Clearance of intracellular aggregation-prone protein fragments
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Amyloid-β is efficiently degraded in the cytoplasm

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Alzheimer’s disease is hallmarked by the accumulation and aggregation of amyloid-β (Aβ) peptides in extracellular plaques. However, intraneuronal accumulation of Aβ is thought to be an early and crucial event in Alzheimer’s disease and is associated with impairment of the ubiquitin-proteasome system. Aβ peptides are generated in the secretory and endocytic pathway, but Aβ can be translocated into the cytoplasm. To examine the fate of cytoplasmic Aβ peptides, we used both GFP-Ub-Aβ fusion constructs that efficiently release Aβ peptides in the cytoplasm upon expression and quenched Aβ peptides that become fluorescent upon degradation. Here, we show that cytoplasmic Aβ peptides were efficiently degraded in living cells even upon inhibition of several classes of proteases either or not simultaneously. However, we found several peptidases that were responsible for Aβ degradation in vitro, including tripeptidyl peptidase II and metalloproteases. In vitro, Aβ degradation was inhibited by its aggregation likely because peptidases can only degrade monomeric peptides. In conclusion, our data indicates that moderate cytoplasmic Aβ levels can be cleared efficiently, but too slow degradation of Aβ induces aggregates that cannot be degraded by peptidases.
Alzheimer’s disease (AD) is the most common form of dementia, affecting 26.6 million people worldwide. The pathological hallmarks in brain tissue of AD patients are extracellular senile plaques (SPs) containing fibrillar amyloid-β (Aβ) peptides, intracellular neurofibrillary tangles (NFTs) containing hyperphosphorylated tau and loss of synapses and neurons. Aβ is derived from the widely-expressed type 1 membrane protein amyloid precursor protein (APP), which is sequentially cleaved by β- and γ-secretases. Aβ is produced in a variety of subcellular compartments, including the ER, Golgi apparatus, and the endosomal-lysosomal pathway (LaFerla et al., 2007; Selkoe, 2001). Aβ peptides of variable lengths are released, predominantly of 40 (Aβ_{40}) or 42 amino acids (Aβ_{42}) (Thinakaran and Koo, 2008). The two additional amino acids at the C-terminus of Aβ_{42} make it more aggregation prone and therefore Aβ_{42} is considered to be involved in the initiation of AD (Jarrett et al., 1993).

Autosomal dominant forms of AD are often caused by mutations that increase Aβ production, in particular Aβ_{42}. Since Aβ production is not increased in sporadic forms of AD, the increased levels of Aβ may be the result of decreased activity of Aβ-degrading enzymes during aging. Indeed, levels of insulin-degrading enzyme (IDE) and neprilysin (NEP) decrease with age (Caccamo et al., 2005). These metalloproteases degrade Aβ in the extracellular matrix in vivo (Farris et al., 2003; Iwata et al., 2001). Many other proteases, such as endothelin-converting enzyme (ECE) and plasmin are also reported to degrade Aβ extracellularly (Eckman et al., 2003; Tucker et al., 2000; Wang et al., 2006). Furthermore, overexpression of IDE, NEP or sustained plasmin activity in APP transgenic mice retards plaque formation and cytopathology (Jacobsen et al., 2008; Leissring et al., 2003).

Extracellular Aβ deposits have long been considered to be the primary cause of AD. However, intracellular Aβ_{42} accumulation has been detected in neurons prior to the appearance of extracellular Aβ deposits (D’Andrea et al., 2001; Gouras et al., 2000; Ohyagi et al., 2005; Wirths et al., 2001). Intraneuronal Aβ_{42} is associated with cytotoxicity and dysfunction of mitochondria, lysosomes, multivesicular bodies and synapses (Almeida et al., 2006; Busciglio et al., 2002; Glabe, 2001; Oddo et al., 2003; Shie et al., 2003; Takahashi et al., 2002; Zhang et al., 2002). This suggests that intraneuronal Aβ accumulation is an early and crucial event in the development of AD.

Aβ has been found in the cytoplasm of post-mortem brains of AD patients and in AD transgenic mice (Billings et al., 2005; Gouras et al., 2000), whereas overexpression of APP or ER-targeted Aβ_{42} resulted in cytoplasmic Aβ (Buckig et al., 2002; Grant et al., 2000). Furthermore, it has been demonstrated that cytoplasmic Aβ can induce apoptosis in cultured neuronal cells (Zhang et al., 2002). Aβ is generated in the secretory and endocytic pathway. Translocation of Aβ into the cytoplasm can occur via various routes (Li et al., 2007). ER-associated degradation (ERAD) is normally involved in the translocation of misfolded proteins from the ER to the cytoplasm and the subsequent degradation of these proteins by the ubiquitin-proteasome pathway (Scheper and Hoozemans, 2009). However, it is also able to transport ER-generated Aβ peptides into the cytoplasm (Buckig et al., 2002; Schmitz et al., 2004). Aβ can passively leak into the cytoplasm along the secretory pathway as well. Extracellular Aβ can enter the cytoplasm via passive diffusion through the plasma membrane or via internalization via endosomes which increases...
membrane permeability of lysosomes. Furthermore, Aβ can be actively taken up via receptors such as α7 nicotinic acetylcholine receptors, apolipoprotein E (ApoE) receptors, and N-methyl-D-aspartic acid (NMDA) receptors (LaFerla et al., 2007).

