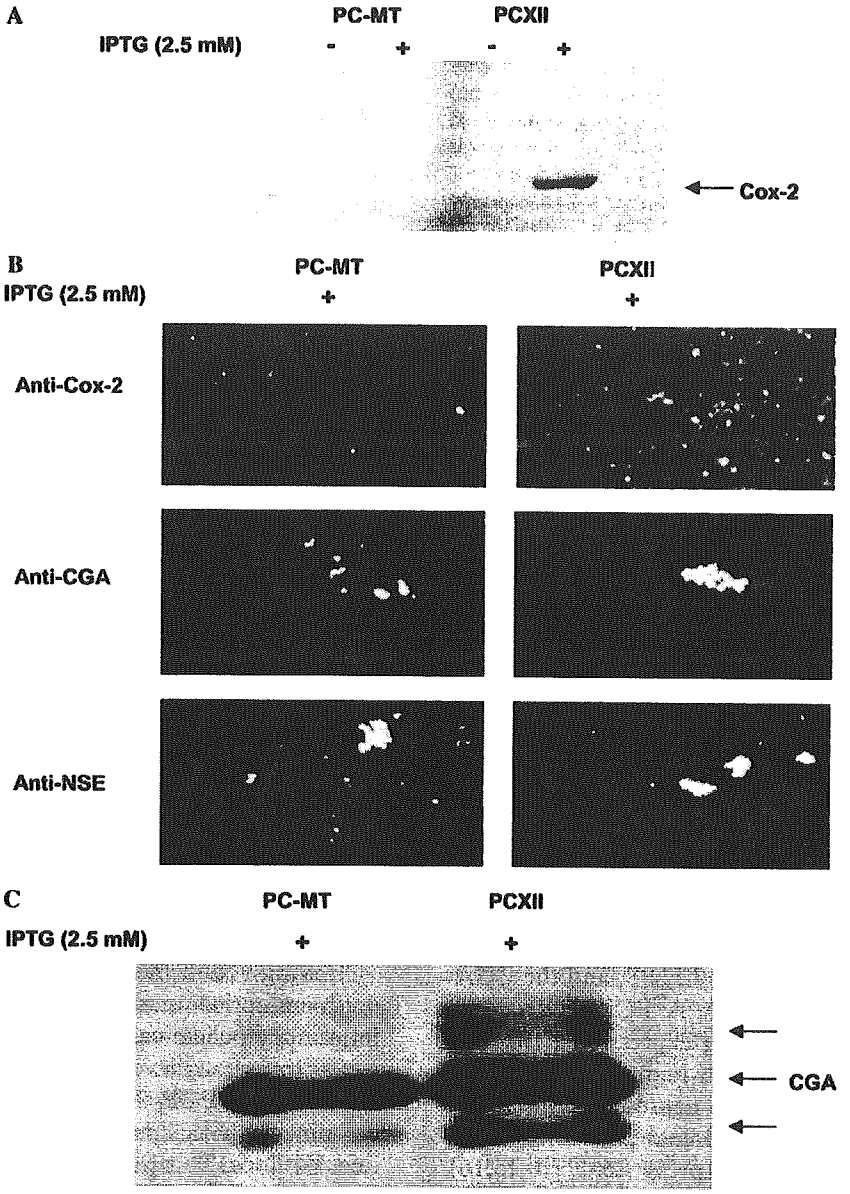

Fig. 2. Immunohistochemical and immunoblot analysis of Cox-2-dependent CGA upregulation in PCXII cells. (A) Immunoblot analysis of IPTG-inducible Cox-2 expression in PC-MT and PCXII cells. (B) Immunohistochemical analysis of Cox-2, CGA, and NSE expression in PC-MT and PCXII cells. (C) Immunoblot analysis of CGA expression in PC-MT and PCXII cells.

this would allow the putative effect of Bcl-2 on CGA-processing to be examined.

For each of the six neuroendocrine markers, five independent experiments were carried out on all four cell lines and images were recorded. Expression of all six neuroendocrine markers was confirmed in both the mock-transfected and Bcl-2 over-expressing cell lines (Figs. 3–8). Not surprisingly, as these represent ubiquitous neuroendocrine markers expressed in the majority of neuroendocrine cells and tissues [6–8,17], high levels (3+) of CGA, PGP 9.5, and NSE were demonstrated in all cell lines (Table 1). PP and the CGA-derived peptides IP and VST-I were also detected in all four cell lines, albeit at lower levels of expression (Table 1; [17]).

On the advice of a statistician, expression of the neuroendocrine markers by the two control cell lines was compared by means of the Mann–Whitney *U* test. Similarly, neuroendocrine marker expression in the two Bcl-2 over-expressing cell lines was compared by means of this statistical test. This analysis confirmed that no significant differences in the expression of any of the six peptides existed between paired control or paired Bcl2 over-expressing cell lines. Having thus ruled out potential clonal influences on neuropeptide expression levels, Mann–Whitney *U* analysis was carried out using grouped data from the control, and also from the Bcl-2 over-expressing, cell lines. Critically, no significant differences in expression were observed between control

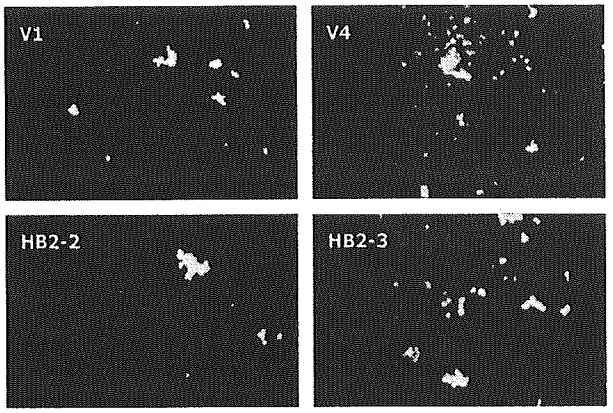

Fig. 3. Immunocytochemical analysis of NSE expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

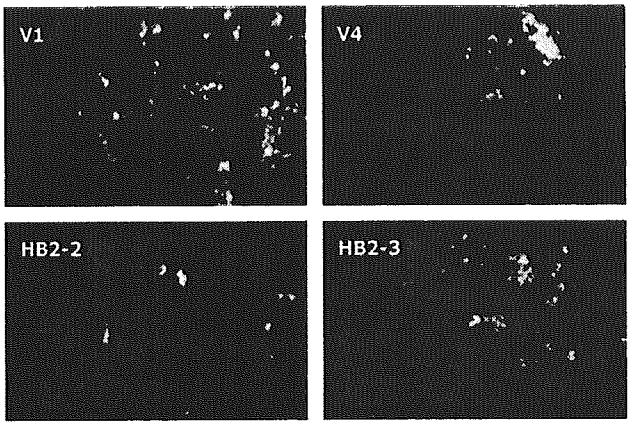

Fig. 4. Immunocytochemical analysis of CGA expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

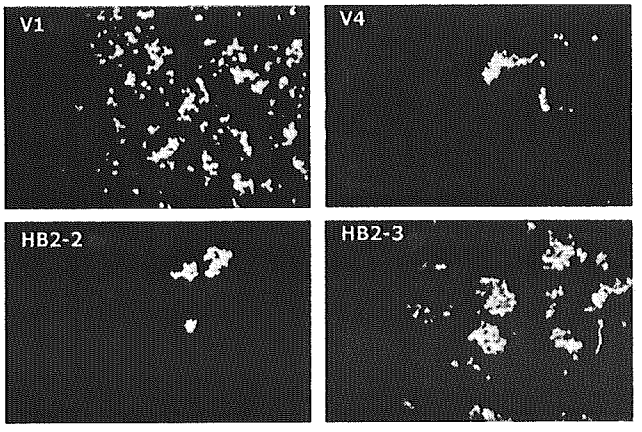

Fig. 5. Immunocytochemical analysis of PGP 9.5 expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

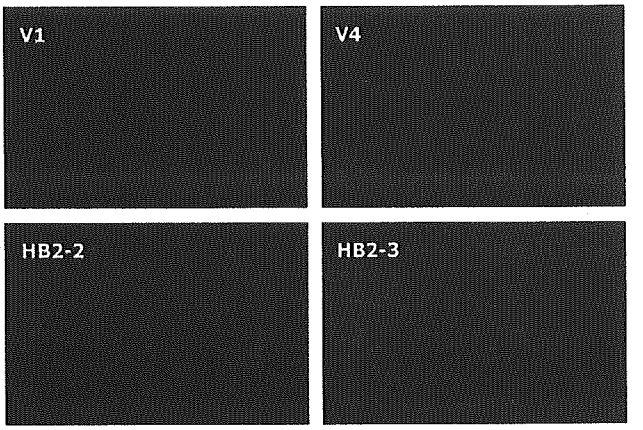

Fig. 6. Immunocytochemical analysis of PP expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

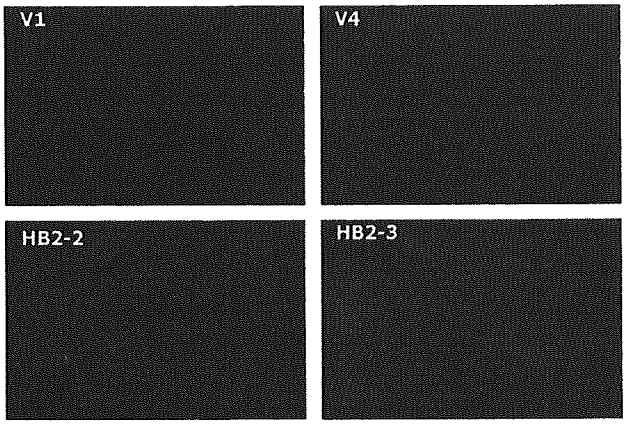

Fig. 7. Immunocytochemical analysis of IP expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

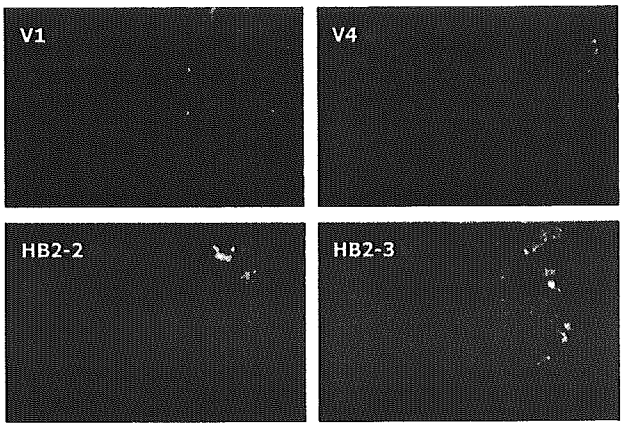

Fig. 8. Immunocytochemical analysis of VST-1 expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

and Bcl-2 over expressing cells for any of the six neuropeptides examined (Table 2). Given the similarity in levels of neuroendocrine marker expression between the two pairs of cell lines—with the exception of IP (control vs Bcl-2 over expressing lines; 2+ vs 1+; $p = 0.07$)—the finding that there were no differences between the CGA, NSE, PGP 9.5, PP, and VST-I expression levels was not unexpected (Table 2). Taken together, these results indicate that neither neuroendocrine marker expression nor

Table 1
Immunohistochemical assessment of neuroendocrine marker expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines

Peptide	V1	V4	HB2-2	HB2-3
CGA	+++	+++	+++	+++
NSE	+++	+++	+++	+++
PGP 9.5	+++	+++	+++	+++
PP	+	+	+	+
IP	++	++	+	+
VST-1	++	++	++	++

Shown are the immunopositivity grades from a representative experiment ($n = 5$).

