



An investigation into the role of Bcl-2 in neuroendocrine differentiation

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Abstract

Introduction: In addition to its role in apoptosis suppression, Bcl-2 has been reported to be co-expressed with neuroendocrine markers in several tissues, leading to speculation that this oncoprotein may promote neuroendocrine differentiation.

Aim: This study investigated whether Bcl-2 modulated neuroendocrine biopeptide expression.

Methods: Levels of chromogranin A, neurone specific enolase, protein gene peptide 9.5, pancreatic polypeptide, and the chromogranin-derived peptides, intervening peptide and vasostatin-1 were examined by immunocytochemistry in rat pheochromocytoma (PC12) cell lines genetically engineered to over-express Bcl-2 and their mock-transfected controls. Intensity of fluorescence was graded using a semi-quantitative scale from (–) indicating negative expression to (++++) indicating intense positivity.

Results: Mann–Whitney *U* analysis indicated that no significant differences in expression existed between control and Bcl2 over-expressing cell lines for any of the six peptides examined.

Conclusions: The results of this study do not support the hypothesis that Bcl-2 promotes the acquisition of a neuroendocrine phenotype.

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Bcl-2 is an anti-apoptotic cellular protein [1]. Studies have shown that elevated expression of this protein is associated with several malignancies [1–4]. Models of over-expression suggest that Bcl-2-dependent inhibition of apoptosis is important in tumour formation [1–4]. In addition to its well-characterised role in the suppression of programmed cell death, evidence exists that Bcl-2 is expressed both in true neuroendocrine cells and in cells that have acquired a neuroendocrine phenotype [5–7], leading to speculation that this protein may not be involved solely in cytoprotection. For example, the anterior pituitary neuroendocrine cells of the adult rhesus

monkey represent the only brain cell population consistently exhibiting a non-microglial pattern of Bcl-2 expression [5]. Bcl-2 and neuroendocrine marker co-expression has also been demonstrated in a range of histologically distinct lung tumours [6]. In this study Bcl-2 expression was found to be closely associated with the neuroendocrine differentiation of tumour cells, leading the authors to suggest that this protein may be involved in cell development and differentiation [6]. Similarly, in colon carcinoma cells and neighbouring crypt cells, elevated Bcl-2 expression has been found to correlate with the expression of a number of bioactive neuroendocrine markers. Double immunostaining techniques were used to retrospectively analyse whether advanced colorectal carcinomas and tumour neighbouring mucosa produced both Bcl-2 and a range of gastrointestinal neuropeptides.

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These workers provided evidence that Bcl-2 immunoreactivity correlated with the expression of key neurohormonal polypeptides and amines including vasointestinal polypeptide (VIP), pancreatic polypeptide (PP), and somatostatin in both carcinoma tissue and in the neighbouring mucosa, and suggested that in this setting Bcl-2 may promote endodermal stem cell neuroendocrine differentiation [7]. Similarly, Azzoni et al. [8] reported that in the normal oxyntic mucosa Bcl-2 was consistently expressed by a distinct population of neuroendocrine cells located predominantly in the central mucosal layer, suggesting a role for the protein in the migration of maturing endocrine cells. These authors also found that increased probability of gastric carcinoid development was paralleled by greater Bcl-2 expression in hyperplastic oxyntic endocrine cells. Indeed, they found that the ratio of Bcl-2 to chromogranin A (CGA)-positive cells was highest in atrophic fundal gastritis, a condition conferring the highest risk of carcinoid development, and lowest in sporadic Zollinger–Ellison syndrome, a condition characterised by virtually no risk [8].

In addition to studies reporting a correlation between the levels of expression of Bcl-2 and those of neuroendocrine biopeptides, this oncoprotein has also been reported to be physically associated with the neuroendocrine-associated reticulon (RTN) family member RTNxs [9]. Tagami et al. [9] found that the interaction between Bcl-2 and RTNxs sequestered the former in the endoplasmic reticulum, thereby reducing its anti-apoptotic activity. While this study employed cell culture models of Bcl-2 over-expression, the putative modulation of levels of RTNxs, its family members, or indeed any other neuroendocrine markers, by this oncoprotein was not reported. The aim of the current study was, therefore, to examine the relative levels of a panel of neuroendocrine markers in an established cell culture model of Bcl-2 over-expression in order to determine whether Bcl-2 positively modulates levels of these biopeptides.

