C-Terminal Maturation Fragments of Presenilin 1 and 2 Control Secretion of APPα and Aβ by Human Cells and Are Degraded by Proteasome

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Abstract

Background: Most early-onset forms of Alzheimer's disease are due to missense mutations located on two homologous proteins named presenilin 1 and 2 (PS1 and PS2). Several lines of evidence indicate that PS1 and PS2 undergo various post-transcriptional events including endoproteolytic cleavages, giving rise to 28–30 kD N-terminal (NTF) and 18–20 kD C-terminal (CTF) fragments that accumulate in vivo. Whether the biological activity of presenilins is borne by the processed fragments or their holoprotein precursor remains in question. We have examined the putative control of βAPP maturation by CTF-PS1/PS2 and the catabolic process of the latter proteins by the multicatalytic complex, proteasome.

Materials and Methods: We transiently and stably transfected HEK293 cells with CTF-PS1 or CTF-PS2 cDNA. We examined these transfectants for their production of Aβ40, Aβ42, and APPα by immunoprecipitation using specific polyclonals. The effect of a series of proteases inhibitors on the immunoreactivity of CTF-PS1/PS2 was examined by Western blot. Finally, the influence of proteasome inhibitors on the generation of βAPP fragments by CTF-expressing cells was assessed by combined immunoprecipitation and densitometric analyses.

Results: We showed that transient and stable transfection of CTF-PS1 and CTF-PS2 cDNAs in human cells leads to increased secretion of APPα and Aβ, the maturation products of βAPP. Furthermore, we demonstrated that two proteasome inhibitors, lactacystin and Z-IE(Ot-Bu)A-Leucinal, prevent the degradation of both CTFs. Accordingly, we established that proteasome inhibitors drastically potentiate the phenotypic increased production of APPα and Aβ elicited by CTF-PS1/PS2.

Conclusion: Our data establish that the C-terminal products of PS1 and PS2 maturation exhibit biological activity and in particular control βAPP maturation upstream to α-and β/γ-secretase cleavages. This function is directly controlled by the proteasome that modulates the intracellular concentration of CTFs.

Introduction

Familial forms of Alzheimer's disease are due to inherited mutations on genes, the loci of which have been identified on chromosomes 21, 14, and 1 (for review see ref. 1). The gene products of chromosomes 1 and 14 have been shown to be responsible for most early-onset forms of Alzheimer's disease and have been identified as presenilins 2 and 1 (PS2 and PS1), respectively (2–4). PS1 and PS2 are highly homologous transmembrane proteins (5,6) located mainly in the endoplasmic reticulum (7–11) and Golgi apparatus (10,11). These proteins undergo various post-transcriptional modifications (for reviews see refs. 12,13) and are particularly susceptible to endoproteolytic cleavages giving rise to N-terminal (NTF) and C-terminal (CTF) fragments (14–17). This cleavage by an as-yet unknown protease seems to be a major event since intact PS

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holoproteins are poorly detectable in the brain of transgenic mice and affected patients and the two proteolytic fragments appear to accumulate with a 1/1 stochiometry (15). Interestingly, NTF anf CTF undergo phosphorylation events (9,18), behave as targets of caspase-mediated proteolysis (19–21), and can physically interact (12,13). Whether these post-transcriptional events reflect regulatory mechanisms aimed at modulating putative NTF and CTF biological activity remains to be established.

We have examined whether the expression of CTF-PS1/PS2 modulates the processing of βAPP in HEK293 cells. We show that both CTF-PS1 and CTF-PS2 expression leads to increased secretion of Aβ and APPα. Furthermore, we establish that both PS maturated fragments are catabolized by the proteasome. Accordingly, two proteasome inhibitors drastically potentiate the CTF-mediated increase in the recovery of both βAPP maturation products. Altogether, our data indicate that CTF-PS1 and CTF-PS2 are biologically active and in particular, control βAPP maturation. This function appears to be directly modulated by the proteolytic catabolism elicited by the proteasome.

