Mutations in the Transmembrane Domain of APP Altering γ-Secretase Specificity†

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Received May 7, 1997; Revised Manuscript Received September 3, 1997⊗

ABSTRACT: Alzheimer's disease (AD) β-amyloid peptide (Aβ and βA4) is derived from the amyloid precursor protein (APP) by the subsequent action of the so-far unidentified β- and γ-secretases. γ-secretase, which generates the C-terminus of Aβ, cleaves within the transmembrane domain of APP, preferentially after Aβ-residue 40 (Aβ 40) but also after residue 42 (Aβ 42). This Aβ 42 represents the major subunit of the plaques in AD. Since the position of γ-secretase cleavage is crucial for understanding the pathogenic pathway, we investigated the effect of different point mutations at Thr43 on γ-secretase specificity in SPA4CT (SPC99)-expressing COS7 cells. These constructs only require γ-cleavage for Aβ release. We observed that all Thr43 mutations altered the specificity of γ-secretase. Small hydrophobic residues favored the generation of Aβ 42, leading to an increase in the 42/40 ratio of Aβ (1.6−2.8-fold). The increase was even stronger (5.6−5.8-fold) when combined with the familial mutation Val46Phe. Thus, these constructs might be highly valuable for the generation of animal models for AD. Processing of full-length APP or SPA4CT yielded the same 42/40 ratio of Aβ (4.7%). Both constructs, bearing the familial AD mutation Val46Phe, led to a similar increase in the 42/40 ratio (3.3- versus 3.6-fold). The p3 fragment, produced by α- and γ-secretase, showed 42/40 ratios similar to Aβ when derived from wild-type and mutant proteins. These results suggest that the different Aβ- and p3-species are generated by γ-cleavage activities with a similar enzymatic mechanism.

The main protein component of amyloid plaques found in the brain of patients with Alzheimer's disease (AD)[1] is β-amyloid (Aβ and βA4) (Glenner & Wong, 1984; Masters et al., 1985), a 4 kDa peptide which is derived from the larger amyloid precursor protein (APP) (Kang et al., 1987). APP processing by the so-far unidentified protease activities termed α-, β-, γ-, and δ-secretases leads to a variety of different soluble and membrane-bound proteins (Figure 1) [for a review, see Evin et al. (1994), Selkoe (1994), and Simons et al. (1996)]. The α-secretase process most likely occurs in a post-Golgi-compartment and results in the cleavage of APP within the Aβ domain. This yields secretory APP (sAPPα), comprising the entire N-terminal part of APP, and the remaining C-terminal fragment p3CT. Alternatively, APP can be cleaved by β-secretase at the N-terminus of Aβ, generating a shortened, soluble APP (sAPPβ) and a C-terminal fragment of 99 residues (A4CT and C99). This membrane protein A4CT contains the entire Aβ domain, the transmembrane domain and the cytoplasmic tail of APP (Dyrks et al., 1988) and is the precursor for Aβ (Figure 1) (Higaki et al., 1995).

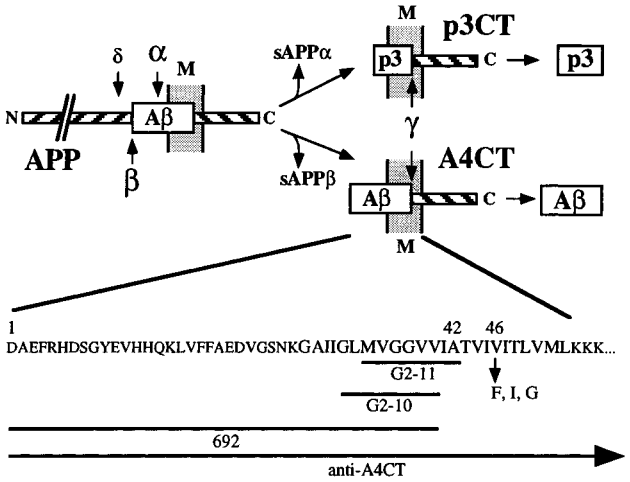

FIGURE 1: Processing of APP by α-, β-, γ-, and δ-secretases. Schematic representation of APP and its proteolytic C-terminal fragments. Cleavage sites of α-, β-, γ-, and δ-secretases are shown. The amino acid sequence of the Aβ domain and the transmembrane domain is given in the one letter code with the residues of the membrane domain in a larger size than the residues outside of the membrane. The mutations at residue 46 are responsible for a familial form of AD. The antibodies used in this study have been raised against the indicated peptides (see Experimental Procedures). M, membrane (shaded); sAPPα and sAPPβ, secreted APP, generated by α- or β-secretase cleavage, respectively.

Both membrane-bound C-terminal fragments of APP, A4CT and p3CT, are cleaved by γ-secretase within their transmembrane domains at the C-terminus of Aβ or p3, thus releasing the 40 and 42 residue Aβ peptide (Aβ 40 and Aβ 42) and the 16 residue shorter p3 peptide (p3 40 and p3 42) into the medium (Figure 1) (Citron et al., 1996; Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992).

†This work was supported by the Deutsche Forschungsgemeinschaft (through SFB 317) and the Fonds der Chemischen Industrie of Germany.

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⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1997.

[1] Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; FAD, familial Alzheimer's disease; Aβ 40, Aβ-species with different N-termini ending at residue 40; Aβ 42, Aβ-species with different N-termini ending at residue 42; p3 40, peptide p3 ending at residue 40 of A4CT; p3 42, peptide p3 ending at residue 42 of A4CT; A4CT, C-terminal 99 residues of APP; if A4CT is derived from SPA4CT, the N-terminus is extended by Leu-Glu; SPA4CT, signal peptide of APP followed by A4CT; p3CT, C-terminal 83 residues of APP; wt, wild-type.