Various Aβ degrading enzymes are known to be active in the extracellular environment, but less is known about degradation of intracellular Aβ peptides. The proteasome is the major pathway for intracellular protein degradation, which degrade proteins into peptides (Craiu et al., 1997). Within seconds after their generation by proteasomes, peptides are recycled into amino acids by downstream aminopeptidases (Reits et al., 2003). Several groups have reported proteasomal impairment in AD post-mortem brains (Keck et al., 2003; Keller et al., 2000) and inhibition of proteasomal activity by intracellular Aβ (Almeida et al., 2006). Furthermore, inhibition of proteasomal activity results in accumulation of Aβ (Lopez Salon et al., 2003; Schmitz et al., 2004; Tseng et al., 2008). The proteasome can degrade long peptides like Aβ, but Aβ may be degraded by downstream aminopeptidase as well. Most peptidases can only handle small peptides up to 15 amino acids, but some peptidases like tripeptidyl peptidase II (TPPII) can degrade longer peptides as well (Reits et al., 2004). Since Aβ peptides have a heterogeneous sequence, various classes of proteases may be involved in their degradation when present in a monomeric form in the cytoplasm.

In the present study, we mimicked Aβ release in the cytoplasm using a GFP-ubiquitin (Ub)-Aβ_{40/42} fusion construct. When this construct is expressed in cells, it causes immediate release of Aβ_{40/42} peptides from GFP-Ub due to cleavage by Ub C-terminal hydrolases (De Vrij, 2005; Johnson et al., 1995). We also applied a quenched Aβ peptide that becomes fluorescent upon degradation. Here, we show that cytoplasmic Aβ peptides are rapidly degraded and inhibition of proteasomes, IDE or most peptidases did not result in accumulation of Aβ peptides in the cytoplasm. However, inhibition of metalloproteases and knockdown of TPPII reduced the degradation rates of Aβ peptides in vitro.

**Cytoplasmic Aβ is Efficiently Degraded**

To study the fate of monomeric Aβ_{40} and Aβ_{42} in the cytoplasm, fluorescently-tagged Ub was fused N-terminally to Aβ_{40} or Aβ_{42}, resulting in GFP-Ub-Aβ constructs. When these constructs are expressed in eukaryotic cells, Aβ is released within the cytoplasm by efficient cleavage of Ub C-terminal hydrolases between the C-terminal glycine of Ub and the N-terminal aspartic acid of Aβ (Figure 1A; De Vrij, 2005; Johnson et al., 1995). These GFP-Ub-Aβ fusion constructs produce Aβ peptides that have the same amino acid sequence as Aβ peptides generated in vivo, without additional amino acids or tags. Importantly, a starting methionine is not present, which may affect its stability according to the N-end rule (Varshavsky, 1996). Additional control constructs included GFP-UbG76V-Aβ_{40}, which cannot be cleaved by Ub C-terminal hydrolases (Butt et al., 1988; Johnson et al., 1992), a scrambled version of Aβ with the same amino acids but in a different order (GFP-Ub-Aβ_{scr}) and an Aβ-GFP fusion protein (Ub-Aβ_{42}-GFP).

Expression of the various GFP-Ub-Aβ constructs and subsequent release of Aβ was verified by Western blotting. GFP-Ub derived from cleaved GFP-Ub fusion proteins was present in
all lanes when GFP-Ub or GFP-Ub-Åβ40/42 were expressed (Figure 1B). Even after prolonged exposure, no additional bands were detected that could represent uncleaved GFP-Ub-Åβ (data not shown), indicating that GFP-Ub-Åβ fusion proteins were efficiently cleaved. Expression of GFP-UbG76V-Åβ40 or Ub-Åβ42-GFP resulted in a GFP-positive band at 42 kDa or 32.5 kDa, respectively, representing uncleaved UbG76V-Åβ40 or Åβ42-GFP, respectively (Figure 1B). Subsequent blotting against Åβ did not show positive bands when expressing GFP-Ub-Åβ40/42, with the exception of non-cleavable GFP-UbG76V-Åβ40 and Ub-Åβ42-GFP (arrows; Figure 1B). This data is in agreement with the data by Hol and colleagues, who also could not detect Åβ peptides after similarly expressing Åβ peptides in cells using HA-Ub-Åβ constructs (De Vrij, 2005). Together, these findings suggest that Åβ peptides are efficiently released from GFP-Ub after expression, leading to subsequent degradation of released monomeric Åβ peptides. As Åβ42-GFP could be detected on Western blot, suggests that expression of Åβ as a fusion protein stabilized the Åβ peptide. A separately added synthetic Åβ was detected at 4 kDa (arrowhead; Figure 1B), suggesting that the small size of these peptides did not limit their detection on Western blots.