Materials and methods

The cell lines employed in the current study were rat adrenal pheochromocytoma (PC12) cells genetically engineered to constitutively over-express Bcl-2 (PC12 HB2-2 and PC12 HB2-3) and their mock-transfected controls (PC12 V1 and PC12 V4) [10]. In addition, a well-characterised PC12 model of Cox-2 over-expression (PCXII) and their mock-transfected controls (PC-MT) were employed in optimisation experiments [11]. In PCXII cells exogenous Cox-2 expression is induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG, 2.5 mM) to the cell culture media [11].

All cell lines were cultured on sterile plasticware obtained from Iwaki, Japan. Cell culture media including Dulbecco's modified Eagle's media (DMEM), heat-inactivated horse serum (HS), and foetal calf serum (FCS) were obtained from Invitrogen Life Technologies, CA, USA. Trypsin, versene, and all supplements used during culture were obtained from Sigma Aldrich, Missouri, USA. Rat-tail collagen type I was from Roche Diagnostics, Germany.

For immunocytochemistry, cells were cultured in collagen-coated BD Falcon slides which were obtained from BD Biosciences, UK.

The primary antibodies for immunocytochemistry included anti-Cox-2 (SC-1745, Santa Cruz Biotechnologies, USA), anti-CGA (20085, Diasorin, USA), and anti-Protein Gene Peptide 9.5 (PGP 9.5) from UltraClone, UK. The remaining primary antibodies used in immunocytochemistry [namely anti-vasostatin (VST-1), anti-intervening peptide (IP), anti-neuron specific enolase (NSE), and anti-pancreatic polypeptide (PP)] were produced in-house [12–14]. Swine anti-rabbit fluorescein isothiocyanate (SWAR-FITC) was obtained from DakoCytomation, Denmark (F0205), the biotinylated donkey anti-rabbit (BAR)-streptavidin-FITC system (RPN 1004 and 1232, respectively) came from Amersham Biosciences, UK, and rabbit anti-goat fluorescein isothiocyanate (RAG-FITC) was also supplied by DakoCytomation (F0233).

For experiments all cells were routinely subcultured in growth media onto collagen-coated slides. The following day growth media were replaced with restriction media (DMEM, 0.5% horse serum/calf serum). In the case of PC-MT and PCXII cells restriction media also routinely included IPTG (2.5 mM). Media were removed from cells 18–24 h later and these were washed three times in PBS (0.15 M sodium chloride, pH 6.0, containing 0.03 M sodium dihydrogen orthophosphate-1-hydrate and 0.09 M anhydrous disodium hydrogen orthophosphate). The cells were then fixed in 4% paraformaldehyde (30 min, 4 °C) after which they were washed three times in PBS. Primary antibodies, namely anti-Cox-2 (1:100) anti-CGA (1:500), anti-NSE (1:50), anti-PGP 9.5 (1:400), anti-IP (1:400), anti-PP (1:200), and anti-VST-1 (1:200), were then applied as appropriate. These primary antibodies were diluted in PBS containing 0.5% bovine serum albumin Fraction V, 0.5% Triton X-100, and 0.015 M sodium azide (antibody diluent). Brain cortex, brain cerebellum, pancreas head, and ileal carcinoid tissue sections were employed as positive controls for the expression of NSE, PGP 9.5, PP, and VST-1, respectively, with adrenal medulla tissue sections serving as positive control for CGA and IP. For immunocytochemistry, negative controls included were a secondary antibody only (antibody diluent) control and the appropriate serum control (1:500). The cells were then placed in an immunostaining chamber and left at 4 °C overnight.

Following this incubation the cells were washed with PBS (3 × 5 min). The appropriate secondary antibodies were then applied to the cells, namely SWAR-FITC for anti-IP, anti-PP, and anti-VST-1 (1:100, for 1 h at room temperature in darkness), RAG-FITC for anti-Cox-2, and BAR-streptavidin for anti-CGA, anti-NSE, and anti-PGP 9.5. The latter was applied in a two-step process. First cells were incubated with BAR (1:100 for 30 min at room temperature). Following a PBS wash, the cells were then incubated in streptavidin-FITC (1:200 for 30 min in darkness at room temperature).

