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# THE PRODUCTION RATIOS OF AICD $\epsilon$ 51 AND A $\beta$ 42 BY INTRAMEMBRANE PROTEOLYSIS OF $\beta$ APP DO NOT ALWAYS CHANGE IN PARALLEL

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## RUNNING TITLE

Variety of  $\epsilon$ -cleavage of  $\beta$ APP

## Abstract

**Background:** During intramembrane proteolysis of  $\beta$ APP by presenilin (PS)/ $\gamma$ -secretase,  $\epsilon$ -cleavages at the membrane-cytoplasmic border precede  $\gamma$ -cleavages at the middle of the transmembrane domain. Generation ratios of A $\beta$ 42, a critical molecule for Alzheimer disease (AD) pathogenesis, and the major A $\beta$ 40 species may be associated with  $\epsilon$ 48 and  $\epsilon$ 49 cleavages, respectively. Medicines to down-regulate A $\beta$ 42 production have been investigated by many pharmaceutical companies. Therefore, the  $\epsilon$ -cleavages, rather than the  $\gamma$ -cleavage, may be more effective upstream targets for decreasing the relative generation of A $\beta$ 42. Thus, one may evaluate compounds by analyzing the generation ratio of the AICD species ( $\epsilon$ -cleavage-derived), instead of that of A $\beta$ 42.

**Methods:** Cell-free  $\gamma$ -secretase assays were performed to observe *de novo* AICD production. Immunoprecipitation/MALDI-TOF MS analysis was performed to detect the N-termini of AICD species. A $\beta$  and AICD species were measured by ELISA and immunoblotting techniques.

**Results:** Effects on the  $\epsilon$ -cleavage by AD-associated pathological mutations around the  $\epsilon$ -cleavage sites (*i.e.*,  $\beta$ APP V642I, L648P and K649N), were analyzed. The V642I and L648P mutations caused an increase in the relative ratio of  $\epsilon$ 48 cleavage as expected from previous reports. Cells expressing the K649N mutant, however, underwent a major  $\epsilon$ -cleavage at the  $\epsilon$ 51 site. These results suggest that  $\epsilon$ 51, as well as  $\epsilon$ 48 cleavage, is associated with A $\beta$ 42 production. Only AICD $\epsilon$ 51, though, and not A $\beta$ 42 production, dramatically changed with modifications to the cell-free assay conditions. Interestingly, the increase in the relative ratio of the  $\epsilon$ 51 cleavage by the K649N mutation was not cancelled by these changes.

**Conclusion:** Our current data indicate that the generation ratio of AICD $\epsilon$ 51 and A $\beta$ 42 do not always change in parallel. Thus, to identify compounds that decrease the relative ratio of A $\beta$ 42 generation, measurement of the relative level of A $\beta$ 42-related AICD species (*i.e.*, AICD $\epsilon$ 48 and AICD $\epsilon$ 51) might not be useful. Further studies to reveal how the  $\epsilon$ -cleavage precision is decided are necessary before it will be possible to develop drugs targeting  $\epsilon$ -cleavage as a means for decreasing A $\beta$ 42 production.

**Key words:** Alzheimer Disease,  $\beta$ APP,  $\gamma$ -cleavage,  $\epsilon$ -cleavage, presenilin/ $\gamma$ -secretase, "dual-cleavage" mechanism, AICD $\epsilon$ 51

## INTRODUCTION

The transmembrane domain of  $\beta$ -Amyloid Protein Precursor ( $\beta$ APP) is proteolysed by presenilin (PS)/ $\gamma$ -secretase <sup>1</sup>. Analysis of the resultant products has revealed that the proteolysis proceeds by at least two-distinct cleavages. The “ $\epsilon$ -cleavage” liberates its intracellular domain (*i.e.*, AICD) into the cytoplasm, while the “ $\gamma$ -cleavage” releases Alzheimer disease (AD)-associated Amyloid  $\beta$ -protein ( $A\beta$ ) <sup>2-6</sup>.