Figure 1. Cytoplasmic Åβ peptides are efficiently degraded. (A) Schematic representation of cleavage of GFP-Ub-Åβ by Ub C-terminal hydrolases directly after Ub, thereby separating GFP-Ub from Åβ peptides. (B) Cytoplasmic cell lysates of HEK cells expressing GFP-Ub, GFP-Ub-Åβ40/42/Scr, the uncleavable GFP-UbG76V-Åβ40 or Ub-Åβ42-GFP for 48 hours were analyzed by Western blotting together with synthetic Åβ peptides. Blots were subsequently stained for Åβ, including a higher exposure of a part of the Western blot at the height of Åβ peptides (upper panel), GFP (middle panel) and actin (lower panel). Arrowheads indicate Åβ peptides, arrows UbG76V-Åβ40 (42 kDa) or Åβ42-GFP (32.5 kDa), respectively, and asterisks endogenous APP and aspecific bands. (C) Confocal images of differentiated SH-SY5Y cells transfected with various GFP-Ub-Åβ fusion constructs were fixed after 48 hours and subsequently immunostained for Åβ using the antibody 6E10. Scalebar: 20 µm.
The various GFP-Ub-Aβ constructs were expressed in differentiated SH-SY5Y neuronal cells. Subsequent staining for Aβ only resulted in Aβ-positive cells when the non-cleavable GFP-UbG76V-Aβ$_{40}$ or the Aβ$_{42}$-GFP fusion protein were expressed (Figure 1C). Expression levels of all GFP-Ub-Aβ fusion constructs were similar, as shown by their GFP levels, indicating that the generation of monomeric Aβ$_{40}$ and Aβ$_{42}$ leads to their efficient and rapid degradation by cytoplasmic proteases.

PROTEASE INHIBITION DOES NOT REDUCE INTRACELLULAR DEGRADATION OF Aβ

The absence of Aβ peptides upon expression of GFP-Ub-Aβ$_{40/42}$ indicates that the Aβ peptides are rapidly degraded. We inhibited the proteasome, IDE and various classes of peptidases, which all reside mainly within the cytoplasm (Authier et al., 1996; Reits et al., 2003). HEK cells expressing GFP-Ub-Aβ$_{40/42}$ for 24 hours were incubated for a subsequent 20 hours in the presence of the reversible proteasome inhibitor MG-132, the irreversible IDE inhibitor bacitracin, or various peptidase inhibitors that cover the major classes of peptidases. Inhibition of proteasomes or IDE did not result in any detectable Aβ peptides (Figure 2), indicating that these proteases were not the primary Aβ-degrading enzymes. Treatment with the irreversible cysteine protease inhibitor E64, the irreversible aspartyl protease inhibitor pepstatin A, the reversible aminopeptidase inhibitor bestatin, the irreversible serine protease inhibitor phenylmethylsulfonyl (PMSF), or the irreversible metalloprotease inhibitor phenanthroline also did not prevent rapid clearance of Aβ, as no Aβ peptides were detected on Western blots (Figure 2). Expression levels of GFP-Ub-Aβ were not affected by addition of any of these inhibitors, as GFP staining representing GFP-Ub levels were similar in untreated cells and cells treated with inhibitors (Figure 2). Since Aβ can be a substrate of various proteases due to its heterogeneous sequence, a cocktail containing all the individual inhibitors was added to cells as well. However, even simultaneous inhibition of various classes of proteases did not increase Aβ peptide levels (Figure 2), suggesting that Aβ was still efficiently degraded in the cytoplasm.

![Figure 2. Protease inhibitors do not prevent efficient clearance of cytoplasmic Aβ peptides.](image)

Western blot analysis of HEK cells expressing GFP-Ub, GFP-Ub-Aβ$_{40}$ or GFP-Ub-Aβ$_{42}$ that had been treated at 24 hours for a subsequent 24 hours with the following protease inhibitors: 10 µM E64, 1 µM pepstatin A, 5 µM bestatin, 100 µM PMSF, 1 µM AAF-CMK, 1 µM MG-132, 200 µM bacitracin, 500 µM phenanthroline and a cocktail containing all inhibitors. Cytoplasmic cell lysates were analyzed by Western blotting and subsequently stained for Aβ (upper panel), GFP (middle panel) and actin (lower panel).
THE METALLOPROTEASE INHIBITOR PHENANTHROLINE REDUCES Aβ DEGRADATION

