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Alcadein Cleavages by Amyloid β-Precursor Protein (APP) α- and γ-Secretases Generate Small Peptides, p3-Alcs, Indicating Alzheimer Disease-related γ-Secretase Dysfunction*[S]

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Alcadeins (Alcs) constitute a family of neuronal type I membrane proteins, designated Alc_{α}, Alc_{β}, and Alc_{γ}. The Alcs express in neurons dominantly and largely colocalize with the Alzheimer amyloid precursor protein (APP) in the brain. Alcs and APP show an identical function as a cargo receptor of kinesin-1. Moreover, proteolytic processing of Alc proteins appears highly similar to that of APP. We found that APP α-secretases ADAM 10 and ADAM 17 primarily cleave Alc proteins and trigger the subsequent secondary intramembranous cleavage of Alc C-terminal fragments by a presenilin-dependent γ-secretase complex, thereby generating "APP p3-like" and non-aggregative Alc peptides (p3-Alcs). We determined the complete amino acid sequence of p3-Alc_{α}, p3-Alc_{β}, and p3-Alc_{γ}, whose major species comprise 35, 37, and 31 amino acids, respectively, in human cerebrospinal fluid. We demonstrate here that variant p3-Alc C termini are modulated by FAD-linked presenilin 1 mutations increasing minor β-amyloid species Aβ42, and these mutations alter the level of minor p3-Alc species. However, the magnitudes of C-terminal alteration of p3-Alc_{α}, p3-Alc_{β}, and p3-Alc_{γ} were not equivalent, suggesting that one type of γ-secretase dysfunction does not appear in the phenotype equivalently in the cleavage of type I membrane proteins. Because these C-terminal alterations are detectable in human cerebrospinal fluid, the use of a substrate panel, including Alcs and APP, may be effective to detect γ-secretase dysfunction in the prepathogenic state of Alzheimer disease subjects.

Alcadein (Alc)[5] proteins comprise a family of evolutionarily conserved, type I membrane proteins that are predominantly expressed in neuronal tissues. Alc has been independently identified as a binding protein for the neuron-specific adaptor protein X11L (X11-like) (1) and as a postsynaptic Ca^{2+}-binding protein, where it is known by the name calsyntenin (2). Alc functions as a cargo-receptor for the kinesin-1 motor that mediates anterograde transport of APP (3, 4), and a mutation in a nematode ortholog of the Alc gene is reported to cause a defect in associative learning (5, 6). Thus, Alc plays important roles in vesicular transport at the subcellular level and in learning behavior at the organismal level. Alc exists as four isoforms in mammals: $Alc_{\alpha 1}$ (971 amino acids in humans), $Alc_{\alpha 2}$ (981 amino acids in humans), Alc_{β} (956 amino acids in humans), and Alc_{γ} (955 amino acids in humans) (1). Alc_{α}, Alc_{β}, and Alc_{γ} are

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[5] The abbreviations used are: Alc, alcadein; AD, Alzheimer disease; Aβ, amyloid β-protein; ADAM, a disintegrin and metalloproteinase; APP, amyloid β-precursor protein; sAPP, soluble large extracellular N-terminal domain of APP truncated at the primary cleavage site; sAlc, soluble large extracellular N-terminal domain of Alc truncated at the primary cleavage site; p3-Alc, small peptide generated by serial primary and secondary cleavages of Alc; CSF, cerebrospinal fluid; CTF, C-terminal fragment of APP or Alc truncated at the primary cleavage site; BACE, β-site APP-cleaving enzyme; MEF, mouse embryonic fibroblast; PS, presenilin; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; MS, mass spectrometry; MS/MS, tandem MS.

encoded by independent genes, whereas $Alc_{\alpha 1}$ and $Alc_{\alpha 2}$ are splice variants derived from the Alc_{α} gene.

In neurons, Alc proteins are complexed to X11L molecules, which, in turn, are complexed with the amyloid β-precursor protein (APP), a type I transmembrane protein that is believed to play a seminal role in the pathogenesis of familial and sporadic Alzheimer disease (reviewed for AD in Refs. 7–9 and for X11L in Refs. 10 and 11). In the absence of X11L, both Alc and APP proteins are rapidly cleaved in a coordinated manner (12). Levels of the endogenous APP metabolite, amyloid-β protein (Aβ), are elevated in the brains of X11L-deficient mice, indicating that the APP-X11L interaction is physiologically important in the regulation of APP metabolism in the brain *in vivo* (13, 14). Alc proteins are also cleaved successively by secretases and release soluble Alc ectodomain (sAlc, corresponding to the soluble APP ectodomain (sAPP)) and p3-Alc (corresponding to the APP fragment, p3) (12). Taken together with similarities and/or identities in their structure, cellular distribution, and neural function, the physiological and pathophysiological metabolic fate of Alc would be predicted to parallel that of APP (1, 3, 12).

In this study, we report that all three members of the Alc family (Alc_{α}, Alc_{β}, and Alc_{γ}) are cleaved by ADAM 10 and ADAM 17, which have been identified as the α-secretases for APP (15–17). Subsequent cleavage of the remaining Alc C-terminal fragments involves the presenilin-1 (PS1)-dependent γ-secretase, and this reaction liberates into cell-conditioned medium and into cerebrospinal fluid (CSF) a short peptide, p3-Alc, previously designated "β-Alc." Our other analysis using CSF from three groups of human subjects ($n = 158$) indicates that p3-Alc_{α} variant ratio (minor p3-Alc_{α}38/major p3-Alc_{α}35) correlated with the Aβ42/40 ratio in the sporadic AD (clinical dementia rating 0.5 + 1 patients) but not elderly non-demented and other neurological disease controls.[6] Therefore, the detailed biochemical analysis for the cleavages of Alc proteins is significant for understanding the features of p3-Alc peptides in human subjects. We found that various FAD-linked PS1 mutations appeared, at different magnitudes, with the alterations of C termini of p3-Alcs, suggesting that one type of γ-secretase dysfunction appears in various phenotypes upon cleavage of Alc proteins and APP. In other words, one type of γ-secretase dysfunction largely alters the cleavage of one Alc species and APP, but the same dysfunction slightly alters the cleavage of another Alc species. When the cleavage phenotypes appear on APP to increase pathogenic Aβ42, the subject may experience the onset of AD. Testing the hypothesis that AD-related variant processing of p3-Alc peptide might yield surrogate markers for γ-secretase dysfunction is important. In this study, we characterized all p3-Alc species generated from Alc_{α}, Alc_{β}, and Alc_{γ} in detail.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Stable Cell Lines Expressing PS1—The human Alcadein cDNAs we used were $hAlc_{\alpha 1}$ (GenBank™ accession number AY753301), $hAlc_{\beta}$ (NM_014718), and $hAlc_{\gamma}$ (NM_022131). The FLAG sequence was inserted between Leu^{38} and Glu^{39} to generate pcDNA3-FLAG-$hAlc_{\alpha 1}$, between Lys^{26} and Pro^{27} to generate pcDNA3.1-FLAG-$hAlc_{\beta}$ and between Gln^{29} and Arg^{30} to generate pcDNA3.1-FLAG-$hAlc_{\gamma}$. The pcDNA3-FLAG-APP_{695} (18), pcDNA3-ADAM10-HA, and pcDNA3-ADAM17-HA (19) have been described previously. The plasmid encoding human PS1 cDNA, pcDNA3-PS1wt, was described previously (12). FAD-linked mutations were introduced by PCR-based site-directed mutagenesis to generate pcDNA4-PS1M146L, pcDNA3.1-PS1L166P, pcDNA4-PS1A246E, pcDNA4-PS1R278T, pcDNA4-PS1L286V, and pcDNA4-PS1A434C. HEK293 cells were transfected with these plasmids, and cells stably expressing PS1 were cloned.

Cells, Transfection, and Western Blot Assay—Mouse embryonic fibroblasts (MEFs) derived from ADAM 10 homozygous (−/−) and heterozygous (+/−) gene knock-out mice were described previously (20). HEK293, Neuro 2a, and MEFs ($0.3–1.0 \times 10^6$) were subjected to gene transfection with the indicated amounts of various combinations of plasmids in Lipofectamine 2000 or Lipofectamine according to the manufacturer's protocol (Invitrogen). After transfection for 24 h, the medium was changed, and the cells were cultured for a further ~24 h. In order to analyze secreted proteins, sAlc and sAPP were recovered from the conditioned medium by immunoprecipitation with an anti-FLAG antibody and Protein G-Sepharose. To analyze cellular proteins, the cells were harvested and lysed in Hepes-buffered saline with Triton X-100 (12). The cell lysates and the immunoprecipitates were analyzed by Western blotting with the indicated antibodies, detected by ECL (GE Healthcare), and quantified using the VersaDoc imaging System (Bio-Rad).