Materials and Methods

Design of CTF-PS1 and CTF-PS2 cDNAs

The cDNA encoding CTF-PS1 (ct-1) was engineered by introducing the Kosak sequence of PS1 upstream of the ATG codon encoding the PS1 methionine in position 292 (oligo: 5'-CAT-AGG-ATC-CGT-TGC-TCC-AAT-GGT-GTG-GTT-GGT-GAA-TAT-GGC-AGA-A-3'). An additional BamH1 restriction site was also added, adjacent to the Kosak sequence for further subcloning of the construction in pcDNA3 (ct-1). The cDNA construction coding for CTF-PS2 (ct-2) was engineered similarly, with the Kozak sequence of PS2 upstream of the ATG codon encoding the methionine in position 298 and an additional Kpn restriction site adjacent to the Kozak sequence (oligo: 5'-ATC-TGG-TAC-CGG-CAG-GGC-TAT-GGT-GTG-GAC-GGT-TGG-CAT-GGC-GAA-G-3').

Stable Transfections of HEK293 Cells

HEK293 cells were grown as previously described (22). Stable transfectants were obtained by calcium phosphate precipitation with 1 μg of empty pcDNA₃ vector, ct-1, or ct-2 and called

Mock, CPS1, and CPS2, respectively. CPS1 and CPS2 were identified after Western blot analysis of electrophoresed proteins by means of the αPS1Loop or αPS2Loop antibodies (15) as described previously (23,24). HEK293 cells expressing wild-type-βAPP₇₅₁ (referred to as WT) were obtained as described previously (22).

Transient Transfections of HEK293 Cells

Mock or WT stable transfectants were transiently transfected with 2 μg of ct-1, ct-2, or empty vector by means of DAC30 according to manufacturer recommendations (Eurogentec). Transfection efficiency was checked by Western blot with αPS1Loop or αPS2Loop antibodies. Analysis of βAPP maturation products was performed 48 to 72 hr after transfection.

Immunoprecipitation and Detection of APPα and Aβ

Transiently or stably transfected cells were maintained in the above F12/DMEM-supplemented medium then washed, and secretion of APPα and Aβ was initiated for 5 hr at 37°C in the absence or with presence of proteasome inhibitors. Aliquots of media were recovered, diluted in an equal volume of RIPA buffer, and incubated overnight with a 3000-fold dilution of 207 antibody (APPα) or with a 350-fold dilution of FCA18 (total Aβ) as previously described (25). Samples were centrifuged, and the pellets were washed three times with RIPA 1× containing NaCl (350 mM), rinsed with TBS buffer, then resuspended in the loading buffer, electrophoresed on 8% SDS-PAGE (APPα) or Tris-tricine gels (Aβ), and Western blotted for 1–3 hr. Nitrocellulose sheets were capped with skim milk (5% in TBS buffer) and exposed overnight to a 200-fold dilution of mAb10D5C (APPα) or 1 μg/ml of WO2 (Aβ) antibodies. The nitrocellulose sheets were rinsed with TBS buffer then incubated with adequate anti-IgGs, revealed, and quantified as previously described (25).