Although various protease inhibitors did not prevent complete degradation of Aβ, they may have an effect on the rate of degradation. To examine whether inhibition of particular peptidases affects the cytoplasmic half-life of Aβ, we designed an Aβ_{40} peptide containing a fluorophore (F) at position 22 that was quenched by a Dabcyl group (Q) on position 12 (Figure 3A). The quencher and fluorophore thus flanked the KLVFF sequence present in the middle of Aβ peptides, which is a critical region for Aβ to aggregate (Tjernberg et al., 1996). The quenched Aβ peptide only becomes fluorescent upon separation of quencher and fluorophore, hence after degradation of Aβ. Quenched Aβ peptides were added to cytoplasmic fractions of mildly lysed cells, preventing contamination with extracellular membrane-bound proteases or lysosomal proteases. Fluorescence was measured in time. An increase in fluorescent signal was detected upon addition of quenched Aβ peptides to cell lysates, whereas no increase in fluorescence was observed when the same amount of peptides was added to PBS (Figure 3B). This indicates that quenched Aβ is degraded by cytoplasmic proteases. Addition of proteasome or IDE inhibitors did not affect the degradation of Aβ peptides (Figure 3B). Furthermore, the majority of the peptidase inhibitors E64, bestatin, PMSF, pepstatin A or the inhibitor of puromycin-sensitive peptidase PAQ-22 showed no effect on the half-life Aβ (Figure 3C). A cocktail containing E64, bestatin, PMSF and pepstatin A did not affect degradation of Aβ peptides either (Figure 3C). However, addition of the metalloprotease inhibitor phenanthroline clearly affected the degradation rate of Aβ (Figure 3C), suggesting that Aβ is degraded by metalloproteases. While IDE is a metalloprotease, the IDE inhibitor itself did not affect its degradation, suggesting that other metalloproteases degrade Aβ peptides. Phenanthroline is suggested to interfere

![Figure 3](image-url)

Figure 3. Inhibition of metalloproteases reduces degradation rates of Aβ peptides. (A) Model of detecting degradation of Aβ peptides using internally quenched Aβ peptides. Fluorescence can only be detected when the quencher group (Q) is separated from the fluorescein group (F) by protein degradation. Quenched Aβ peptides were added to PBS or 15 µg supernatant of mildly lysed HEK cells that were pre-incubated with no inhibitor, 10 µM MG-132 or 200 µM bacitracin (B) or with various peptidase inhibitors, 50 µM E-64, 50 µM bestatin, 100 µM PMSF, 10 µM pepstatin A, a cocktail containing the four inhibitors, 500 µM phenanthroline or 50 µM PAQ (C) for 30 min on ice. Degradation of quenched Aβ peptides in cell lysates was detected by measuring fluorescence increase in time. Representative degradation curves are shown.
with fluorescence according to the manufacturer. However, addition of phenanthroline to cell lysates containing fully degraded, fluorescent Aβ did not affect fluorescence levels, excluding the interference of phenanthroline with spectrophotometric analysis (data not shown).

**KNOCK-DOWN OF TPPII REDUCES Aβ PEPTIDE DEGRADATION**

Most peptidases have limited activity in degradation of peptides exceeding 10-15 amino acids, but the peptidase TPPII can degrade fragments longer than 20 amino acids (Reits et al., 2004). TPPII has both exo- and endo-peptidase activity and prefers lysine and arginine residues for endo-peptidase activity (Reits et al., 2004). Both amino acids are present in the Aβ sequence. To test whether TPPII is involved in Aβ degradation, we used TPPII-deficient mouse embryonic fibroblasts (MEFs) generated by Rock and colleagues. The degradation rate of quenched Aβ peptides was clearly reduced using cell lysates of TPPII-deficient MEFs, whereas Aβ was efficiently degraded in cell lysates of wild-type MEFs (Figure 4A). This indicates that TPPII is involved in degradation of quenched Aβ peptides. Furthermore, the aspecific irreversible TPPII inhibitor Ala-Ala-Phe-chloromethylketone (AAF-CMK) reduced degradation rates of Aβ using cell lysates of wild-type MEFs (Figure 4B). In TPPII-deficient cells, the inhibitor had hardly an effect (Figure 4B). Whereas TPPII seems to be involved in the degradation of Aβ peptides, its inhibition by AAF-CMK in HEK cells did not lead to accumulation of Aβ peptides upon expression of GFP-Ub-Aβ constructs, because Aβ was not detected on Western blots (Figure 2). The same counted for metalloproteases, because phenanthroline did not cause Aβ detection on Western blots.

Since Aβ is degraded in the absence of TPPII, other proteases are involved in its degradation as well, such as metalloproteases. To verify whether metalloproteases and TPPII act independently or sequentially on the degradation of Aβ, the metalloprotease inhibitor phenanthroline was added to TPPII-deficient cells and degradation rates of quenched Aβ peptides were measured. Metalloprotease inhibitors had an additive effect in TPPII-deficient cells (Figure 4C), suggesting that TPPII and metalloproteases act independently in the degradation of Aβ peptides.