There are some variations in both the  $\gamma$ - and  $\epsilon$ -cleavages of  $\beta$ APP <sup>6-8</sup>. The major N-termini of AICD species consist of leucine-49, valine-50 and leucine-52 ( $A\beta$ -numbering), while the major C-termini of  $A\beta$  species are comprised of valine-40 and alanine-42. (Figure 1A) <sup>6</sup>. Among these, highly aggregatable  $A\beta$ 42 is the major component of senile plaques in AD brains <sup>9</sup>.

Are there any relationships between the  $\epsilon$ - and  $\gamma$ -cleavages? How do these cleavages occur? Ihara and colleagues have tried to address these questions and recently revealed that  $\epsilon$ -cleavage precedes  $\gamma$ -cleavage in *in vitro*  $\gamma$ -secretase assays <sup>10</sup>.  $\beta$ APP-CTF stubs,  $\beta$ APP membrane-tethered remnants following  $\beta$ -cleavage, first undergo  $\epsilon$ -cleavage <sup>10</sup>. The  $\epsilon$ -cleavage liberates AICD from the membrane and produces a membrane-bound 48/49 amino-acid-long  $A\beta$  species that undergoes further C-terminal truncation by PS/ $\gamma$ -secretase <sup>11</sup>. Stepwise cleavages remove every three amino-acid residues from the C-terminus of the long  $A\beta$  species, which finally secretes  $A\beta$ 40/42 <sup>12-14</sup>. For example, mutant PS causes increased both  $\epsilon$ 48 and  $\gamma$ 42 cleavages <sup>8</sup>. Thus, the  $\gamma$ -cleavage seems to occur in an  $\epsilon$ -cleavage-dependent manner <sup>10</sup>. Moreover, these results indicate that the production process for pathological  $A\beta$ 42 is distinct from that of  $A\beta$ 40 <sup>15</sup>. That is, the major  $\epsilon$ 49 cleavage causes the production of  $A\beta$ 40, while a minor  $\epsilon$ 48 cleavage causes production of pathological  $A\beta$ 42 <sup>14</sup>.

Modulation of  $\gamma$ -secretase function to specifically inhibit  $A\beta$ 42 production is one of the promising strategies for developing drugs to modify the disease course of AD <sup>16</sup>. Given the possible correlation between the  $\epsilon$ - and  $\gamma$ -cleavages, we think that targeting the up-stream  $\epsilon$ -cleavages will be a novel and more efficient method for developing  $A\beta$ 42-lowering drugs. To test if precision of the  $\epsilon$ -cleavage can be used as a novel target for drug development, we investigated the  $\epsilon$ -cleavage pathway, particularly  $\epsilon$ 51 cleavage, which has previously not been well-characterized <sup>7</sup>.



## RESULTS

### **The $\beta$ APP K649N Belgian mutant increased both the relative ratio of AICD $\epsilon$ 51 and A $\beta$ 42 production in a cell-free $\gamma$ -secretase assay.**

To test if the  $\epsilon$ 51 cleavage precedes the  $\gamma$ 42 cleavage, we analyzed the effects of three  $\beta$ APP mutants (V642I<sup>17</sup>, L648P<sup>18</sup>, and K649N<sup>19</sup>) around the  $\epsilon$ -site. The L648P and K649N mutants ( $\beta$ APP695 numbering) are located downstream of the  $\epsilon$ 51 site, and the V642I mutant is located upstream of the  $\epsilon$ 48 site (Figure 1A). Each of the three mutants is familial AD-associated and, therefore, increases the relative ratio of A $\beta$ 42 production. We raised K293 cells stably expressing each of the mutants, prepared the crude membrane fractions<sup>20</sup> and performed the cell-free  $\gamma$ -secretase assays<sup>7,21</sup>.

