGV V IA TVI I ITLVML

[a] All APP constructs were also tested, containing additionally a N3Q mutation (E599Q). For example, APP(WT) (N3Q) is shown. For investigating glutamate cyclization by human glutaminyl cyclase, the constructs APP(NLE) (KM595/596NL, ΔD597, ΔA598, V642I) and APP(NLQ) (KM595/596NL, ΔD597, ΔA598, E599Q, V642I) were used.

mammalian cell culture by expression of APP and QC to show whether QC is capable of generating Aβ$_{3(pE)-42}$ following amyloidogenic processing of APP. The results, thus, should provide new cell-based screening systems for small molecule QC inhibitors and validate QC inhibition as a strategy for preventing pGlu-Aβ formation.

EXPERIMENTAL PROCEDURES

Peptides. Aβ peptides were purchased from AnaSpec (San Jose, CA) or synthesized as described previously (*17*).

Vectors. The cDNAs of human APP695(Swedish/London), APP(NLE), and APP(NLQ) was generated as described previously (*22, 23*). The E599Q point mutation was introduced into the FAD- and WT-containing APP cDNAs. The cDNAs were ligated into the *Not*I site of vector pcDNA 3.1(+) (Invitrogen) (Scheme 1). Additionally, the human QC was inserted into vector pcDNA 3.1(+) using *Hin*dIII and *Not*I restriction sites. For generation of a human QC−EGFP fusion protein, the enhanced green fluorescent protein was inserted into the *Xho*I site of vector pcDNA 3.1(+) and subsequently fused to human QC cDNA lacking the stop codon using the *Hin*dIII and *Not*I sites. All constructs were confirmed by sequencing and isolated for cell culture purposes using the EndoFree Plasmid Maxi Kit (Qiagen).

Cell Culture and Transfection. Human embryonic kidney cells (HEK293) and human glioma cell line LNZ308 were cultured in DMEM (10% FBS) in a humidified atmosphere of 5% CO₂ at 37 °C. Cells were transfected with APP695 variants with Lipofectamine2000 (Invitrogen), essentially as described previously (*22*). The next day, cells were either analyzed for APP expression using Western blotting and immunohistochemistry or incubated for 24 h in assay medium (DMEM, without phenol red, without FBS). The supernatant was collected and readily mixed with Complete Mini protease inhibitor cocktail (Roche) supplemented with additional 1 mM AEBSF (Roth)

to prevent unspecific degradation by proteases. The samples were stored at −80 °C until the assay.

Concentration of Aβ Peptides. Prior to Western blot analysis of Aβ, the conditioned media were collected. Aβ was concentrated using Hydrosart centricons (Sartorius) with a 1 kDa cutoff. For immunoprecipitation, monoclonal antibody 4G8 (Chemicon), detecting total Aβ, was added to the cell culture medium and the mixture incubated under continuous shaking for 24 h at 4 °C. The next day, sheep anti-mouse IgG dynalbeads (Invitrogen) were added to the solution and the mixture was incubated for an additional 24 h at 4 °C. Afterward, Aβ peptides were dislodged by boiling in urea−PAGE gel buffer for 5 min and analyzed by Western blotting. For ELISA detection, the beads were incubated in a methanol/formic acid solution for 1 h. The supernatant was neutralized by addition of 200 mM phosphate buffer (pH 8.0) and EIA ELISA diluent buffer (IBL-Hamburg) and subsequently probed on the desired ELISA plate.