Western Blot Analysis of βAPP and CTFs Immunoreactivity

Stably transfected cells were treated as above then lysed in 50 mM Tris-HCl, pH 7.5, containing 150 mM of NaCl and protease inhibitors (5 mM EDTA, 1 mM leupeptin, 1 μM pepstatin and 1 mM AEBSF). Analysis for βAPP and CTF-PS1/CTF-PS2 contents was performed by means of BR188 antibody or αLoopPS1/PS2 as described previously (26).
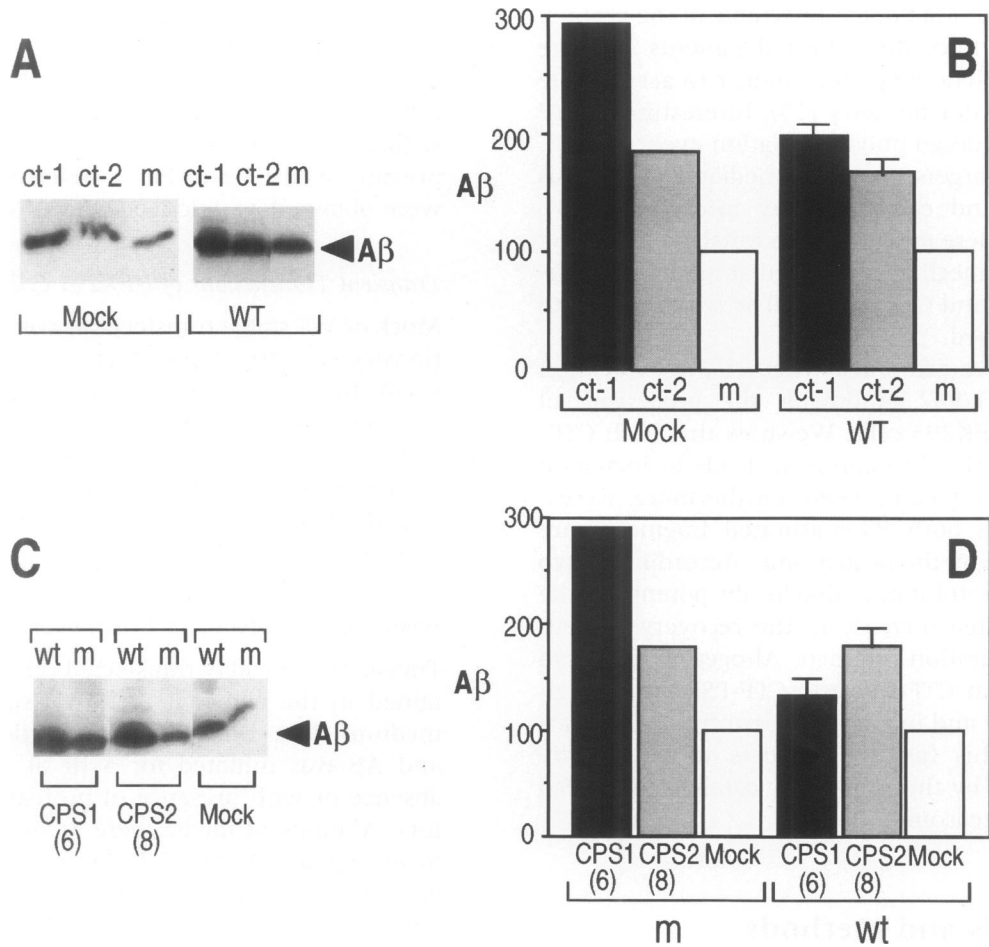
Fig. 1.   Influence of CTF-PS1 and CTF-PS2 on Aβ secretion. Stable transfectants expressing wt-βAPP$_{751}$ (WT) or empty pcDNA$_3$ (Mock) are transiently transfected with empty pcDNA3 (m) or coding for CTF-PS1 (ct-1) or CTF-PS2 (ct-2) fragments (A). Secreted Aβ was immunoprecipitated with FCA18 antibody then detected with mAbWO2 as described in Materials and Methods. Bars in B correspond to densitometric analysis of secreted Aβ (normalized for ct-1 and ct-2 expression) and are expressed as the percent of control corresponding to Mock and WT cells transiently transfected with empty pcDNA (m). Values are the mean ± SEM of 3 independent experiments. In C, stably transfected HEK293 cells overexpressing CTF-PS1 (CPS1-6), CTF-PS2 (CPS2-8), or with empty pcDNA$_3$ (Mock) were examined for Aβ secretion after transient transfection with wt-βAPP$_{751}$ (wt) or empty pcDNA$_3$ (m). Total Aβ was obtained after immunoprecipitation with FCA18 and revelation with mAbWO2 as described in Materials and Methods. Bars in D correspond to densitometric analysis of Aβ expressed as the percent of control corresponding to stable Mock cells transiently transfected with pcDNA$_3$ (m) or βAPP$_{751}$ (wt).