Antibodies—Anti-Alc_{α} polyclonal rabbit UT135 antibody was raised against a peptide that was composed of Cys plus the sequence between positions 839 and 851 (NPHPFAVVPSTAT+C) of human $Alc_{\alpha 1}$. The anti-Alc_{α} monoclonal antibody 3B5 was raised against a peptide that was composed of Cys plus the sequence between positions 821 and 826 (C+FVHPEH). The anti-Alc_{β} polyclonal rabbit UT143 antibody was raised against a GST-fusion protein containing the sequence between 819 and 847 (FLHRGHQPPPEMAGHSLASSHRNSMIPSA) of human Alc_{β}. The anti-Alc_{γ} polyclonal antibody UT166 was raised against a peptide composed of Cys plus the sequence between positions 823 and 834 (C+IQHSSVVPSIAT) of human Alc_{γ}. These Alc-specific antibodies were raised against the extracellular juxtamembrane region of Alc family proteins and were specific for their respective p3-Alc targets with the exception of UT166, which exhibited cross-reactivity to p3-Alc_{α} (data not shown). These antibodies were used to isolate and detect p3-Alc. The regions recognized by the specific antibodies are shown in supplemental Fig. S1. The anti-Alc_{α} and anti-Alc_{β} cytoplasmic domain antibodies UT83 and UT99 were described previously (12). The monoclonal anti-FLAG (M2, Sigma) and anti-HA (12CA5, BD Biosciences) antibodies were purchased from vendors as noted. Anti-mouse and anti-rabbit IgG peroxidase-linked species-specific whole antibodies were purchased from GE Healthcare.

[6] S. Hata, S. Fujishige, Y. Araki, M. Taniguchi, K. Urakami, E. Peskind, H. Akatsu, M. Araseki, R. Martins, M. Maeda, A. Levey, K. Chung, T. Montine, J. Leverenz, A. Fagan, A. Goate, R. Bateman, D. Holtzman, T. Yamamoto, T. Nakaya, S. Gandy, and T. Suzuki, submitted for publication.

MALDI-TOF/MS and -MS/MS Analysis of p3-Alc Secreted into the Cultured Medium and Human CSF—HEK293 cells ($8–9 \times 10^6$) were transfected with the plasmids (6 μg) pcDNA3-hAlc$_{\alpha 1}$, pcDNA3.1-hAlc$_\beta$, or pcDNA3.1-hAlc$_\gamma$ in Lipofectamine 2000 for 24 h. The p3-Alc$_\alpha$, p3-Alc$_\beta$, and p3-Alc$_\gamma$ that were secreted into the medium (6 ml) were recovered by immunoprecipitation with the polyclonal anti-p3-Alc$_\alpha$ U135 (4 μg, affinity-purified), polyclonal anti-p3-Alc$_\beta$ UT143 (100 μl of serum), and polyclonal anti-p3-Alc$_\gamma$ UT166 (100 μl of serum) antibodies, respectively, and Protein G-Sepharose beads. The beads were sequentially washed with Wash buffer I (10 mM Tris-HCl (pH 8.0), 140 mM NaCl, 0.1% (w/v) *n*-octyl-D-glucoside, 0.025% (w/v) sodium azide) and Wash buffer II (10 mM Tris-HCl (pH 8.0), 0.025% (w/v) sodium azide), and then samples were eluted with trifluoroacetic acid/acetonitrile/water (1:20:20) saturated with sinapinic acid. The dissolved samples were dried on a target plate, and MALDI-TOF/MS analysis was performed using a UltraflexII TOF/TOF (Bruker Daltonics, Bremen, Germany). Molecular masses were calibrated using the peptide calibration standard (Bruker Daltonics).

For experiments with human samples (CSF was furnished by Choju Medical Institute and Tottori University), a mixture of human CSF (0.3–1.0 ml) from 5–10 individuals (70–90-year-old AD patients with clinical dementia rating 0.5, 1, or 2) was subjected to immunoprecipitation with the above antibodies. The beads were washed, and samples were eluted as described above. The dissolved samples were dried on a target plate, and MALDI-TOF/MS analysis was performed as described above.

The quantitative accuracy of mass spectrometric analysis with immunoprecipitation was confirmed by studies with a mixture of synthetic p3-Alc$_\alpha$35 and p3-Alc$_\alpha$39 peptides and a mixture of synthetic p3-Alc$_\beta$37 and p3-Alc$_\beta$40 peptides (supplemental Fig. S2). Molecular masses of p3-Alc species measured with MALDI-TOF/MS were compared with theoretical values to show the accuracy of mass spectrometric analysis (supplemental Table 1).

In all immunoprecipitation studies prior to MALDI-TOF/MS analysis, protease inhibitor mixture (5 μg/ml chymostatin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin) was added in samples (cell media, CSF, cell/brain lysates) to prevent nonspecific proteolysis (12).

Aβ40 and Aβ42 levels were quantified with sandwich enzyme-linked immunosorbent assay systems (Immuno-Biological Laboratories, Takasaki, Japan). Informed consent for the use of human CSF in this study was obtained from the patients and their families.

RESULTS

Identification of the Proteinases That Initiate Processing of Alc$_\alpha$, Alc$_\beta$, and Alc$_\gamma$—We have previously shown that the initial cleavage of Alc occurs in the extracellular juxtamembrane region and liberates the Alc ectodomain (sAlc) (12). The cell-associated C-terminal Alc fragment (AlcCTF) is proteolyzed by an intramembrane-cleaving protease, and both Alc cleavages are apparently catalyzed by the identical secretase enzymes that process APP (12). ADAM 10 is the enzyme that usually underlies the constitutive α-site cleavage of APP (16, 17); therefore, we initially examined whether this ADAM also cleaved Alc. FLAG-Alc$_\alpha$, FLAG-Alc$_\beta$, FLAG-Alc$_\gamma$, or FLAG-APP was expressed in MEFs from ADAM 10-deficient, homozygous (−/−) and heterozygous (+/−) knock-out mice (20). We recovered sAlc isoforms by immunoprecipitation of conditioned media with an anti-FLAG antibody; the immunoprecipitates and cell lysates were analyzed by Western blotting with M2 (Fig. 1, *A–D*).

As expected, we found that the primary cleavage of APP was deficient in homozygous (−/−) but not in heterozygous (+/−) ADAM 10-deficient MEF cells (Fig. 1*D*); secretion of sAPP by −/− cells was 40% below the level of sAPP in ADAM $10^{+/-}$ cells (compare *lane 2* with *lane 1* in *D*). Release of sAlc$_\alpha$, sAlc$_\beta$, and sAlc$_\gamma$ was deficient in ADAM $10^{-/-}$ cells to a similar extent (Fig. 1, *A–C*). These observations strongly suggest that Alc$_\alpha$, Alc$_\beta$, and Alc$_\gamma$ are subjected to primary cleavage by ADAM 10 in a fashion similar to the α-secretase processing of APP.

The cleavage of Alc family proteins by ADAM 10 was rescued following expression of HA-tagged ADAM 10 in ADAM $10^{-/-}$ cells (Fig. 1, *E–H*). In this experiment, we initially confirmed that sAPP secretion was restored by the expression of an exogenous cDNA for ADAM 10 (Fig. 1*H*). Secretion of sAPP from ADAM $10^{-/-}$ cells that were expressing exogenous ADAM 10 increased ~1.7-fold as compared with the level of sAPP that was secreted from ADAM $10^{-/-}$ cells. As expected, we also found that the level of intracellular mature APP holoprotein (which is the substrate of ADAM 10 that gives rise to sAPP) was diminished in ADAM $10^{-/-}$ cells following expression of exogenous ADAM 10. The wild type secretion patterns of sAlc$_\alpha$, sAlc$_\beta$, and sAlc$_\gamma$ were restored in ADAM $10^{-/-}$ cells following expression of exogenous ADAM 10 (Fig. 1, *E–G*). In a dose-dependent fashion, these cells exhibited a 1.8–2.2-fold increase in sAlc in the medium when compared with sAlc secretion from ADAM $10^{-/-}$ cells (Fig. 1, *E–G*). Taken together, these results suggest that ADAM 10 is an important secretase that proteolyzes Alc$_\alpha$, Alc$_\beta$, and Alc$_\gamma$.