Figure 4. TPPII and metalloproteases affect degradation rates of Aβ peptides. Quenched Aβ peptides were added to lysates of wild-type (wt) or TPPII-/- MEF cell lysates (A) that were pre-incubated with either 5 μM AAF-CMK (B) or 500 μM phenanthroline (C). Representative degradation curves are shown.
AGGREGATION DEPLETES THE POOL OF DEGRADABLE Aβ

The Aβ degradation curves showed lower plateau levels in TPPII-deficient cells and when metalloproteases were inhibited, although variations between plateau levels was observed (compare Figure 4A with 4C). This suggests that a slower rate of degradation of Aβ peptides also results in lower amounts of totally degraded Aβ. This is surprising, because identical amounts of Aβ peptides were added to cell lysates. Degradation rates can be different depending on the added inhibitors, but should result in similar end-levels of fluorescence upon complete degradation. To examine whether degrading proteases became clogged or impaired when degrading Aβ peptides, Aβ peptides were added again to cell lysates that had already reached a degradation plateau level. The additional Aβ peptides were degraded in all conditions (Figure 5A), indicating that the limited breakdown of Aβ is not due to protease impairment or clogging.

An alternative explanation for the lower fluorescence levels is increased aggregation of Aβ peptides, as aggregated Aβ cannot be degraded by proteasomes and peptidases. As a result, inhibition of proteases that leads to reduced degradation rates of quenched Aβ peptides, also leads to less degradation of quenched Aβ, and thus reduced fluorescent plateau levels. To test this possibility, we pre-incubated quenched Aβ peptides for 2 hours before addition to cell lysates. When added to cell lysates, reduced fluorescence levels were found with pre-incubated Aβ peptides (Figure 5B), suggesting that Aβ rapidly aggregated, thereby preventing degradation. Addition of the same amount of quenched Aβ peptides to half the amount of cell lysate caused a reduction in both degradation rates and plateau levels (Figure 5C). As only half the amount of peptidases was present, the rate of degradation was reduced as well. The reduced degradation rate allowed aggregation of Aβ peptides, leading to lower fluorescence levels. Together, these data show that cytoplasmic peptidases can only degrade monomeric Aβ peptides efficiently, whereas aggregated forms are resistant to degradation. This also explains the observed lower fluorescent plateau levels when Aβ peptides were slower degraded.

Figure 5. Aggregation prevents degradation of quenched Aβ peptides. (A) To examine whether differences in final plateau levels was due to impaired protease activity, a second amount of quenched Aβ peptides were added after 5 hours (arrow). Degradation of the first amount of quenched Aβ peptides had reached its plateau level by this time. (B) Quenched Aβ peptides were pre-incubated for 0 or 2 hours before being added to cell lysates. (C) The same amount of quenched Aβ peptides were added to either 15 µg or 7.5 µg lysates of wild-type or TPP-deficient MEFs. Representative degradation curves are shown.
Aggregation of Aβ depletes the pool of peptides that can be degraded and may subsequently induce proteasomal impairment and toxicity.

**Discussion**

Both Alzheimer’s disease and polyglutamine (polyQ) disorders such as Huntington’s disease are neurodegenerative disorders that are initiated by aggregation of protein fragments (Orr and Zoghbi, 2007; Selkoe, 2001). These toxic protein fragments are Aβ peptides and truncated forms of polyQ-expanded proteins, respectively. These protein fragments aggregate both in vitro and in vivo (Chafekar et al., 2007; Scherzinger et al., 1997; Wacker et al., 2004). Previously, we showed that expanded polyQ peptides are degradation-resistant and accumulate upon expression in cells (Raspe et al., 2009). In contrast, similarly released aggregation-prone Aβ peptides using GFP-Ub-Aβ_{40/42} constructs were efficiently degraded by cytoplasmic proteases. This difference between Aβ and polyQ peptides may be caused by two differences between these proteins, namely peptide length and sequence. PolyQ diseases are generally caused by polyQ repeats exceeding 40 glutamines (Orr and Zoghbi, 2007). Only expression of proteins with a polyQ tract above the threshold induces aggregation, which is also observed when expressing GFP-Ub-polyQ peptides of different lengths (Raspe et al., 2009). Expression of Q48 peptides results in aggregation in a low percentage of cells compared to expression of Q65 or Q112 peptides. Whereas Q65 and Q112 peptides are easily detected on Western blot, Q48 peptides could not be detected (unpublished results). This suggests that cytoplasmic peptidases can degrade peptides within the range of 40-50 glutamines. This may be similar in the case of Aβ peptides with a length of 40-42 amino acids. Secondly, the difference in sequence between polyQ and Aβ peptides may favor clearance of Aβ peptides that contain multiple amino acids that can be targeted by various peptidases. For example, the presence of basic residues within the sequence should enable TPPII to digest amyloid peptides by its endo-peptidase activity (Geier et al., 1999).