Antibodies

FCA18 (27) and WO2 (28) specifically interact with the N-terminus of Aβ. The 207 antibody (donated by Drs. M. Savage and B. Greenberg, Cephalon, Westchester, NY) recognizes the N-terminus of βAPP and APPα. 10D5C (provided by Dr. D. Schenk, Athena Neurosciences) specifically recognizes the C-terminus of APPα. αPS1Loop and αPS2Loop (provided by Dr. G. Thinakaran, Johns Hopkins University, Baltimore) specifically interact with the hydrophilic loop of PS1 and PS2 located between their predicted sixth and seventh transmembrane domains. BR188 (supplied by Dr. M. Goedert, Cambridge, England) recognizes the C-terminus of mature and immature βAPP.

Results

Stably mock-transfected HEK293 cells (Mock) were transiently transfected with empty pcDNA3 (m), ct-1, or ct-2 (Fig. 1A, left panel). CTF-PS1
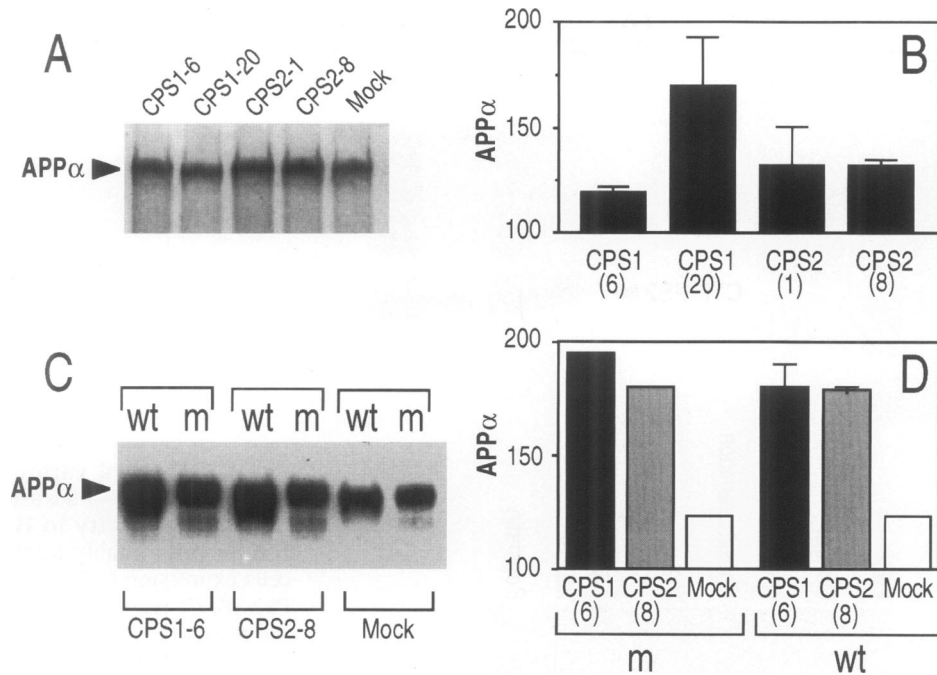
Fig. 2. Influence of CTF-PS1 and CTF-PS2 overexpression on APPα secretion. Stably transfected HEK293 cells overexpressing CTF-PS1 (CPS1-6 and CPS1-20), CTF-PS2 (CPS2-1 and CPS2-8), or empty pcDNA₃ (Mock) were examined for APPα secretion from endogenous βAPP (A, B) or after transient transfection (C, D) with βAPP751 (wt) or pcDNA3 (m). APPα was detected after immunoprecipitation with 207 antibody and western blot with mAB10D5C as described in Materials and Methods. Bars in B and D represent the APPα densitometric analysis of experiments A (normalized for CTF-PS1/PS2 expressions) and C (normalized for wt expression), respectively. Values are expressed as the percent of control APPα secretion obtained with Mock cells (B) or Mock-transfected cells (D) transiently transfected with empty vector (m) or wt-βAPP751 (wt). Values are the means ± SEM of independent experiments.

and CTF-PS2 increased the recovery of total secreted Aβ (Fig. 1A, left panel) by about 200 to 300% (Fig. 1B) over control (Mock/m) cells, indicating that transient transfection of ct-1 and ct-2 modulates the Aβ production derived from endogenous βAPP. The same phenotypic increase in Aβ secretion was observed (Fig. 1A, right panel) with stably transfected wt-βAPP751-expressing cells (WT) transiently transfected with ct-1 and ct-2 (about 200% over control WT/m).

