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

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Alcadeins (Alcs) constitute a family of neuronal type I membrane proteins, designated Alcα, Alcμ, and Alcγ. 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 Alcs 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 intramembrane cleavage of Alc C-terminal fragments by a presenilin-dependent γ-secretase complex, thereby generating “APP p3-like” and non-aggregating Alc peptides (p3-Alcs). We determined the complete amino acid sequence of p3-Alcα, p3-Alcμ, and p3-Alcγ 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-Alcα, p3-Alcμ, and p3-Alcγ 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.
encoded by independent genes, whereas Alcα1 and Alcα2 are splice variants derived from the Alcα 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 (Alcα, Alcβ, and Alcγ) 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-Alcα variant ratio (minor p3-Alcα38/major p3-Alcα35) 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 Alcα, Alcβ, and Alcγ, in detail.

**EXPERIMENTAL PROCEDURES**

*Plasmid Construction and Stable Cell Lines Expressing PS1—*
The human Alcadein cDNAs we used were hAlcα (GenBank™ accession number AY753301), hAlcβ (NM_014718), and hAlcγ (NM_022131). The FLAG sequence was inserted between Leu38 and Glu39 to generate pcDNA3-FLAG-hAlcα1, between Lys26 and Pro37 to generate pcDNA3.1-FLAG-hAlcβ, and between Gln38 and Arg40 to generate pcDNA3.1-FLAG-hAlcγ. The pcDNA3-FLAG-APP695 (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 × 10⁶) 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-Alcα polyclonal rabbit UT135 antibody was raised against a peptide that was composed of Cys plus the sequence between positions 839 and 851 (NPHFAVYVSTAT+C) of human Alcα1. The anti-Alcα monoclonal antibody 3B5 was raised against a peptide that was composed of Cys plus the sequence between positions 821 and 826 (C+1FVHPEH). The anti-Alcβ polyclonal rabbit UT143 antibody was raised against a GST-fusion protein containing the sequence between 819 and 847 (FLHRGHQPPPEMGH-SLASSHRNSMPSA) of human Alcβ. The anti-Alcγ polyclonal antibody UT166 was raised against a peptide composed of Cys plus the sequence between positions 823 and 834 (C+1QHSSVPSIAT) of human Alcγ. 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-Alcα (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-Alcα and anti-Alcβ 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. Uраками, E. Peskind, H. Akatsu, M. Araseki, R. Martins, M. Maeda, A. Levey, K. Chung, T. Montine, J. Legerenz, 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 x 10⁶) were transfected with the plasmids (6 μg per well) pcDNA3-Alcα, pcDNA3.1-Alcβ, or pcDNA3.1-Alcγ in Lipofectamine 2000 for 24 h. The p3-Alcα, p3-Alcβ, and p3-Alcγ that were secreted into the medium (6 ml) were recovered by immunoprecipitation with the polyclonal anti-p3-Alcα U135 (4 μg, affinity-purified), polyclonal anti-p3-Alcβ UT143 (100 μl of serum), and polyclonal anti-p3-Alcγ 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 Choko 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. As expected, we found that the primary cleavage of APP was deficient in homozygous (−/−) but not in heterozygous (+/−) AD AM10-deficient MEF cells (Fig. 1D); 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 sAlcα, sAlcβ, and sAlcγ was deficient in ADAM 10−/− cells to a similar extent (Fig. 1, A–C). These observations strongly suggest that Alcα, Alcβ, and Alcγ 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. 1H). 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 sAlcα, sAlcβ, and sAlcγ 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 proteolyses Alcα, Alcβ, and Alcγ.