In order to find out which proteases are involved in the degradation of Aβ peptides, we either inhibited proteasomes, IDE or several classes of peptidases. Surprisingly, all inhibitors alone or in combination did not cause any significant accumulation of Aβ peptides in living cells. This may be explained by the heterogeneous structural character of Aβ peptides, enabling degradation by various proteases simultaneously. The quenched peptides enabled us to determine the effect of peptidase inhibitors on their degradation, which turned out to be more sensitive and informative than determining Aβ levels by Western blot analysis after expression of GFP-Ub-Aβ constructs and subsequent inhibition of several proteases. When using these quenched Aβ peptides, both TPPII and metalloproteases appeared to be involved in the degradation of Aβ peptides in vitro. As mentioned earlier, the endo-peptidase activity of TPPII may be well suited to degrade Aβ peptides into smaller peptides. These smaller peptides may be subsequently recycled into amino acids by other aminopeptidases. It already has been shown before that metalloproteases degrade Aβ peptides, as increased accumulation of Aβ peptides was observed upon inhibition of metalloproteases (Naidu et al., 1995; Sudoh et al., 2002).
Efficient degradation of the quenched Aβ peptide *in vitro* appeared to be hindered in time by its aggregation. This was not observed when expressing GFP-Ub-Aβ_{40/42}, which resulted in monomeric Aβ peptides that apparently were degraded before dimerization and aggregation could take place. This may be in contrast with micro-injection and *in vitro* degradation studies that introduce a bolus of Aβ peptides into the cytoplasm facilitating dimerization and aggregation. Together, this suggests that when Aβ peptides are not efficiently cleared they start to accumulate and aggregate. The presence of oligomeric structures may also explain why aggregation and toxicity was observed upon micro-injection of Aβ peptides in living cells (Zhang et al., 2002). Two previous studies used similar constructs to express non-tagged Aβ peptides. One study used HA-Ub-Aβ and could not detect Aβ peptides in cells as well (De Vrij, 2005). Another study used GFP-Ub-Aβ_{42} constructs to express Aβ peptides in SH-SYSY cells. It was found that Aβ_{42} peptides accumulated in half of the cells and this accumulation induced proteasomal impairment (Lee et al., 2006). However, proteasomal impairment was detected using the short-lived proteasome reporter GFPu, which recently was shown to accumulate in response to proteotoxic stress such as aggregation (Salomons et al., 2009). Different levels of Aβ peptide expression may explain these contradictory results. High expression levels of Aβ peptides may exceed the capacity of cells to efficiently degrade these peptides, leading to their accumulation and eventually inducing toxicity and proteasomal impairment.

When Aβ accumulates, stimulation of macroautophagy may be an alternative pathway to degrade aggregated Aβ in the cytoplasm. Macroautophagy is the bulk degradation of mainly long-lived proteins, protein complexes and cell organelles (Mizushima et al., 2008). However, aggregates can be degraded via autophagy as well, as stimulation of macroautophagy reduces aggregation and is protective in polyQ disease models (Berger et al., 2006). Because macroautophagy is impaired in AD brain and AD mice (Nixon et al., 2005; Yu et al., 2005), increasing autophagic clearance of Aβ peptides by improving autophagosomal-lysosomal fusion may be a good therapeutic strategy, instead of inducing macroautophagy.

Aβ is not generated in the cytoplasm, but Aβ has been found to be present in cytoplasm and nucleus in degenerating neurons in Tg mice and AD brain (D’Andrea et al., 2001; Gouras et al., 2000; Ohyagi et al., 2005). Furthermore, cytoplasmic Aβ is able to induce apoptosis in transgenic mice, *Drosophila*, neuronal and nonneuronal cell lines and in primary human neurons (LaFerla et al., 1995; Lee et al., 2006; Ling et al., 2009; Ohyagi et al., 2005; Yan et al., 1997; Zhang et al., 2002). Aβ was shown to inhibit proteasomal activity, as determined by the degradation of the fluorogenic proteasome peptide substrate Suc-LLVY-AMC (Almeida et al., 2006; Tseng et al., 2008). Although these studies suggest that cytoplasmic Aβ can induce toxicity and proteasomal impairment, we show that monomeric Aβ residing in the cytoplasm is efficiently degraded. It should be noted that in all studies except in this study and the study performed by Hol and colleagues (De Vrij, 2005), Aβ peptides were used either containing a starting methionine (M-Aβ) or an ER signal peptide (sAβ). This may affect stability as endogenous generated Aβ and also Aβ peptides used in the present study, start with an aspartic acid and should therefore be rapidly degraded according to the N-end rule (Varshavsky, 1992).