APP is also cleaved by another α-secretase, ADAM 17 (15, 16, 21), although ADAM 17 is largely expressed in glial cells rather than neurons (22). Cleavage of the Alc family was examined in Neuro 2a cells overexpressing an HA-tagged ADAM 17. We initially confirmed that ADAM 17 cleaved APP in these cells (supplemental Fig. S3). As expected, increased expression of ADAM 17 enhanced sAPPα secretion, indicating that, in our cells, exogenously expressed ADAM17 was active in proteolysis of APP. The secretion of sAlc$_\alpha$, sAlc$_\beta$, and sAlc$_\gamma$ from Neuro 2a cells expressing exogenous ADAM 17 was then assayed and quantified using experimental conditions that were identical to those used for the analysis of APP. We found that the secretion of sAlc (sAlc$_\alpha$, sAlc$_\beta$, and sAlc$_\gamma$) increased in a dose-dependent manner in response to the exogenous expression of exogenous ADAM 17. These observations suggest that, like ADAM 10, ADAM 17 also cleaves Alc$_\alpha$, Alc$_\beta$, and Alc$_\gamma$ as well as APP. Taken together, these data show that Alc and APP are metabolized by the same two APP α-secretases.

Identification of the Primary and Secondary Cleavage Sites of Alc—ADAM 10 and ADAM 17 have been identified as the α-secretases that cleave the peptide bond between Lys^{612} and Leu^{613} of APP$_{695}$, thereby destroying the Aβ domain (Fig. 2). This cleavage at the juxtamembranous region triggers a sec-

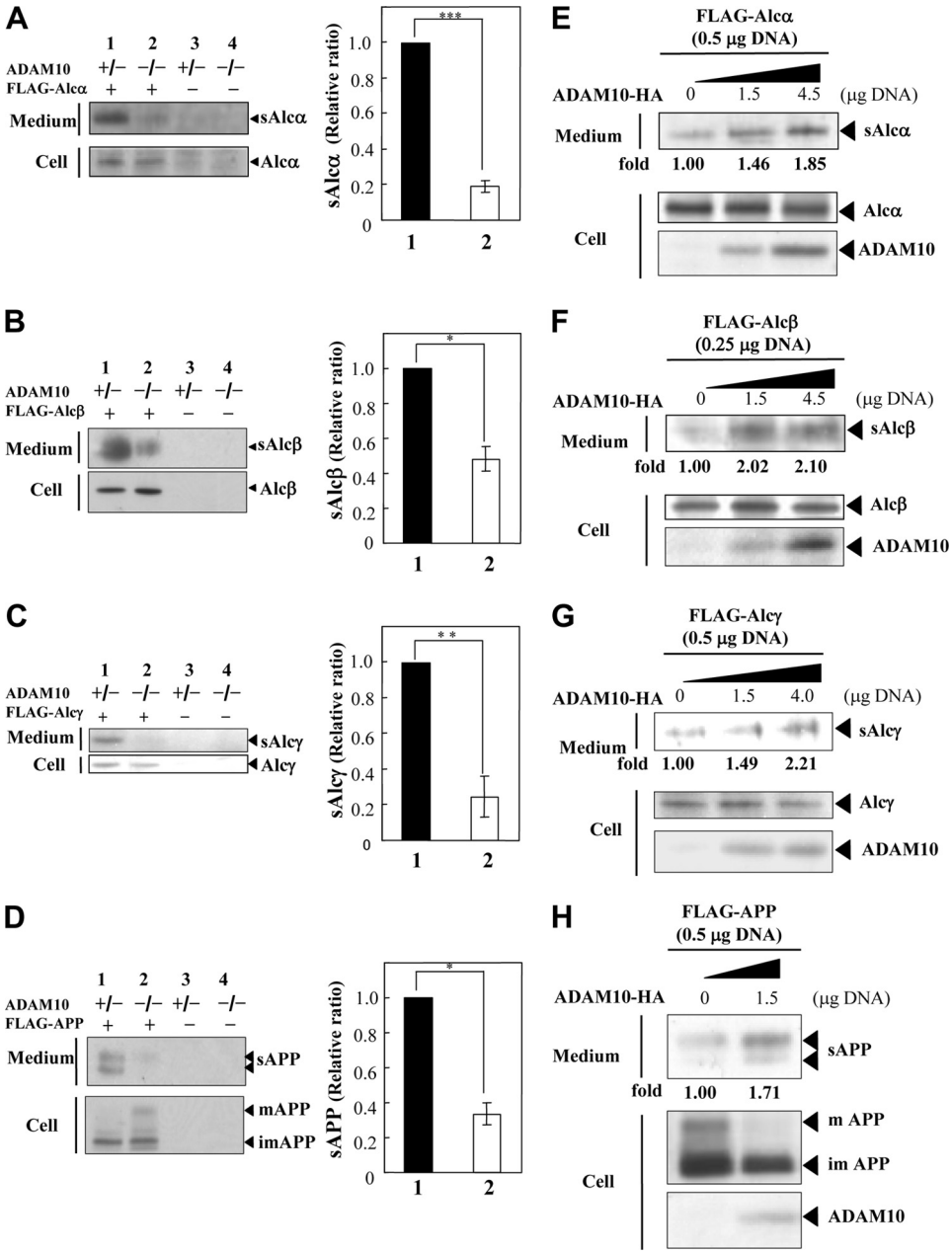


FIGURE 1. **Evidence that Alc family proteins and APP undergo identical processing by ADAM 10 α-secretase.** *A–D*, generation of sAlc and sAPP in ADAM 10-deficient cells. ADAM 10 homozygous (−/−, *lanes 2* and *4*) and heterozygous (+/−, *lanes 1* and *3*) deficient MEFs were transiently transfected with 1.5 μg of pcDNA3-FLAG-hAlc$_{\alpha 1}$ (+ in *A*), pcDNA3.1-FLAG-hAlc$_{\beta}$ (+ in *B*), pcDNA3.1-FLAG-hAlc$_{\gamma}$ (+ in *C*), and pcDNA3-FLAG-APP$_{695}$ (+ in *D*), or vector alone (− in *A–D*). Culture medium (1 ml) was immunoprecipitated with an anti-FLAG M2 antibody. The immunoprecipitates of conditioned medium (*Medium*) and cell lysate (*Cell*; 20 μg of protein) were analyzed by Western blotting with M2 (*left panels*). The levels of sAlc and sAPP in *lane 2* are indicated as ratios relative to the levels shown in *lane 1*, which was assigned a reference value of 1.0 (values represent means ± S.E.). The *asterisks* indicate statistical significance as determined by Student's *t* test ($n = 3$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$) (*right panels*). *E–H*, rescue of the primary α-secretase type cleavage of Alc family proteins and APP in ADAM 10-deficient cells by expression of exogenous ADAM 10. ADAM 10-deficient MEFs were transiently transfected with the indicated amount of pcDNA3-ADAM10-HA in the presence of the indicated amounts of pcDNA3-FLAG-hAlc$_{\alpha 1}$ (*E*), pcDNA3.1-FLAG-hAlc$_{\beta}$ (*F*), pcDNA3.1-FLAG-hAlc$_{\gamma}$ (*G*), or pcDNA3-FLAG-APP$_{695}$ (*H*). Empty vector was also added to standardize the amount of plasmid used. Conditioned culture medium (*Medium*; 1 ml) was immunoprecipitated with the anti-FLAG M2 antibody and analyzed by Western blotting with the same antibody. Cell lysates (*Cell*; 20 μg of protein) were analyzed by Western blotting with M2 for Alc family proteins and APP or with the anti-HA antibody for ADAM 10. The levels of sAlc and sAPP are indicated as -fold changes with respect to the levels detected when ADAM 10-HA was not expressed; this was assigned a reference value of 1.0. *mAPP*, mature APP; *imAPP*, immature APP.

ondary intramembranous γ-cleavage, which results in the secretion of a nonamyloidogenic 3-kDa peptide (p3) composed of 24–26 amino acids (for a review, see Refs. 7 and 8). We have previously reported that Alc family proteins, like APP family proteins, are also cleaved within the intramembranous region. In our earlier study, we showed that the small peptide, previously designated "β-Alc," was secreted by the secondary cleavage of alcadein C-terminal fragments (CTFs) by γ-secretase, but the exact cleavage sites were not previously identified (12). Now we have demonstrated that the peptide previously designated "β-Alc" is generated by the successive action of α- and γ-secretases. Furthermore, β-secretase/β-APP site-cleaving enzyme (BACE) (23) is not likely to be involved in the primary cleavage of Alc$_{\alpha}$, Alc$_{\beta}$, and Alc$_{\gamma}$ (supplemental Fig. S4). In HEK293 cells overexpressing human BACE1, the generation of sAPPβ and CTFβ, the products of APP cleaved by BACE, increased (supplemental Fig. S4*A*), whereas no remarkable changes were observed in the cleavages of Alc$_{\alpha}$, Alc$_{\beta}$, and Alc$_{\gamma}$ (supplemental Fig. S4*B*) in quality. The results suggest that Alcs are not a preferential substrate for BACE. Therefore, the β-Alc peptide has been renamed "p3-Alc" so as to maintain consistency with an APP-based nomenclature for the peptides produced by the proteolytic processing of Alc.