Western Blot Analysis. The detection of APP was performed applying Tris-glycine−PAGE as described previously (*22*). The electrophoretic separation of N-terminally modified Aβ peptides was carried out using 15% urea−PAGE gels (*24*). Proteins were transferred to a nitrocellulose membrane under semidry conditions. Subsequently, the membrane was blocked using 3% (w/v) dry milk in TBS-T [20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.05% (v/v) Tween 20]. APP and Aβ were detected using a polyclonal anti-APP antibody (Cell Signaling) and monoclonal anti-Aβ(1−16) antibody 6E10 (Chemicon), respectively. Pyroglutamyl-modified Aβ was detected applying antibody 8E1 (IBL-Hamburg). For visualization, blot membranes were incubated with secondary antibodies (anti-rabbit for APP and anti-mouse for Aβ), both conjugated with horseradish peroxidase (Cell Signaling) in TBS-T containing 5% (w/v) dry milk, and subsequently developed using the SuperSignal West Pico System (Pierce) according to the manufacturer's protocol.
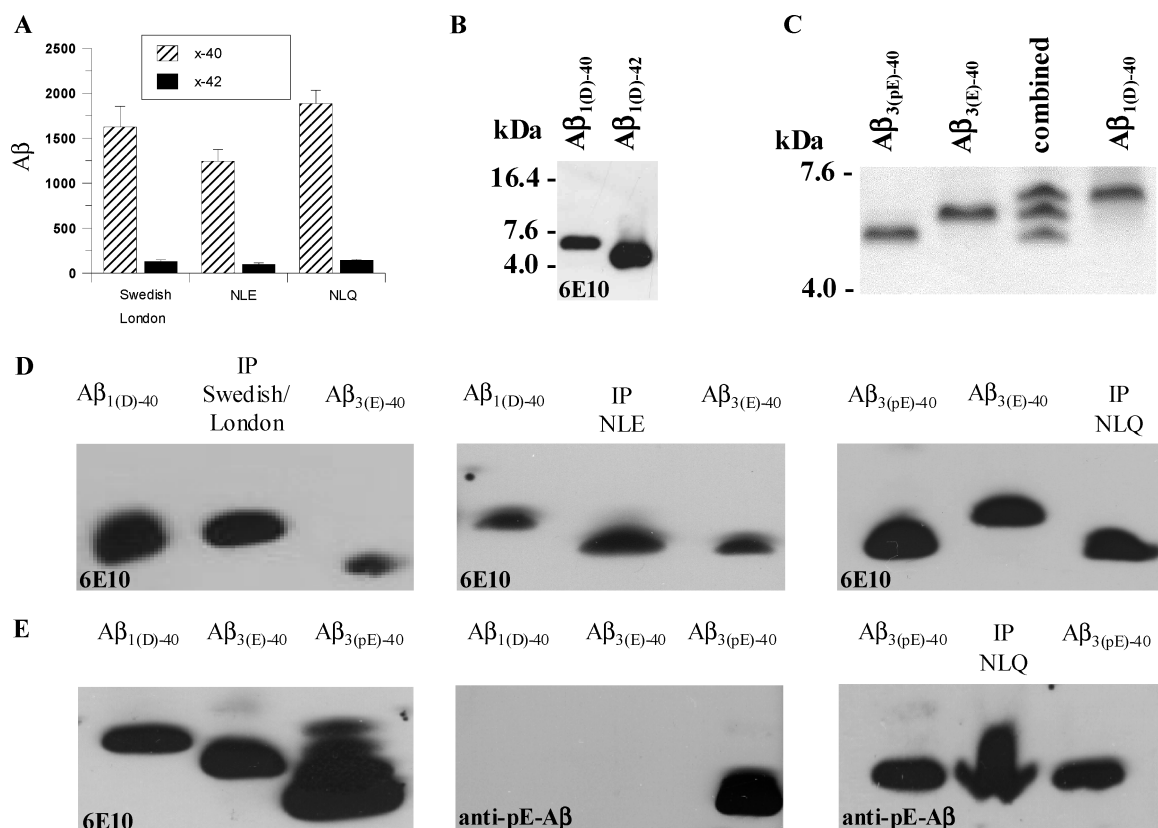
FIGURE 1: (A) Analysis of Aβ_{x-40} (hatched bars) and Aβ_{x-42} (black bars) secreted by HEK293 cells, which were transfected with APP(Swedish/London), APP(NLE), and APP(NLQ) using an ELISA detecting either Aβ_{x-40} or Aβ_{x-42} (total Aβ). The Aβ concentration was normalized to cell count (picograms per milliliter per 1 × 10^6 cells) ($n = 6$). (B) Urea−PAGE followed by Western blot analysis of 10 ng of A$\beta_{1(D)-40}$ in comparison to 10 ng of A$\beta_{1(D)-42}$ standard peptides. (C) Urea−PAGE followed by Coomassie staining of different N-terminal variants of Aβ_{40} (3 μg each). (D) Western blot of Aβ species secreted by HEK293 cells expressing APP(Swedish/London), APP(NLE), and APP(NLQ) in comparison to 10 ng of standard Aβ peptides. (E) In addition, an antibody specific for A$\beta_{3(pE)-x}$ was implemented, showing the generation of A$\beta_{3(pE)-40}$ after expression of APP(NLQ), compared to A$\beta_{3(pE)-40}$ standard peptides (10 ng).

Immunohistochemistry. Cells were washed twice with D-PBS (Invitrogen) and fixed using ice-cold methanol for 10 min, followed by three washing steps using D-PBS for 10 min at room temperature. For staining of the Golgi complex, HEK293 cells were incubated with rabbit anti-mannosidase II polyclonal antibody (Chemicon), applying a 1:50 dilution of the antibody in D-PBS at room temperature for 3 h. For APP and Aβ staining, HEK293 cells were incubated at room temperature with mouse anti-β-amyloid monoclonal antibody 6E10 (Calbiochem) for 3 h using a 1:50 dilution of the antibody in D-PBS. Subsequently, the cells were washed three times with D-PBS for 10 min. The immunostained Golgi complex and APP were tagged by applying IgG secondary antibodies, which were conjugated with Rhodamin-RedX (Dianova). The samples were incubated at room temperature in the dark for 45 min. Afterward, cells were washed three times with D-PBS for 5 min at room temperature. Finally, the fixed and stained samples were mounted with citifluor and covered with a microscope slide. The cells were observed with oil immersion using a confocal laser scanning microscope (Carl-Zeiss).

Quantification of Aβ Peptides and QC Activity. Glutaminyl cyclase activity was measured using the substrate H-Gln-βNA as described previously (*25*). The assay reaction was started by addition of the QC-containing cell culture supernatant and evaluated using a Novostar reader for microplates (BMG-Labtechnologies). QC activity was determined from a standard curve of β-naphthylamine under assay conditions.

Aβ_{40} and Aβ_{42} concentrations were determined using specific sandwich ELISAs detecting total Aβ_{x-40} and Aβ_{x-42}, full-length A$\beta_{1(D)-40}$ and A$\beta_{1(D)-42}$, or the N-terminally pyro-glutamated variants A$\beta_{3(pE)-40}$ and A$\beta_{3(pE)-42}$ (all IBL-Hamburg) according to the manufacturer's instructions.

Investigation of Intracellular pGlu Formation. HEK293 cells were transfected with vector APP(NLE) alone or in combination with a vector encoding the native human QC. Additionally, HEK293 cells were transfected with APP(NLE) alone, and recombinant human QC was added to the cell culture medium. After 24 h, samples were collected and the Aβ concentration was determined using ELISAs.