We have shown that cytoplasmic Aβ can be degraded by several proteases, including TPPII and metalloproteases, but a number of questions remain to be resolved. Does expression
of GFP-Ub-Aβ in TPPII-deficient cells lead to accumulation of Aβ peptides and will this be prevented by TPPII overexpression? More importantly, is the observed reduction in fluorescent levels of quenched Aβ peptides upon inhibition of various peptidases caused by aggregation? This question can be answered by using circular dichroism and electron microscopical analysis. Accumulation of Aβ peptides may be accompanied by proteasomal impairment and toxicity. Furthermore, degradation kinetics of quenched Aβ should also be studied in living cells.

In conclusion, monomeric Aβ peptides are rapidly degraded by various cytoplasmic proteases, including TPPII and metalloproteases. Degradation of Aβ peptides is prevented by its aggregation and aggregated Aβ peptides may induce proteasomal impairment and toxicity.

DNA constructs. GFP-Ub and GFP-Ub-Aβ constructs were obtained in several steps. First, Ub was generated by PCR from GFP-Ub (Dantuma et al., 2006) using forward primer 5’-CGCGGA-TCCATGCAGATCTTCGTGAAG-3’ containing a BamHI site and reverse primer 5’-CGGGAATTCTAGCATCCCACCTCGAGACGGAG-3’. The PCR product contained besides Ub, the first nucleotides of Aβ followed by Mph1103I and EcoRI restriction sites at the 3’ region. This construct was ligated into pSuper puro (OligoEngine) using BamHI and EcoRI. Aβ40/42 were obtained from pCEP4-APP695 Val-Phe (kindly provided with Wyp Scheper) using forward primer 5’-GCCTGCAGAATTCCGACATGA-3’ containing an PstI site and reverse primers 5’-ATAGTTTAGCGGCCGCTCAGACAACCGCCAC-3’ or 5’-ATAGTTTAGCGGCCGCTCAGACAACCGCCAC-3’, respectively, containing an Apal site. Aβ40/42 was ligated into the pSuper puro-Ub using PstI and NotI for Aβ and Mph1103I and NotI for pSuper puro-Ub, respectively. Finally, Ub and Ub-Aβ40/42 were ligated into pEGFP-C3 (Clontech) using Sall and Apal. Mutant UbG76V-Aβ was generated by introducing a point mutation within glycine 76 using Quikchange II Site directed Mutagenesis (Stratagene) with forward primer 5’-TCCGTCTCAGAGGTGTGGATGCAGAATTCCG-3’ and reverse primer 5’-CGGAATTCTGCATCCACCTCTGAGACGGA-3’ following manufacturers protocol. Ub-Aβ40/42 was obtained by PCR from HA-Ub-Aβ scr (kindly provided by Elly Hol; Malin et al., 2001) using forward primer 5’-GCGGAGCTCATGCGGCGCTGTTGAGATCGAATCC3’ and reverse primers 5’-ATAGTTAGCGGCCGCTCAGACAACCGCCAC-3’ or 5’-ATAGTTAGCGGCCGCTCAGACAACCGCCAC-3’, respectively, containing an Apal site and reverse primer 5’-GTG CCA AAT GTG GAA TTC TGA GGG CCC GCG 3’ containing an Apal site. Ub-Aβ40/42 was subsequently cloned into EGFP-C3 using SacI and Apal.

Cell culture and transfection. Human Embryonic Kidney (HEK293T) cells and the human melanoma cell line Mel JuSo were cultured in Iscove’s Modified Eagle Medium (IMDM; Gibco) supplemented with 10% fetal calf serum (FCS), 25 mM Heps and penicillin/streptomycin/glutamine (Gibco). SH-SY5Y human neuroblastoma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% FCS and penicillin/streptomycin/glutamine. SH-SY5Y cells were differentiated with 10 μM retinoic acid (Sigma) at 72 hours prior to plating. HEK and SH-SY5Y cells were maintained at 37°C in 5% CO₂. Wild-type and TPPII+ mouse embryonic fibroblast (MEF) cells were cultured in DMEM supplemented with 20% FCS and penicillin/streptomycin/glutamine and maintained at 37°C in 10% CO₂. Cells were dissociated using trypsinization. HEK293T cells were plated (0.2x10⁶ cells) in a 6-well and transfected after 24 hours with Fugene6 (Roche) or polyethylenimine (PEI; Polysciences). SH-SY5Y cells were plated (0.15x10⁶ cells) onto glass coverslips (24mm; Fisher scientific) in a 6-well. Differentiated cells were transient transfected with various GFP-Ub-Aβ constructs at 24 hours after plating using Dreamfect Gold (OZ Biosciences).

Cells were treated with various inhibitors, concentrations are indicated in the figure legends, 1,10-phenanthroline (Sigma), AAF-CMK (Enzo lifesciences AG), bacitracin (Sigma), bestatin (Enzo lifesciences AG), E64 (Sigma), MG-132 (Sigma), pepstatin A (Sigma) and PAQ-22 (Wako Chemicals), PMSF (Sigma).