Following incubation, the cells were washed in PBS in the dark for 20 min. Coverslips were then applied to the slides using 0.1 M PBS/glycerol (1:9; v/v) containing 2.5% 1,4 diazabicyclo(2,2,2)octane (antifade). The slides were viewed under the fluorescence microscope and images were taken. The fluorescent microscope used was fitted with exciter filters that produced blue light (495 nm) for the excitation of fluorescein.

Quantification of the fluorescence intensity emitted by the FITC tag was undertaken by two independent observers. Fluorescence intensity was scored on 4 mark scale ranging—negative (–), weakly positive (+), moderately positive (++) or intensely positive (+++). For each of the six neuroendocrine markers, five independent experiments were carried out on all four cell lines and images were recorded.

For immunoblotting, cell lines were routinely subcultured from confluent T25 flasks at a split ratio of 1:10 in order to achieve 70–80% confluency at time of cell lysis. Growth media were replaced with restriction media, –/+ 2.5 mM IPTG, 18–24 h prior to cell lysis. Routinely, cells were placed on ice and restriction media were removed. Cells were then washed three times with ice-cold PBS. Following the complete removal of PBS, cells were lysed in hypotonic lysis

buffer, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes), pH 7.5, containing 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride, and 200 µM orthovanadate (100 µl) as described previously [11]. Samples standardised for protein were separated by SDS–polyacrylamide gel electrophoresis using 5% and 8% acrylamide stacking and resolving gels, respectively, and immunoblotted for Bcl-2, Cox-2 or CGA expression according to the manufacturer's instructions (anti-Bcl-2, Catalogue No. 610538, BD Transduction Labs, Oxford, UK; 1:500, anti-Cox-2 Catalogue No. SC-1745, Santa Cruz, Biotechnology, USA, 1:200, and anti-CGA, Catalogue No. SC-1488, Santa Cruz, Biotechnology, USA, 1:200, in Tris buffered saline, pH 7.8, containing 5% [v/v] BSA). Blots were visualised using an ECL Western blotting detection kit, also exactly according to the manufacturer's instructions.

Statistical analysis. For each of the six neuroendocrine markers, five independent experiments were carried using the two control (V1/V4) and two Bcl-2 over-expressing (HB2-2/-3) cell lines. On the advice of a statistician, expression of the neuroendocrine markers by the control (V1/V4) cell lines was compared by means of the Mann–Whitney *U* test. Similarly, neuroendocrine marker expression in the Bcl-2 over-expressing (HB2-2/-3) cell lines was compared by means of this statistical test. Mann–Whitney *U* analysis was then carried out using grouped data from the control and Bcl-2 over-expressing cell lines.

Results and discussion

In view of the reports implicating the anti-apoptotic oncoprotein Bcl-2 in the acquisition/promotion of a neuroendocrine phenotype, the current study sought to determine whether forced expression of this protein was associated with modulated levels of the neuroendocrine markers, CGA, NSE, PGP 9.5, PP, and the CGA-derived peptides VST-1 and IP. A PC12 cell culture model of constitutive Bcl-2 over-expression ([10]; Fig. 1) was used in conjunction with immunocytochemical methods. Since PC12 cells are a neuroendocrine line—derived originally from a rat adrenal pheochromocytoma—which do not basally express Bcl-2 [10,15,16], they represent an ideal model in which to examine the putative effects of exogenously expressed Bcl-2 on neuroendocrine peptide expression. Moreover, the use of immunohistochemical methods in the present study facilitated the semi-quantitative determination of marker expression, some of which would have proven difficult to detect by immunoblotting due to their low molecular weight.