To further document the influence of the overexpression of CTF-PS1/2 on the β/γ-derived product of βAPP maturation, clones stably mock-transfected (Mock) or overexpressing CTF-PS1 (CPS1) and CTF-PS2 (CPS2) were transiently transfected with empty pcDNA₃ (m) (Fig. 1C). Both CTF-expressing clones secreted higher amounts of Aβ than Mock/m control cells (Fig. 1D). Transient transfection of Mock, CPS1, and CPS2 clones with wt-βAPP751 cDNA (wt) led to increased Aβ secretion, the production of which was higher in CPS1 and CPS2 than in Mock-transfected cells (Fig. 1C, D).

We examined the putative influence of CTF-PS1 and CTF-PS2 expression on the secretion of the α-secretase-derived physiological product of βAPP maturation, APPα. Two independent clones of CPS1 and CPS2 produced higher amounts of APPα than Mock-transfected cells (Fig. 2A, B). Transient transfection of Mock, CPS1, and CPS2 (Fig. 2C) with empty pcDNA3 (m) or wt-βAPP751 cDNA (wt) confirmed that the CTF-PSs-expressing cells secrete more APPα than the Mock-transfected cells (Fig. 2D).

The effect of the proteasome inhibitor Z-IE(Ot-Bu)A-Leucinal on CTF-PS1 and CTF-PS2 immunoreactivity in stable transfectants was also examined. Figure 3 indicates that immunoreactivity of both CTF-PS1 (Fig. 3A) and CTF-PS2 (Fig. 3B)is highly enhanced by Z-IE(Ot-Bu)A-Leucinal. The involvement of the proteasome in CTF degradation is further demonstrated by the enhancement of CTF immunoreactivity upon treatment with lactacystin, a very potent and selective proteasome blocker (Fig. 3C). It should be noted that a smir of high-molecular-weight proteins is detectable upon proteasome inhibi-
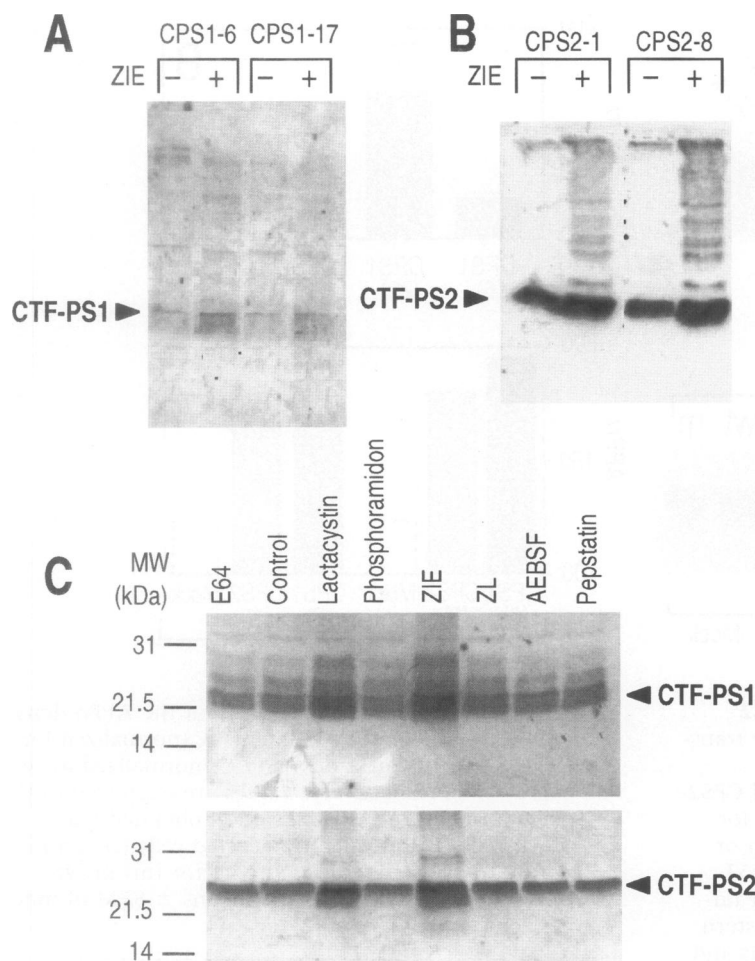
Fig. 3. Effect of various protease inhibitors on CTF-PS1 and CTF-PS2 immunoreactivity in HEK293 transfected cells. Stably transfected HEK293 cells expressing CTF-PS1 (A, C) or CTF-PS2 (B, C) were incubated for 5 hr in the presence of the proteasome inhibitors Z-IE(Ot-Bu)A-leucinal (ZIE, $2.5 \times 10^{-5}M$) and lactacystin ($5 \times 10^{-6}M$) or with *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64, $10^{-4}M$), 4-(2-amido-ethyl) benzenesulfonyl fluoride (AEBSF, 10^{-4} M), phosphoramidon (10^{-5} M), pepstatin A (10^{-5} M), and Z-L-Leucinal (ZL, 2.5×10^{-5} M). Cells were lysed, then immunoreactivity of CTF-PS was revealed with αPS1Loop and αPS2Loop antibodies as described in Materials and Methods.