Immunohistochemistry and confocal microscopy. SH-SY5Y cells were fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature at 48 hours after transfection. After washing with PBS cells
were permeabilized for 30 minutes in PBS containing 1% Triton X-100 and 1% FCS (TNBS). Cells were subsequently stained for Aβ for 1.5 hours at room temperature using primary antibody 6E10 (1:500; Signet), followed by AlexaFluor (1:200; Invitrogen). Confocal analysis was performed using a Leica TCS SP2 confocal system equipped with an Ar/Kr laser with a 63x objective.

Immunoblotting. HEK293T cells were harvested at 48 hours after transfection and lysed with ice-cold 2x Tris-buffer (100 mM tris-HCl pH 7.5, 300 mM NaCl, 10 mM EDTA; pH 8.0, 1% Triton X-100) supplemented with complete mini protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma). Cells were incubated for 30 minutes on ice and centrifuged for 15 minutes at 14,000 rpm at 4°C. Protein concentrations were determined using the Bradford protein assay (BioRad). Protein lysates were separated on either SDS-PAGE gels (stained against GFP and actin) or Tris-tricine-SDS gels (stained against Aβ). Using Tris-tricine-SDS gels, a better detection of low molecular weight proteins and peptides is obtained. Equal protein levels in sample buffer (3 mM Tris-HCl pH 6.8, 5% glycerol, 1% SDS, 2.5% 2-mercaptoethanol, 0.005% (w/v) OrangeG (SDS-PAGE), or (0.45 mM Tris-HCl pH 8.45, 10% glycerol, 4% SDS, 0.005% (w/v) OrangeG (Tris-tricine-SDS)) were boiled 5 minutes at 98°C and loaded on 12.5% SDS-PAGE gels, or 10% Tris-tricine-SDS, respectively. As a control, 50 ng synthetic Aβ42 (Sigma) was also loaded on Tris-tricine-SDS gels. After electrophoresis, proteins were transferred either onto a 0.45 µm pore size nitrocellulose membrane filter (Schleicher & Schuell) using a semi-dry transfer system (SDS-PAGE) or onto a 0.2 µm pore size nitrocellulose membrane filter (Schleicher & Schuell) using wet blotting at 4°C for 3 hours (Tris-tricine-SDS). Blots probed against Aβ were boiled for 5 min in Tris-buffered saline (TBS) before blocking, to better detect Aβ peptides. Blots were blocked in 5% dry milk in TBS, incubated with primary antibodies against GFP (1:1,000; Invitrogen), β-actin (1:10,000; Sigma) and Aβ (1:1,000; Signet) in TBS containing 0.1% Tween-20, and subsequently with secondary antibodies IRDye 680 or IRDye 800 (1:10,000; LI-COR Biosciences). Signal was detected using the Odyssey imaging system (Westburg).

Peptide synthesis. An Aβ40 peptide containing fluorophore and quencher was synthesized by solid phase strategies using an automated multiple peptide synthesizer (Syrol, MultiSyntech). A fluorescein (Fl) was introduced at amino acid 22 by covalent coupling of fluorescein-5-iodoacetamide (5-IAF, Fluka) to the cysteine. Quenching of Fl fluorescence (f) was performed by a dabcyl group (q) that had been introduced in the peptide at amino acid 12 by coupling of Fmoc-L-Lys(Dabcyl)-OH (NeoMPS), resulting in the following peptide DAEFRHDSGY EqHHQKLVFF AfDVGSNKGA IIGLMVGGVV. Peptides were purified by size exclusion chromatography and RP-HPLC (>95% pure) and showed the expected molecular mass as determined by mass spectrometry (Maldi Tof, Voyager, ABI). Quenched Aβ40 was dissolved in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma), aliquoted, dried under vacuum in a SpeedVac (Eppendorf) and stored at -20°C as described before (Chafekar et al., 2007).

Degradation assay. HEK293T cells were lysed in 25 µM digitonin (Sigma) in KMH buffer (110 mM KAc, 2 mM MgAc and 20 mM Hepes-KOH, pH 7.2) for 30 minutes on ice and centrifuged for 15 minutes at 14,000 rpm at 4°C to release the cytoplasmic fraction. Protein concentrations were determined with a Bradford protein assay and 15 µg cytoplasmic cell lysate was added to 96-well. Various protease inhibitors were added to cell lysates (as indicated in figure legends) and incubated for 30 minutes at 4°C. Immediately prior to use, HFIP-treated aliquots of Aβ40 were resuspended in DMSO followed by sonication for 10 minutes, 1 µL of quenched Aβ40 was added per well. Degradation of the peptide was analyzed using the FLUOstar OPTIMA (BMG Labtec.).

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accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer’s disease.


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