Immunoblot analysis of the four cell lines confirmed that under basal conditions the PC12 HB2-2 and PC12 HB2-3 cell lines expressed Bcl-2, whilst the control cell lines, PC12 V1 and V4, did not (Fig. 1; [10,15,16]). It was next necessary to validate the methodologies chosen in order to confirm that immunohistochemical protocols would be sufficiently sensitive to detect differential neuroendocrine marker expression. In parallel with the current study expression array analysis in our laboratory involving a second, and equally well-characterised, PC12 model of Cox-2 over-expression [11] had indicated that levels of CGA were 2-fold higher in PCXII than their control (PC-MT) counterparts (Connolly et al., unpublished observations). Immunocytochemical and immunoblotting analyses were subsequently carried out to verify this observation. These experiments confirmed that, following the induction of Cox-2 expression in response to IPTG in PCXII cells (Fig. 2A), levels of CGA were elevated compared to those found in their control cell line (Figs. 2B and C). Interestingly, levels of NSE were similar in the two cell lines indicating that the effect of Cox-2 on CGA expression was specific and not due to a global enhancement of neuroendocrine marker expression/differentiation. Critically for the purposes of the current study, the finding that Cox-2-dependent modulation of CGA expression was clearly detected by immunocytochemistry (Fig. 2B) validated the use of this technique for the detection of putative Bcl-2-dependent modulation of neuroendocrine marker expression in PC12 HB2-2/HB2-3 cells and their mock-transfected controls.

Having confirmed that increased expression of neuroendocrine marker expression could be detected immunocytochemically using a PC12 cell model of over-expression, levels of the neuroendocrine markers CGA, NSE, PGP 9.5, PP, IP, and VST-1 were next examined by this method in the HB2-2, HB2-3, V1, and V4 lines. This particular panel of markers was chosen since CGA, NSE, PGP 9.5, and PP represent established neuroendocrine-specific proteins/peptides whose co-expression with Bcl-2 has been demonstrated in a range of tissues [6–8] and whose expression in PC12 cells has also been reported [17]. The expression of the CGA-derived peptides IP and VST-1 was also examined since, as well as representing additional, somewhat less ubiquitous, neuroendocrine biopeptides, it was reasoned that

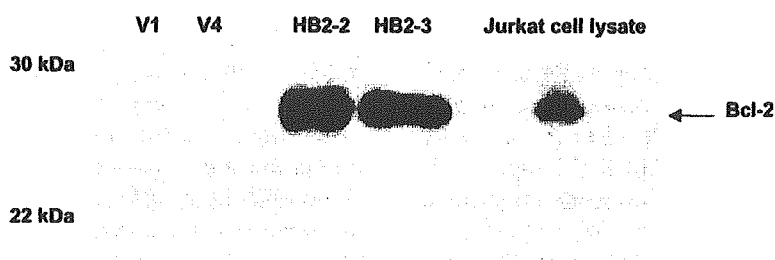


Fig. 1. Immunoblot analysis of Bcl-2 expression in control (PC12 V1 and V4) and Bcl-2 over-expressing (PC12 HB2-2 and HB2-3) cell lines.