The recent production of anti-Alc$_{\alpha}$, anti-Alc$_{\beta}$, and anti-Alc$_{\gamma}$ antibodies raised against the respective extracellular juxtamembrane sequences has enabled us to recover p3-Alc$_{\alpha}$, p3-Alc$_{\beta}$, and p3-Alc$_{\gamma}$ secreted into the culture medium by HEK293 cells expressing Alc$_{\alpha}$, Alc$_{\beta}$, and Alc$_{\gamma}$. The molecular masses of p3-Alc$_{\alpha}$, p3-Alc$_{\beta}$, and p3-Alc$_{\gamma}$ were determined by analyses with MALDI-TOF/MS. The spectrum of p3-Alc$_{\alpha}$ showed two major peaks with molecular masses of 3804.8 and 4007.0 (supplemental Fig. S5*A*, indicated by *arrows*). The spectrum of p3-Alc$_{\beta}$ showed two

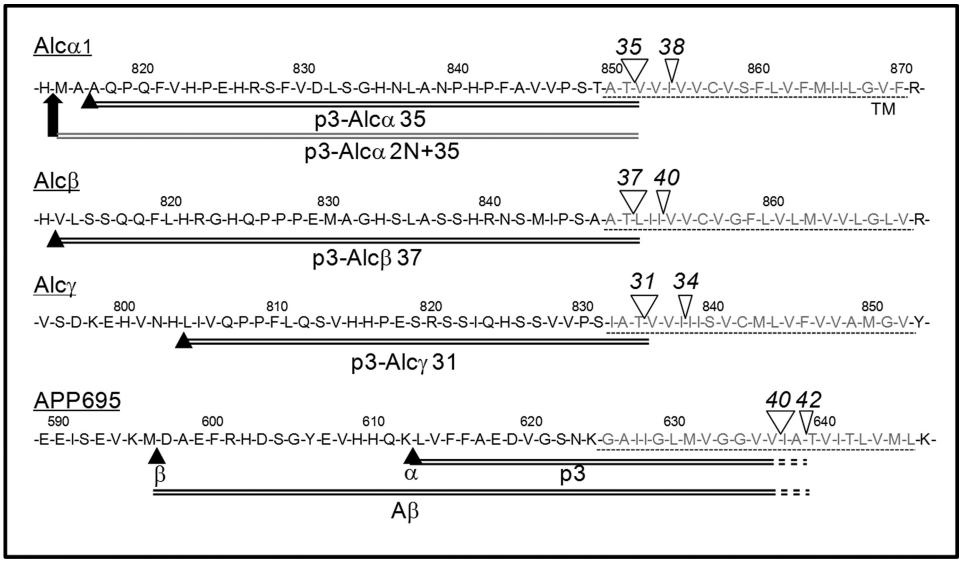


FIGURE 2. **Amino acid sequence of p3-Alc$_\alpha$, p3-Alc$_\beta$, and p3-Alc$_\gamma$.** The amino acid sequences of the major p3-Alc species are shown. p3-Alc$_\alpha$35, p3-Alc$_\beta$37, and p3-Alc$_\gamma$31, are indicated as *double-underlined letters* along with the sequences of p3 and Aβ40 of APP and the sequence of p3-Alc$_\alpha$2N+35. The major primary (*closed arrowheads*) and secondary (*open arrowheads*) cleavage sites of Alc$_{\alpha1}$, Alc$_\beta$, and Alc$_\gamma$ are indicated together with those of APP (α, the cleavage site by α-secretase or ADAM 10/17; β, the cleavage site by β-secretase or BACE). Another primary cleavage site of Alc$_{\alpha1}$ is also indicated (*arrow*). *Numbers* on amino acids indicate their positions. *Gray letters* with a *broken underline* along with the sequence indicate the putative transmembrane region suggested by the Swiss-Prot protein knowledge base. The N terminus of p3-Alc$_\alpha$ is Ala^{817} as determined by MALDI-TOF/MS analysis; however, that of p3-Alc$_\alpha$2N+"X" is Met^{815}. The p3-Alc$_\alpha$35 with N-terminal Ala^{817} is a major species in CSF, whereas the p3-Alc$_\alpha$2N+35 with N-terminal Met^{815} is a major species in HEK293 cells (see supplemental Figs. S5*A*, S6*A*, and S7*A*). The N terminus of p3-Alc$_\beta$ is Val^{813}, which was determined by MALDI-MS/MS analysis (see supplemental Figs. S5*B* and S6*B*). The N terminus of p3-Alc$_\gamma$ is Leu^{804} (supplemental Fig. S5*C*); this coincides with the N-terminal sequence of Alc$_\gamma$ CTF that was determined using a gas phase peptide sequencer.

major peaks with molecular masses of 3964.0 and 4303.3 (supplemental Fig. S5*B*). The spectrum of p3-Alc$_\gamma$ showed one major peak with a molecular mass of 3377.8 Da and a minor peak with a molecular mass of 3689.2 Da (supplemental Fig. S5*C*).

The amino acid sequences of the respective major peak products were determined by MALDI-MS/MS analysis; the amino acid sequence of p3-Alc$_\alpha$ was composed of 35 and 37 amino acids, that of p3-Alc$_\beta$ was composed of 37 and 40 amino acids, and that of p3-Alc$_\gamma$ was composed of 31 and 34 amino acids (supplemental Fig. S5, *middle* and *right*; the sequences are also *double-underlined* in Fig. 2). Thus, the major p3-Alc$_\alpha$ species, p3-Alc$_\alpha$35 and p3-Alc$_\alpha$2N+35, that were secreted from HEK293 cells expressing Alc$_{\alpha1}$ were peptides that include the sequence from Ala^{817} to Thr^{851} and from Met^{815} to Thr^{851} of human Alc$_{\alpha1}$. The major p3-Alc$_\beta$ species, p3-Alc$_\beta$37 and p3-Alc$_\beta$40, that were secreted from HEK293 cells expressing Alc$_\beta$ were peptides that included the sequences from Val^{813} to Thr^{849} and from Val^{813} to Ile^{852} of human Alc$_\beta$. The major p3-Alc$_\gamma$ species, p3-Alc$_\gamma$31 and p3-Alc$_\gamma$34, that were secreted from HEK293 cells expressing Alc$_\gamma$ were peptides that included the sequences from Leu^{804} to Thr^{834} and from Leu^{804} to Ile^{837} of human Alc$_\gamma$. These p3-Alc$_\alpha$35, p3-Alc$_\beta$37, and p3-Alc$_\gamma$31 species are also the major species in human CSF (see supplemental Fig. S6).

In our previous report, we used a gas phase protein sequencer to identify Ala^{816} (numbering for the Alc$_{\alpha1}$ isoform) as the N-terminal amino acid of the Alc$_\alpha$ CTF (12). We therefore expected that ADAM 10 and ADAM 17 would cleave Alc$_\alpha$ at the peptide bond between Met^{815} and Ala^{816}. In the present study, we used MALDI-MS/MS to show that the N-terminal amino acid is Ala^{817} for p3-Alc$_\alpha$35 and Met^{815} for p3-Alc$_\alpha$2N+35) (supplemental Fig. S5*A*). The N-terminal Met^{815} and/or Ala^{816} of Alc$_\alpha$ CTF generated by primary cleavage may be removed by an N-terminal exopeptidase during p3-Alc$_\alpha$ secretion from cells.