As shown in Figure 1B, the K649N  $\beta$ APP mutant caused marked increase in the relative ratio of AICD $\epsilon$ 51 production. However, the other two mutants caused completely different effects on the cleavage. The L648P mutant produced a barely detectable level of AICD $\epsilon$ 51, while in the V642I mutant cells, the ratio of AICD $\epsilon$ 51 production was comparable to that of wild-type (wt) expressing cells. It is of note that, instead of increased AICD $\epsilon$ 51 production, these V642I and L648P mutants substitutively increased the relative ratio of AICD $\epsilon$ 48 production. Next we measured A $\beta$  species secretion by the stable cells in conditioned media using ELISA (Figure 1C). As expected, we observed a significant increase in the ratio of A $\beta$ 42 to total A $\beta$  secretion in the conditioned medium of the mutant cells. This data indicates that the K649N mutant increased the ratio of A $\beta$ 42 production through up-regulation of the  $\epsilon$ 51 cleavage, while the V642I and L648P mutants increased A $\beta$ 42 production through the  $\epsilon$ 48 cleavage. Based on these results, we suggest that not only the  $\epsilon$ 48 but also the  $\epsilon$ 51 cleavage precedes A $\beta$ 42 production, possibly by sequential three amino-acid C-terminal truncation<sup>14</sup> (Figure 1D).

### **Incubation in higher pH does not cancel the K649N $\beta$ APP mutant effects.**

We previously found that the precision of  $\epsilon$ -cleavage changes depending on the buffer pH<sup>7,21</sup>. The relative ratio of AICD $\epsilon$ 51 production is the most sensitive to such changes. Therefore, we next determined whether the relative ratio of AICD $\epsilon$ 51 and/or A $\beta$ 42 production by the K649N mutant is affected by changing the buffer pH during the cell-free assay. As expected, incubation in the higher pH (pH 7.4 vs pH 6.0) buffer decreased the relative ratio of AICD $\epsilon$ 51 generation in both the K649N mutant and wt  $\beta$ APP membrane fraction. However, the pH effect was not so strong as to cancel the AICD $\epsilon$ 51 up-regulation effect by the K649N mutant (Figure 2A). We further analyzed the pH effects on the increase in the relative ratio of A $\beta$ 42 production by the mutant

(Figure 2B). Surprisingly, the assay pH elevation did not cause any changes in the relative ratio of A $\beta$ 42 generation. Therefore, unlike the effects of the K649N mutant on the  $\epsilon$ 51- and  $\gamma$ 42-cleavages, the elevation of the buffer pH causes a decrease in the relative ratio of AICD $\epsilon$ 51 production but does not cause any changes in A $\beta$ 42 production. The data suggests that two distinct mechanisms may contribute to the determination of the relative ratio of AICD $\epsilon$ 51 production.

**Alkali pre-treatment of the crude membrane fraction cancels the effect of higher pH cell-free incubation on  $\epsilon$ -cleavage.**

Since the  $\epsilon$ 51 cleavage occurs at the membrane-cytosol interface, we considered that membrane-bound substances might induce the pH-dependent effects on AICD $\epsilon$ 51 production. Many substances detach from the membrane upon treatment with alkali solution<sup>22</sup>. To test this theory, we washed the wt  $\beta$ APP membrane fraction in a pH 11 solution (see "Materials and Methods") then conducted the cell-free assay at pH 6.0. The relative ratio of AICD $\epsilon$ 51 production markedly decreased (Figure 3A), while that of the A $\beta$ 42 did not (Figure 3B). The phenomena are reminiscent of the effects of raising the pH of the incubation buffer (see Figure 2). Thus, we further considered that the decrease in the AICD $\epsilon$ 51 production resulting from the use of a higher incubation buffer pH might also be due to detachment of substances from the membrane. When the membrane fraction was incubated in a pH 7.4 buffer after alkali treatment, we could no longer observe the pH-dependent incubation buffer effects on the AICD $\epsilon$ 51 ratio (Figure 3C). Collectively, though incubation at lower pH buffer increased in the AICD $\epsilon$ 51 ratio (Figure 2A), the effects was cancelled by the alkali pre-treatment (Figure 3A). These results suggest that substances removed by the alkali treatment might induce the changes in the relative ratio of AICD $\epsilon$ 51 production.