RESULTS

Generation of A$\beta_{3(pE)-40/42}$ Peptides in Cell Culture. On the basis of several different APP constructs (Scheme 1), the N- and C-terminal heterogeneity of Aβ peptides generated by HEK293 cells was assessed. Expression of all APP variants led to a significant increase in the Aβ_{x-40} and Aβ_{x-42} concentration (not shown), which was in good agreement with previous findings (*26, 27*), suggesting that the HEK293 expression system is well-suited for analysis of A$\beta_{x-40/42}$ formation. The Aβ concentrations were negligible in conditioned media of untransfected or mock transfected cells.

To prove QC-mediated pGlu-Aβ formation occurred after amyloidogenic processing of APP, we expressed APP(Swedish/London) and its modified variants APP(NLE) and
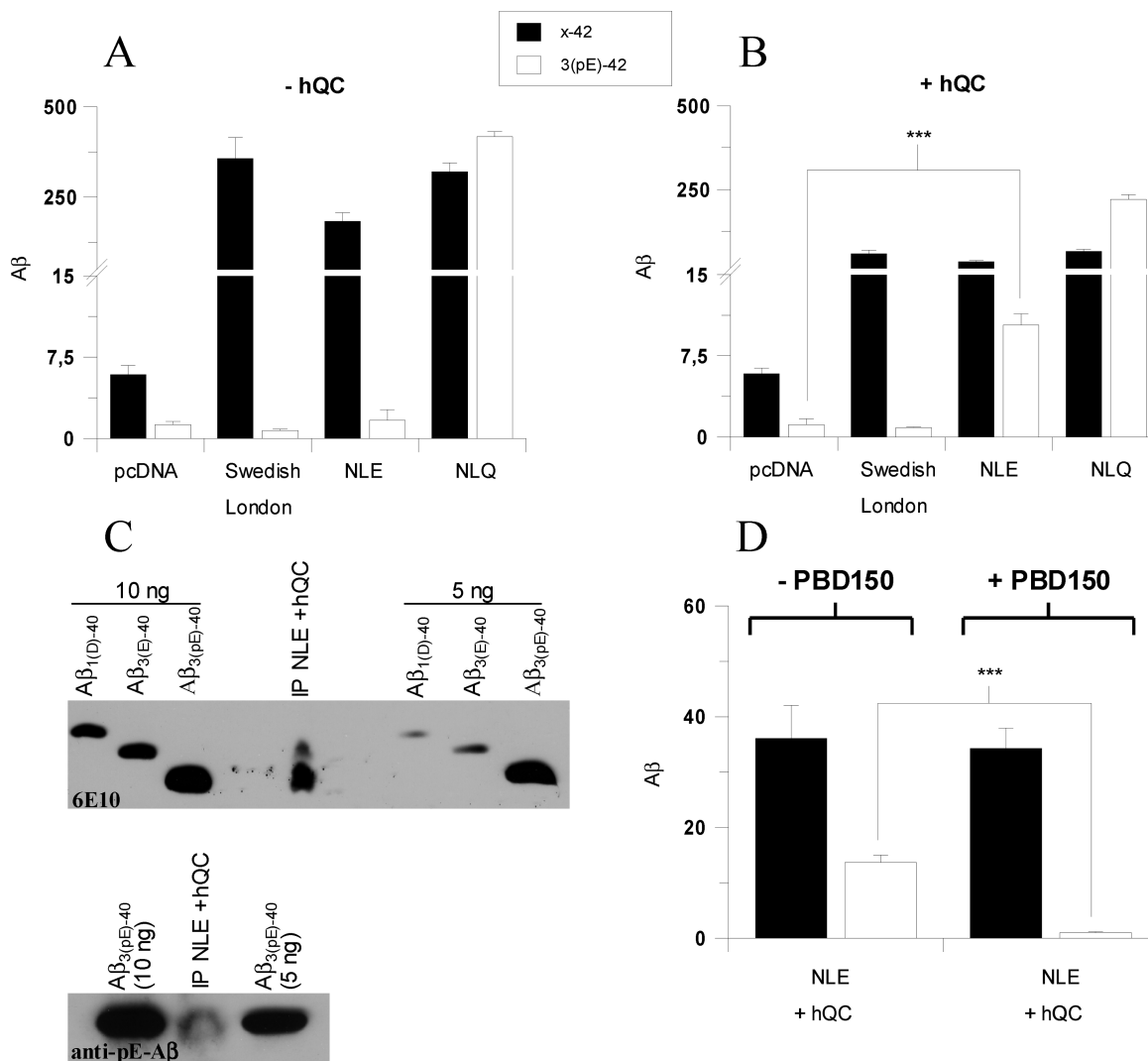
FIGURE 2: Analysis of pGlu formation after expression of vectors pcDNA, APP(Swedish/London), APP(NLE), and APP(NLQ) in HEK293 cells using an ELISA in the absence (A) or presence (B) of cotransfection with human QC. $A\beta$ concentrations were normalized to cell count (picograms per milliliter per 1×10^6 cells) (asterisks, $P < 0.001$; Student's t test; $n = 3$). (C) QC-dependent pGlu formation of APP(NLE) was corroborated using Western blot analysis detecting total-$A\beta$ (6E10) or pGlu-modified $A\beta$ (anti-pE-$A\beta$) (application in comparison to standard $A\beta$ peptides). (D) The formation of $A\beta_{3(pE)-42}$ after cotransfection of APP(NLE) with human QC was inhibited by application of the QC-specific inhibitor PBD150 (10 μM). $A\beta$ concentrations were normalized to cell count (picograms per milliliter per 1×10^6 cells) (asterisks, $P < 0.001$; Student's t test; $n = 6$).