Table 2
Statistical analysis of neuroendocrine marker expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines

Peptide	V1/V4 vs HB2-2/-3
CGA	$p = 1.00$
NSE	$p = 1.00$
PGP 9.5	$p = 1.00$
PP	$p = 1.00$
IP	$p = 0.07$
VST-1	$p = 1.00$

CGA processing appears to be modulated in response to Bcl-2 over-expression.

To our knowledge this is the first study to investigate whether Bcl-2 actually causes, rather than is simply associated with, enhanced neuroendocrine differentiation. It is also the first study to specifically quantify expression levels of neuroendocrine markers relative to those of Bcl-2. Obviously the results from the current study do not support the hypothesis that, in addition to its apoptosis-suppressing properties, Bcl-2 also promotes the acquisition of a neuroendocrine phenotype by positively modulating the expression of key neuroendocrine markers. As such the current results are at variance with previous reports that have suggested a link between Bcl-2 expression and enhanced neuroendocrine differentiation [6–8]. None of these reports have, however, demonstrated an inter-dependence between Bcl-2 expression and the development of a neuroendocrine phenotype, nor have they quantified relative levels of expression. For example, the association between Bcl-2 and neuroendocrine marker expression described by Ohmori et al. [7] was based on double immunostaining for Bcl-2 and several neuroendocrine markers in colon cancer tissue. While this, and similar studies [6,8], have confirmed that both Bcl-2 and various neuroendocrine proteins/peptides are present within the same cell, significantly, in none of these studies has a causal relationship been established.

In summary, the results of the current study do not support the hypothesis that Bcl-2 plays a role in the development of a neuroendocrine phenotype. In view of these findings it would appear that the reported expression of Bcl-2 in neuroendocrine cells [5–8] relates either to the well-documented anti-apoptotic actions of this oncoprotein or to additional, as yet undiscovered, functions. Previous studies have shown that forced Bcl-2 expression suppresses apoptosis of PC12 cells [15,16] and it has been speculated that the observed expression of Bcl-2 in oxyntic endocrine cells may promote cytosurvival during their maturation and downward migration from isthmal/neck-localised stem cells [8]. Numerous studies have documented Bcl-2 expression in neuroendocrine tumours such as phaeochromocytomas [18], thymic neuroendocrine tumours [19], and gastroenteropancreatic neuroendocrine tumours [20]. Investigations into the function of this oncoprotein in the pathology of neuroendocrine tumour development are, however, sparse and whether in this setting its actions differ from the apoptosis-inhibitory role it plays in more common cancers remains to be established.

Acknowledgments

The authors thank Mrs. Kathy Pogue and Mrs. Maureen McDevitt for their excellent technical support and advice. This study was funded by a grant (EAT/1889/01 to A.M. and B.J.) from the NIHPSS.

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Reconstitution of γ-secretase by truncated presenilin (PS) fragments revealed that PS C-terminal transmembrane domain is critical for formation of γ-secretase complex

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The presenilin (PS) complex, including PS, nicastrin (NCT), APH-1 and PEN-2, is essential for γ-secretase activity. Previously, the PS C-terminal tail was shown to be essential for γ-secretase activity. Here, to further understand the precise mechanism underlying the activation of γ-secretase regulated by PS cofactors, we focused on the role of the PS1 C-terminal region including transmembrane domain (TM) 8 in γ-secretase activity. For this purpose, we co-expressed C-terminally truncated PS1 (PS1ΔC) completely lacking γ-secretase activity and the PS1 C-terminal short fragment in PS-null cells, because the successful reconstitution of γ-secretase activity in PS-null cells by the co-expression of PS1ΔC and the PS1 C-terminal short fragment would allow us to investigate the role of the PS1 C-terminal region in γ-secretase activity. We found that the exogenous expression of the PS1 C-terminal short fragment with NCT and APH-1 completely rescued a defect of the γ-secretase activity of PS1ΔC in PS-null cells. With this reconstitution system, we demonstrate that both TM8 and the PS1 C-terminal seven-amino-acid-residue tail are involved in the formation of the active γ-secretase complex via the assembly of PS1 with NCT and APH-1.

Introduction

Amyloid β-protein (Aβ) is the major component of amyloid plaques that are a characteristic feature of the neuropathology of Alzheimer's disease (AD) (Selkoe 2001). Presenilin (PS1 and PS2) is an integral membrane protein that constitutes γ-secretase complex, required for the intramembranous proteolytic cleavage of β-amyloid precursor protein (APP) and the resulting production of Aβ (for review, see Selkoe 2001). Aβ has two major C-terminal variants, the Aβ that ends at residue 40 (Aβ40) and the Aβ that ends at residue 42 (Aβ42). Significantly, all AD-associated mutations in *PS* genes increase the relative production of Aβ42 which is more amyloidogenic than Aβ40, although the exact mechanism is not known (for review, see Selkoe 2001).

Recent accumulating evidence has also revealed that PS mediates not only γ-secretase activity (De Strooper *et al.* 1998), but it is also required for several intramembranous cleavages, including the cleavages of notch, CD44, ErbB-4, alcadein (Araki *et al.* 2004) and cadherin (for review, see De Strooper 2003). These results suggest that PS-mediated intramembranous cleavage plays a critical role in several biological functions. PS has a putative eighth transmembrane domain (Li & Greenwald 1998), and full-length PS is endoproteolytically processed into two fragments, the N-terminal fragment (NTF) and

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DOI: 10.1111/j.1365-2443.2005.00914.x

the C-terminal fragment (CTF) between transmembrane domain (TM) 6 and TM7 (Thinakaran *et al.* 1996). The cellular level of processed PS is tightly limited (Ratovitski *et al.* 1997; Thinakaran *et al.* 1997), and the processed PS resides in a high-molecular-weight complex that includes mature glycosylated NCT, APH-1 and PEN-2 (for review, see De Strooper 2003). Several lines of evidence clearly established that NCT, APH-1 and PEN-2 (collectively named PS cofactors in this study) are required for PS endoproteolysis and the formation of the active γ-secretase complex (Francis *et al.* 2002; Edbauer *et al.* 2003; Kimberly *et al.* 2003; Takasugi *et al.* 2003). However, it remains to be elucidated how PS cofactors regulate γ-secretase activity and PS endoproteolysis.

PS contains two conserved, essential aspartate residues in adjacent TM6 and TM7 that may define a novel aspartyl protease active site (Wolfe *et al.* 1999; Steiner *et al.* 2000; Li *et al.* 2000b; Weihofen *et al.* 2002). However, the precise catalytic mechanism underlying the formation of the γ-secretase complex, including the roles of TMs and PS cofactors in γ-secretase activity, is not completely understood. Previously, it was shown that a short C-terminal tail of PS is required for PS endoproteolysis and/or γ-secretase activity (Tomita *et al.* 1999; Shirotani *et al.* 2000). Here, to gain deep insights into the mechanism underlying the formation of active γ-secretase, we focused on the role of the PS1 C-terminal region including TM8 in γ-secretase activity. For this purpose, we co-expressed C-terminally truncated PS1 (PS1ΔC) completely lacking γ-secretase activity and the PS1 C-terminal short fragment in PS-null cells. Previously, it was shown that the co-expression of PS1 NTF and CTF restored γ-secretase activity in PS-null cells (Laudon *et al.* 2004; Shiraishi *et al.* 2004). However, it was not known whether the exogenous expression of PS1 C-terminal short fragment can rescue a defect in the γ-secretase activity of PS1ΔC. In this study, we found that the exogenous expression of the PS1 C-terminal short fragment with NCT and APH-1 completely rescued a defect of the γ-secretase activity of PS1ΔC in PS-null cells. With this reconstitution system, we demonstrate that both TM8 and the PS1 C-terminal seven-amino-acid-residue tail are involved in the formation of the active γ-secretase complex via the assembly of PS1 with NCT and APH-1.

Results

To clarify the role of the PS1 C-terminal region including C-terminal TM8 in γ-secretase activity, we constructed C-terminally truncated PS1 (PS1ΔC) and the PS1 C-terminal short fragment. As shown in Fig. 1A, PS1ΔC66 lacks PS1 C-terminal 66 amino acid residues including TM8, and PS1ΔC37, which was truncated downstream of TM8, lacks C-terminal 37 amino acid residues including the PALP sequence (Tomita *et al.* 2001). We also constructed cDNA encoding the PS1 C-terminal short fragment starting at methionine. C68 and C37 correspond to the fragments of the first methionine plus PS1 C-terminal 68 and 37 amino acid residues, respectively. PS1ΔC66 and PS1ΔC37 exhibited the complete loss of Aβ generation and PS endoproteolysis in PS-null cells, as observed in the PS1 mutant with other short C-terminal truncations (Bergman *et al.* 2004) (Fig. 1B).