**Alkali pre-treatment of the crude membrane fraction did not cancel the effects of the K649N mutant on the  $\epsilon$ -cleavage.**

As shown in Figure 1, the K649N  $\beta$ APP mutation causes up-regulation of both the AICD $\epsilon$ 51 and A $\beta$ 42 ratio, while alkali pre-treatment causes down-regulation of only the AICD $\epsilon$ 51 ratio (Figure 3). These data indicate that changes in the AICD $\epsilon$ 51 ratio caused by the mutation and by the treatment occur by two distinct processes. A further experiment was conducted to confirm whether the K649N mutation cause a change in the relative ratio of AICD $\epsilon$ 51 production through the effect of the alkali treatment (Figure 4A). Following treatment of the K649N mutant membrane fraction in the alkali solution, the cell-free assay was performed at pH 6.0. As shown in Figure

4A, even after the alkali treatment, the K649N mutant membrane produced a relatively higher level of AICD $\epsilon$ 51 than that of the wt fraction (Figure 3A). Moreover, the elevated A $\beta$ 42 ratio was not changed by the pre-treatment (Figure 4B).

## DISCUSSION

In the present study we determined that there are at least two factors that change the precision of  $\epsilon$ -cleavage: (i) a process induced by a pathological  $\beta$ APP mutation and (ii) another process induced by possibly unidentified substances removed from the membrane fraction by alkali pre-treatment. In the case of  $\beta$ APP mutations, the relative ratio of  $\epsilon$ 51 and  $\epsilon$ 48 production increases in parallel with the ratio of AD-associated A $\beta$ 42.

It has been reported that  $\epsilon$ -cleavage precedes  $\gamma$ -cleavage and  $\gamma$ -cleavage seems to occur in an  $\epsilon$ -cleavage-dependent manner<sup>10</sup>. Considering these reports and our own preliminary results, it seemed possible that measurement of the relative ratio of AICD $\epsilon$ 48/AICD $\epsilon$ 51 production might help develop A $\beta$ 42-lowering anti-AD drugs. Further study revealed, however, that the relative level of AICD $\epsilon$ 51 production is drastically affected by the removal of unidentified substances from the membrane as a result of alkali pre-treatment. Interestingly, the alkali pre-treatment did not cause any changes in the relative ratio of A $\beta$ 42 generation. These results indicate that changes in the precision of  $\epsilon$ -cleavage do not always cause parallel alterations in the precision of  $\gamma$ -cleavage, even though  $\epsilon$ -cleavage occurs upstream of the  $\gamma$ -cleavage. Therefore, although measuring the levels of AICD species is a potentially attractive new target for developing A $\beta$ 42 lowering compounds, challenges still must be overcome before screening methods for such compounds can be established. For example, the paradoxical mechanism discussed above must first be understood before an assay in which the  $\epsilon$ -cleavage precision accurately reflects the  $\gamma$ -cleavage precision can be developed.

How does alkali pre-treatment result in a decreased ratio of AICD $\epsilon$ 51 production? One may consider the presence of unknown substances which (i) transiently associate with the PS/ $\gamma$ -secretase and affect its intramembrane cleavage precision, or (ii) truncate a couple of N-terminal amino-acid residues of AICD produced by the  $\epsilon$ -cleavage. The second possibility is reminiscent of ACE activity to truncate the C-terminus of A $\beta$ 42<sup>23</sup>. Of course, the possibility that alkali pre-treatment might change the character of PS/ $\gamma$ -secretase itself also cannot be excluded.

## CONCLUSION

Our current data suggest that the precision of  $\epsilon$ -cleavage do not always changes in parallel with the precision of  $\gamma$ -cleavage, even though  $\epsilon$ -cleavage occurs upstream of the  $\gamma$ -cleavage. Thus, to measure the levels of AICD species might be an attractive new target for developing A $\beta$ 42 lowering compounds, there still remain some

challenges.

## MATERIALS AND METHODS

### Cell culture and cDNA constructs

cDNAs of  $\beta$ APP V642I, L648P and K649N mutants were generated by PCR-based mutagenesis using a Quickchange mutagenesis kit (Stratagene) or KOD plus (Toyobo) with wt  $\beta$ APP695 cDNA as a template. K293 cells were transfected and cultured as previously described <sup>24</sup>.