S0006-2960(97)01071-4 CCC: $14.00

The major Aβ-species secreted into human cerebrospinal fluid and by cultured cells expressing APP wt ends at residue 40 of Aβ; the minor species ends at residue 42. In cells expressing APP with the familial mutation at Val717 (Figure 1), γ-cleavage at Ala42 is increased resulting in elevated concentrations of Aβ 42 intra- as well as extracellularly (Suzuki et al., 1994; Tienari et al., 1997). The underlying mechanism is not known. Aβ 42 has been shown to aggregate *in vitro* much faster than Aβ 40 (Jarrett et al., 1993) and is also the major subunit of amorphous and neuritic plaques in AD and Down's syndrome (Gravina et al., 1995; Iwatsubo et al., 1994; Kang et al., 1987). Moreover, it has recently been shown that p3 42 is a major constituent of Down's syndrome cerebellar preamyloid (Lalowski et al., 1996). Thus, γ-cleavage after Aβ residue 42 leads to the pathologically relevant peptides. However, it is now well established that cleavage after residue 42 is a physiological process (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992). The majority of Aβ-species starts at Asp1, but other species with a prolonged or a shortened N-terminus have been reported [for a review, see Evin et al. (1994)]. Aβ 40 and Aβ 42 therefore include all Aβ-species with N-terminal heterogeneity ending at residue 40 or 42, respectively.

The exact cellular localization of γ-secretase is not known, but there is evidence that in kidney cells the γ-cleavage activity occurs in early endosomes or in a post-Golgi-compartment (Dyrks et al., 1993; Haass et al., 1993), whereas in neurons, additional intracellular sites with γ-cleavage activity were identified (Tienari et al., 1997). Although γ-cleavage occurs in the membrane domain of A4CT and p3CT, it is not clear whether the cleavage occurs within the phospholipid bilayer, at the surface of the bilayer, or after removal of the corresponding protein fragments from the membrane.

The sequence specificity of γ-secretase is far from being understood. The cleavage event after residue 42 might represent a second cleavage site for a single γ-secretase in addition to the main cleavage after residue 40. On the other hand, different γ-secretases could be involved in generating the Aβ 40- and Aβ 42-species as has been suggested in a recent study (Citron et al., 1996). Other investigators (Tischer & Cordell, 1996) have shown that replacing the transmembrane domain of APP by that of the human EGF receptor still leads to the generation of an Aβ-like peptide. This suggests that γ-secretase does not have a specific recognition site in the primary sequence of its substrate molecules but rather cleaves within stretches of hydrophobic residues. In addition to hydrophobic residues, the membrane domain of APP also comprises two Thr residues (at positions 714 and 719 of APP770 or 43 and 48 of A4CT, Figure 1). The Thr43 is directly at the γ-secretase cleavage site, where the pathologically important Aβ 42 is generated, suggesting that the hydroxyl group of Thr43 might be crucial for the cleavage after residue 42.

In the present study, we investigated the amino acid requirement at residue 43 for γ-cleavage at the peptide bond 42−43. We studied the effects of the point mutations Thr43Ser, Thr43Ala, Thr43Phe, Thr43Pro, and Val46Phe and of the double mutations Thr43Ala + Val46Phe and Thr43Gly + Val46Phe (numbering of A4CT, Figure 2) on the release of Aβ and p3 in A4CT-expressing COS7 cells. In addition, we determined how these mutations affect the ratio of Aβ 42/40 and hence the cleavage specificity of Alzheimer's disease γ-secretase.

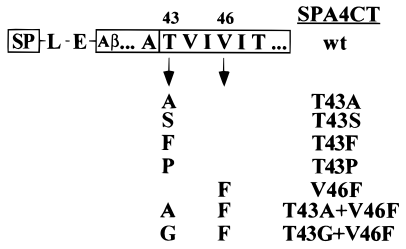

FIGURE 2: SPA4CT mutations used for the study of γ-secretase cleavage specificity. SPA4CT consists of the signal peptide (SP) of APP followed by Leu and Glu and the C-terminal 99 amino acids (A4CT and C99) of APP. Amino acids are shown in the one letter code.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections. COS7 cells were cultured according to the protocol described previously for SH-SY5Y cells (Dyrks et al., 1993). Cell culture media were obtained from Sigma. The pCEP4 vector (Invitrogen) with the APP or SPA4CT cDNA inserts was transfected into COS7 cells using Lipofectin (Gibco BRL) as described elsewhere (Dyrks et al., 1993). Stable transfectants were selected using 270 μg/mL Hygromycine (Boehringer Mannheim). For each construct, two independent transfections were tested in cell culture for same expression levels, same amounts of generated Aβ and same Aβ 42/40 ratio.

Antibodies. The rabbit antiserum 692 was raised against synthetic peptide Aβ 1−40. The monoclonal antibodies G2−10 (specific for Aβ ending at residue 40) and G2−11 (specific for Aβ ending at residue 42) were raised against synthetic peptides (Ida et al., 1996). The polyclonal antibody anti-A4CT was raised against A4CT (Dyrks et al., 1993).