We first investigated whether the co-expression of PS1ΔC66 and PS1 cofactors restore Aβ generation in PS-null cells (Fig. 2A, left 5 lanes). As shown in Fig. 2A (lanes 1–5), no Aβ generation was observed, suggesting that PS1ΔC66 failed to form the active γ-secretase complex with PS cofactors. However, the exogenous expression of C68 with the APH-1b and NCT completely rescued a defect in the γ-secretase activity of PS1ΔC66, although the expression of C68 without PS cofactors did not affect γ-secretase activity (Fig. 2A, lanes 6–8). The endogenous level of PEN-2 was found to be slightly increased in the co-expression of PSΔC66, C68, APH-1b and NCT, but the endogenous level of PEN-2 was not as high as that of PS null cells expressing full-length PS (Fig. 2B). Therefore, the exogenous expression of PEN-2 was expected to further enhance the reconstituted γ-secretase activity. However, a further increase in γ-secretase activity was not observed when PEN-2 was additionally co-expressed with C68, APH-1b and NCT (Fig. 2A, lane 9). The limiting factors for γ-secretase activity in this reconstitution system were found to be both APH-1 and NCT as shown in Fig. 2C. The rescue in a defect in PS1ΔC66 endoproteolysis was also observed by the co-expression of C68, APH-1b and NCT (Fig. 2A, lane 8), and PS1ΔC66 endoproteolysis was further stimulated by the expression of PEN-2 with C68, APH-1b and NCT (Fig. 2A, lane 9). Previously, the C-terminal short tail of PS was shown to be required for PS endoproteolysis and PS stabilization (Tomita *et al.* 1999; Shirotani *et al.* 2000). Therefore, we next investigated whether the expression of C68 lacking the last seven amino acid residues (C68ΔC7) with PS cofactors also rescues the defects in γ-secretase activity and PS1ΔC66 endoproteolysis. The result showed that the co-expression of C68ΔC7 and PS cofactors did not restore γ-secretase activity and endoproteolysis (Fig. 2A, lanes 10 and 11), indicating that the rescue by the co-expression of C68 and PS cofactors is completely dependent on the presence of

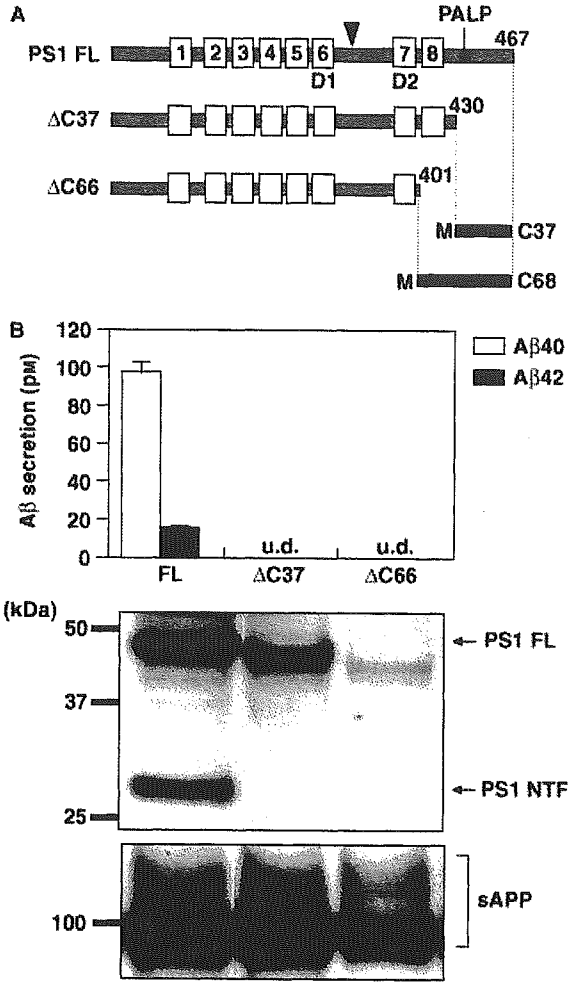


Figure 1 Schematic diagram of C-terminally truncated forms of PS1 (PS1ΔC) and PS1 C-terminal fragments expressed in (A) PS-null cells, and (B) a defect in Aβ generation and endoproteolysis of PS1ΔC. (A) A schematic diagram of C-terminally truncated forms of PS1 used in this study is shown. The last 37 (ΔC37) or 66 (ΔC66) amino acid residues of PS1 were deleted. PS1 C-terminal fragments starting at Met (M) encoded by the cDNAs used in this study are also shown. C37, Met plus the PS1 C-terminal 37 amino acid residues; C68, Met plus the PS1 C-terminal 68 amino acid residues. Boxes with numbers illustrate putative transmembrane domains (TM). D1 and D2, two aspartates essential for γ-secretase activity; PALP, the PALP sequence (56); FL, full-length; arrowhead, the site of endoproteolytic processing; number denotes the position of an amino acid residue at the C-terminus. (B) APP695 and the full-length (FL) PS1 or PS1ΔC were retrovirally expressed in PS-null cells (2×10^5); Aβ40 and Aβ42 secreted from cells during a 48-h culture were quantified by ELISA (the upper panel). u.d., Aβ was not detected (< 10 pM). Values are means ± SD of two independent dishes ($n = 2$). CHAPSO-solubilized lysates (10 μg) were immunoblotted with the anti-PS1 NTF antibody (the middle panel). Soluble APP secreted from cells were immunoblotted with the anti-APP antibody, 22C11 (the bottom panel).

the C-terminal seven-amino-acid-residue tail (Fig. 2, lanes 10 and 11). It was also noted that the extent of Aβ generation from the reconstituted γ-secretase induced by the co-expression of PS1ΔC66, C68 and PS cofactors was higher than that from the co-expression of PS1 FL and PS cofactors.

To further investigate the effects of the co-expression of PS1ΔC66 and C68 on the assembly of the active γ-secretase complex, we performed the co-immunoprecipitation experiment using an anti-PS1 NTF antibody (Fig. 3). As shown in Fig. 3A, APH-1b and NCT failed to co-immunoprecipitate with PS1ΔC66 (Fig. 3A, lanes 1–4), but they were co-immunoprecipitated when C68 was co-expressed (Fig. 3A, lanes 5 and 6); however, PEN-2 co-immunoprecipitated with PS1ΔC66 even when exogenous C68 was not expressed (Fig. 3, lanes 4 and 6). It is also noted that the level of mature NCT in the complex reconstituted with PS1ΔC66 and C68 was lower than that when PS1 FL with cofactors are expressed (Fig. 3, lanes 1 and 2; lanes 5 and 6). We also confirmed that C68 has the domain(s) for the binding of NCT and APH-1 by co-immunoprecipitation in the cells expressing C68 in the absence of the PS1ΔC66 (Supplementary Fig. S1).

In addition, when C68ΔC7 was expressed, no assembly of PS1ΔC66 with PS cofactors was observed (Fig. 3A, lanes 7 and 8). We also found that the level of C68ΔC7 bound to PS1ΔC66 was lower than that of C68 bound to PS1ΔC66 (Fig. 3A, bottom panel). The co-immunoprecipitation experiment using the anti-NCT antibody also demonstrated that the smaller amount of C68ΔC7 co-immunoprecipitates with NCT than that of C68 (Fig. 3B). This result indicates that the assembly of C68, not only with PS1ΔC66, but also with NCT, requires the presence of the C-terminal seven-amino-acid-residue tail. This result agrees with a result of a recent study showing that PS1 C-terminus binds to NCT (Kaether *et al.* 2004). Taken together, the rescue of the defects in γ-secretase activity and PS1ΔC66 endoproteolysis by the expression of C68 with PS cofactors is accompanied by the rescue of a defect in the assembly of PS1ΔC66 with APH-1b and NCT.

We next determined the role of TM8 in γ-secretase activity with this reconstitution system of γ-secretase activity. For this purpose, we co-expressed PS1ΔC66 and C37 that lacks TM8 in PS-null cells (Figs 1A and 4A). As shown in Fig. 4A, the co-expression of C37 and PS cofactors did not rescue the defects in γ-secretase activity and PS1ΔC66 endoproteolysis. This result suggests that the TM8 region of C68 is necessary for the functional rescue of inactive PS1ΔC66. However, we cannot exclude the possibility that C37 does not have an ability to rescue

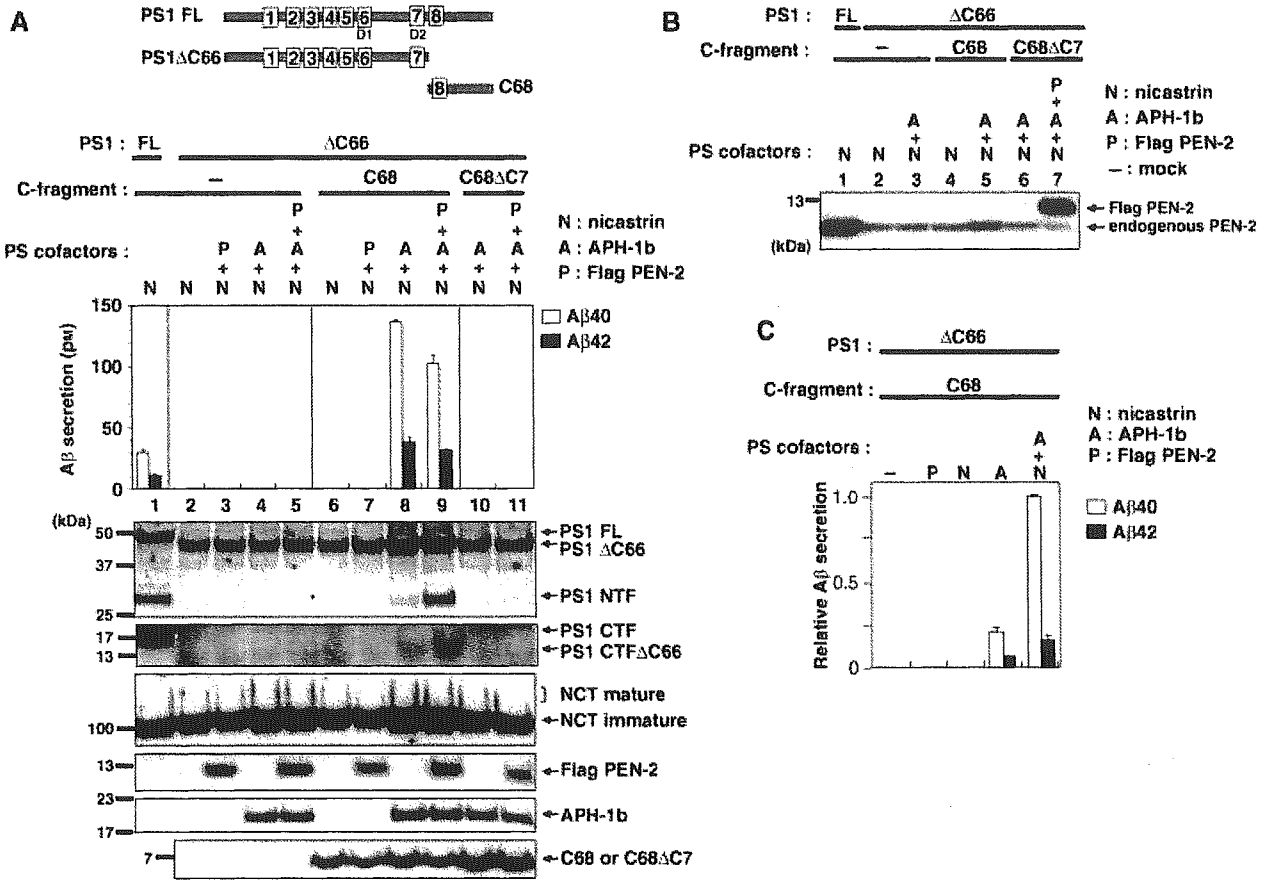


Figure 2 The expression of C68 with PS cofactors rescued defects in γ-secretase activity and endoproteolysis of PS1ΔC66. (A) After APP695 and the indicated exogenous PS cofactors were retrovirally expressed with PS1ΔC66 and C68 or C68ΔC7 in PS-null cells, Aβ secreted from these cells during a 48-h culture was quantified by ELISA, and CHAPSO-solubilized lysate (10 μg) was immunoblotted with the anti-PS1 NTF antibody (for the detection of PS1 FL, PS1ΔC66 and PS1 NTF), anti-PS1 loop monoclonal antibody (for the detection of PS1 CTF and CTFΔC66), anti-APH-1b antibody, anti-FLAG antibody (for the detection of PEN-2), anti-NCT antibody and the anti-PS1C-20 antibody (for the detection of C68 or C68ΔC7). C-fragment, C-terminal short fragment; –, mock transfection (pMX). Values are means ± SD of two independent dishes (n = 2). Data are representative of three independent experiments. (B) The same lysates prepared from the PS-null cells retrovirally expressing the indicated truncated PS and PS cofactors as described in Figure 2A were immunoblotted with anti-PEN-2 antibody for the detection of endogenous PEN-2. (C) After APP695 and the indicated exogenous PS cofactors (–, mock transfection) were retrovirally expressed with PS1ΔC66 in PS-null cells, Aβ secreted from these cells during a 48-h culture was quantified by ELISA. The Aβ levels were expressed as relative to Aβ40 levels obtained from PS-null cells expressing PS1ΔC66 with NCT and APH-1b.