APP(NLQ) (Scheme 1) in HEK293 cells. The processing of the latter constructs should result in rapid liberation of $A\beta_{3(E)-40/42}$ and $A\beta_{3(Q)-40/42}$, i.e., precursors of $A\beta_{3(pE)-40/42}$. The expression of APP(Swedish/London), APP(NLE), and APP(NLQ) resulted in comparable $A\beta_{x-40/42}$ concentrations in the cell culture supernatant (Figure 1A). In addition, the secreted $A\beta$ peptides were analyzed using urea−PAGE according to the method of Kalfki et al. (24), followed by Western blot analysis. In agreement with the earlier observations using urea−PAGE, $A\beta_{1(D)-42}$ migrates faster than $A\beta_{1(D)-40}$ (Figure 1B). Furthermore, the method enables the separation of $A\beta_{1(D)-40}$, $A\beta_{3(E)-40}$, and $A\beta_{3(pE)-40}$ (Figure 1C). According to the Western blot analysis, the expression of APP(Swedish/London) led to $A\beta$ peptides starting with aspartate 1 [$A\beta_{1(D)-40/42}$]. In contrast, transfection of APP-(NLE) resulted primarily in $A\beta_{3(E)-40/42}$, as revealed by the faster migration in urea−PAGE, whereas the transfection of APP(NLQ) exclusively generated $A\beta_{3(pE)-40/42}$ (Figure 1D). The dominant formation of $A\beta_{3(pE)}$ by expression of APP-(NLQ) is most likely caused by rapid QC-catalyzed cycliza-

tion of $A\beta_{3(Q)-40/42}$, possibly accompanied by spontaneous cyclization of glutamine (Figure 1E) (22, 23). In conclusion, the results suggest that β- and γ-secretase appropriately process the APP variants APP(NLE) and APP(NLQ).

$A\beta_{3(pE)}$ Formation Is Catalyzed by QC. Since the expression of APP(Swedish/London) and its derivatives APP(NLE) and APP(NLQ) led to the secretion of $A\beta$ peptides starting with distinct N-terminal amino acids (aspartic acid, glutamic acid, or glutamine), these vectors were analyzed for $A\beta_{x-40/42}$ and $A\beta_{3(pE)-40/42}$ generation after transfection into HEK293 cells. Again, expression of APP(Swedish/London), APP(NLE), and APP(NLQ) resulted in generation of $A\beta_{x-40/42}$ peptides, but significant $A\beta_{3(pE)-40/42}$ formation could be detected only in the case of APP(NLQ), which is catalyzed by endogenous QC, present in HEK293 cells (22) (Figures 1E and 2A). However, formation of $A\beta_{3(pE)-42}$ was observed after cotransfection of APP(NLE) with human QC (Figure 2B). This finding was further corroborated by Western blot analysis after immunoprecipitation of $A\beta$ peptides from the cell culture supernatant of HEK293 cells coexpressing
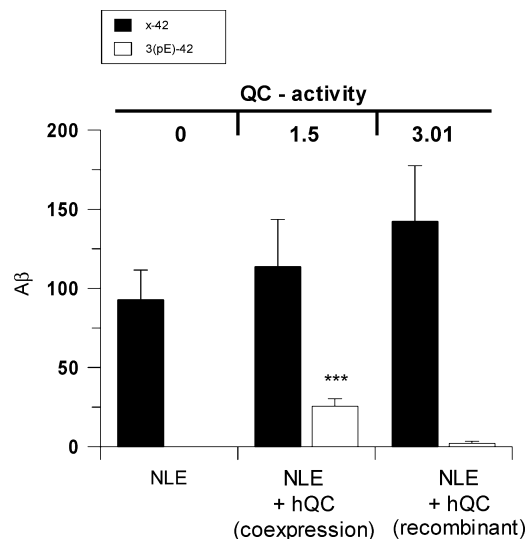
FIGURE 3: Determination of QC-dependent intracellular and extracellular Aβ$_{3(pE)-42}$ formation in HEK293 cells. Expression of APP(NLE) in the absence or presence of coexpression with native human QC or expression of APP(NLE) and subsequent application of recombinant human QC to the cell culture medium. Determination of the amount of Aβ$_{x-42}$ (black bars) and Aβ$_{3(pE)-42}$ (white bars) by an ELISA. QC activity (in micromolar per minute per 1 × 10^6 cells) was measured using a fluorescence assay (asterisks, P < 0.001; Student's t test; n = 6).

APP(NLE) and human QC (Figure 2C). The detection of Aβ$_{3(pE)-42}$ after cotransfection of APP(NLE) and human QC typically resulted in 5−20% pGlu formation at the N-terminus of Aβ (Figure 2B, replication not shown). Interestingly, if the Aβ peptides where immunoprecipitated, the

Aβ$_{3(pE)}$ band was more prominent than the unmodified Aβ$_{3(E)}$ signal, when using antibody 6E10 for detection. However, when the anti-pE-Aβ antibody was applied, a rather weak pGlu-Aβ signal was obtained (Figure 2C).

In an accompanying experiment, the efficacy of the QC inhibitor PBD150 in suppressing the formation of Aβ$_{3(pE)}$ was evaluated. Therefore, APP(NLE) and human QC were coexpressed in the absence and presence of 10 μM PBD150. The inhibitor did not affect the secretion of total Aβ$_{x-42}$. However, the extent of Aβ$_{3(pE)-42}$ generation was significantly lower, when PBD150 was applied (Figure 2D).