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 sAlcα, sAlcβ, and sAlcγ 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 sAlcα, sAlcβ, and sAlcγ 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 Alcα, Alcβ, and Alcγ, 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 Lys612 and Leu613 of APPε10, thereby destroying the ABβ domain (Fig. 2). This cleavage at the juxtamembranous region triggers a sec...
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The recent production of anti-Alcα, anti-Alcβ, and anti-Alcγ antibodies raised against the respective extracellular juxtamembrane sequences has enabled us to recover p3-Alcα, p3-Alcβ, and p3-Alcγ secreted into the culture medium by HEK293 cells expressing Alcα, Alcβ, and Alcγ (supplemental Fig. S4B) in quality. The results suggest that Als 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.

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 (−/−) and heterozygous (+/−) deficient MEFs were transiently transfected with 1.5 μg of pcDNA3-FLAG-hAlcα (+ in A), pcDNA3.1-FLAG-hAlcβ (+ in B), pcDNA3.1-FLAG-hAlcγ (+ in C), and pcDNA3-FLAG-APPΔCTF+ (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 for Alc family proteins, and APP or with the anti-HA antibody for ADAM 10. 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-hAlcα (E), pcDNA3.1-FLAG-hAlcβ (F), pcDNA3.1-FLAG-hAlcγ (G), or pcDNA3-FLAG-APPΔCTF+ (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.
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![Diagram](image)

FIGURE 2. Amino acid sequence of p3-Alcα, p3-Alcβ, and p3-Alcγ. The amino acid sequences of the major p3-Alc species are shown: p3-Alcα, p3-Alcβ, and p3-Alcγ, are indicated as double-underlined letters along with the sequences of p3 and Aβ40 of APP and the sequence of p3-Alcα2N+35. The major primary (closed arrowheads) and secondary (open arrowheads) cleavage sites of Alcα, Alcβ, and Alcγ, are indicated with those of APP (a, the cleavage site by α-secretase or ADAM 10/17; β, the cleavage site by β-secretase or BACE). Another primary cleavage site of Alcα, 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-Alcγ is Ala817 as determined by MALDI-TOF/MS analysis; however, that of p3-Alcα2N+35 is Met815. The p3-Alcγ35 with N-terminal Met815 is a major species in HEK293 cells. The p3-Alcα2N+35 with N-terminal Met815 is a major species in HEK293 cells (see supplemental Figs. S5A, S5B, and S5A). The N terminus of p3-Alcα, is Val813, which was determined by MALDI-MS/MS analysis (see supplemental Figs. S5B and S6B). The N terminus of p3-Alcγ, is Leu834 (supplemental Fig. S5C); this coincides with the N-terminal sequence of Alcγ CTF that was determined using a gas phase peptide sequencer.

The amino acid sequences of the respective major peak products were determined by MALDI-MS/MS analysis; the amino acid sequence of p3-Alcα was composed of 35 and 37 amino acids, that of p3-Alcγ was composed of 37 and 40 amino acids, and that of p3-Alcγ 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-Alcγ species, p3-Alcα35 and p3-Alcα2N+35, that were secreted from HEK293 cells expressing Alcα35 were peptides that include the sequence from Ala817 to Thr851 and from Met815 to Thr851 of human Alcα. The major p3-Alcβ species, p3-Alcβ37 and p3-Alcβ40, that were secreted from HEK293 cells expressing Alcβ were peptides that included the sequences from Val813 to Thr851 and from Val813 to Ile852 of human Alcβ. The major p3-Alcγ species, p3-Alcγ35 and p3-Alcγ37, that were secreted from HEK293 cells expressing Alcγ were peptides that included the sequences from Leu803 to Thr851 and from Leu803 to Ile837 of human Alcγ. These p3-Alcα35, p3-Alcα37, and p3-Alcγ35 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 Ala816 (numbering for the Alcα isoform) as the N-terminal amino acid of the Alcα CTF (12). We therefore expected that ADAM 10 and ADAM 17 would cleave Alcα at the peptide bond between Met815 and Al816. In the present study, we used MALDI-MS/MS to show that the N-terminal amino acid is Ala817 for p3-Alcα35 and Met815 for p3-Alcα2N+35 (supplemental Fig. S5A). The N-terminal Met815 and/or Ala816 of Alcα CTF generated by primary cleavage may be removed by an N-terminal exopeptidase during p3-Alcα secretion from cells.