a defect in γ-secretase activity. Therefore, we next determined whether C37 has an ability to rescue inactive PS1ΔC, or whether TM8 is necessary for the functional rescue of inactive PS1ΔC in PS-null cells. For this purpose, we investigated whether the co-expression of C37 and PS1ΔC37 truncated downstream of TM8 (Figs 1A and 4B) restores γ-secretase activity in PS-null cells. As shown in Fig. 4B, the defects in γ-secretase activity and PS1ΔC37 endoproteolysis were completely rescued by the expression of C37 with APH-1b and NCT, although they were not rescued by the expression of exogenous PS cofactors without C37 (Fig. 4B, lanes 2–9). These results indicate that TM8 is necessary for the rescue of γ-secretase activity and PS1 endoproteolysis by the co-expression of PS1ΔC and the PS1 C-terminal fragment with PS cofactors in PS-null cells. The rescue by the co-expression of C37 and PS cofactors was also completely dependent on the presence of the C-terminal seven-amino-acid-residue tail (Fig. 4, lanes 10 and 11). It is also noted that the level of C37 is higher when APH-1 and NCT were co-expresssed, compared with that when APH-1 was not expressed (Fig. 4A, lanes 2–5 and Fig. 4B, lanes 6–9).

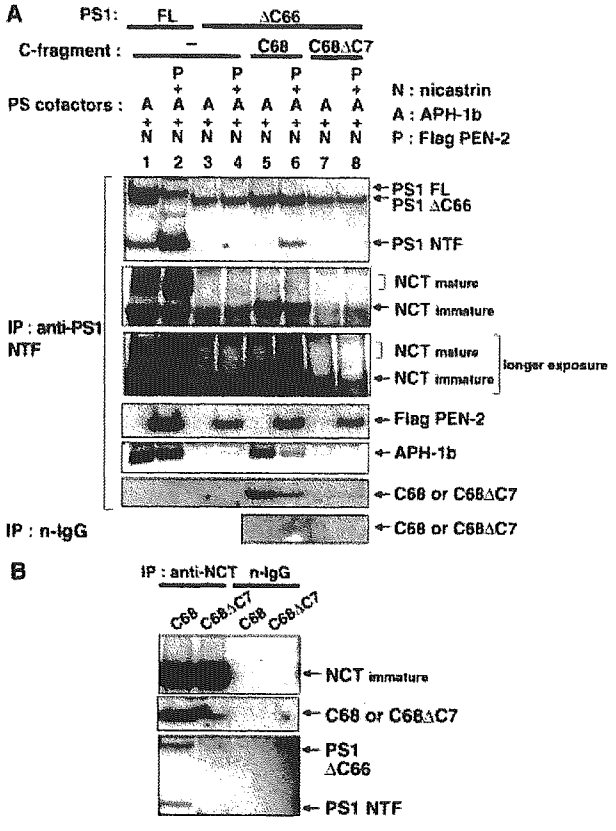


Figure 3 The expression of C68 rescued a defect in the assembly of PS1ΔC66 with APH-1 and NCT. (A) After APP695 and the indicated exogenous PS cofactors were retrovirally expressed with PS1ΔC66 and C68 or C68ΔC7 in PS-null cells, CHAPSO-solubilized lysate (1 mg) was immunoprecipitated (IP) with the anti-PS1 NTF antibody (H-70) and then immunoblotted with the anti-PS1 NTF antibody (Chemicon International, Inc.), anti-APH-1b antibody, anti-FLAG antibody, anti-NCT antibody and anti-PS1C-20 antibody. n-IgG, normal IgG (control for the anti-PS1NTF antibody). (B) The CHAPSO-solubilized lysate (1 mg) used in lanes 6 and 8 of A was immunoprecipitated (IP) with the anti-NCT antibody and then immunoblotted with the anti-NCT antibody, anti-PS1C-20 antibody (for the detection of C68 or C68ΔC7) and the anti-PS1 NTF antibody. n-IgG, normal IgG (control for the anti-NCT antibody). Data are representative of two independent experiments.

Thus, APH-1 is likely to stabilize the C37 fragment, although, at present, the exact reason for this is not known. We next determined whether TM8 is involved in the assembly of the active γ-secretase complex. As shown in Fig. 5, APH-1b and NCT were co-immunoprecipitated using the anti-PS1 NTF antibody when PS1ΔC37 and C37 were co-expressed, although the anti-PS1 antibody did not co-immunoprecipitate APH-1b and NCT when PS1ΔC66 and C37 were co-expressed. PEN-2 was also co-immunoprecipitated in both cases (Fig. 5, lanes 2 and 4). Interestingly, C37 was also co-immunoprecipitated with PS1ΔC37, PS1 CTFΔC37, NCT, APH-1, and PEN-2, indicating that all truncated PS fragments, including CTFΔC37, constitute the active γ-secretase complex (Fig. 5). These results indicate that TM8 is involved in the PS1 complex assembly with NCT and APH-1b, and in PS endoproteolysis. Although C68 has the domain(s) for the binding of NCT and APH-1 (Supplementary Fig. S1), at present, we failed to determine by co-immunoprecipitaiton whether C37 has such domains, because C37 was unstable in the absence of PS1ΔC37 (data not shown). However, a previous study using a chimeric protein of CD4 TM domain followed by PS1 C-terminal fragment corresponding to the exact C37 region in our study (Kaether *et al.* 2004), strongly suggested that the C37 region has the domain(s) for the binding of NCT and APH-1. All results are summarized in Table 1.

Discussion

Although several lines of evidence have established the notion that PEN-2, APH-1 and NCT, in addition to PS, are required for the formation of the active γ-secretase complex (Francis *et al.* 2002; Edbauer *et al.* 2003; Kimberly *et al.* 2003; Takasugi *et al.* 2003), it remains unclarified how these three PS cofactors activate γ-secretase. To gain deep insights into the mechanism underlying the formation of the active γ-secretase complex, we reconstituted γ-secretase activity in PS-null cells by the co-expression of C-terminal truncated PS1 and the PS1 C-terminal short fragment. Using this reconstitution system, we found that both PS1 TM8 and the PS1 C-terminal last seven-amino-acid-residue tail are critical for γ-secretase activity and the assembly of the PS1 complex with APH-1 and NCT.

The PS C-terminal tail includes a hydrophobic stretch, which is a potential domain for the interaction with some proteins such as PDZ-domain-containing proteins (Saras *et al.* 1997; Tomita *et al.* 1999). Previously, it was pointed out that the PS C-terminal tail is important for PS endoproteolysis and PS stabilization (Tomita *et al.* 1999; Shirotani *et al.* 2000). Recently, it has also been shown that a short deletion of the C-terminal region from PS1 causes marked impairments in PS1 endoproteolysis and γ-secretase activity in PS-null cells (Bergman *et al.* 2004), indicating that the PS1 C-terminal region is critical for γ-secretase activity. However, it is not precisely known whether the PS C-terminal region is a functional domain for the formation of active γ-secretase or whether a short deletion at the C-terminus causes a conformational change leading to a loss of γ-secretase

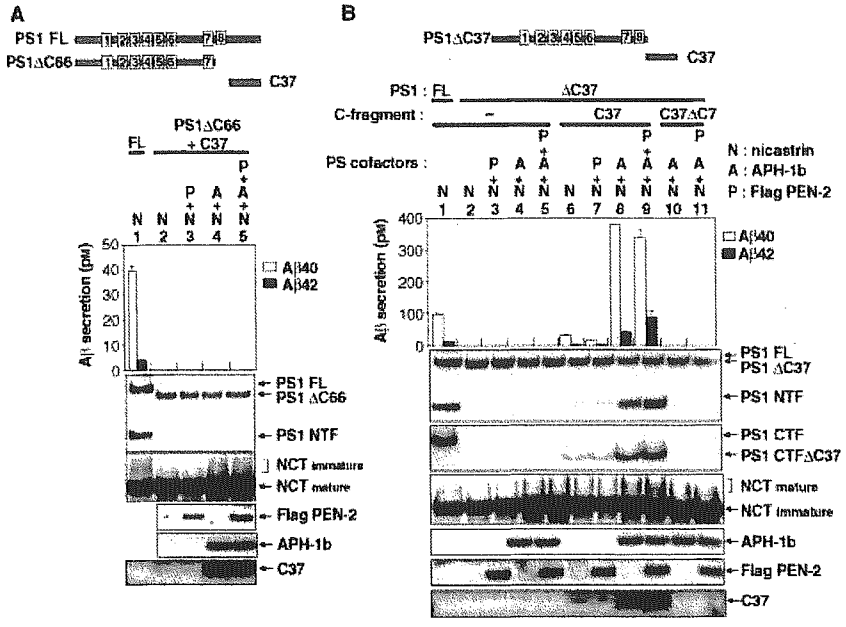


Figure 4 The rescue of a defect in γ-secretase activty of PS1ΔC by the expression of PS1 C-terminal fragment with PS cofactors requires the PS1 TM8 region. After APP695 and the indicated exogenous PS cofactors were retrovirally expressed with (A) PS1ΔC66 and C37 or (B) PS1ΔC37 and C37 in PS null cells, Aβ secreted from these cells during a 48-h culture was quantified by ELISA, and CHAPSO-solubilized lysate (10 μg) was immunoblotted with the anti-PS1 NTF antibody (for detection of PS1 FL and PS1 ΔC37), anti-PS1 loop antibody (for detection of PS1 CTF and PS1 CTFΔC37), anti-APH-1b antibody, anti-FLAG antibody (for the detection of PEN-2), and the PS-C3 antibody (for detection of C37; note: this antibody does not immunoreact with C37ΔC7). –, mock transfection (pMX). Values are means ± SD of two independent dishes ($n = 2$). Data are representative of three independent experiments. The difference in Aβ level from PS1 FL between A and B is due to the difference in virus titer used for transfection.

activity. The successful reconstitution of γ-secretase activity in PS-null cells by the co-expression of C-terminally truncated PS1 and the PS1 C-terminal short fragment demonstrated that the PS1 C-terminal region has a distinct functional domain for the formation of active γ-secretase, and enabled us to investigate its role in γ-secretase activity.