QC-Dependent Aβ$_{3(pE)}$ Formation Is Favored Intracellularly. Previous investigations of the pH dependency of QC-catalyzed cyclization of glutamic acid in vitro revealed an optimum under mildly acidic conditions. Localizing the environment under which the cyclization occurs in the cell-based system was another goal. Cotransfection of APP(NLE) with human QC led to N-terminal cyclization of glutamate and, because of secretion of the enzyme, to an increased QC activity within the cell culture medium (not shown). Therefore, there was a need for elucidation of whether an intracellular colocalization of human QC and APP(NLE) was required for Aβ$_{3(pE)}$ formation or the QC activity within the medium was responsible. The APP(NLE) construct was expressed either singly or in combination with human QC. In parallel samples, recombinant human QC (*28*) was applied to the culture medium of cells expressing APP(NLE) to clarify whether extracellular QC activity contributes to the glutamate cyclization. As expected, the single expression of APP(NLE) led only to the detection of Aβ$_{x-42}$. In contrast, cotransfection of APP(NLE) with human QC resulted in
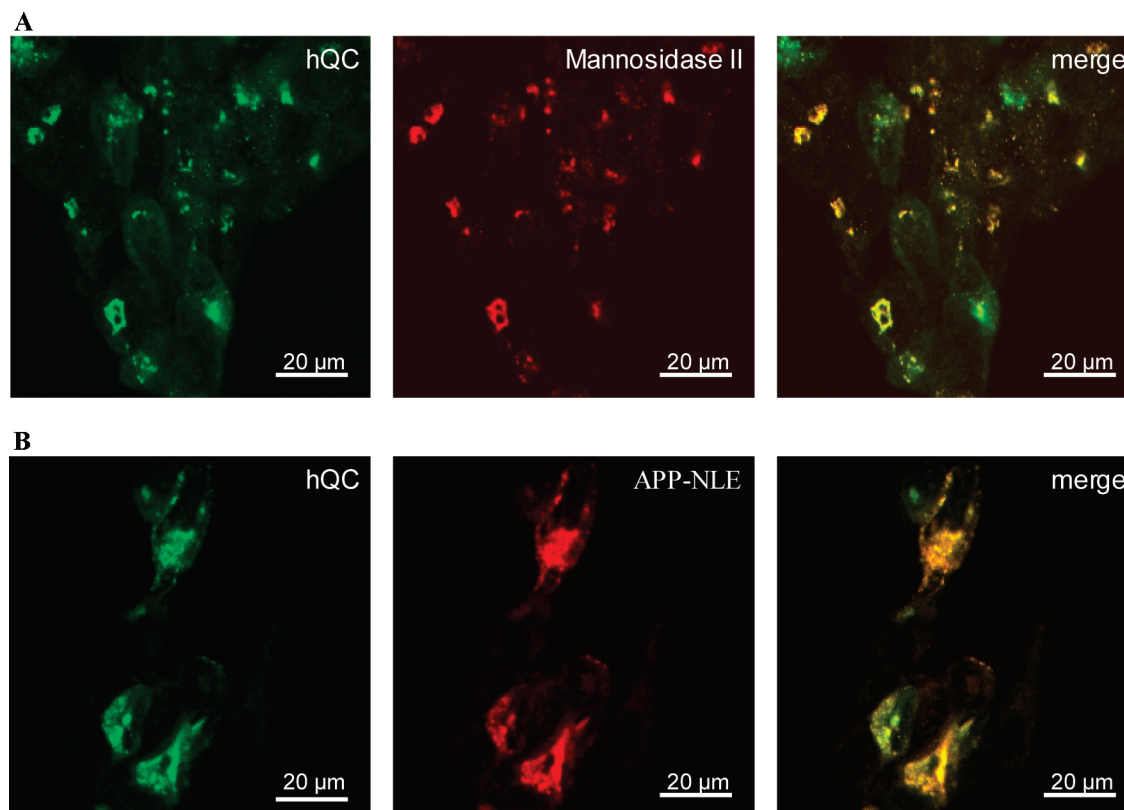

FIGURE 4: (A) Immunohistochemical staining of Mannosidase II (red) in HEK293 cells for identification of the Golgi zone and comparison to the expression pattern obtained with the hQC−EGFP fusion protein (green) in HEK293. An overlay of hQC and Mannosidase II suggests colocalization within the Golgi zone (merge). (B) Expression of hQC−EGFP protein (green) and APP(NLE) (red) in HEK293 cells. An overlay of hQC and APP(NLE) suggests a colocalization at least within the Golgi compartment (merge).
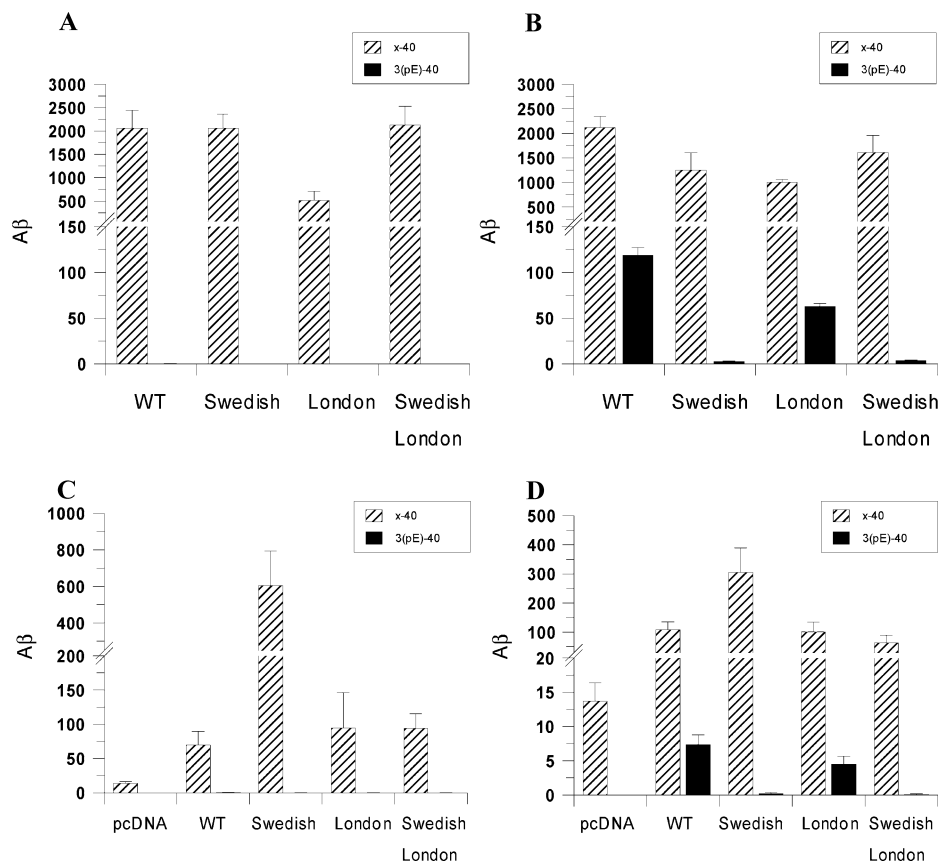
FIGURE 5: N-Terminal variants generated by the expression of FAD mutants and APP(WT) in HEK293 cells (A and B) and LNZ308 cells (C and D). The formation of $A\beta_{3(pE)-40}$ was investigated in the absence (A and C) and presence (B and D) of an E599Q (N3Q) mutation. The $A\beta$ concentration was determined using ELISAs specific for $A\beta_{x-40}$ and $A\beta_{3(pE)-40}$ and normalized to cell count (picograms per milliliter per 1×10^6 cells).