tion (see Fig. 3B), which could reflect protection of ubiquitinated forms of CTF as was shown for presenilin 1 (23) and presenilin 2 (29).

Other protease blockers unable to inhibit the proteasome activity do not modify CTF-PS1/2 recovery (Fig. 3C). Thus, Z-L-Leucinal (calpain and cathepsin B inhibitor), E64 (thiol and serine protease inhibitor), AEBSF (serine protease inhibitor), phosphoramidon (endopeptidase 3.4.24.11 inhibitor), and pepstatin (acid protease inhibitor) do not protect CTF-PS1 and CTF-PS2 from degradation in HEK293 cells (Fig. 3C).

If CTF-PS1/2 degradation by the proteasome is physiologically relevant, one would expect to potentiate their stimulatory effect on Aβ and APPα secretion upon proteasome inhibition. Z-IE(Ot-Bu)A-Leucinal augments the APPα secretion triggered by CPS1 and CPS2 transfectants (Fig. 4A, B). This inhibitor also potentiates the secretion of Aβ by wt-βAPP$_{751}$-expressing transfectants (WT) transiently transfected with ct-1 and ct-2 constructions (Fig. 4C, D). Therefore, it

can be concluded that inhibition of the proteasome potentiates the effect of CTF-PS1 and CTF-PS2 on both α- and β/γ-secretase-derived products of βAPP maturation in HEK293 cells.