We were unable to determine the N-terminal amino acid sequence of the Alcγ CTF using a gas phase protein sequencer; however, Val813, which was identified by MALDI-MS/MS analysis of p3-Alcγ37 in this study (supplemental Figs. S5B and S6B), is likely to be the N-terminal amino acid of Alcγ CTF. We also determined the N-terminal sequence of Alcγ CTF (Leu804-Ile-Val-Gln-Pro-Pro-Phe-Leu-Gln812) using a gas phase protein sequencer. This result was identical to the result obtained from the MALDI-MS/MS analysis (supplemental Fig. S5C), indicating that the primary cleavage site of Alcγ is the N-terminal amino acid between His803 and Leu804. The major 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 and γ-secretase.

In our previous study (11), we determined the γ-secretase cleavage sites in APP and γ-secretase-dependent cleavage sites in APP that generate Aβ using a gas phase protein sequencer. In the present study, we have determined the γ-secretase cleavage sites in Alcα and Alcβ using a gas phase protein sequencer. We have previously reported that the N-terminal amino acid of Alcα is Ala816 (numbering for the Alcα isoform) as the N-terminal amino acid of the Alcα CTF (12). We therefore expected that ADAM 10 and ADAM 17 would cleave Alcα at the peptide bond between Met815 and Al816. In the present study, we used MALDI-MS/MS to show that the N-terminal amino acid is Ala817 for p3-Alcα35 and Met815 for p3-Alcα2N+35 (supplemental Fig. S5A). The N-terminal Met815 and/or Ala816 of Alcα CTF generated by primary cleavage may be removed by an N-terminal exopeptidase during p3-Alcα secretion from cells.

We were unable to determine the N-terminal amino acid sequence of the Alcγ CTF using a gas phase protein sequencer; however, Val813, which was identified by MALDI-MS/MS analysis of p3-Alcγ37 in this study (supplemental Figs. S5B and S6B), is likely to be the N-terminal amino acid of Alcγ CTF. We also determined the N-terminal sequence of Alcγ CTF (Leu804-Ile-Val-Gln-Pro-Pro-Phe-Leu-Gln812) using a gas phase protein sequencer. This result was identical to the result obtained from the MALDI-MS/MS analysis (supplemental Fig. S5C), indicating that the primary cleavage site of Alcγ is the peptide bond between His803 and Leu804. The major 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 and γ-secretase.
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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, A278T, L286V, or A434C mutations or vector alone (Mock) were co-transfected with Alcα, Alcβ, and Alcγ or without plasmid (−).

p3-Alcα, p3-Alcβ, and p3-Alcγ were recovered from the culture medium by immunoprecipitation with anti-Alcα (UT135), anti-Alcβ (UT143), and anti-Alcγ (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 APP695 transiently (Fig. 4A, lower right).

As expected, HEK293 cells stably expressing wild type PS1 (WT) or vector alone (Mock) plus Alcα generated p3-Alcα species with C-terminal end of Thr851 (p3-Alcα2N+35) as the major species (Fig. 3A). We determined the amino acid sequences of this p3-Alcα species with MALDI-MS/MS analysis and confirmed that this is p3-Alcα2N+35 and not p3-Alcα37. This p3-Alcα2N+35 was a minor p3-Alcα species in human CSF in which p3-Alcα35 was major (left panel in supplemental Figs. S5A and S6A). In any case, HEK293 cells expressing PS1 (WT) generated largely p3-Alcα species with the C-terminal Thr851 (right panel in supplemental Figs. S5A and S6A).