PS1ΔC66 has two aspartate residues in TM6 and TM7, which are essential for γ-secretase activity, but PS1ΔC66 lacks the entire C-terminal region, including TM8 and the region immediately downstream from TM8. PS1ΔC66 exhibited the complete loss of γ-secretase activity and endoproteolysis in PS-null cells; however, we found that the exogenous expression of a C-terminal fragment starting from TM8, that is, C68, completely rescued these defects when APH-1 and NCT were co-expressed. The limiting factors for γ-secretase activity in this reconstitution system were found to be APH-1 and NCT, not PEN-2 (Fig. 2C), and the restored endogenous expression level of PEN-2 is sufficient to reconstitute the γ-secretase activity of inactive PS1ΔC66. Indeed, this interpretation was supported by our result showing that PS1ΔC66 had a marked defect in the binding of NCT and APH-1, but PS1ΔC66 did not have a significant defect in the binding of PEN-2. In addition, the rescue of γ-secretase activity was completely accompanied by a rescue of the defect in the assembly of PS1ΔC66 with APH-1 and NCT. It was also noted that these rescues were completely dependent on the presence of the C-terminal last seven-amino-acid-residue tail of C68. This result is completely consistent with the previous result showing that the extreme C-terminus of PS1 is essential for the assembly of active γ-secretase (Bergman *et al.* 2004). From these results, we concluded that the active γ-secretase complex is reconstituted by the exogenous co-expression of PS1ΔC66, C68 and PS cofactors APH-1 and NCT.

Recently, it has been shown that the PS1 C-terminus is involved in the interaction with NCT and APH-1 (Bergman *et al.* 2004). We also showed that the association of C68ΔC7 with PS1ΔC66 and NCT was lower than that of C68 (Fig. 3B). Therefore, the failure of C68 lacking the C-terminal seven-amino acid residues (C68ΔC7) to rescue the formation of the active γ-secretase complex was likely to be caused by the lower association of C68ΔC7 with PS1ΔC66 and NCT, and possibly with APH-1, than that of C68 (Fig. 3B).

PS endoproteolysis is not always associated with γ-secretase activity, because mutant PS1 Δexon 9 is not endocleaved (Thinakaran *et al.* 1996). However, this mutant PS1 has γ-secretase activity (Wolfe *et al.* 1999). Therefore, the necessity of PS endoproteolysis for γ-secretase activity has not been firmly established. Our result also showed that the reconstituted γ-secretase activity induced by the truncated PS fragments is not associated with the extent of PS endoproteolysis

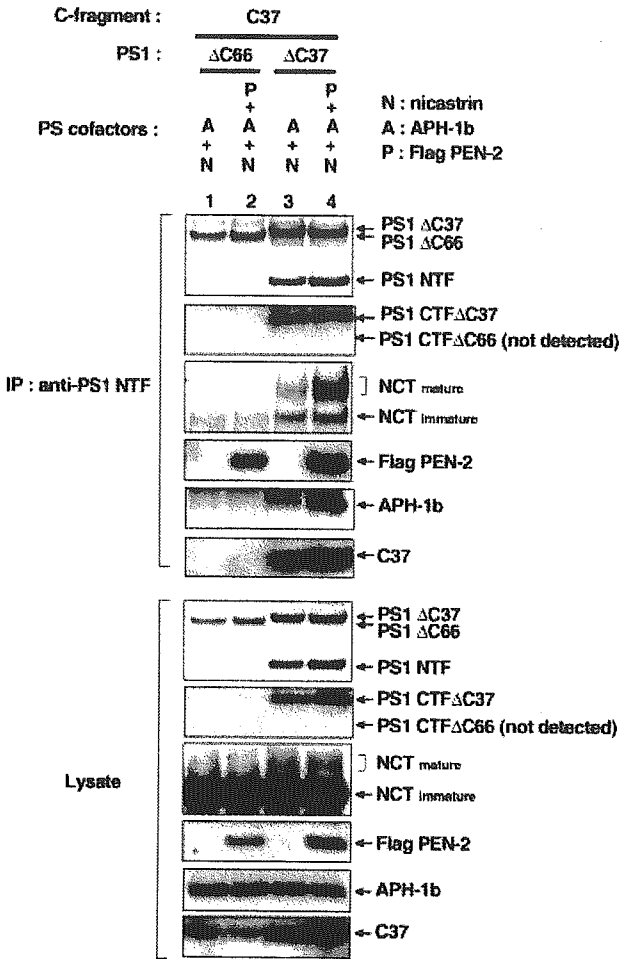


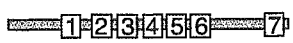
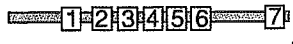
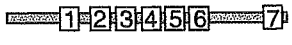
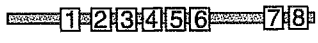
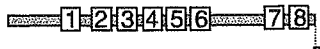
Figure 5 The assembly of the γ-secretase complex requires the PS1 TM8 region. After APP695 and the indicated exogenous PS cofactors were retrovirally expressed with PS1ΔC66 and C37 or with PS1ΔC37 and C37 in PS-null cells, CHAPSO-solubilized lysate (1 mg) was immunoprecipitated (IP) with the anti-PS1 NTF antibody (H-70) and then immunoblotted (IB) with the anti-PS1 NTF antibody (Chemicon International, Inc.; for detection of PS1ΔC37, PS1ΔC66 and PS1 NTF), anti-PS1 loop monoclonal antibody (for detection of PS1 CTFΔC37), anti-APH-1b antibody, anti-FLAG antibody, anti-NCT antibody and PS-C3 antibody (for detection of C37). CHAPSO-solubilized lysate (10 μg) was also immunoblotted with the same antibodies (the bottom four panels). Data are representative of two independent experiments. Note: PS1 NTF and PS1 CTFΔC66 in lanes 1 and 2 were not detected, because the expression of PS1ΔC66 with C37 and PS cofactors did not cause the endoproteolysis of PS1ΔC66.

although the stimulation of PSΔC endoproteolysis by the expression of exogenous PEN-2 was observed in this reconstitution system (Figs 2A, 3A, and 4B). In addition, the reconstituted γ-secretase activity by the truncated PS fragments was dependent on APH-1 level, while γ-secretase activity from FL PS1 is not dependent on the APH-1 level (Supplementary Fig. S2). At present, we do not know the exact reason for the difference in the exogenous PS cofactors required for the stimulation of γ-secretase activity between the expression of PS FL and that of the truncated PS fragments. One possible explanation is that PS FL requires PS endoproteolysis for the conformational change from an inactive form to an active form, and this step is the limiting step for the activation of PS FL; however, the truncated PS fragments might not require PS endoproteolysis for forming the active complex, probably because the complex of the truncated PS fragments has a lower structural integrity than that of the PS FL. Instead, the truncated PS fragments required APH-1 and NCT rather than the stimulation of PS endoproteolysis by PEN-2 expression for the proper conformation and/or the proper trafficking of the complex for reconstituted γ-secretase activity. The difference in the structural integrity could also generate the difference in γ-secretase activity between the PS complex and the complex reconstituted by the truncated PS fragments, as observed in this study.

As previously reported (Herreman *et al.* 2003; Nyabi *et al.* 2003), the maturation of NCT is separable from γ-secretase activity, strongly suggesting that the difference in the extent of maturation of NCT in the PS complex does not affect γ-secretase activity. Indeed, our result showed that the γ-secretase activity reconstituted with PS1ΔC66 and C68 was not associated with the full maturation of NCT. Although, at present, the exact mechanism for the poor maturation of NCT in the reconstituted truncated PS complex is unknown, we speculate that the intracellular site, where the assembly of truncated PS fragments with PS cofactors occurs, may be slightly different from the case of wild-type (wt) PS, because PS that lacks the C-terminal portion does not reside in the ER as previously reported (Kaether *et al.* 2004), whereas full-length PS resides in the ER. The assembly of full-length wt PS with PS cofactors is likely to occur in the ER (Capell *et al.* 2005; Niimura *et al.* 2005), which is followed by the transport of the complex into the Golgi compartment, where the terminal sugar modification of NCT occurs. However, if the truncated PS fragments and the over-expressed immature NCT exist in the distal/post-Golgi compartment, they form the active γ-secretase complex; the gylcosylation of NCT in the complex could be less than that in the wt PS complex.

To determine whether the TM8 of C68 is required for the rescue of a defect in the γ-secretase activity of PS1ΔC66, we investigated whether the C37 fragment, which is immediately downstream of TM8 and therefore

Table 1 Summary of functional rescue of C68 and C37 for inactive PS1ΔC in PS-null cells

	Transgene (+ exogenous three PS cofactors)	γ-secretase activity	Assembly of PS1ΔC with NCT	APH-1	PEN-2
1	PS1 FL 	+	+	+	+
2	PS1ΔC66 	–	–	–	+
3	PS1ΔC66 				
	 C68	+	+	+	+
	 C68ΔC7	–	–	–	+
4	PS1ΔC66 				
	C37	–	–	–	+
5	PS1ΔC37 	–	–	–	+
6	PS1ΔC37 				
	 C37	+	+	+	+
	C37ΔC7	–	* –	* –	* +

1, 2 and 3, summary of the results of Figs 2 and 3; 4, 5 and 6, summary of the results of Figs 4 and 5. +, rescued; –, not rescued; * data not shown.

lacks TM8, can similarly rescue a defect in the γ-secretase activity of PS1ΔC66. The result showed a failure of the rescue; however, the expression of C37 with APH-1 and NCT rescued a defect in the γ-secretase activity of PS1ΔC37 that has TM8, but lacks the C-terminal region immediately downstream of TM8. These results clearly demonstrate that TM8 is involved in the formation of an active γ-secretase. Indeed, C37 expression did not rescue a defect in the assembly of PS1ΔC66 with NCT and APH-1, but it significantly rescued a defect in the assembly of PS1ΔC37 with NCT and APH-1, indicating that TM8 is involved in the assembly of PS1ΔC37 with NCT and APH-1. Thus, we concluded that TM8 is also required for the formation of an active PS1 complex with NCT and APH-1 (Table 1).

Previously, it was shown that the TM of NCT is involved in the assembly of an active γ-secretase complex, and that the cytoplasmic domain of NCT is dispensable for γ-secretase complex formation (Capell *et al.* 2003). It was also shown that the GXXXG motif in the TM of APH-1 is critical for the assembly of the γ-secretase complex (Lee *et al.* 2004), strongly suggesting that the TM of APH-1 is involved in the assembly of this complex. Taken together with our results, the TM8 of PS1 is likely to interact with NCT or APH-1 or both through their transmembrane. In addition, our data also demonstrate that the PS1 C-terminal seven-amino-acid-residue tail is critical for the assembly of the γ-secretase complex in the reconstitution system (Table 1). A previous study has shown that the PS1 C-terminus probably binds to the TM of NCT (Kaether *et al.* 2004). Therefore, both the C-terminus and PS1 TM8 appear to bind to the TM of NCT. Although C68 and C37 are likely to have the domains for the interaction of NCT and APH-1 (Supplementary Fig. S1; Kaether *et al.* 2004), how the TM8 in concert with C-terminus interact with NCT and APH-1 to form the active PS complex remains to be determined in future studies. The formation of an intermediate subcomplex of APH-1 and NCT as previously shown (LaVoie *et al.* 2003) also could be a prerequisite for the interaction of PS TM8 and the C-terminus with APH-1/NCT.