Metabolic Labeling and Immunoprecipitation. After 45 min of preincubation in methionine-free MEM, stably transfected COS7 cells were incubated for 16 h in methionine-free MEM containing 10% fetal bovine serum and 133 μCi/mL [^{35}S]methionine (Amersham). The conditioned media were centrifuged at 4 °C for 1 minute at 4000*g* and divided into three samples with a volume of 1 mL each. Aβ and p3 were immunoprecipitated with antibody 692 (dilution 1:67, 2 h incubation time) and 4 mg of protein-A Sepharose (Pharmacia) or with G2−10 (12.5 μg/mL, 6 h incubation time) or G2−11 (17.3 μg/mL, 6 h incubation time) and 100 μg of protein-G Agarose (Boehringer Mannheim Inc.), respectively. The cell lysates were prepared by suspending the cells in 0.5 mL of SOL (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P40, and 2 mM phenylmethanesulfonyl fluoride) (Dyrks et al., 1993). After 20 min on ice, the samples were centrifuged at 10000*g* for 5 min. The supernatants were preincubated for 30 min with 3 mg of protein-A Sepharose. After a brief centrifugation, A4CT was immunoprecipitated from the supernatant with antibody anti-A4CT (dilution 1:32, 90 min incubation time) and 4 mg of protein-A Sepharose. To investigate the expression of A4CT and the removal of the signal peptide, COS7 cells were pulse-labeled for 10 min (as described above). The immunoprecipitated proteins were separated on 10% Tris-Tricine gels (Schägger & von Jagow, 1987). The intensity of the bands was quantified using a Fuji phosphorimager (BAS 1000).

Plasmid Construction. Mutated cDNA constructs encoding A4CT T43A + V46F, T43A, and T43S were generated by three polymerase chain reactions (PCR) using the vector pHD/APP695 V642F as template.[2] The oligonucleotides used as primers and carrying the mutations are as follows (numbering refers to A4CT): 5′-CATAGCGGCAGTGATCGTCATCAC-3′ (T43A sense), 5′-GATGACGATCACTGCCGCTATGACAAC-3′ (T43A antisense), 5′-GTCATAGCGGCAGTAATATTCATCACC-3′ (T43A + V46F sense), 5′-GATGAATATTACTGCCGCTATGACAAC-3′ (T43A + V46F antisense), 5′-GTCATAGCTAGCGTGATCGTCATCAC-3′ (T43S sense), 5′-GATGACGATCACGCTAGCTATGACAAC-3′ (T43S antisense), 5′-GTCATAGCCGGCGTAATATTCATC-3′ (T43G + V46F sense), 5′-GAATATTACGCCGGCTATGACAACACC-3′ (T43G + V46F antisense), 5′-GTTGTCATAGCACCGGTAATCGTCATCAC-3′ (T43P sense), 5′-ACGATTACCGGTGCTATGACAACAC-3′ (T43P antisense), 5′-GTCATAGCATTCGTGATCGTCATCACC-3′ (T43F sense), 5′-CGATCACGAATGCTATGACAACACCG-3′ (T43F antisense). Further primers used are 5′-TTGACAAATATCAAGACGGAG-3′ (NT) and 5′-ATGCACTAGTTTGATACAGCT-3′ (CT). The first PCR used the corresponding antisense primer and primer NT, the second PCR the corresponding sense primer and primer CT. The third PCR, using both fragments from the first and second PCR as well as the primers NT and CT, generated the mutated full-length sequence of A4CT, which was subcloned into the *Sma*I digested pUC18 vector according to the manufacturers protocol of the Sure Clone Ligation Kit (Pharmacia). The plasmid pUC18/A4CT V46F was generated with a single PCR using primers NT and CT.

The *Eco*RI/*Kpn*I fragments of the pUC18/A4CT constructs were cloned into pBS/SPA4CT wt digested with *Eco*RI/*Kpn*I to generate the mutated pBS/SPA4CT plasmids. pBS/SPA4CT wt was obtained cloning the *Sac*I/*Hin*dIII fragment of pSP65/SPA4CT (Dyrks et al., 1992) into the pBS vector digested with *Sac*I/*Hin*dIII. The plasmid pBS/APP695 V642F was generated by cloning the *Nru*I/*Eco*RI fragment of pCEP4/APP695 (Dyrks et al., 1993) into pBS/SPA4CT V46F that was digested with *Nru*I/*Eco*RI. The eukaryotic expression vectors pCEP4/SPA4CT T43A, T43S, T43A + V46F, and V46F were obtained by cloning the *Sma*I/*Spe*I fragment of the pBS/SPA4CT plasmids into pCEP4 vector (Invitrogen) that was digested with *Nhe*I/*Pvu*II. The resulting plasmids encode SPA4CT (APP signal peptide-Leu-Glu-A4CT) carrying the indicated mutations. pCEP4/APP695 V642F was generated cloning the *Sma*I/*Spe*I fragment of pBS/APP695 V642F into pCEP4 digested with *Nhe*I/*Pvu*II. The plasmid pHD/APP695 V642F[2] was generated by PCR mediated mutagenesis using plasmid pHD/APP695 (Hartmann et al., 1996). pBS/SPA4CT rev. was obtained by cloning the *Kpn*I/*Nhe*I fragment of pCEP4/SPA4CT (Dyrks et al., 1993) in the pBS/SPC99 vector (Tienari et al., 1996) that was digested with *Kpn*I/*Xba*I. Plasmids pBS/SPA4CT with the mutations T43F, T43P, and T43G + V46F were generated using the Quik Change Site Directed Mutagenesis Kit (Stratagene), the corresponding sense and antisense primers and pBS/SPA4CT rev. as DNA template. The *Kpn*I/*Spe*I fragments of these constructs were cloned into the pCEP4 vector that was digested with *Kpn*I/*Nhe*I. The identity of the constructs obtained by PCR was confirmed by DNA sequencing.

[2] Czech, C. Ph.D. Thesis, University of Heidelberg, Germany, 1994.