formation of $A\beta_{3(pE)-42}$. Furthermore, the addition of recombinant human QC to the cell culture medium of APP(NLE)-expressing cells generated only minor amounts of $A\beta_{3(pE)-42}$ compared to the human QC cotransfection (Figure 3). The determination of QC activity after incubation for 24 h on the cells showed a 2-fold higher activity, when recombinant QC was applied, in comparison to the coexpression of APP(NLE) and human QC. This result supports the assumption that cyclization of glutamate is favored in intracellular compartments. This was further substantiated by an immunohistochemical analysis of the subcellular distribution of human QC and APP(NLE) in HEK293 cells. A cDNA construct was generated encoding human QC, which was fused to the enhanced green fluorescent protein (EGFP). The expression of the QC−EGFP fusion protein led to a vesicular staining within the expressing cells. HEK293 cells were counterstained using an anti-Mannosidase II antibody as a marker protein for the Golgi complex. Superimpositions of the resulting images show that human QC colocalizes within the Golgi complex with Mannosidase II, substantiating the localization of QC in the secretory compartments (Figure 4A). Coexpression of human QC−EGFP fusion protein with APP(NLE) clearly supports partial colocalzation in the secretory pathway, as revealed by a counterstaining with anti-β-amyloid antibody 6E10 (Figure 4B).

Formation of N-Truncated Aβ Peptides Is Influenced by the β-Secretase Cleavage Site. The removal of the dipeptide Asp-Ala from the N-terminus of $A\beta_{1(D)-x}$ must precede the formation of $A\beta_{3(pE)}$ in vivo. It remains unclear whether the

pGlu precursor $A\beta_{3(E)}$ is sequentially liberated by β-secretase and an aminopeptidase or is generated directly by endoproteolysis of APP by a yet unknown mechanism. The impact of FAD mutations on the N-terminal composition of Aβ peptides was investigated using ELISAs, which discriminate between intact N-terminal $A\beta_{1(D)-40/42}$ or N-truncated $A\beta_{x-40/42}$ peptides. Introduction of the KM595/596NL Swedish mutation into the APP695 sequence led preferentially to the generation of Aβ molecules possessing an intact N-terminus. However, the K595/M596 WT sequence at the β-secretase cleavage site produced prominent amounts of Aβ peptides differing from $A\beta_{1(D)}$ at the N-terminus. Only 21% of $A\beta_{x-40}$ corresponded to $A\beta_{1(D)-40}$ and 46% of $A\beta_{x-42}$ to $A\beta_{1(D)-42}$, if APP(WT) was expressed. In contrast, the Swedish mutation within the APP sequence provoked generation of Aβ peptides with an intact N-terminus (not shown).