### Membrane preparation

The crude membrane fraction was prepared as previously described with a slight modification <sup>7,21</sup>. In the present study, the homogenization buffer contained 0.25 M sucrose and 50 mM HEPES (pH 7.4) containing a protease inhibitor cocktail (Roche). To prepare the alkaline pre-treated membrane, the membrane fraction was suspended in a 50 mM bicarbonate buffer (pH 11.0) and incubated at 4 °C for 1 h. The suspension was then centrifuged at 100,000  $\times$  g for 1 h followed by washing once with a 50 mM Mes buffer (pH 6.0).

### Cell-free $\gamma$ -secretase assay

The cell-free  $\gamma$ -secretase assay was performed as previously described with a modification <sup>7,21</sup>. The reaction buffer in the present study contained a 150 mM citrate buffer (pH 6.0), 50 mM MES (pH 6.0), 167 mM NaCl and a protease inhibitor mixture comprised a 5x complete protease inhibitor cocktail (Roche), 0.5 mM DIFP (WAKO), 1  $\mu$ g/ml TLCK (Sigma-Aldrich), 10  $\mu$ g/ml antipain (Peptide Institute), 10  $\mu$ g/ml leupeptin (Peptide Institute), 5 mM 1,5 phenanthroline (Sigma-Aldrich), 10  $\mu$ M amastatin (Peptide Institute), 10  $\mu$ M bestatin (WAKO), 1  $\mu$ M thiorphan (Sigma-Aldrich), 10  $\mu$ M phosphoramidon (Peptide Institute) and 1  $\mu$ M pepstatin A (Peptide Institute). To prepare the pH 7.4 buffer, 50 mM HEPES (pH 7.4) was used instead of the citrate and MES buffers.

### Immunoprecipitation/MALDI MS (IP-MS) analysis

IP-MS analysis followed by cell-free incubation was carried out as previously described <sup>7,21,25</sup>. The heights of the MS peaks and molecular weights were calibrated using angiotensin and bovine insulin  $\beta$ -chain as standards (Sigma-Aldrich).

### ELISA analysis for A $\beta$

A $\beta$ 40 and A $\beta$ 42 levels in conditioned media were quantified by ELISA (WAKO).

### Immunoblotting of A $\beta$

SDS-solubilized proteins were separated by SDS-PAGE using an 8 M Urea gel <sup>24</sup> and transferred to a nitrocellulose membrane. Immunoblotting of A $\beta$  species using 82E1 (IBL) was performed as previously described <sup>26</sup>.

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The authors declare no competing financial interests.

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## FIGURE LEGENDS

### Figure 1. Effect of familial AD-associated $\beta$ APP mutations around the $\epsilon$ -cleavage site.

**A**, Schematic diagram of intramembrane cleavage sites of  $\beta$ APP and the familial AD mutations used in the present study. The amino acid sequence around the juxta membrane region of human  $\beta$ APP is described ( $A\beta$  numbering). Filled inverted triangles indicate the cleavage sites. Substituted amino acids of the familial AD mutations are indicated in open rectangles. The site of each mutant is also indicated using APP695 numbering.

**B**, Mass spectra of *de novo* AICD species in the cell-free assay. Crude membrane fractions obtained from wt  $\beta$ APP and the indicated  $\beta$ APP mutant cells were used.

**C**, Relative secreted  $A\beta_{42}$  to  $A\beta_{40}$  ratio in the conditioned media of wt  $\beta$ APP and the indicated  $\beta$ APP mutant cells. The asterisks indicate statistical significance (\* $P < 0.05$ , \*\* $P < 0.001$ , one-way analysis of variance (ANOVA) and Tukey-Kramer method). Error bars indicate standard error of the mean (SEM).

**D**, Hypothesis for explaining increased  $\gamma_{42}$  cleavage in each mutant  $\beta$ APP (upper panels) and differential production of  $A\beta_{40}$  and  $A\beta_{42}$  (lower panels).