We were unable to determine the N-terminal amino acid sequence of the Alc$_\beta$ CTF using a gas phase protein sequencer; however, Val^{813}, which was identified by MALDI-MS/MS analysis of p3-Alc$_\beta$37 in this study (supplemental Figs. S5*B* and S6*B*), is likely to be the N-terminal amino acid of Alc$_\beta$ CTF. We also determined the N-terminal sequence of Alc$_\gamma$ CTF (Leu^{804}–Ile-Val-Gln-Pro-Pro-Phe-Leu-Gln^{812}) using a gas phase protein sequencer. This result was identical to the result obtained from the MALDI-MS/MS analysis (supplemental Fig. S5*C*), indicating that the primary cleavage site of Alc$_\gamma$ is the peptide bond between His^{803} and Leu^{804}. The primary cleavage sites for the Alc family are shown in Fig. 2 (*black arrowhead* and *arrow*); these sites are also compared with the primary cleavage sites (α- and β- sites) of APP$_{695}$.

MALDI-TOF/MS and -MS/MS analyses enabled us to determine the secondary cleavage sites of Alc$_\alpha$, Alc$_\beta$, and Alc$_\gamma$ (supplemental Figs. S5 and S6). The major secondary cleavage site of Alc$_\alpha$ is the peptide bond between Thr^{851} and Val^{852}; cleavage at this site generates p3-Alc$_\alpha$35 and p3-Alc$_\alpha$2N+35. The major secondary cleavage site of Alc$_\beta$ is the peptide bond between Thr^{849} and Leu^{850}; cleavage at this site generates p3-Alc$_\beta$37. Another secondary cleavage site of Alc$_\beta$ is the peptide bond between Ile^{852} and Val^{853}, which generates p3-Alc$_\beta$40. The major secondary cleavage site of Alc$_\gamma$ is the peptide bond between Thr^{834} and Val^{835}, which generates p3-Alc$_\gamma$31. Another secondary cleavage site of Alc$_\gamma$ includes the peptide bond between Ile^{837} and Ile^{838}, which generates p3-Alc$_\gamma$34. Secondary cleavage sites (*open arrowheads*) of Alc family proteins, determined by MALDI-MS/MS analysis, are also shown in Fig. 2, together with the major secondary γ-secretase-dependent cleavage sites of APP that generate Aβ40 and Aβ42.

Modulation of the γ-Cleavage Sites of Alc by FAD-linked PS1 Mutations—We have previously reported that secondary cleavage of Alc$_\alpha$ is performed by a PS1-dependent γ-secretase (12). PS1 is known to be the catalytic component of the γ-secretase complex, and several mutations in the PS1 and PS2 genes cause FAD. Several FAD-linked PS1 mutations are known to alter the

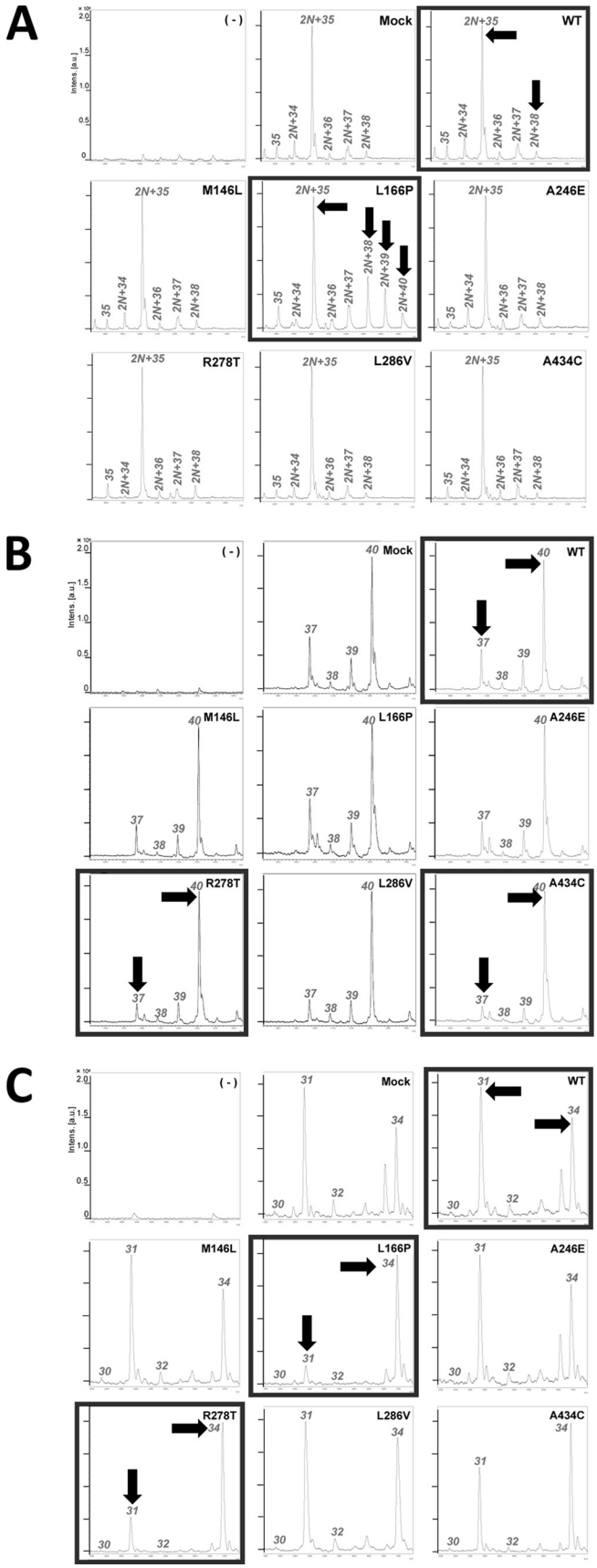


FIGURE 3. **Displacement of the intramembranous cleavage sites of Alc_{α}, Alc_{β}, and Alc_{γ} in cells expressing FAD-linked mutations in PS1.** *A*, representative MS spectra of p3-Alc_{α} secreted from cells expressing wild type PS1 or FAD-linked PS1 mutants. HEK293 cells with (*WT*) or without (*Mock*) the stable expression of wild type PS1, FAD-linked PS1 M146L, L166P, A246E, R278T, L286V, and A434C mutations were transfected with or without (−) pcDNA3-h$Alc_{\alpha 1}$. The culture medium (6 ml) of cells expressing $Alc_{\alpha 1}$ was immunoprecipitated with UT135, and the immunoprecipitates were subjected to MALDI-TOF-MS analysis. *35*, p3-Alc_{α}35; *2N+34*, p3-Alc_{α}2N+34; *2N+35*, p3-Alc_{α}2N+35; *2N+36*, p3-Alc_{α}2N+36; *2N+37*, p3-Alc_{α}2N+37; *2N+38*, p3-Alc_{α}2N+38; *2N+39*, p3-Alc_{α}2N+39; *2N+40*, p3-Alc_{α}2N+40. *B*, representative MS spectra of p3-Alc_{β} secreted from cells expressing wild type PS1 or FAD-linked PS1 mutants. HEK293 cells were transfected as described above with or without (−) pcDNA3-hAlc_{β}. The culture medium (6 ml) of cells expressing Alc_{β} was immunoprecipitated with UT143, and the immunoprecipitates were subjected to MALDI-TOF-MS analysis. *37*, p3-Alc_{β}37; *38*, p3-Alc_{β}38; *39*, p3-Alc_{β}39; *40*, p3-Alc_{β}40. *C*, representative MS spectra of p3-Alc_{γ} secreted from cells expressing wild type PS1 or FAD-linked PS1 mutants. HEK293 cells were transfected as described above with or without (−) pcDNA3-hAlc_{γ}. The culture medium (6 ml) of cells expressing Alc_{γ} was immunoprecipitated with UT166, and the immunoprecipitates were subjected to MALDI-TOF-MS analysis. *30*, p3-Alc_{γ}30; *31*, p3-Alc_{γ}31; *32*, p3-Alc_{γ}32; *34*, p3-Alc_{γ}34.

cleavage of APP β CTFs and increase the generation of C-terminally altered Aβ species, such as Aβ42 (24–27). Because Alc and APP are cleaved by the identical γ-secretase, we sought to determine whether Alc family proteins demonstrate displaced secondary cleavage sites in the presence of FAD-linked PS1 mutations. HEK293 cells stably expressing wild type PS1 (*WT* in Fig. 3) or PS1 carrying either FAD-linked M146L, L166P, A246E, R278T, L286V, or A434C mutations or vector alone (*Mock*) were co-transfected with Alc_{α}, Alc_{β}, and Alc_{γ} or without plasmid (−).

p3-Alc_{α}, p3-Alc_{β}, and p3-Alc_{γ} were recovered from the culture medium by immunoprecipitation with anti-Alc_{α} (UT135), anti-Alc_{β} (UT143), and anti-Alc_{γ} (UT166) antibodies and analyzed by MALDI-TOF/MS (Fig. 3). The expression of PS1 was confirmed by Western blotting with anti-PS1 N- and C-terminal antibodies (data not shown), and the effect of the FAD-linked mutations on the Aβ42/Aβ40 ratio was examined in the medium of HEK293 cell lines, each of which expressed PS1 stably plus APP_{695} transiently (Fig. 4*A*, *lower right*).