To investigate the influence of the β-cleavage on $A\beta_{3(pE)-42}$ formation, we introduced a novel E599Q (N3Q) point mutation into APP(WT), APP(Swedish), APP(London), and APP(Swedish/London), allowing the sensitive and specific detection of Aβ species, which are N-terminally truncated. Upon N-terminal cleavage, glutamine is readily cyclized to pGlu as observed with the APP(NLQ) construct (Figure 2A,B). Thus, the N3Q mutation within the applied FAD-APP mutants and APP(WT) serves as a monitoring mutation for the generation of N-truncated Aβ peptides starting with a pGlu residue in position 3. All APP variants were expressed in the absence (Figure 5A,C) and presence (Figure 5B,D) of the N3Q mutation in HEK293 cells (Figure 5A,B) and
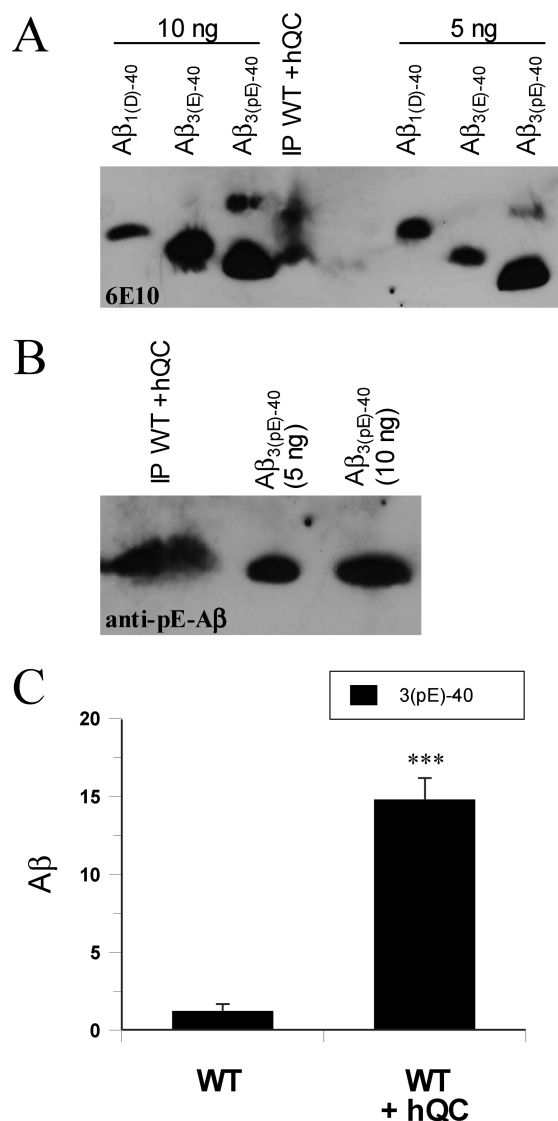
FIGURE 6: QC-dependent pGlu formation of APP(WT) was investigated by Western blotting after immunoprecipitation of Aβ peptides (application in comparison to standard Aβ peptides) using antibody 6E10, detecting total-Aβ (A) and antibody detecting pGlu-modified Aβ (B). (C) QC-dependent pGlu formation investigated using an ELISA after concentration of Aβ-containing cell media using centrifugal devices [single transfection of APP(WT) or cotransfection of APP(WT) with hQC] (Aβ concentration in picograms per milliliter) (asterisks, $P < 0.001$; Student's t test; $n = 5$).

LNZ308 cells (Figure 5C,D). The cell culture medium was analyzed for potential $A\beta_{3(pE)-40}$ formation. Intriguingly, the expression of APP variants containing the N3Q mutation led only in the case of APP(WT) and APP(London) to significant amounts of $A\beta_{3(pE)-40}$, whereas the presence of the Swedish mutation resulted only in scarce amounts of $A\beta_{3(pE)-40}$. This result was also observed when $A\beta_{3(pE)-42}$ was analyzed, pointing to differences in the liberation of Aβ peptides from APP molecules bearing the APP(WT) and APP(Swedish) sequence at the β-secretase cleavage site.

These significant differences in the liberation of N-truncated Aβ peptides prompted us to investigate the formation of $A\beta_{3(pE)}$ from APP(WT). As described for the APP(NLE) construct, cotransfection of APP(WT) and human QC was implemented to facilitate the formation of $A\beta_{3(pE)}$. On the basis of the urea−PAGE Western blot analysis, two different Aβ forms were detected (Figure 6A). The lower

band corresponds to $A\beta_{3(pE)-40}$, whereas the upper band migrates slower than $A\beta_{1(D)-40}$, again suggesting an N-terminus differing from that of full-length Aβ, observed for APP(WT) expression. In addition, the results obtained by IP−Western blot analysis were validated by application of a pGlu-specific antibody (Figure 6B) and by concentration of the supernatant in centrifugal devices, followed by an ELISA, revealing significant $A\beta_{3(pE)-40}$ formation after cotransfection of APP(WT) and human QC (Figure 6C).

DISCUSSION

The Aβ peptides of amyloid deposits in brains of patients with Alzheimer's disease display a profound N- and C-terminal heterogeneity (*7, 11, 29*). Cleavage of γ-secretase causes primarily the C-terminal differences. Because neuritic plaques are mainly composed of $A\beta_{42}$, and the deposition of $A\beta_{42}$ precedes that of $A\beta_{40}$, $A\beta_{42}$ is thought to be more amyloidogenic than $A\beta_{40}$ (*27, 30*). The role and formation of N-terminal modifications, however, are more poorly understood. In particular, it is known that truncated Aβ peptides possessing a pGlu residue at the N-terminus are highly abundant in affected brains of patients with Alzheimer's disease and Down syndrome (*11, 12, 14*). Furthermore, the amyloidogenic peptides ABri in familial British dementia (FBD) and ADan in familial Danish dementia (FDD) possess an N-terminal pGlu residue, and pGlu formation appears to be crucial for the deposition of the ADan peptides in vivo (*31, 32*). Moreover, these pGlu-modified peptides have been shown to seed the aggregation of $A\beta_{1(D)-42}$ (*17*). Therefore, the prevention of pGlu formation might represent a new concept for the causal treatment of Alzheimer's disease and other pGlu-related amyloidoses.

However, the generation of pGlu peptides in AD, FBD, and FDD remained elusive, leaving room for speculation about their generation. In addition, despite evidence of an early role of pGlu-Aβ in the development of Alzheimer's disease, the formation of pGlu-Aβ peptides is frequently considered as a spontaneous secondary reaction occurring late in the progression of the disease (*33*). It should be noted that the uncatalyzed cyclization of N-terminal glutamic acid occurs exceptionally slowly, with half-lifes of years to decades under in vivo conditions (*43, 44*). In addition, since Aβ anabolism and catabolism make up a homeostasis showing a high rate of daily turnover, it is conceivable to assume an enzyme-catalyzed formation of pGlu-Aβ peptides. On the basis of artificial peptide substrates, recent in vitro studies provided the first evidence that glutamate cyclization at the N-terminus of Aβ might be due to catalysis of QC (*21, 22*). These results are highlighted here by the proof that the formation of $A\beta_{3(pE)-40/42}$ from glutamate occurs after amyloidogenic processing of APP. Most importantly, we describe for the first time the generation of $A\beta_{3(pE)}$ after expression of APP(WT), which is present in the vast majority of all AD cases, substantiating the assumption that QC might be a novel drug target for the treatment of pGlu-related amyloidoses.