### Figure 2. Effect of cell-free incubation pH levels on the precision of $\epsilon/\gamma$ -cleavages.

**A**, Mass spectra of AICD generated in the cell-free assay performed at the indicated pH (upper and middle panels). Peak heights of AICD $\epsilon_{49}$  and  $\epsilon_{51}$  were measured and the ratios of AICD $\epsilon_{49}$  to  $\epsilon_{51}$  were calculated (lower panel). The asterisks indicate statistical significance (\* $P < 0.05$ , \*\* $P < 0.001$ , one-way ANOVA and Tukey-Kramer method). Error bars indicate SEM.

**B**, Levels of  $A\beta$  generated at the indicated pH. Levels of  $A\beta_{40}$  and 42 were measured by western blotting and the  $A\beta_{42}$  to 40 ratios calculated. The asterisks indicate statistical significance. Error bars show SEM.

### Figure 3. Effect of alkali pre-treatment on the precision of $\epsilon/\gamma$ -cleavages of wt $\beta$ APP.

**A**, Mass spectra of AICD generated in the cell-free assay with and without alkali pre-treatment. Peak heights of AICD $\epsilon_{49}$  and  $\epsilon_{51}$  were measured and the AICD $\epsilon_{49}$  to  $\epsilon_{51}$  ratios calculated. The asterisk indicates statistical significance (\* $P < 0.05$ , paired t-test). Error bars indicate SEM.

**B**, Levels of  $A\beta$  generated in the cell-free assay following alkali pre-treatment. Levels of  $A\beta_{40}$  and 42 were measured by western blotting with and without alkali pre-treatment and the  $A\beta_{42}$  to 40 ratios calculated.

**C**, Mass spectra of AICD generated in the cell-free assay at the indicated pH following

alkali pre-treatment.