As expected, HEK293 cells stably expressing wild type PS1 (*WT*) or vector alone (*Mock*) plus Alc_{α} generated p3-Alc_{α} species with C-terminal end of Thr^{851} (p3-Alc_{α}2N+35) as the major species (Fig. 3*A*). We determined the amino acid sequences of this p3-Alc_{α} species with MALDI-MS/MS analysis and confirmed that this is p3-Alc_{α}2N+35 and not p3-Alc_{α}37. This p3-Alc_{α}2N+35 was a minor p3-Alc_{α} species in human CSF in which p3-Alc_{α}35 was major (*left panel* in supplemental Figs. S5*A* and S6*A*). In any case, HEK293 cells expressing PS1 (*WT*) generated largely p3-Alc_{α} species with the C-terminal Thr^{851} (*right panel* in supplemental Figs. S5*A* and S6*A*).

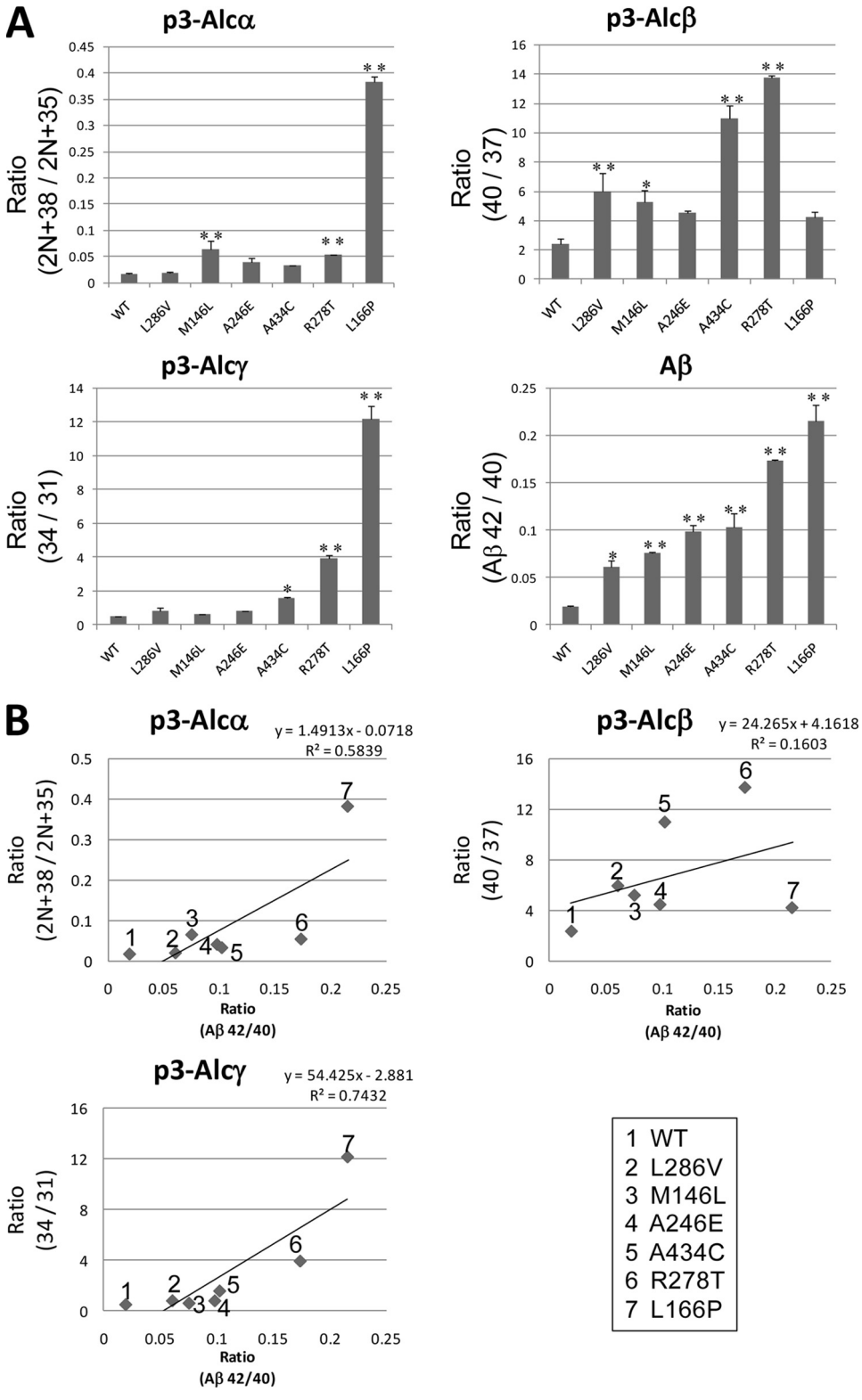
In contrast, HEK293 cells expressing FAD-linked PS1 mutants generated qualitatively altered p3-Alc_{α} (Fig. 3*A*). This was especially remarkable in the medium of cells expressing PS1 carrying the L166P mutation; there were increases in the levels of p3-Alc_{α}2N+38 that included a peptide of Met^{815}–Ilu^{854} (Fig. 4*A*, *upper left*). The p3-Alc_{α}2N+39 that included a peptide of Met^{815}–Val^{855}, and p3-Alc_{α}2N+40 that included a peptide of Met^{815}–Val^{856} were also increased (*arrows* in Fig. 3*A*). This L166P mutation is known to increase Aβ42/40 ratio greatly (Fig. 4*A*, *lower right*). Other FAD-linked PS1

mutations, such as R278T, A434C, and A246E, showed a moderate effect to increase Aβ42/40, whereas these mutations appeared to have little or almost no effect on the increase of minor species, including p3-Alcα2N+38 (Fig. 4*A*, compare *upper left* to *lower right*).

We then investigated the generation of p3-Alc$_\beta$ and p3-Alc$_\gamma$ from HEK293 cells expressing Alc$_\beta$ or Alc$_\gamma$ together with PS1 carrying FAD-linked mutations (Fig. 3, *B* and *C*, and Fig. 4*A*). HEK293 cells stably expressing wild type PS1 (*WT*) or vector alone (*Mock*) plus Alc$_\beta$ generated major p3-Alc$_\beta$37 and p3-Alc$_\beta$40 (see Fig. 2 for the amino acid sequence). HEK293 cells expressing FAD-linked PS1 mutants, especially R278T and A434C, demonstrated remarkable decreases in levels of p3-Alc$_\beta$37 (*arrows* in Fig. 3*B*). We then created minor species/major species ratios for p3-Alc$_\beta$40 to p3-Alc$_\beta$37, which were compared with the Aβ42/40 ratio (Fig. 4*A*, compare *upper right* to *lower right*). In cultured cells, p3-Alc$_\beta$40 is a major species rather than p3-Alc$_\beta$37, whereas p3-Alc$_\beta$37 is a major species on CSF (compare Fig. 3*B* to Fig. 5*B*). Therefore, in the case of p3-Alcβ, we sought the p3-Alc$_\beta$40/p3-Alc$_\beta$37 ratio as minor/major ratio. In contrast to the ratio p3-Alc$_\alpha$2N+38/p3-Alc$_\alpha$2N+35, L166P did not show remarkable change for the ratio p3-Alc$_\beta$40/p3-Alc$_\beta$37.

HEK293 cells stably expressing wild type PS1 or vector alone (mock) plus Alc$_\gamma$ generated p3-Alc$_\gamma$31 and p3-Alc$_\gamma$34 as major peptide metabolites (see Fig. 2 for the amino acid sequence). HEK293 cells expressing FAD-linked PS1 mutants, especially L166P and R278T, demonstrated remarkable decreases in the levels of p3-Alc$_\gamma$31 along with increases in the levels of p3-Alc$_\gamma$34 (*arrows* in Fig. 3*C*). The alteration of the p3-Alc$_\gamma$34/p3-Alc$_\gamma$31 ratio showed some similarity to that of the p3-Alc$_\alpha$2N+38/p3-Alc$_\alpha$2N+35 ratio in various PS1 mutations but differed from the alteration of the p3-Alc$_\beta$40/p3-Alc$_\beta$37 ratio (Fig. 4*A*, compare *lower left* to *upper right*).