Discussion

We have previously established that the proteasome contributes to the maturation of endogenous βAPP in human cells. Thus, proteasome inhibitors increase the recovery of both Aβ and APPα in naive HEK293 cells (22). This led us to hypothetize that a cellular intermediate located upstream to the α- and β/γ-secretases cleavages and behaving as substrate of the proteasome plays an important role in βAPP maturation. The search for such an effector led us to suggest that PS1 and PS2 could fulfill such a role. First, we showed that overexpression of PS1 (26) and PS2 (24) elicit an increased secretion of both Aβ and APPα. Second, we demonstrated that PS1 and
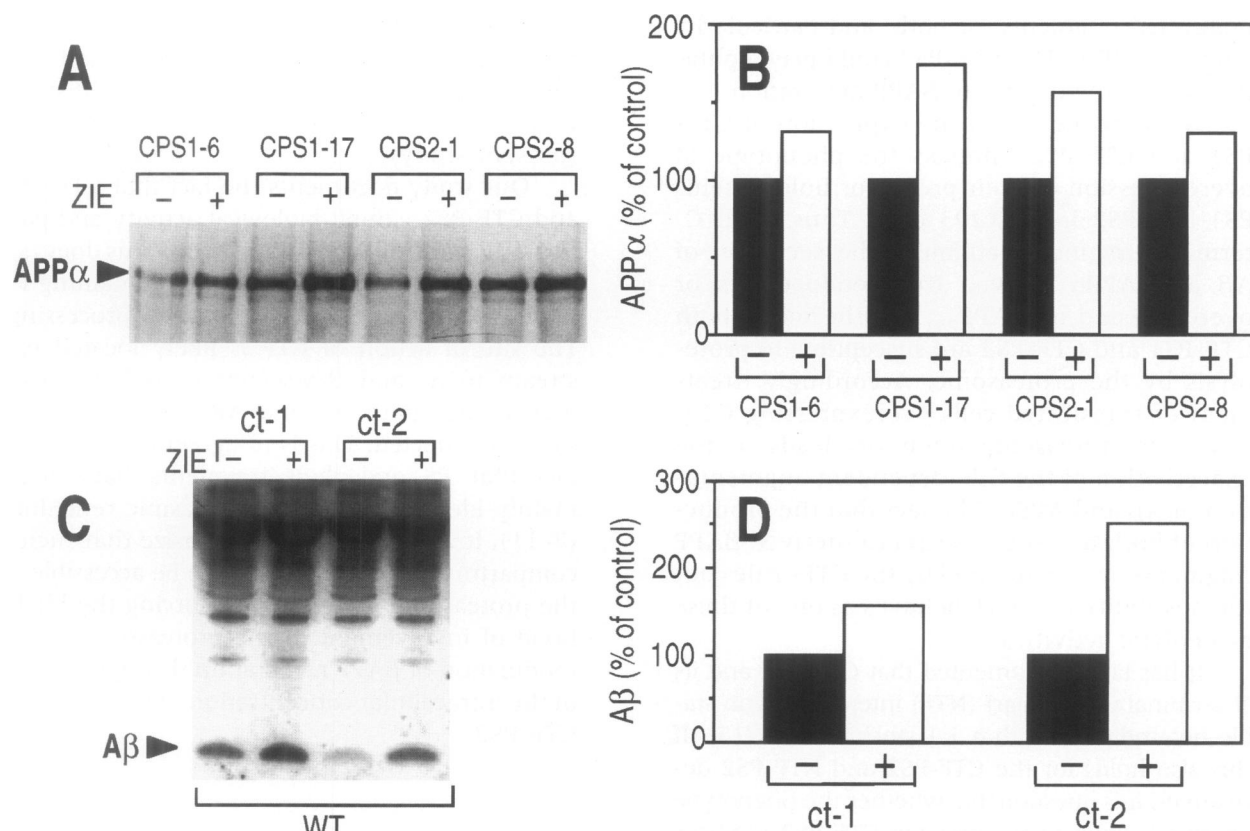
Fig. 4. Effect of Z-IE(Ot-Bu)A-leucinal on Aβ and APPα secretion by stably transfected HEK293 expressing CTF-PS1 and CTF-PS2. Stable transfectants overexpressing CTF-PS1 (CPS1-6 and CPS1-17) and CTF-PS2 (CPS2-1 and CPS2-8) were grown as described in Materials and Methods then APPα secretion was initiated in the absence (−) or presence (+) of Z-IE(Ot-Bu)A-leucinal. APPα was immunoprecipitated after 5 hr by means of 207 antibody and revealed with mAb10D5C (A). Stable transfectants overexpressing wt-βAPP751 (WT) were transiently transfected with CTF-PS1 (ct-1) or CTF-PS2 (ct-2) cDNAs. Secreted total Aβ was obtained after immunoprecipitation with FCA18 and revealed with mAbWO2 as described in Materials and Methods (C). Densitometric analyses of APPα (B) or Aβ (D) [normalized for CTF-PS1/2 (A) or ct-1/2 (C)] secretions are expressed as the percent of control corresponding to secretion obtained in absence of inhibitor. Values are the mean of independent experiments carried out on the indicated independent clones.

PS2 behave as excellent substrates of the proteasome (23,24), which is in agreement with other studies (29,30). Third, we established that the proteasome inhibitors exacerbate the PS1/PS2-induced increased recovery of both βAPP maturation products (23,24). Interestingly, proteasome inhibitors also exacerbate the phenotypic alterations of βAPP maturation triggered by FAD-linked PS mutations (23,24). Therefore, we suggested that PS1 and PS2 control βAPP maturation upstream to secretases, and that this function is directly modulated by catabolic events triggered by the proteasome.