Because the proposed catalytic aspartate residues are embedded in TM6 and TM7, it remains to be clarified how hydrolysis that is required for γ-secretase activity occurs within the hydrophobic environment. Interestingly,

recent studies have shown the possibility that PS has a ninth TM in the C-terminal region (Henricson *et al.* 2005; Laudon *et al.* 2005). If this is the case, the C37 in our study is supposed to harbor the novel TM9. Although, at present, the precise roles of the TM8 and possibly also TM9 are unknown, one possible role is that the C-terminal TM(s) contribute to the formation of the catalytic space between TM6 and TM7 within the hydrophobic environment of the lipid bilayer, because these TM(s) are found to be necessary for γ-secretase activity and the association with APH-1/NCT, that are the essential cofactors for γ-secretase activity.

In this study, we established the reconstitution of γ-secretase activity by truncated PS fragments and PS cofactors. With this reconstitution system, we demonstrated that both PS1 TM8 and the PS1 C-terminal last-seven-amino-acid-residue tail are critical for γ-secretase activity and the assembly of the PS1 complex with APH-1 and NCT. More precise studies of how TM8 and the C-terminal tail are involved in the assembly of the γ-secretase complex may help clarify the regulation of γ-secretase activity.

Experimental procedures

Antibodies, reagents and cell lines

The monoclonal antibody 6E10 specific to human Aβ1-17 was purchased from Senetek (St. Louis, MO, USA). The other Aβ antibodies have all been characterized previously (Asami-Odaka *et al.* 1995). The anti-APP N-terminal antibody 22C11 was purchased from Sigma (St. Louis, MO, USA). A rat anti-PS1 antibody (for NTF of PS1) and a mouse anti-PS1 loop monoclonal antibody were purchased from Chemicon International, Inc. (Temecula, CA, USA). A goat anti-PS1 C-20 antibody (for PS1 C-terminal 20 amino acid residues) and a rabbit anti-PS1 H-70 antibody (for PS1 N-terminal 70 amino acid residues) were purchased from Santa Cruz Biotechnology, Inc. A rabbit PS-C3 antibody was prepared against the synthetic peptide corresponding to the C-terminal 15 amino acid residues of PS1. An anti-FLAG antibody was purchased from Sigma. A rabbit anti-human APH-1b antibody was prepared against the synthetic peptide corresponding to the C-terminal 17 amino acid residues of APH-1b. An anti-nicastrin antibody was purchased from Sigma. A rabbit anti-PEN-2 antibody (for the detection of the endogenous PEN-2) was purchased from Zymed Laboratory Inc. PS1/PS2 double-deficient murine fibroblasts (PS-null cells) and wild-type murine fibroblasts immortalized with a large T antigen were maintained as previously described (Herreman *et al.* 2000; Sai *et al.* 2002).

Plasmids and retrovirus-mediated gene expression

cDNAs encoding PS1ΔC were generated from pMX-PS1 by the PCR method. The primer sequences used for the PCR were as follows: a sense primer: 5′-TGCAGAATTCATGACAGAGTTACCTGCA-3′; and anti-sense primers: 5′-CATGCTCGAGTCATTTCTTGAAAATGGCAAGGAG-3′ (PS1CΔ37: the last 37 amino acids deletion), and 5′-CATGCTCGAGTCAACTGGCTGTTGCTGAGGCTTT-3′ (PS1ΔC66: the last 66 amino acids deletion). The PCR products were digested with *Eco*RI and *Xho*I inserted into pMX (Onishi *et al.* 1996). cDNA encoding PS1 C-terminal 37 (C37: residues 431–467) or 68 (C68: residues 400–467) amino acid residues starting at Met, which was added by the *Eco*RI site followed by Kozak consensus sequence at the 5′ end and the *Xho*I site at the 3′ end was generated by the PCR method using the following primers: sense primers, 5′-TGCAGAATTCCACCATGGCATTGCCAGCTCTTCCA-3′ (C37) and 5′-TGCAGAATTCCACCATGGCCAGTGGAGACTGGAAC-3′ (C68); and an anti-sense primer, 5′-CATGCTCGAGCTAGATATAAAATTGATGG-3′. The resultant cDNAs were inserted into pMX at *Eco*RI and *Xho*I. cDNAs encoding C37ΔC7 and C68ΔC7 were generated by the PCR method using the following primers: a sense primer for C37 or C68; and an anti-sense primer: 5′-CATGCTCGAGCTATAATTGGTCCATAAAAGGCTG-3′. The resultant cDNAs were inserted into pMX at *Eco*RI and *Xho*I.

pMX-F-PEN-2 is a *Bam*HI-*Sal*I fragment carrying the sequence encoding the N-terminal FLAG-tagged PEN-2 and Kozak consensus sequence (CCACC) at the 5′ end of FLAG-PEN-2 inserted into the *Bam*HI and *Sal*I sites of pMX as previously described (Shiraishi *et al.* 2004). pMX-NCT, pMX-PS1, and pMX-APP695 were constructed as previously described (Komano *et al.* 2002). APH-1b (Francis *et al.* 2002) was generated from the cDNA library prepared from HEK293 cells using a sense primer, GCGAATTCTTTCCGCGGTGGCCATGACT and anti-sense primer, GCAGATCTGAAGTGCTGGTTCCCTGAGG. The PCR product was digested with *Eco*RI and inserted into pMX at the *Eco*RI site.

All resulting constructs were verified by DNA sequencing. Retrovirus-mediated gene expression in cells was carried out as previously reported (Onishi *et al.* 1996; Komano *et al.* 2002). The infection efficiency was nearly 100% in this study, as estimated in a control experiment using pMX-GFP (retroviral vector carrying GFP).

Aβ detection and co-immunoprecipitation techniques

Aβ level was determined by ELISA as previously described (Asami-Odaka *et al.* 1995). The capture antibody used was BNT77. The detector antibodies used were horseradish peroxidase (HRP)-conjugated BA27 (for Aβ40) and HRP-conjugated BC05 (for Aβ42). ELISA data were statistically analyzed by ANOVA using StatView-J.4.11 (Abacus Concepts, Inc., Berkeley, CA, USA). Cultured cells were lyzed in 1% CHAPSO buffer [1% CHAPSO, 150 mM NaCl, 10 mM Tris/HCl (pH 7.5), 2 mM EDTA, a protease inhibitor cocktail]. CHAPSO-solubilized proteins were co-immunoprecipitated with PS1 by incubating with the anti-PS1 NTF antibody and 100 μL of 20% protein-G Sepharose (Pharmacia) slurry with rotation at 4 °C overnight, as

previously described (Sudoh *et al.* 1998; Li *et al.* 2000a). The immunoprecipitates were solubilized in SDS sample buffer (0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 8 M urea) and subjected to SDS-PAGE.

Acknowledgements

This study was supported by the Program for the Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research; by a Grant-in-Aid for Scientific Research on Priority Areas (C)-Advanced Brain Science Project (to K.Y.); by a Grant-in-Aid 15659023 and 16390029 from the Ministry of Education, Culture, Sports, Sciences and Technology; by a grant from the Takeda Medical Research Foundation; by a Grant for Dementia and Bone Fracture from the Ministry of Health, Labor and Welfare, Japan. We thank Dr B. De Strooper (Katholieke Universiteit Leuven and Flanders Interuniversity, Herestraat, Belgium) for providing PS1/PS2 double-deficient fibroblasts and wild-type fibroblasts.

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Received: 2 August 2005
Accepted: 3 October 2005

Supplementary material

The following supplementary material is available for this article online:

Figure S1 Interaction of C68 with NCT and APH-1

Figure S2 Aβ generation in PS-null cells expressing truncated PS fragments is APH-1-dependently increased

Journal of Neurochemistry, 2006, **96**, 1491–1499 doi:10.1111/j.1471-4159.2006.03668.x

Homocysteine-induced endoplasmic reticulum protein (Herp) is up-regulated in sporadic inclusion-body myositis and in endoplasmic reticulum stress-induced cultured human muscle fibers

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Abstract

Herp is a stress-response protein localized in the endoplasmic reticulum (ER) membrane. Herp was proposed to improve ER-folding, decrease ER protein load, and participate in ER-associated degradation (ERAD). Intra-muscle-fiber ubiquitinated multiprotein-aggregates containing, among other proteins, either amyloid-β (Aβ) or phosphorylated tau are characteristic of sporadic inclusion-body myositis (s-IBM). ER stress and proteasome inhibition appear to play a role in s-IBM pathogenesis. We have now studied Herp in s-IBM muscle fibers and in ER-stress-induced or proteasome-inhibited cultured human muscle fibers. In s-IBM muscle fibers: (i) Herp was strongly immunoreactive in the form of aggregates, which co-localized with Aβ, GRP78, and β2 proteasome subunit; (ii) Herp mRNA and protein were increased. In ER-stress-induced cultured human muscle fibers: (i) Herp immunoreactivity was diffusely increased; (ii) Herp mRNA and protein were increased. In proteasome-inhibited cultured human muscle fibers: (i) Herp immunoreactivity was in the form of aggregates; (ii) Herp protein was increased, but its mRNA was not. Accordingly, in s-IBM muscle fibers: (i) increase of Herp might be due to both ER-stress and proteasome inhibition; (ii) co-localization of Herp with Aβ, proteasome, and ER-chaperone GRP78 could reflect its possible role in processing and degradation of cytotoxic proteins in ER.

Keywords: inclusion-body myositis; homocysteine-induced endoplasmic reticulum protein; endoplasmic reticulum stress; unfolded/ misfolded proteins; proteasome inhibition; amyloid-β.
J. Neurochem. (2006) **96**, 1491–1499.

The endoplasmic reticulum (ER) functions include processing, folding, and exporting of newly synthesized proteins into the secretory pathway (Ellgaard and Helenius 2001). An efficient system of molecular chaperones in the ER is required to assure proper folding of misfolded abnormal and native proteins (Kaufman 1999). Unfolded proteins accumulating in the ER lead to ER-stress (Rutkowski and Kaufman 2004; Shen *et al.* 2004). This elicits the unfolded protein response (UPR), a functional mechanism by which cells attempt to protect themselves against ER-stress (Mori 2000; Rutkowski and Kaufman 2004). The UPR involves: (i) attenuating translation to reduce protein overload and subsequent accumulation of unfolded proteins (Mori 2000; Liu and Kaufman 2003); (ii) transcriptional induction of ER

Received August 10, 2005; revised manuscript received October 19, 2005; accepted November 11, 2005.