Six FAD-linked PS1 mutants (L166P, R278T, A434C, A246E, M146L, and L286V) increased the Aβ42/40 ratio at various magnitudes (Fig. 4*A*, *lower right*), whereas some of them did not show a similar effect to alter the minor species/major species ratio in the p3-Alc species. To analyze the correlation coefficients of γ-cleavage alteration between APP and Alc, the Aβ42/40 ratio was plotted to certain p3-Alc minor/major ratios



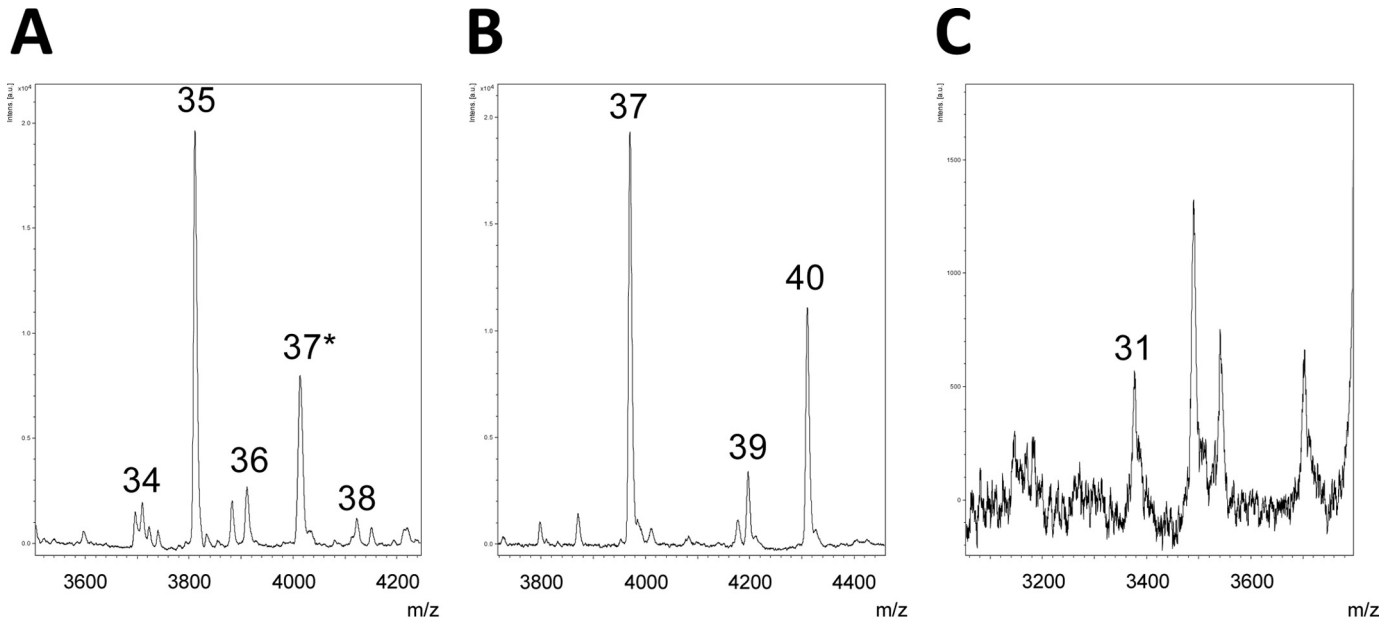


FIGURE 5. **Representative MS spectra of p3-Alc peptides in human CSF.** Human p3-Alc$_\alpha$ (*A*), p3-Alc$_\beta$ (*B*), and p3-Alc$_\gamma$ (*C*) species in CSF. The 300 μl (*A* and *B*) or 1 ml (*C*) of human CSF mixture was subjected to immunoprecipitation with UT135 (*A*, 8 μg of IgG fraction), UT143 (*B*, 100 μl of serum), and UT166 (*C*, 100 μl of serum) antibodies, respectively. The precipitates were analyzed for molecular mass with MALDI-TOF/MS. *A*, *34*, p3-Alc$_\alpha$34; *35*, p3-Alc$_\alpha$35; *36*, p3-Alc$_\alpha$36; *37**, a mixture of p3-Alc$_\alpha$37 and p3-Alcα2N+35 (see supplemental Fig. S7*B*); *38*, p3-Alc$_\alpha$38; *39*, p3-Alc$_\alpha$39. *B*, *35*, p3-Alc$_\beta$35; *36*, p3-Alc$_\beta$36; *37*, p3-Alc$_\beta$37; *38*, p3-Alc$_\beta$38; *39*, p3-Alc$_\beta$39; *40*, p3-Alc$_\beta$40. *C*, *31*, p3-Alc$_\gamma$31.

(Fig. 4*B*). We have confirmed that the p3-Alc minor/major ratio of peak area detected by MALDI-TOF/MS analysis correlated well to those of theoretically calculated values in quantity (supplemental Fig. S2). As expected from the Fig. 4*A* on the basis of visual inspection, p3-Alc$_\beta$ showed a property different from p3-Alc$_\alpha$ and p3-Alc$_\gamma$ in correlation coefficient to Aβ42/40. The p3-Alc$_\alpha$2N+38/p3-Alc$_\alpha$2N+35 and p3-Alc$_\gamma$34/p3-Alc$_\gamma$31 ratios showed a strong correlation to the Aβ42/40 ratio ($R^2 >$ 0.5), whereas the p3-Alc$_\beta$40/p3-Alc$_\beta$37 ratio showed a positive but weak correlation to the Aβ42/40 ratio. These results suggest that phenotypes of γ-secretase dysfunction appeared in the altered cleavages of APP and/or Alc family proteins in variety. The magnitudes of C-terminal alteration of p3-Alc$_\alpha$, p3-Alc$_\beta$, and p3-Alc$_\gamma$ along with Aβ were not equivalent, suggesting that one type of γ-secretase dysfunction does not appear in the phenotype equivalently in the cleavage of type I membrane proteins.

The p3-Alc Species in Human CSF—Secreted Aβ species are detectable in human CSF, and it has been proposed that they might be useful as possible biomarkers or endophenotypes for the diagnosis and classification of AD (28–31). Above, we discussed how p3-Alc speciation and Aβ42/40 were modulated by certain FAD mutant PS1 molecules, which reflect γ-secretase dysfunction (Figs. 3 and 4). In our separate study, we sought to characterize CSF p3-Alc/Aβ relationships with a special interest in determining whether sporadic AD CSF might display a covariance between p3-Alc and Aβ as a potential indicator of underlying γ-secretase dysfunction.[6] We herein examined whether the p3-Alc$_\alpha$, p3-Alc$_\beta$, and p3-Alc$_\gamma$ species that were identified in cell study were present in human CSF. The p3-Alc species were recovered from pooled human CSF samples by immunoprecipitation with anti-p3-Alc$_\alpha$ 3B5, anti-p3-Alc$_\beta$ UT143, and anti-p3-Alc$_\gamma$ UT166 antibodies, followed by analyses with MALDI-TOF/MS (Fig. 5 and supplemental Fig. S6).

The UT135 or 3B5 antibody recovered a peptide of molecular mass 3804.6, which was assigned the identity of p3-Alc$_\alpha$35 by MALDI-MS/MS analysis (Fig. 5*A* and supplemental Fig. S6*A*).