According to the previous investigations of substrate specificity, the QC-catalyzed cyclization of glutamate requires a protonated γ-carboxyl group and an unprotonated α-amino group. The highest concentrations of these species are found under mildly acidic conditions around pH 6.0 (*21*). Similar pH conditions have been described for the secretory compartments
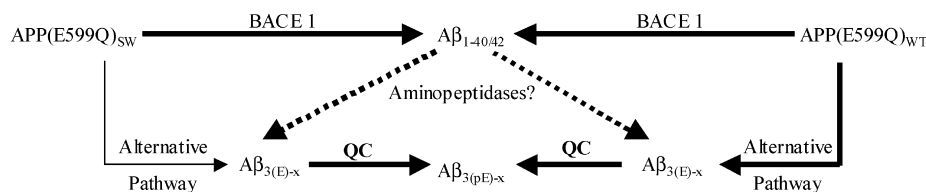
FIGURE 7: Proposed mechanism for the generation of N-terminally truncated Aβ peptides. Aβ is naturally liberated by N-terminal proteolysis due to BACE I, leading to A$\beta_{1(D)-40/42}$. This full-length Aβ species can be truncated by aminopeptidases. However, significant differences were observed for the generation of A$\beta_{3(pE)-x}$ between APP(E599Q)$_{WT}$ and APP(E599Q)$_{SW}$. Obviously, an alternative pathway exists, which is more pronounced for the APP(E599Q)$_{WT}$ variant. This leads to the generation of N-truncated A$\beta_{3(E)-x}$ species, which can be further cyclized by QC to obtain A$\beta_{3(pE)-x}$. Whether the alternative pathway represents different subcellular sites of BACE I-mediated Aβ liberation or a BACE I-independent mechanism has to be further addressed.

(37). As summarized in Figure 4, APP and QC are colocalized at least within the Golgi complex, where a spatially high concentration of both QC and Aβ or the respective β-CTFs can be expected (34–36). In this regard, these data suggest that the colocalization of QC and Aβ enhances pGlu-Aβ formation (Figure 3) and further support a catalyzed generation of this peptide species. In addition, the accumulation of pGlu-Aβ might also contribute to the intracellular aggregation of Aβ, which is frequently detected in patients with Down syndrome and Alzheimer's disease (38–41), in terms of generating the initial insoluble seeds for further Aβ deposition. The seeding capability of pGlu-Aβ was recently investigated in vitro, supporting the possibility that pGlu-Aβ can initiate seeding of full-length Aβ peptides (17).

Although these results strongly imply a QC-catalyzed formation of A$\beta_{3(pE)}$, a molecular pathway of APP processing leading finally to the substrate A$\beta_{3(E)-x}$ has never been investigated in detail. To examine the generation of A$\beta_{3(pE)}$ from APP processing, we introduced a novel monitoring mutation [APP(E599Q)], which leads to instant pGlu formation following the release of the N-terminal amino acids of Aβ. Intriguingly, the results suggest that the WT sequence at the β-secretase cleavage results in the production of N-truncated Aβ species, whereas the Swedish mutation leads preferentially to full-length A$\beta_{1(D)-x}$ peptides (Figure 7). Apparently, the endoproteolytic processing of APP(WT) and APP(Swedish) by β-secretase differs not only in our analyzed model system. Data from studies in transgenic animals overexpressing the APP(Swedish) mutation, e.g., Tg2576, have revealed conspicuous differences with regard to the Aβ composition (42). AD patients display up to 50% of pGlu-Aβ deposited as an early Aβ species. These mouse models, in stark contrast, show only minor amounts of pGlu-Aβ [up to 0.5% (unpublished data)] occurring late in the life span of Tg2576 (12−15 months of age) (S. Schilling et al., manuscript in preparation). Most intriguingly, Tg2576 shows only mild, if any, cognitive deficits, whereas animal models accumulating larger amounts of pGlu-Aβ display strong cognitive decline and hippocampal neuron loss (20).

In conclusion, the data presented here provide for the first time evidence that (i) cyclization of glutamic acid generating A$\beta_{3(pE)-40/42}$ is facilitated by QC after amyloidogenic processing of APP, (ii) the localization of QC and APP and the significant formation of A$\beta_{3(pE)-40/42}$ after coexpression point to a primarily intracellular pGlu generation, and (iii) the generation of N-truncated Aβ, accounting for the majority of Aβ in AD and DS, is possibly mediated by an alternative pathway of APP processing. The latter result is reflected in the unexpected finding of tremendous A$\beta_{3(pE)-40/42}$ formation in an APP(E599Q) variant, suggesting that the WT sequence

at the β-cleavage site leads to Aβ molecules that are prone to cyclization by QC (Figure 7).

The results thus strongly imply that the majority of the pGlu peptides deposited in AD and DS brains are formed by enzymatic catalysis following processing of APP(WT). QC activity, mediated by the neurotoxic potential of A$\beta_{3(pE)-40/42}$, might be involved in the first intracellular events of the amyloid cascade, potentially driving the aggregation process of Aβ. The enhanced aggregation propensity and stability of pyroglutamated Aβ, in turn, might trigger the aggregation of other Aβ species, which are generated by β- and γ-secretase at higher levels. Accordingly, the prevention of pGlu-Aβ formation might represent a novel concept for a causal treatment of Alzheimer's disease.

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