RESULTS

γ-Secretase Tolerates Mutations at Residue 43. The membrane domain of APP consists mainly of hydrophobic residues but also comprises two Thr residues (Figure 1), one of them next to Ala 42 of the long Aβ-species Aβ 42 (Thr residue 43 of A4CT, residue 714 of APP770). First, we investigated whether efficient γ-cleavage requires at this position an amino acid with a hydroxyl group in the side chain (Ser and Thr). We used A4CT (C99) as model (Figure 2) and mutated the corresponding residue Thr43 to Ser, Ala, Phe, and Pro. Secondly, we compared the processing of these mutated constructs with the processing of A4CT wt and A4CT V46F. Thirdly, we studied the processing of two double mutants, A4CT T43A + V46F and A4CT T43G + V46F. All constructs were stably transfected as SPA4CT in COS7 cells (Figure 2). SPA4CT consists of the N-terminal signal peptide of APP followed by the amino acids Leu and Glu and A4CT (APP signal peptide-Leu-Glu-A4CT) (Dyrks et al., 1993).

The cells were tested for the expression level of SPA4CT. After overnight labeling, the expression levels of the wild-type and mutated proteins did not differ significantly (data not shown). The signal peptide of SPA4CT was efficiently removed, yielding A4CT with the two additional amino acids Leu and Glu at the N-terminus as reported earlier for SPA4CT wt (Dyrks et al., 1992, 1993) (data not shown).

The peptides Aβ, p3.5, and p3 were immunoprecipitated with antibody 692 from the conditioned medium of metabolically labeled COS7 cells (Figure 3A). The N-terminus of both p3.5 and p3 could be generated by the same α-secretase (Haass & Selkoe, 1993), and thus, we use the term p3 for both species, p3.5, and p3. There is less p3.5 generated than p3, so that the p3.5 bands are hardly visible for some of the constructs in Figure 3A. Nevertheless, the bands could be quantified using a sensitive phosphorimager. All mutated A4CT constructs were found to be processed to Aβ and p3 in the same way as A4CT wt (Figure 3A). Only for the Thr43Pro mutation the amounts of Aβ and p3 were drastically reduced (Figure 3A), probably due to a structural change in the mutated A4CT substrate molecule, thereby inhibiting efficient binding to γ-secretase. Aβ derived from the A4CT constructs migrates at a higher apparent molecular mass than Aβ derived from APP695 (Figure 3A), which is known to be due to the two additional residues Leu and Glu at the N-terminus of Aβ (Figure 2) (Dyrks et al., 1993). The mobility of the p3 bands is independent of its source. Cells expressing APP show an additional band of 6 kDa which results presumably from δ- and subsequent γ-cleavage of APP (Simons et al., 1996) and that cannot be generated in SPA4CT-expressing cells, where the corresponding sequence is not present (Figure 3A). In COS7 cells that were transfected with the vector alone, Aβ was not detected and p3 only in low amounts (Figure 3A). This p3 must be derived from the endogenous APP of COS7 cells. Quantification of the SPA4CT-derived Aβ bands in repeated experiments did not reveal significant differences between protein amounts from wild-type and mutated A4CT constructs, except for the Thr43Pro mutation (data not shown). Thus, the single (Thr43Ser, Thr43Ala, Thr43Phe,

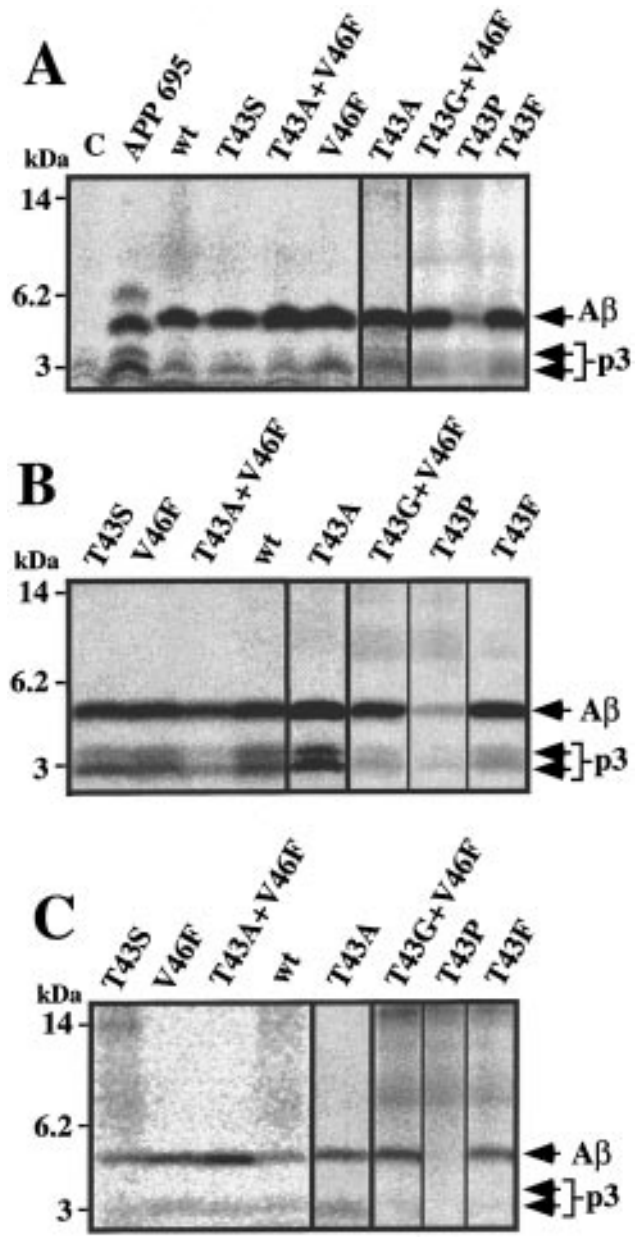

FIGURE 3: Influence of the SPA4CT mutations on substrate turnover and on γ-secretase cleavage specificity. Proteins from 16 h conditioned medium of COS7 cells labeled with [^{35}S]methionine were immunoprecipitated with antibodies against Aβ and p3. Cells were stably transfected with the indicated SPA4CT constructs. (A) Immunoprecipitation with antibody 692, raised against synthetic peptide Aβ 1−40. (B) Immunoprecipitation with antibody G2−10, specific for Aβ- and p3-peptides ending in residue 40. (C) Immunoprecipitation with antibody G2−11, specific for Aβ- and p3-peptides ending in residue 42. C, control (cells transfected with vector pCEP4 without an insert); APP695, COS7 cells transfected with APP695.