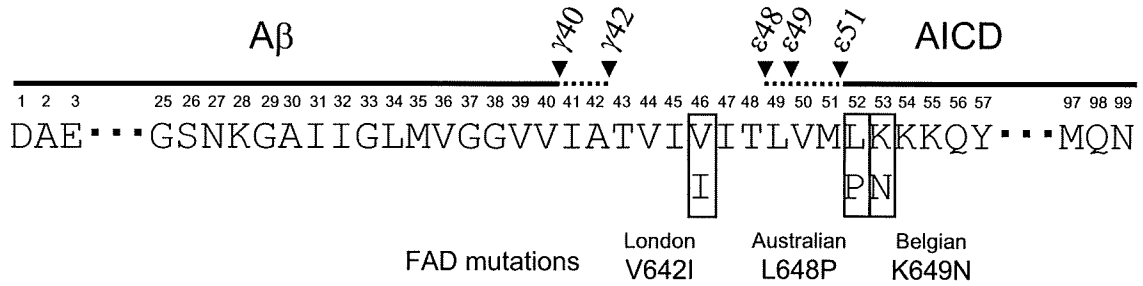
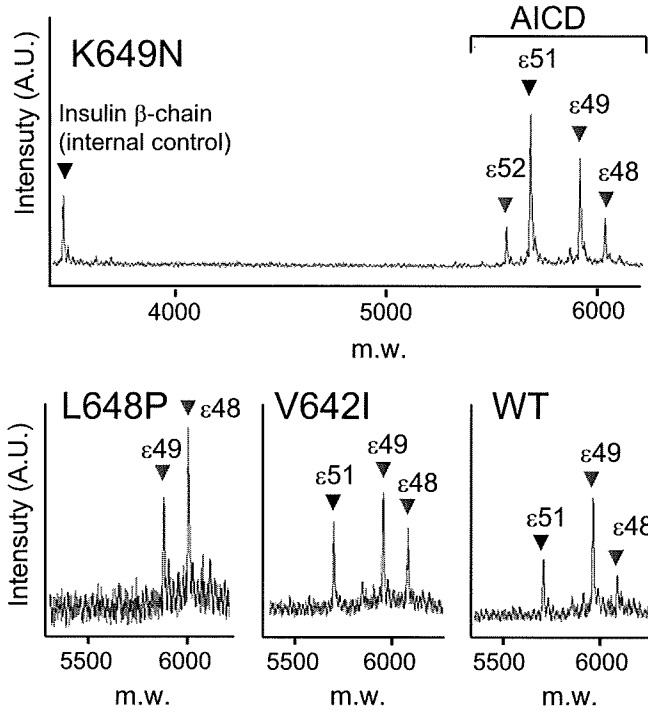
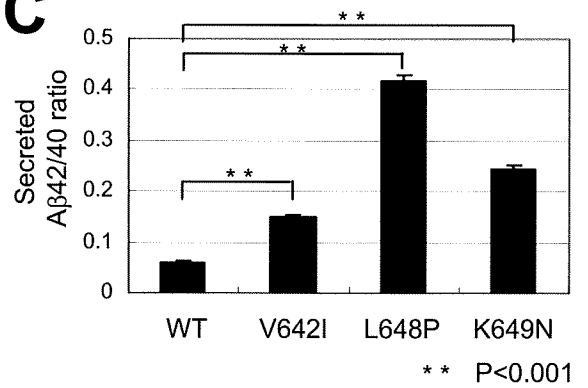
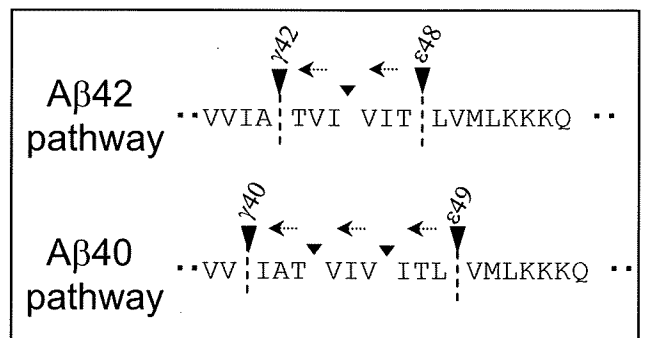
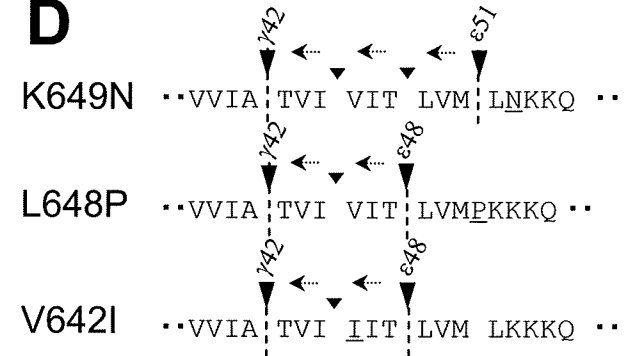
**Figure 4. Effect of alkali pre-treatment on the precision of  $\epsilon/\gamma$ -cleavages of  $\beta$ APP K649N Belgian mutant.**

**A,** Mass spectra of AICD generated in the cell-free assay with and without alkali pre-treatment.

**B,** Levels of A $\beta$  generated in the cell-free assay following alkali pre-treatment.

**Table 1. Molecular species of AICD generated in the cell-free assay.**

AICD species		m/z		
		Calculated [M+H]	observed [M+H]	
			mean	SD
AICD $\epsilon$ 51 (52-99)	wt	5677.79	5678.38	0.64
	V642I	5677.79	5678.30	0.70
	K649N	5663.74	5663.96	0.23
AICD $\epsilon$ 49 (50-99)	wt	5907.9	5908.35	0.29
	V642I	5907.9	5908.49	0.21
	L648P	5891.87	5892.48	0.20
	K649N	5893.84	5894.10	0.27
AICD $\epsilon$ 48 (49-99)	wt	6020.98	6021.36	0.40
	V642I	6020.98	6021.59	0.42
	L648P	6004.96	6005.59	0.33
	K649N	6006.93	6007.51	0.17
AICD $\epsilon$ 52 (53-99)	K649N	5550.65	5551.01	0.27

**A****B****C****D**

Mori et al., Figure 1