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Abbreviations used: Aβ, amyloid-β; AβPP, amyloid-β precursor protein; AD, Alzheimer disease; ATF4, activating transcription factor 4; BiP/GRP78, immunoglobulin heavy chain-binding protein/glucose-regulated protein 78; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); Herp, homocysteine-induced ER protein; s-IBM, sporadic inclusion-body myositis; ULD, ubiquitin-like domain; UPR, unfolded protein response; XBP-1, X-box-binding protein 1.

chaperone proteins, which increase folding capacity of the ER and prevent protein aggregation (Mori 2000; Liu and Kaufman 2003); (iii) removing misfolded proteins from the ER through retrograde transport coupled to their degradation by 26S proteasome, called ER-associated degradation (ERAD) (Travers *et al.* 2000).

Herp (homocysteine-induced ER protein) is a novel 54 kDa protein recently described as a stress-response protein localized in the ER membrane (Kokame *et al.* 2000). Under ER-stress, Herp is the most highly inducible protein (Yamamoto *et al.* 2004). Unlike other stress-induced ER chaperones, Herp is an integral membrane protein whose N- and C- terminals both face the cytoplasm (Kokame *et al.* 2000; Sai *et al.* 2002). Its N-terminus has an ubiquitin-like domain (ULD) (Kokame *et al.* 2000), which is probably involved in Herp degradation via the ubiquitin–proteasome pathway (Sai *et al.* 2003). Cellular functions of Herp are gradually being discovered. It was suggested that during ER stress, Herp plays a neuroprotective role in brain by stabilizing ER Ca^{2+} homeostasis and maintaining mitochondrial function (Chan *et al.* 2004). Herp was also proposed to improve ER folding capacity, decrease ER protein load (Hori *et al.* 2004), and participate in the ERAD (Yamamoto *et al.* 2004; Schroder and Kaufman 2005). Herp was also proposed to prevent apoptosis triggered by ER stress (Chan *et al.* 2004; Hori *et al.* 2004). Furthermore, Herp was reported to interact with presenilins and, through this mechanism, to increase production of amyloid-β (Aβ) (Sai *et al.* 2002), which is considered to be cytotoxic in sporadic inclusion-body myositis (s-IBM) muscle and in Alzheimer disease (AD) brain (Askanas and Engel 2001, 2003; Selkoe 2001; Cuello 2005).

s-IBM, the most common progressive degenerative muscle disease of persons age 50 years and older, is of unknown etiology and pathogenesis (Askanas and Engel 2001, 2002). It leads to severe disability and there is no successful treatment (Askanas and Engel 2001, 2003). The main light-microscopic features of s-IBM muscle biopsies include: (i) vacuolated muscle fibers, (ii) intramuscle-fiber multiprotein aggregates, and (iii) various degrees of mononuclear-cell inflammation (Askanas and Engel 2001, 2003). An intriguing feature is the similarity of the s-IBM muscle-fiber phenotype to that of AD brain, including accumulation of Aβ, phosphorylated tau, and several other Alzheimer characteristic proteins (Askanas and Engel 2001, 2003). Two major types of intracellular inclusion-bodies in s-IBM muscle contain either Aβ or phosphorylated tau (Askanas and Engel 2001, 2002). Both types of inclusions are congophilic, indicating that they contain proteins in alternate conformation (unfolded or misfolded) that are assembled into the β-pleated sheet configuration of amyloid (Askanas and Engel 2001, 2003). The cytoplasmic inclusion-bodies are present mainly in vacuole-free regions of vacuolated muscle fibers, and in muscle fibers not vacuolated at the level of the inclusion-bodies.

Aβ and phosphorylated-tau inclusions contain several other accumulated proteins, some of which are present in both (Askanas and Engel 2001, 2003). Some, such as α- synuclein, have, similarly to Aβ and tau, a propensity to unfold, misfold, and form β-pleated-sheet amyloid (Borden 1998).

We have recently demonstrated in s-IBM muscle fibers that several ER chaperones are accumulated in the form of aggregates and their total expression is increased, suggesting that ER stress and UPR play a role in the s-IBM pathogenesis (Vattemi *et al.* 2004). Those ER chaperones physically associate with Aβ, suggesting that they may play a role in Aβ folding and/or attempted disposal of Aβ in s-IBM muscle fibers (Vattemi *et al.* 2004). Our most recent study demonstrated significant proteasomal inhibition and aggresome formation in s-IBM fibers (Fratta *et al.* 2005). Those studies further support our previous proposal that unfolding and misfolding of proteins play a role in formation of the multiprotein-aggregates (inclusions) within s-IBM muscle fibers (Askanas and Engel 2002, 2003).

In the present study, we asked whether Herp might play a role in the s-IBM pathogenesis. We examined Herp expression in s-IBM muscle fibers on the mRNA and protein levels, and Herp light- and electronmicroscopic immunolocalization, including its co-immunolocalization with Aβ, ER chaperone BiP/GRP78 (immunoglobulin heavy chain-binding protein/glucose-regulated protein 78), 20S β2 proteasome subunit, and ubiquitin. To explore the mechanisms involved in regulating Herp in human muscle fibers, we utilized ER stress-induced and proteasome-inhibited cultured human muscle fibers.

Experimental procedures

Muscle biopsies

Immunocytochemical studies were performed on 10-μm-thick unfixed sections of fresh-frozen diagnostic muscle biopsies obtained (with informed consent) from 34 patients with these diagnoses: 13 s-IBM; four polymyositis; one dermatomyositis; two morphologically non-specific myopathy; two mitochondrial myopathy; one peripheral neuropathy; two amyotrophic lateral sclerosis; two oculopharyngeal muscular dystrophy, one non-IBM vacuolar myopathy, and six normal muscle. Diagnoses were based on clinical and laboratory investigations, including our routinely performed 18-reaction diagnostic histochemistry of the biopsies. All s-IBM biopsies had muscle fibers with vacuoles on Engel trichrome staining (Engel and Cunningham 1963), 15–21 nm paired helical filaments by SMI-31 immunoreactivity (Askanas *et al.* 1996a) and by electronmicroscopy, and Congo-red positivity using fluorescence enhancement (Askanas *et al.* 1993b).

Light- microscopic immunocytochemistry

Fluorescence immunocytochemistry was performed as described (Askanas *et al.* 1993a, 1996a, 2000; Vattemi *et al.* 2003, 2004)

using a well-characterized polyclonal antibody against Herp, diluted 1 : 100 (Kokame *et al.* 2000). Double-immunofluorescence utilized an antibody against Herp combined with one of the following: (i) mouse monoclonal antibody 6E10 (Signet, Dedham, MA, USA), diluted 1 : 100, which morphologically recognizes Aβ in both AD brain (Kim *et al.* 1990) and s-IBM muscle (Askanas *et al.* 2000); (ii) mouse monoclonal antibody recognizing BiP/GRP78 (BD Transduction Laboratories, San Diego, CA, USA) diluted 1 : 20; (iii) mouse monoclonal antibody recognizing 20S proteasomal subunit β2 (Affinity Research Products Ltd, UK) diluted 1 : 50; (iv) mouse monoclonal antibody recognizing ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 50.

To block non-specific binding of antibody to Fc receptors, sections were pre-incubated with normal goat or rabbit serum diluted 1 : 10, as described (Askanas *et al.* 1993a, 2000). Controls for staining specificity were (i) omission of the primary antibody or (ii) its replacement with non-immune sera or irrelevant antibody.

Immuno-electronmicroscopy

Double-label gold immuno-electronmicroscopy was performed on 10-μm unfixed frozen biopsy sections adhered to the bottom of 35-mm Petri dishes, as detailed (Askanas *et al.* 1993a, 1996a, 2000). In brief, a primary antibody against Herp was used in combination with an antibody against Aβ, 20S proteasome, or BiP/GRP78. After incubation with the appropriate secondary antibodies conjugated to 5 nm and 15 nm gold particles, sections were processed for electronmicroscopy as described (Askanas *et al.* 1993a, 1996a, 2000).

Immunoblotting

Muscle biopsies of seven s-IBM and seven age-matched control patients were immunoblotted, as recently detailed (Vattemi *et al.* 2003, 2004). Briefly, 10-μm-thick frozen sections were collected in ristocetin-induced platelet agglutination (RIPA) buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 0.1% sodium dodecyl sulfate) containing phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany). All samples were rapidly homogenized on ice. Protein concentration was measured using the Bradford method (Vattemi *et al.* 2004): 20 μg of protein were loaded onto 10% NuPAGE gels (Invitrogen, Carlsbad, CA, USA), electrophoresed, transferred to nitrocellulose membranes, and immuno-probed with antibodies against Herp. After incubation in the appropriate secondary antibodies, blots were developed using an enhanced chemiluminescence system (ECL) (Amersham Biosciences, Piscataway, NJ, USA). Protein loading was evaluated by the actin band visualized with a mouse monoclonal antibody (Santa Cruz Biotechnology). Quantification of the immunoreactivity was performed by densitometric analysis using NIH Image J 1.310 software.

RNA isolation and reverse transcription–polymerase chain reaction

Total RNA from 10-μm-thick frozen sections of s-IBM and control muscle biopsies was isolated using an RNA isolation kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. RNA samples were treated with Dnase I (Amplification Grade, Invitrogen) and 1 μg of RNA was submitted for cDNA synthesis using an oligo-dT primer (Invitrogen) and the Omniscript RT kit (Qiagen, Valencia, CA, USA). Then 1/10 of the RT reaction was amplified using multiplex PCR in a total volume of 20 μL, with Platinum Taq DNA Polymerase (Invitrogen), utilizing previously described primers for Herp (Kokame *et al.* 2000) and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Lehmann *et al.* 2002) as an internal control. The optimized conditions for Herp and GAPDH amplification were 2 min at 94°C followed by 28 cycles of amplification (94°C for 30 s, 60°C for 30 s, 72°C for 90 s), and the final incubation was 10 min at 72°C. The PCR products were separated on a 2% agarose gel and stained with ethidium bromide. The conditions of the reactions were experimentally checked to ensure that signals were in the linear range of the PCR. Identity of the products was confirmed by sequencing.

Statistical analysis

The statistical significance of differences between groups was determined by Student's *t*-test. The level of significance was set at $p < 0.05$. Data are presented as means ± SEM for all groups.

Cultured human muscle fibers

Primary cultures of normal human muscle were established from satellite cells of portions of diagnostic muscle biopsies from patients who, after all tests were performed, were considered free of muscle disease (Askanas and Engel 1992; Askanas *et al.* 1996b). Experiments were performed on seven culture sets, each established from a different muscle biopsy. All experimental conditions were studied on sister cultures in the same culture sets. Three-week-old cultured muscle fibers were treated for 24 h with either (i) one of two well-known ER stress inducers, N-glycosylation inhibitor tunicamycin (4 μg/mL) or an inhibitor of ER-calcium-ATPase thapsigargin (300 nM) (Back *et al.* 2005; Lee 2005) (both from Sigma Co, St. Louis, MO, USA) or (ii) epoxomicin (1 μM) (Biomol Research Laboratories, Plymouth Meeting, PA, USA), a specific and irreversible proteasome inhibitor (Meng *et al.* 1999). After treatment, control and experimental cultures were fixed for immunostainings, harvested for immunoblot studies, or used for RNA isolation.