and Val46Phe) and double (Thr43Ala + Val46Phe and Thr43Gly + Val46Phe) mutations of A4CT do not affect the turnover rate of A4CT by γ-secretase.

We tested whether the mutated A4CT constructs lead to the production of the same Aβ- and p3-species as found for A4CT wt. The Aβ- and p3-species ending at Aβ residue 40 or 42 (Aβ 42, Aβ 40, p3 42, and p3 40) were immunoprecipitated from the conditioned medium using the Aβ 42- and Aβ 40-specific antibodies G2−11 and G2−10, respectively (Figure 3, panels B and C). All constructs, including A4CT T43P, led to the generation of Aβ 40, Aβ

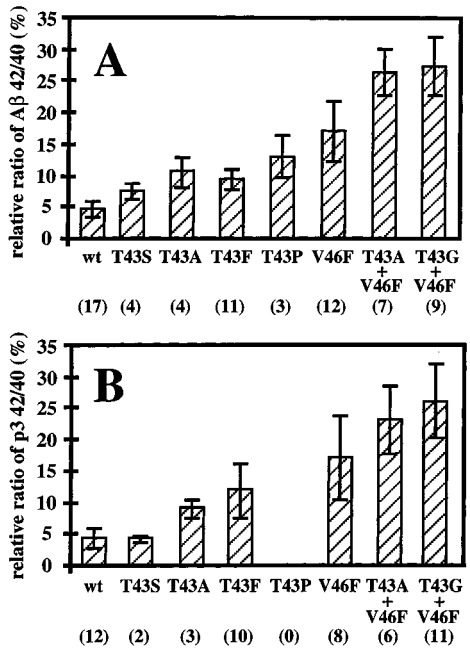

FIGURE 4: Relative ratios of Aβ 42/40 and p3 42/40 derived from mutated SPA4CT constructs. (A) Relative 42/40 ratios of Aβ in the conditioned medium. (B) Relative 42/40 ratios of p3 in the conditioned medium. The bands in Figure 3, panels B and C were quantified by phosphorimaging in the indicated number of independent experiments. Columns represent the mean values. Black error bars give the standard deviation.

42, and p3 40. The peptide p3 42 was detected for all constructs except for A4CT T43P. Given the fact that the overall amount of p3 is very low for this mutation, it is likely that p3 42 is generated but that the amount is below the detection limit. Although the p3 bands in Figure 3C are hardly visible for some of the constructs, the bands could still be quantified using a sensitive phosphorimager. The results clearly show that an efficient cleavage of A4CT by γ-secretase and the generation of both Aβ- and p3-species (Aβ 42, Aβ 40, p3 42, and p3 40) do not require a hydroxyl group at residue 43. Moreover, γ-secretase tolerates at residue 43 large and small as well as hydrophobic and polar residues.

Effect of Thr43 and Val46 Mutations on the Ratio Aβ 42/40 and p3 42/40. The FAD-linked mutations of APP695 at Val642 (corresponding to Val46 of A4CT and Val717 of APP770) influence γ-secretase cleavage specificity in neuronal cells (Suzuki et al., 1994; Tienari et al., 1997). These mutations lead to an increased level of Aβ ending at residue 42. The mechanism underlying this effect is not understood. It is possible that the mutations alter the binding-affinity of γ-secretase to one of the two cleavage sites in the membrane domain of A4CT, i.e., peptide bonds 40−41 and 42−43. According to this hypothesis, the mutations at Thr43 were expected to alter the affinity of γ-secretase to the A4CT-cleavage sites and, thus, to have an effect on the ratio of Aβ 42/40. Moreover, the double mutations T43A + V46F and T43G + V46F should show the combined effects of the single mutations in altering the ratio of Aβ 42/40. To test this hypothesis, the amounts of Aβ 42 and Aβ 40 produced by these constructs (Figure 3, panels B and C) were quantified by phosphorimaging. The ratio of Aβ 42/40 was determined in 4−17 experiments for each construct (Figure 4A and Table 1). In relation to SPA4CT wt (relative Aβ

Table 1: Aβ 42/40 Ratios

construct	Aβ 42/40 (%)[a]	increase relative to wt construct	p (t-test)[b]
	SPA4CT		
wild-type	4.7 ± 1.3	1.0	
T43S	7.7 ± 1.2	1.6	0.01
T43A	10.7 ± 2.3	2.2	<0.02
T43F	9.5 ± 1.5	2.0	<0.001
T43P	13.2 ± 3.3	2.8	ns[c]
V46F	17.1 ± 4.8	3.6	<0.001
T43A + V46F	26.5 ± 3.6	5.6	<0.001
T43G + V46F	27.5 ± 4.7	5.8	<0.001
	APP		
wild-type	4.7 ± 1.2	1.0	
V642F	15.4 ± 1.6	3.3	<0.001

[a] Aβ 42, Aβ with variable N-terminus ending at residue 42 of A4CT; Aβ 40, Aβ with variable N-terminus ending at residue 40 of A4CT. [b] p value represents the significance compared to the wild-type construct and is determined with the two-sided Student's t-test. [c] ns, not significant.