FIGURE 4. **Analysis of conditioned media of cultured cells.** Covariant relationships linking the Aβ42/40 ratio with the ratios of minor p3-Alc species to major p3-Alc species. *A*, changes of the p3-Alc$_\alpha$2N+38 (*minor*) to p3-Alc$_\alpha$2N+35 (*major*) ratio. HEK293 cells with wild type PS1 or FAD-linked PS1 mutants were transfected with pcDNA3-hAlc$_{\alpha1}$, and p3-Alc$_\alpha$ peptides were analyzed by MALDI-TOF-MS analysis in duplicate studies as described in the legend to Fig. 3. The peak area of p3-Alc$_\alpha$2N+38 was compared with that of p3-Alc$_\alpha$2N+35, and ratios (p3-Alc$_\alpha$2N+38/p3-Alc$_\alpha$2N+35) are indicated (*upper left panel*). Changes of p3-Alc$_\beta$40 to p3-Alc$_\beta$37 ratio are shown. HEK293 cells with wild type PS1 or FAD-linked PS1 mutants were transfected with pcDNA3-hAlc$_\beta$, and p3-Alc$_\beta$ peptides were analyzed. In cultured cells, p3-Alc$_\beta$40 is a major peptide rather than p3-Alc$_\beta$37, whereas p3-Alc$_\beta$37 is major in CSF (compare Fig. 3*B* with Fig. 5*B*). Therefore, we sought the p3-Alc$_\beta$40/p3-Alc$_\beta$37 ratio as a minor/major ratio. The peak area of p3-Alc$_\beta$40 was compared with that of p3-Alc$_\beta$37, and ratios (p3-Alc$_\beta$40/p3-Alc$_\beta$37) are indicated (*upper right panel*). Changes of the p3-Alc$_\gamma$34 to p3-Alc$_\gamma$31 ratio are shown. HEK293 cells with wild type PS1 or FAD-linked PS1 mutants were transfected with pcDNA3-hAlc$_\gamma$, and p3-Alc$_\gamma$ peptides were analyzed. We sought the p3-Alc$_\gamma$34/p3-Alc$_\gamma$31 ratio as a minor/major ratio. The peak area of p3-Alc$_\gamma$34 was compared with that of p3-Alc$_\gamma$31, and ratios (p3-Alc$_\gamma$34/p3-Alc$_\gamma$31) are indicated (*lower left panel*). Changes of the Aβ42/Aβ40 ratio (*Aβ42/40*) in the conditioned media of cells expressing PS1 (*WT*) and FAD-linked PS1 mutant, L286V, M146L, A246E, A434C, R278T, and L166P, are shown. HEK293 cells with wild type PS1 (WT) or FAD-linked PS1 mutants were transfected with pcDNA3-APP$_{695}$. The amounts of Aβ40 and Aβ42 secreted into the medium were quantified by sandwich enzyme-linked immunosorbent assay, and the Aβ42/Aβ40 ratio was determined (*lower right panel*). Duplicate assays ($n = 2$) were performed, and the significance of the effects of FAD-linked mutants of PS1 *versus* the wild type PS1 was examined by Dunnett's multiple comparisons test. *, $p < 0.05$; **, $p < 0.01$. *B*, covariant analysis of Aβ42/Aβ40 ratio with ratios of certain minor p3-Alc to the major p3-Alc species. *Graphs* showing the relation of ratios of p3-Alc$_\alpha$2N+38/p3-Alc$_\alpha$2N+35 (*upper left*), p3-Alc$_\beta$40/p3-Alc$_\beta$37 (*upper right*), and p3-Alc$_\gamma$34/p3-Alc$_\gamma$31 (*lower left*) with the Aβ42/40 ratio were indicated. *Numbers* in *panels* indicate the PS1 mutation (*WT* indicates wild type PS1) as shown in the *lower right panel*. *y*, inclination; R^2, correlation coefficient.

This antibody also recovered another peptide, which contained two components, namely p3-Alc$_\alpha$37 (molecular mass 4003.0, a peptide composed of Ala^{817}–Val^{853}) and p3-Alc$_\alpha$ 2N+35 (molecular mass 4006.9, a peptide composed of Met^{815}–Thr^{851}). The two components were distinguishable by reflector mode analysis (supplemental Fig. S7). p3-Alc$_\alpha$34, p3-Alc$_\alpha$36, and p3-Alc$_\alpha$38 were also identified in human CSF (supplemental Fig. S7*A*).

The antibody UT143 recovered peptides of molecular masses 3963.9 and 4303.2 (Fig. 5*B* and supplemental Fig. S6*B*), which were designated p3-Alc$_\beta$37 and p3-Alc$_\beta$40, respectively, according to MALDI-MS/MS analysis. The antibody UT166 recovered a peptide of molecular mass 3377.6 (Fig. 5*C* and supplemental Fig. S6*C*). The species with a molecular mass of 3377.6 (*arrow* in supplemental Fig. S6*C*) could not be analyzed by MALDI-MS/MS for amino acid sequence because the amount of peptide that was recovered by immunoprecipitation was not sufficient to obtain significant signals; however, the molecular mass coincided with that of p3-Alc$_\gamma$31 (see supplemental Fig. S5C). These results demonstrate that the intramembrane cleavage sites of Alc in humans are identical to those determined by our cell-conditioned medium studies and that p3-Alc$_\alpha$35, p3-Alc$_\alpha$37, p3-Alc$_\beta$37, p3-Alc$_\beta$40, and p3-Alc$_\gamma$31 are the major p3-Alc peptides recovered from human CSF.

To confirm the major p3-Alc peptide detected in CSF is the exact major product in brain, we compared p3-Alc$_\alpha$ species in mouse CSF and brain (supplemental Fig. S8). The major p3-Alc$_\alpha$35 in CSF was also major product in the brain, along with the similar profile of p3-Alc$_\alpha$ minor species between CSF and brain. Furthermore, the p3-Alc$_\alpha$ species profile of mouse CSF was identical to those of human CSF (supplemental Fig. S8), suggesting that p3-Alc$_\alpha$35 is major product in mouse and human brain.

DISCUSSION

In this study, we show that the Alc family proteins Alc$_\alpha$, Alc$_\beta$, and Alc$_\gamma$ are cleaved by APP α-secretases ADAM 10 and ADAM 17. Alc is expressed largely in neurons (1); therefore, ADAM 10 is thought to be the most likely candidate for Alc cleavage in the central nervous system because ADAM 17 is predominantly expressed in glial cells (22). Thus, in neurons, Alc and APP are primarily cleaved by the same enzymes when both proteins are liberated from their individual or coordinated complexes with X11L. These results agree with our previous report that APP and Alc are likely to be metabolized in a coordinated fashion (12). Although APP is also cleaved by BACE, Alc proteins are not likely to be major substrates for this enzyme (supplemental Fig. S4).

In addition to the similar mechanisms regulating the primary cleavage of Alc family proteins and APP, we also found that Alc family proteins are cleaved by the same γ-secretase complex as is APP (12). In this study, we have demonstrated that p3-Alc species with altered C termini are secreted together with the increased ratio of Aβ42 to Aβ40 by cells expressing FAD-linked PS1 mutants, although the magnitude of alteration was diversified among Alc family proteins in cells expressing various FAD-linked PS1 mutants. p3-Alc species are not prone to aggregation[7] and are detectable in human CSF; this raises the possibility that some p3-Alc changes in the pathological state might serve in clinical situations as markers of variant protein processing by the dysfunction of γ-secretase.

In the cell culture experiments, we observed that wild type and several pathogenic PS1 mutants modulated p3-Alc$_\alpha$, p-3-Alc$_\beta$, and p3-Alc$_\gamma$ C-terminal speciation at various magnitudes. This indicates that APP is not the only γ-secretase substrate that undergoes variant processing in association with PS1 mutations and suggests that some of the PS1 mutations caused more alteration in the cleavage of Alc protein(s) than APP and *vice versa*.

In studies with cells expressing FAD-linked PS1 mutant, a covariance was observed when Aβ42/40 and certain variant p3-Alc minor/major species ratios, such as p3-Alc$_\alpha$2N+38/p3-Alc$_\alpha$2N+35 or p3-Alc$_\gamma$34/p3-Alc$_\gamma$31, were plotted against each other (Fig. 4*B*). We observed similar phenomena in the CSF of subjects with sporadic AD, in that there existed disease-related covariant signature relationships between p3-Alc$_\alpha$38/p3-Alc$_\alpha$35 ratios and Aβ42/40 ratios.[6] In the case of p3-Alc$_\alpha$38/35 ratios, the covariant signature showed a positive slope in AD subjects, whereas the signatures of both the aged non-demented control subjects and the other neurological disease control subjects were similar to each other and negative in slope, suggesting a pathogenic malfunction of the γ-secretase complex in sporadic AD patients,[6] as observed in a pathogenic state of FAD-linked PS mutations in this study (Fig. 4*B*).

The biophysical mechanisms by which pathogenic PS1 mutations exert their effects are poorly understood, and it is unclear how p3-Alc ratios from the CSF of humans who do not harbor PS1 mutations are altered, as can be seen in this study with FAD-linked PS1 mutant. It is possible that wild type PS1 is behaving as if a PS1 mutation were present as a result of changes in some co-factors that modulate γ-secretase function, a result of the conformational changes of PS1, and/or a result of peripheral environmental changes at the place that γ-secretase is active. Therefore, the present results suggest that qualitative changes of p3-Alc species with altered C termini may be useful in diagnosing dysfunction of γ-secretase prior to the pathogenesis of sporadic Alzheimer disease. Taking these findings together with those of another study of human subjects,[6] we propose a hypothesis that γ-secretase dysfunction may be a feature of the pathogenesis in some population of common sporadic AD and that measurement of unusual p3-Alc fragments may provide surrogate markers to detect clinical γ-secretase dysfunction.

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