Light-microscopic immunocytochemistry

Single and double immunofluorescence on paraformaldehyde-fixed cultures was performed as described (Askanas *et al.* 1996b, 1997; Fratta *et al.* 2005).

Immunoblotting

Cultured cells were harvested in ristocetin-induced platelet agglutination (RIPA) buffer containing phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Roche Diagnostic). The immunoblotting procedure was performed as described above for muscle biopsies.

Reverse transcription–polymerase chain reaction and northern blots

Total RNA from control and experimental cultures was extracted using RNA-bee reagent (Tel Tech, Friendwood, TX, USA). After treatment with Dnase I, 150 ng of RNA was used for one-step synthesis of cDNA and the multiplex PCR reaction (Qiagen). The optimized conditions were 30 min at 50°C, 15 min at 95°C

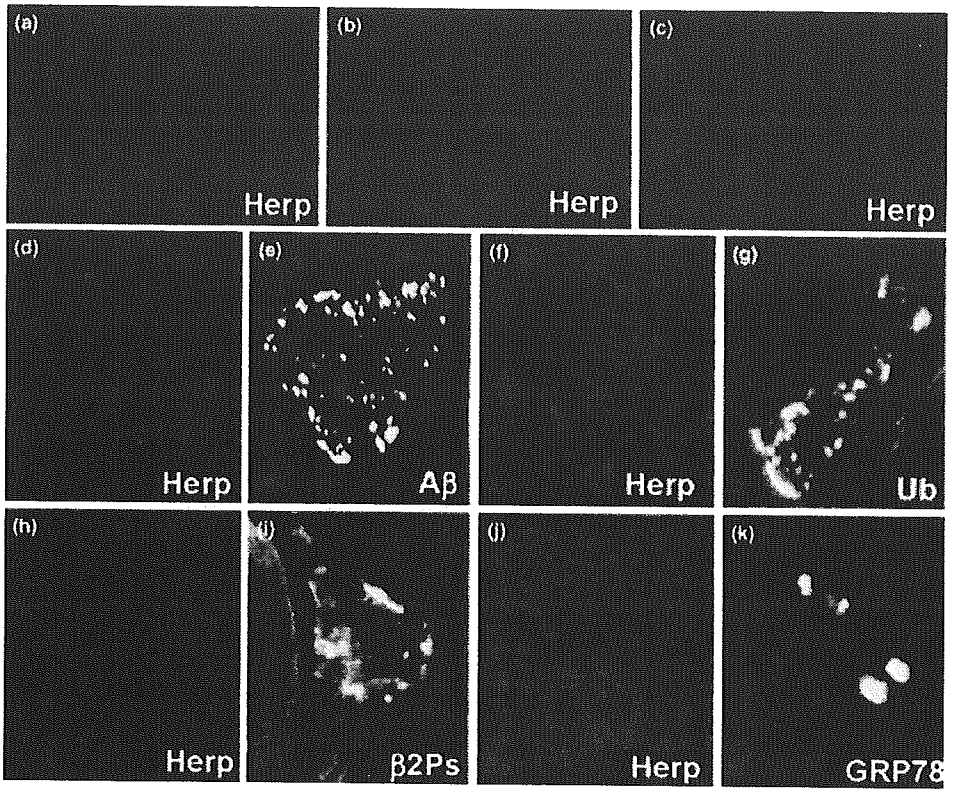

Fig. 1 Immunofluorescence within sporadic inclusion-body myositis muscle fibers. (a–c) Single-label immunofluorescence illustrates immunoreactivity of homocysteine-induced endoplasmic reticulum protein (Herp) in the form of various-sized aggregates. Double-label immunofluorescence illustrates that Herp immunoreactive inclusions (d, f, h, j) co-localized with amyloid-β (Aβ) (e), ubiquitin (g), β2 20S proteasome subunit (i), and Bip/GRP78 ER chaperone (k). Magnification: a–c, f–k, ×1100; d, e ×700. BiP/GRP78, immunoglobulin heavy chain-binding protein/glucose-regulated protein 78; β2Ps, β2 20S proteasome subunit; Ub, ubiquitin.

followed by 28 cycles of amplification (94°C for 30 s, 60°C for 30 s, 72°C for 90 s), and the final incubation of 10 min at 72°C.

For northern blots, aliquots of RNA (10 μg) isolated from experimental and control cultures were denatured and subjected to electrophoresis in 1% agarose gel containing 0.41 M formaldehyde. After electropheretic fractionation, RNA was transferred overnight to a positively charged nylon membrane by capillary blotting and fixed by UV light. A Herp-specific probe was generated by PCR and fluorescein-labelled (Kokame *et al.* 2000). Hybridization and detection procedures using anti-fluorescein-AP conjugate were performed as described (Kokame *et al.* 2000).

Statistical analysis

The statistical analysis was as described above.

Results

Muscle biopsies

Light-microscopic immunocytochemistry

In all s-IBM muscle biopsies, 70–80% of the vacuolated muscle fibers contained, mainly in their non-vacuolated cytoplasm, numerous various-sized aggregates immunoreactive with antibodies against Herp (Figs 1a–c). Approximately 25% of non-vacuolated muscle fibers also contained similar aggregates. By double immunofluorescence, Herp immunoreactive aggregates co-localized with Aβ (Figs 1d and e), ubiquitin (Figs 1f and g), β2 proteasome subunit (Figs 1h and i), and BiP/GRP78 (Figs 1j and k). None of the control non-s-IBM, diseased or normal, human muscle biopsies had muscle fibers containing aggregates immunoreactive with anti-Herp antibody. Eliminating the primary antibodies or replacing them with non-relevant antibodies resulted in non-staining.

Gold-immuno-electronmicroscopy

Immunoreactive Herp was associated with Aβ, 20S β2 proteasome subunit, and BiP/GRP78 within the same structures, namely the 6–10 nm fibrils and amorphous and floccular material (Fig. 2). Herp was not associated with paired helical filaments, which are known to contain phosphorylated tau in IBM muscle fibers (Mirabella *et al.* 1996).

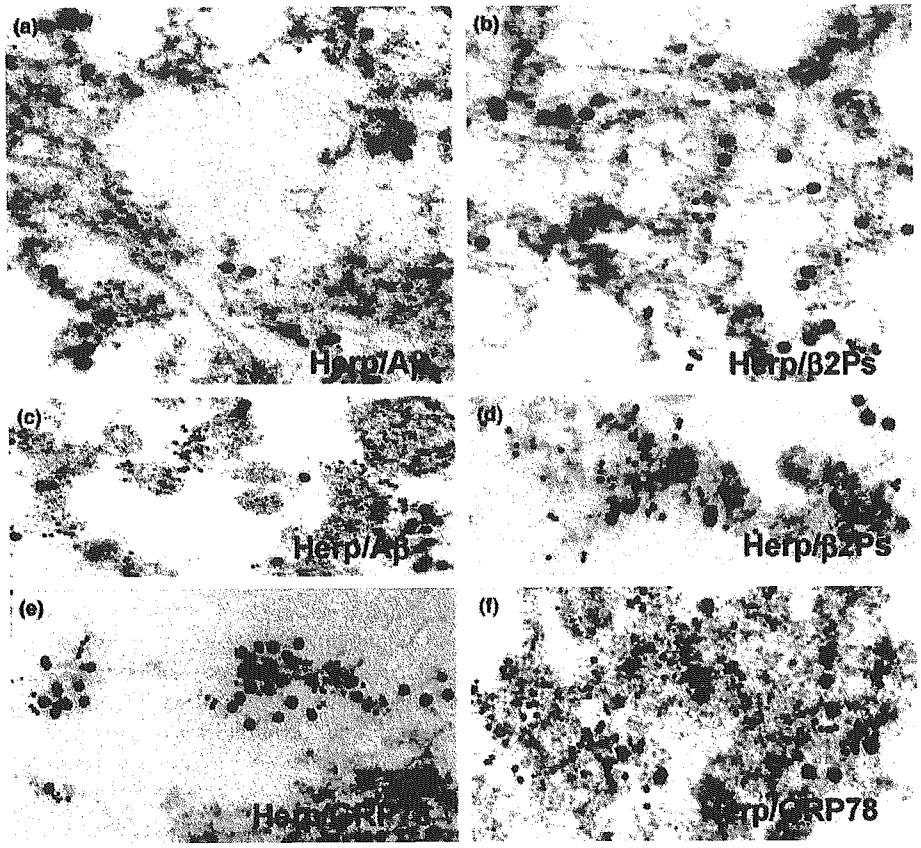

Fig. 2 Double-label gold-immuno-electronmicroscopy in sporadic inclusion-body myositis muscle fibers. This illustrates that both homocysteine-induced endoplasmic reticulum protein (Herp) (5 nm gold particles) and amyloid-β (Aβ) (15 nm gold particles) are associated with 6–10 nm fibrils (a) and floccular material (c). Similarly, Herp (5 nm gold) and β2 20S proteasome subunit (15 nm gold) (b, d) and Herp (5 nm gold) and BiP/GRP78 (15 nm gold) (e, f) are associated with the same 6–10 nm fibrils and floccular material. Magnification: a, c–f, ×51 000; b, ×60 000. BiP/GRP78, immunoglobulin heavy chain-binding protein/glucose-regulated protein 78; β2Ps, β2 20S proteasome subunit; Ub, ubiquitin.

Immunoblots

In normal and s-IBM muscle, Herp migrated as a single 54 kDa band (Fig. 3a), and its expression was much stronger in s-IBM muscle as compared to controls. Omission of the primary antibody resulted in no bands being present (Fig. 3b). Figure 3(c) provides analysis of the data derived from densitometric scans (obtained from seven samples), normalized to β-actin and expressed in arbitrary units. These data indicate that in s-IBM muscle biopsies Herp protein was increased eight-fold ($p < 0.05$) as compared to controls.

RNA expression by reverse transcription–polymerase chain reaction

As determined by RT–PCR analysis, Herp mRNA was prominently increased in s-IBM as compared to controls (Figs 3d and e).

Cultured human muscle fibers

Light-microscopic immunocytochemistry

In control fibers, Herp immunoreactivity was only weak and diffuse (Fig. 4a). ER stress-induced cultured human muscle fibers had strong and diffuse Herp immunoreactivity (Figs 4b and c). In contrast, approximately 80% of the epoxomicin-treated muscle fibers had the Herp protein immunoreactivity in the form of large aggregates (Figs 4d, e, and g), which co-localized with ubiquitin immunoreactivity (Figs 4f and h).

Immunoblots

Treatment with ER-stress inducers or with epoxomicin prominently increased the Herp protein level as illustrated by two representative culture sets (Fig. 5a). Figure 5(b) provides densitometric analysis of the data from seven