Table 2: p3 42/40 Ratios

construct	p3 42/40 (%)[a]	increase relative to wt construct	p (t-test)[b]
	SPA4CT		
wild-type	4.5 ± 1.7	1.0	
T43S	4.3 ± 0.5	1.0	ns[c]
T43A	9.1 ± 1.4	2.0	<0.02
T43F	12.0 ± 4.3	2.7	<0.001
T43P	nd	nd	nd[d]
V46F	17.1 ± 6.7	3.8	<0.01
T43A + V46F	23.2 ± 5.4	5.2	<0.001
T43G + V46F	26.2 ± 5.9	5.9	<0.001
	APP		
wild-type	4.5 ± 1.0	1.0	
V642F	21.0 ± 2.8	4.7	<0.001

[a] p3 42, p3 and p3.5 ending at residue 42 of A4CT; p3 40, p3 and p3.5 ending at residue 40 of A4CT. [b] p value represents the significance compared to the wild-type construct and is determined with the two-sided Student's t-test. [c] ns, not significant. [d] nd, could not be determined.

42/40 ratio = 4.7%), all mutated constructs showed increased ratios of Aβ 42/40 (Table 1). Replacement of Thr43 by Ser led to an increase of Aβ 42/40 by a factor of 1.6 ($p = 0.01$, compared to SPA4CT wt); substitution of Thr43 by Ala produced a stronger increase of Aβ 42/40 by a factor of 2.2 ($p < 0.02$). The Thr43Phe mutation led to an increase of Aβ 42/40 by a factor of 2.0 ($p < 0.001$). Due to the low amounts of Aβ generated from A4CT T43P, the ratio of Aβ 42/40 could only be determined in three out of seven experiments. The increase of Aβ 42/40, found for this mutation A4CT T43P, is not significant compared to SPA4CT wt. The effect of the familial APP mutation Val46Phe, leading to an increase by a factor of 3.6 ($p < 0.001$), was more pronounced than the effect of the Thr43 mutations (Figure 4). The double mutation T43A + V46F showed an even higher increase (5.6-fold, $p < 0.001$) of the ratio of Aβ 42/40 than the point mutations. This increase is an additive effect of the increase measured for the corresponding point mutations (T43A and V46F). A further increase of Aβ 42/40 was found for the double mutation T43G + V46F (5.8-fold, $p < 0.001$).

The ratios of Aβ 42/40, which were measured for the constructs with Thr43 mutations (SPA4CT wt, T43S, T43A, T43F, T43A + V46F, and T43G + V46F), suggested that γ-secretase generates more Aβ 42 if residue 43 is a small amino acid. The wild-type construct with a Thr at residue 43 showed the lowest ratio of Aβ 42/40. When Thr43 was replaced by Ser, the ratio was increased by a factor of 1.6. Ser also has a hydroxyl group in the side chain but is smaller than Thr. A substitution of Thr43 by the even smaller aliphatic amino acid Ala led to a stronger increase by a factor of 2.2. The double mutation T43G + V46F, having the smallest amino acid Gly at residue 43, led to a higher increase than the double mutation T43A + V46F with Ala at position 43. Thus, γ-secretase cleavage after residue 42 and the generation of Aβ 42 are most pronounced if residue 43 is a small hydrophobic amino acid.

To investigate whether p3 is produced by the same or a similar enzymatic activity as Aβ, the relative ratios of p3 42/40 were determined in repeated experiments. Although the p3 bands in Figure 3, panels A and C, are hardly visible for some constructs, the bands could still be quantified using a sensitive phosphorimager. In SPA4CT wt transfected cells, the ratios of 42/40 for Aβ and p3 were found to be similar (Aβ, 4.7%; p3, 4.5%, Tables 1 and 2). In relation to SPA4CT wt, the ratios of p3 42/40 obtained from the mutated constructs were increased to a similar extent as found for the corresponding Aβ ratios (Figure 4B and Table 2). One exception is the Thr43Phe mutation, for which the increase in the p3 42/40 ratio was found to be higher than for the Thr43Ala mutation, whereas for the corresponding Aβ ratios, Thr43Phe showed a smaller increase than the Thr43Ala mutation. As for all constructs, the amount of p3 is less than the amount of Aβ, the intensity of the p3 42 band is near to the detection limit, and the mean standard deviation of the ratios p3 42/40 is higher than for the corresponding Aβ ratios. As the overall amounts of p3 are very low for the T43P mutation, the ratio of p3 42/40 could not be determined.

The ratios of Aβ 42/40 and p3 42/40, as determined here, are expressed in percent and are relative but not absolute ratios as the antibodies have different affinities to the corresponding peptides.

Influence of the Ectodomain of APP on the 42/40 Ratios of Aβ *and p3.* Other investigators have reported an increase by a factor of 1.8, when measuring the increase in the ratio of Aβ 42/40 caused by the Val642Phe mutation using full-length APP695 and the neuronal cell line M17 (Suzuki et al., 1994). We have observed an increase of Aβ 42/40 by a factor of 3.6 for the SPA4CT construct in epithelial kidney COS7 cells of African green monkey. To test whether this difference in the measured ratios was due to the different constructs (SPA4CT versus APP) or due to the different cell lines (COS7 versus M17), we generated COS7 cells expressing APP695 V642F or APP695 wt and measured the 42/40 ratio as above for Aβ and p3 (Figure 5 and Tables 1 and 2). The 42/40 ratios for Aβ and p3 were identical for the wild-type constructs APP wt and SPA4CT wt [Aβ, 4.7% (APP) and 4.7% (SPA4CT); p3, 4.5% (APP) and 4.5% (SPA4CT)]. The Val642Phe mutation led to a similar increase in the 42/40 ratio for both, Aβ and p3, derived from either full-length APP or SPA4CT. The increases of the ratios of Aβ 42/40 were determined to be 3.3 ($p < 0.001$) for APP and 3.6 ($p < 0.001$) for SPA4CT in COS7 cells. The corresponding increases for p3 were 4.7 ($p < 0.001$) for APP and 3.8 ($p < 0.01$) for SPA4CT and, thus, similar to the increases in the ratios of Aβ.