Presenilins undergo several post-transcriptional modifications that include phosphorylation and proteolytic cleavages by unknown proteases as well as by caspase 3 (12,13). Whether the degradation products of PS1/PS2 correspond to intermediate or final catabolites en route to final clearance or, alternatively, to maturated fragments bearing biological activity is not yet established. However, the fact that these products accumulate in vivo in human and transgenic mice brains (15,31) and are able to interact physically raised the possibility that N- and C-terminal PS fragments could display a physiological function. In line with this hypothesis was the observation that the overexpression of a C-terminal fragment of PS2 that can be physiologically generated by proteolysis and alternative transcription was able to rescue HeLa cells from Fas- and TNF-induced apoptosis (32). Therefore, it was still questionable whether PS-related function was indeed due to PS holoproteins, their

maturated fragments, or both, and particularly, whether CTF-PS1 or CTF-PS2 could play a putative role in the control of βAPP maturation.

We show here that the expression of CTF-PS1 and CTF-PS2 mimicks the phenotype of overexpression of their precursor holoproteins PS1 and PS2 in HEK293 cells. Thus, both C-terminal fragments augment the secretion of Aβ and APPα derived from endogenous or overexpressed wt-βAPP$_{751}$. Furthermore, both CTF-PS1 and CTF-PS2 are susceptible to proteolysis by the proteasome. Accordingly, treatment of transfected cells overexpressing CTF-PSs with proteasome inhibitors leads to the exacerbation of the CTF-dependent augmentation of Aβ and APPα. The fact that the production of both α and β/γ-secretase-derived βAPP fragments are augmented by the CTFs rules out the possibility that CTF behaves as one of these proteolytic activities.

It has been documented that CTF-PS1 and its N-terminal counterpart (NTF) interact to form stable heterodimers with a 1:1 stochiometry (15). If this also holds for the CTF-PS2 and NTF-PS2 derivatives, it is questionable whether the phenotype observed when overexpressing CTF-PS2 mimicks the physiological function of endogenously produced CTF-PS2, since the 1:1 stochiometry of endogenous fragments theoretically preclude CTFs from interacting with endogenous NTFs—this fragment would not be available as a free entity. However, it should be noted that several studies have indicated that transient or stable transfection of PS2 led to apoptosis and that transfection of CTF-PS2 cDNA antagonized this effect in various cell systems, suggesting that overexpression of this fragment elicits a physiologically relevant phenotype (32).

Interestingly, Tomita and colleagues also recently examined the effect of overexpression of CTF-PS2 in stably transfected cells and concluded that this did not affect βAPP maturation (33). These discrepancies with our present work can likely be explained by several factors. The CTF constructions used in the Tomita study had different N-termini from ours. Also, the effect of CTF expression was monitored on x-40/42 species that likely include a major contribution of N-terminally truncated fragments derived from either pathogenic or also physiologic cleavages (33), whereas our antibodies were able to monitor total but genuine Aβ. Part of the data in Tomita's study concerns the effect of CTF on x-40/42 fragments derived from the C100 βAPP construction, the process-

ing/routing of which could be different from full-length βAPP. Finally, it can be noted that the respective cells systems are totally distinct [COS and N2a (33) versus HEK293 cells (present study)].

Our study documents the fact that CTF-PS1 and CTF-PS2 exhibit biological activity and particularly, control βAPP maturation. This does not preclude the possibility that intact remaining PS holoproteins also play a role in βAPP processing. The site of action of CTF is likely located upstream to α- and β/γ-secretases cell compartments since both Aβ and APPα secretions are similarly affected. This is in agreement with the fact that PS and their fragments have been mainly identified in the endoplasmic reticulum (7–11). It is interesting to emphasize that such a compartment has been shown to be accessible to the proteasome, therefore reinforcing the likelihood of involvement of the proteasome in the modulation of βAPP maturation through control of the intracellular concentration of CTF-PS1 and CTF-PS2.

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