In contrast, HEK293 cells expressing FAD-linked PS1 mutants generated qualitatively altered p3-Alcα species (Fig. 3A). This was especially remarkable in the medium of cells expressing PS1 carrying the L166P mutation; there were increases in the levels of p3-Alcα2N+38 that included a peptide of Met815–Ile854 (Fig. 4A, upper left). The p3-Alcα2N+39 that included a peptide of Met815–Val855, and p3-Alcα2N+40 that included a peptide of Met815–Val856 were also increased (arrows in Fig. 3A). This L166P mutation is known to increase Aβ42/40 ratio greatly (Fig. 4A, lower right). Other FAD-linked 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, A278T, L286V, and A434C mutations were transfected with or without (−) pcDNA3-hAlcα. The culture medium (6 ml) of cells expressing Alcα was immunoprecipitated with UT135, and the immunoprecipitates were subjected to MALDI-TOF-MS analysis. 35, p3-Alcα35; 2N+34, p3-Alcα2N+34; 2N+35, p3-Alcα2N+35; 2N+36, p3-Alcα2N+36; 2N+37, p3-Alcα2N+37; 2N+38, p3-Alcα2N+38; 2N+39, p3-Alcα2N+39; 2N+40, p3-Alcα2N+40. B, representative MS spectra of p3-Alcα secreted from cells expressing wild type PS1 or FAD-linked PS1 mutants. HEK293 cells were transfected as described above with or without (−) pcDNA3-hAlcα. The culture medium (6 ml) of cells expressing Alcα was immunoprecipitated with UT143, and the immunoprecipitates were subjected to MALDI-TOF-MS analysis. 37, p3-Alcα37; 38, p3-Alcα38; 39, p3-Alcα39; 40, p3-Alcα40. C, representative MS spectra of p3-Alcγ secreted from cells expressing wild type PS1 or FAD-linked PS1 mutants. HEK293 cells were transfected as described above with or without (−) pcDNA3-hAlcγ. The culture medium (6 ml) of cells expressing Alcγ was immunoprecipitated with UT166, and the immunoprecipitates were subjected to MALDI-TOF-MS analysis. 30, p3-Alcγ30; 31, p3-Alcγ31; 32, p3-Alcγ32; 34, p3-Alcγ34.
mutations, such as R278T, A434C, and A246E, showed a moderate effect to increase $\beta_42/40$, whereas these mutations appeared to have little or almost no effect on the increase of minor species, including $\gamma_42N+38$ (Fig. 4A, compare upper left to lower right).

We then investigated the generation of $\gamma$-Alc$_{42}$ and $\gamma$-Alc$_{40}$ from HEK293 cells expressing $\gamma$-Alc$_{42}$ or $\gamma$-Alc$_{40}$ together with PS1 carrying FAD-linked mutations (Fig. 3, B and C, and Fig. 4A). HEK293 cells stably expressing wild type PS1 (WT) or vector alone (Mock) plus $\gamma$-Alc$_{42}$ generated major $\gamma$-Alc$_{42}$ and $\gamma$-Alc$_{40}$ (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 $\gamma$-Alc$_{40}$ (arrows in Fig. 3B). We then created minor species/major species ratios for $\gamma$-Alc$_{40}$ to $\gamma$-Alc$_{42}$, which were compared with the $\alpha_42/40$ ratio (Fig. 4A, compare upper right to lower right). In cultured cells, $\gamma$-Alc$_{40}$ is a major species rather than $\gamma$-Alc$_{42}$, whereas $\gamma$-Alc$_{42}$ is a major species on CSF (compare Fig. 3B to Fig. 5B). Therefore, in the case of $\gamma$-Alc$_{42}$, we sought the $\gamma$-Alc$_{40}$/$\gamma$-Alc$_{42}$ ratio as minor/major ratio. In contrast to the ratio $\gamma$-Alc$_{42}$/p3-Alc$_{40}$, L166P did not show remarkable change for the ratio $\gamma$-Alc$_{40}$/p3-Alc$_{42}$.