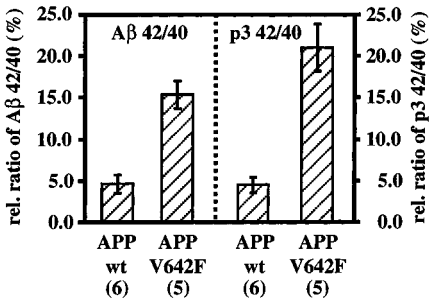

FIGURE 5: Relative 42/40 ratios for Aβ and p3 in APP695 wt- and APP695 V642F-expressing cells. Proteins from 16 h conditioned medium of stably transfected COS7 cells labeled with [^{35}S]-methionine were immunoprecipitated with antibodies specific for the C-terminus of Aβ and p3 (G2−10 and G2−11). Bands were quantified by phosphorimaging of five to six independent experiments. Columns represent the mean values. Black error bars give the standard deviation. The number of experiments is shown in brackets.

These results show that the difference between our data and the data of Suzuki et al. (1994) (3.3-fold versus 1.8-fold increase in the Aβ 42/40 ratio) is due to the different cell lines and not due to the different constructs. Although the Val642Phe mutation leads in both cell lines to an increase in extracellular Aβ 42, the extent of this increase appears to be cell-type specific.

DISCUSSION

γ-Secretase Cleavage Specificity. An important therapeutic target for a rational treatment of AD appears to be γ-secretase, which cleaves the APP-derived protein A4CT within its membrane domain at the C-terminus of Aβ. A major question is the cleavage specificity of γ-secretase, which can be studied although the enzyme has not yet been identified. An appropriate way to study its cleavage specificity is to express mutant precursors for Aβ, e.g., APP or A4CT, in eukaryotic cells and analyze the effect of these mutations on the generation of both Aβ-species Aβ 40 and Aβ 42.

It has been shown that Aβ is produced from A4CT, tagged with a N-terminal signal peptide (SPA4CT and SPC99), as well as from APP (Dyrks et al., 1993). For APP-derived Aβ, it is well-known that it consists mainly of the two species Aβ 40 and Aβ 42, with Aβ 40 being the main species of secretory Aβ (Suzuki et al., 1994) as well as of intracellularly generated Aβ (Tienari et al., 1997). APP-derived p3 presents the same C-terminal heterogeneity as Aβ and occurs as p3 42 and p3 40 (Citron et al., 1996). Using COS7 cells we could show for the first time that both Aβ-species, Aβ 42 and Aβ 40, as well as both p3-species, p3 42 and p3 40, are also generated from SPA4CT. Moreover, Aβ has the same relative 42/40 ratio regardless of whether it is derived from A4CT or APP. The same holds true for the p3 42/40 ratio, which, furthermore, has been found to be nearly identical to that observed for Aβ 42/40.

These results clearly lead to three conclusions. First, the cleavage specificity of γ-secretase, i.e., the ratios of Aβ 42/40 and p3 42/40, is influenced neither by the large part of the APP-ectodomain, which is missing both in A4CT and p3CT, nor by the N-terminal 16 residues of A4CT, which are missing in p3CT. Secondly, SPA4CT is an appropriate model system for the study of γ-secretase. Thirdly, these results suggest that the C-termini of Aβ 40 and p3 40 are generated by the same or a similar enzymatic activity. The same is likely to be the case for the generation of the peptides Aβ 42 and p3 42. This also implies that A4CT and p3CT, although being generated by β- and α-secretase in different compartments, are transported to the same compartment where the γ-secretase activity is located. The γ-secretase which cleaves after residue 40 of Aβ is believed to be distinct from the enzyme that cleaves after residue 42 of Aβ (Citron et al., 1996). However, our results suggest, that the mechanism of γ-cleavage is similar for both γ-secretases.

Molecular Effect of the APP Val717Phe Mutation. One type of familial AD is characterized by mutations in the APP gene at Val717 (Val46 of A4CT), which occur 4 residues C-terminal to the end of Aβ 42 (Figure 1) and affect the cleavage specificity of γ-secretase, by leading to an increased ratio of Aβ 42/40. However, these mutations do not alter the total amount of produced Aβ (Suzuki et al., 1994) and thus do not modulate the turnover rate of the precursor molecule by γ-secretase. The molecular mechanism underlying this change in the cleavage specificity of γ-secretase has not yet been elucidated. It is possible that the mutations alter the binding affinity of γ-secretase to the APP-derived substrate A4CT, thereby favoring a shift of γ-cleavage to the Ala42−Thr43 bond. This explanation implies that both γ-secretases, the one which cleaves A4CT after residue 40 and the one that cleaves after residue 42, are similar enzymes that bind A4CT in a slightly different way, either with the 42- or the 40-cleavage site close to their active sites. The binding affinities of A4CT to the enzyme could be altered by the mutations C-terminal to the γ-cleavage site and thus explain the altered ratios of Aβ 42/40.