HEK293 cells stably expressing wild type PS1 or vector alone (mock) plus $\gamma$-Alc$_{40}$ generated $\gamma$-Alc$_{31}$ and $\gamma$-Alc$_{34}$ 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 $\gamma$-Alc$_{31}$ along with increases in the levels of $\gamma$-Alc$_{34}$ (arrows in Fig. 3C). The alteration of the $\gamma$-Alc$_{34}$/p3-Alc$_{31}$ ratio showed some similarity to that of the $\gamma$-Alc$_{2N+38}$/p3-Alc$_{2N+35}$ ratio in various PS1 mutations but differed from the alteration of the $\gamma$-Alc$_{40}$/p3-Alc$_{42}$ ratio (Fig. 4A, compare lower left to upper right).

Six FAD-linked PS1 mutants (L166P, R278T, A434C, A246E, M146L, and L286V) increased the $\alpha_42/40$ ratio at various magnitudes (Fig. 4A, 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 $\gamma$-cleavage alteration between APP and Alc, the $\alpha_42/40$ ratio was plotted to certain p3-Alc minor/major ratios.
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. 4A on the basis of visual inspection, p3-Alc showed a property different from p3-Alc and p3-Alc in correlation coefficient to A42/40. The p3-Alc2N+38/p3-Alc2N+35 and p3-Alc34/p3-Alc31 ratios showed a strong correlation to the A42/40 ratio (R2 > 0.5), whereas the p3-Alc30/p3-Alc37 ratio showed a positive but weak correlation to the A42/40 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, p3-Alc, and p3-Alc 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 are 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, p3-Alc, and p3-Alc 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, anti-p3-Alc, UT143, and anti-p3-Alc 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-Alc35 by MALDI-MS/MS analysis (Fig. 5A and supplemental Fig. S6A).

**FIGURE 5. Representative MS spectra of p3-Alc peptides in human CSF.** Human p3-Alc (A), p3-Alc (B), and p3-Alc (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, 34; 35, p3-Alc, 35; 36, p3-Alc, 36; 37*, a mixture of p3-Alc, 37 and p3-Alc2N+35 (see supplemental Fig. S7B); 38, p3-Alc, 38; 39, p3-Alc, 39. B, 35, p3-Alc, 35; 36, p3-Alc, 36; 37, p3-Alc, 37; 38, p3-Alc, 38; 39, p3-Alc, 39; 40, p3-Alc, 40, C, 31, p3-Alc, 31.
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This antibody also recovered another peptide, which contained two components, namely p3-Alc37 (molecular mass 4003.0, a peptide composed of Ala817–Val853) and p3-Alc40 (molecular mass 4006.9, a peptide composed of Met815–Thr851). The two components were distinguishable by reflector mode analysis (supplemental Fig. S7). p3-Alc34, p3-Alc36, and p3-Alc38 were also identified in human CSF (supplemental Fig. S7A).

The antibody UT143 recovered peptides of molecular masses 3963.9 and 4303.2 (Fig. 5B and supplemental Fig. S6B), which were designated p3-Alc37 and p3-Alc40, respectively, according to MALDI-MS/MS analysis. The antibody UT166 recovered a peptide of molecular mass 3377.6 (Fig. 5C and supplemental Fig. S6C). The species with a molecular mass of 3377.6 (arrow in supplemental Fig. S6C) 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-Alc31 (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-Alc35, p3-Alc37, p3-Alc37, p3-Alc40, and p3-Alc31 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-Alc34, species in mouse CSF and brain (supplemental Fig. S8). The major p3-Alc35 in CSF was also major product in the brain, along with the similar profile of p3-Alc31 species between CSF and brain. Furthermore, the p3-Alc34 species profile of mouse CSF was identical to those of human CSF (supplemental Fig. S8), suggesting that p3-Alc35 is major product in mouse and human brain.

DISCUSSION

In this study, we show that the Alc family proteins Alc4, Alc34, and Alc 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 aggrega-

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