Four arguments support this hypothesis of two binding sites of A4CT and a similar enzymatic mechanism of γ-cleavage after Aβ 40 and Aβ42. First, the increase in the 42/40 ratio caused by the Val46Phe mutation is similar for both peptides Aβ and p3. Secondly, the increase is similar, regardless of whether Aβ and p3 are derived from APP or SPA4CT. As has already been shown for the wild-type constructs, these results clearly demonstrate again, that the large part of the ectodomain of APP, which is missing in A4CT and p3CT, is not responsible for determining or modulating the cleavage specificity of γ-secretase. Thus, possible sorting signals residing in these domains can also be excluded from modulating the cleavage specificity of γ-secretase. In agreement with these findings, recent experiments in primary hippocampal neurons have shown that the sorting of APP is not influenced by the Val642Phe mutation (Tienari et al., 1996, 1997). Thirdly, mutations close to the cleavage site of γ-secretase other than the Val46Phe mutation do also alter the ratio of Aβ 42/40 but do not affect the total amount of Aβ. The mutations in position 43 analyzed in this study (T43A, T43S, T43F, T43A + V46F, and T43G + V46F) led to an increased ratio of Aβ 42/40. Similar findings were observed for the ratio of p3 42/40, providing further evidence that p3 and Aβ are generated by the same γ-secretase. Fourthly, the 5.6-fold increase found for the double mutation A4CT T43A + V46F shows that the combination of two single point mutations (T43A and V46F) results in an additive increase of the effect of the corresponding point mutations. As both double mutations (A4CT T43A + V46F, T43G + V46F) caused the strongest increase in the ratio of Aβ 42/40 of all mutations analyzed, they appear to be suited for the generation of transgenic animal

models of AD. From our data, we conclude that the Thr43 and the Val46 mutations affect the direct interaction between A4CT and γ-secretase on the molecular level and not other cellular events, such as intracellular sorting mechanisms.

Hydrophobic Active Site of γ-Secretase. Comparison of the ratios of Aβ 42/40, which were measured for the constructs with Thr43 mutations (SPA4CT wt, T43S, T43A, T43F, T43A + V46F, and T43G + V46F), shows that hydrophobic residues (Phe, Ala, and Gly) generally led to a higher ratio than residues with a hydroxyl group (Thr and Ser). This result rules out the hypothesis that the hydroxyl group of Thr 43 is necessary for efficient γ-cleavage after residue 42. Thus, the active site of γ-secretase most probably consists of hydrophobic residues which are most likely involved in hydrophobic interaction with the substrate proteins. Support for this conclusion has come from a recent publication (Tischer & Cordell, 1996), which shows that charged amino acids like Asp at residues 40, 44, or 45 interfere with efficient turnover by γ-secretase and membrane-insertion of the protein.

Our data reveal that within the group of hydrophobic amino acids at residue 43, the Ala mutation showed a higher increase in the ratio of Aβ 42/40 than the Phe mutation. This indicates that γ-cleavage after residue 42 is increased to an even higher extent, if residue 43 is not only a hydrophobic but also a small residue. This point of view is strengthened by the effect of the Thr43Gly + Val46Phe mutation, which led to a higher increase in the ratio of Aβ 42/40 than the double mutation with an Ala at residue 43 (Thr43Ala + Val46Phe).

From our data on the cleavage specificity of γ-secretase, we conclude that A4CT and p3CT follow a common pathway of degradation with the same γ-secretase and that amino acid mutations close to the C-terminus of Aβ are able to alter the binding affinity of γ-secretase to A4CT and thus to also alter the cleavage specificity. However, our data do not exclude the possibility that additional factors, such as the structure of membranes, might have an influence on the cleavage specificity of γ-secretase. One factor might be the thickness of the lipid bilayer which is expected to influence the distance of the γ-secretase cleavage site from the membrane surface (Bretscher & Munro, 1993; Tienari et al., 1997).

Mechanism of γ-Cleavage. So far, it is not known whether γ-secretase cleavage occurs within the phospholipid bilayer or after release of the substrate protein from the membrane. As until now no enzymes are known that are active within the bilayer and as γ-secretase is not yet identified, we also considered an alternative nonenzymatic mechanism for γ-cleavage that is termed threoninolysis. Although this mechanism is an extreme hypothesis, a mechanism similar to threoninolysis, called serinolysis, was found in different organisms for the conversion of several soluble inactive proenzymes to the fully active enzymes (van Poelje & Snell, 1990). Threoninolysis as a putative mechanism of γ-cleavage would require either a Ser or a Thr at residue 43 of A4CT. However, we could show that γ-cleavage still occurs when the wild-type Thr43 is replaced by Ala, Phe, or even Pro. For this reason, we exclude threoninolysis as a mechanism of γ-cleavage. Furthermore, we assume that γ-cleavage is mediated by an enzyme. This has also been supported by the recent finding that peptide aldehydes inhibit the γ-cleavage of A4CT and thus the generation of Aβ (Allsop et al., 1997; Higaki et al., 1995). These aldehydes reveal inhibitor profiles and IC50 values that are consistent with an enzymatic cleavage. Moreover, a recently described new protease, called m-AAA, has been shown to degrade transmembrane domains of proteins at the surface of the inner mitochondrial membrane (Leonhard et al., 1996). This new protease, which is a hetero-oligomeric protein complex, also possesses a chaperone-like activity that may be involved in the partitioning of membrane proteins into a proteinaceous environment where the cleavage reaction takes place (Arlt et al.; 1996, Leonhard et al., 1996). A similarly acting protease might be involved in the γ-cleavage of A4CT.

ACKNOWLEDGMENT

We thank Dr. Felix Wieland for drawing our attention to threoninolysis. We are grateful to the Fonds der Chemischen Industrie of Germany for a fellowship to S.F.L. and to the Erna Struckmann Foundation for support.

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BI971071M