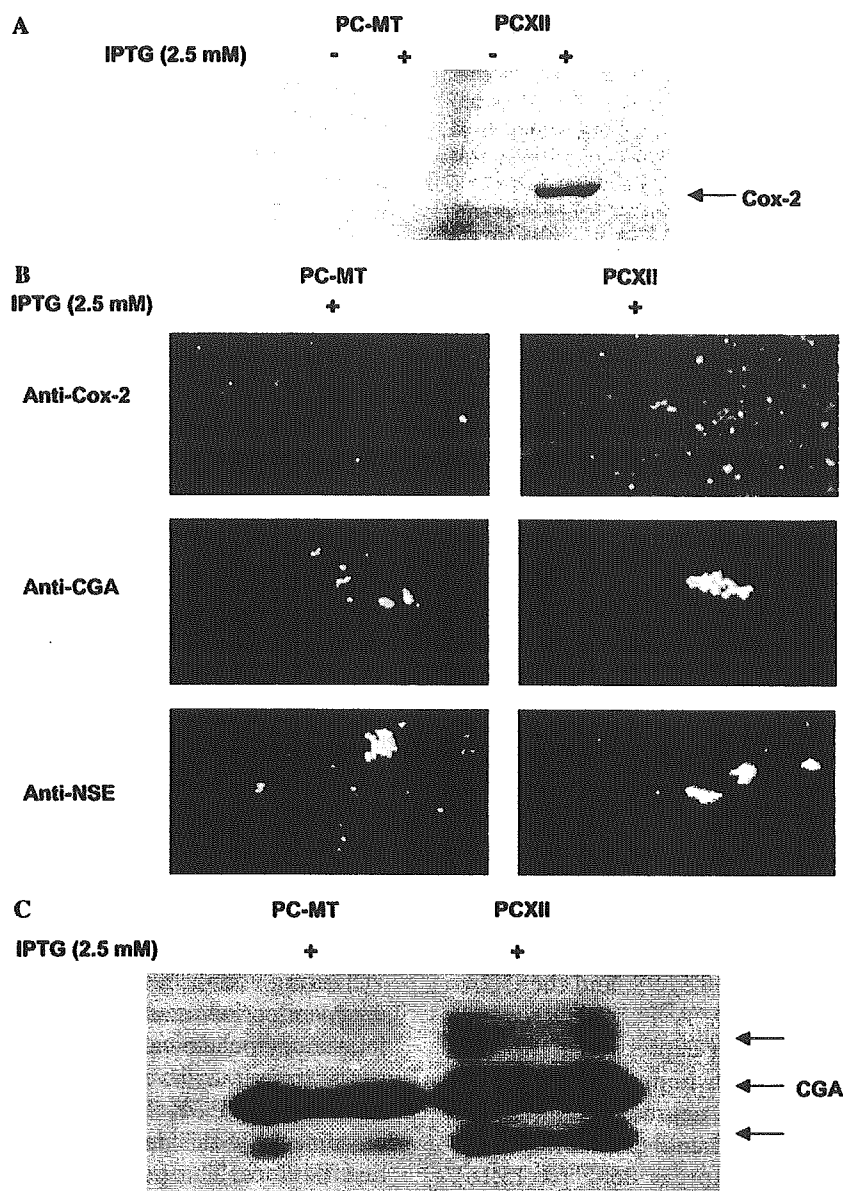


Fig. 2. Immunohistochemical and immunoblot analysis of Cox-2-dependent CGA upregulation in PCXII cells. (A) Immunoblot analysis of IPTG-inducible Cox-2 expression in PC-MT and PCXII cells. (B) Immunohistochemical analysis of Cox-2, CGA, and NSE expression in PC-MT and PCXII cells. (C) Immunoblot analysis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

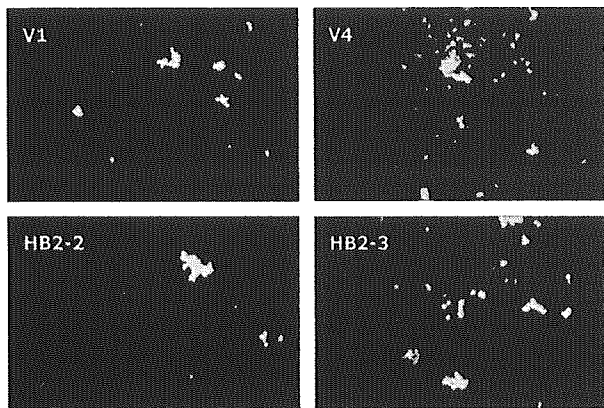


Fig. 3. Immunocytochemical analysis of NSE expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

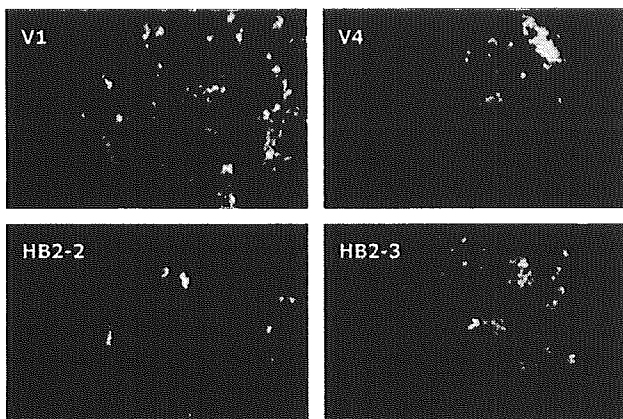


Fig. 4. Immunocytochemical analysis of CGA expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

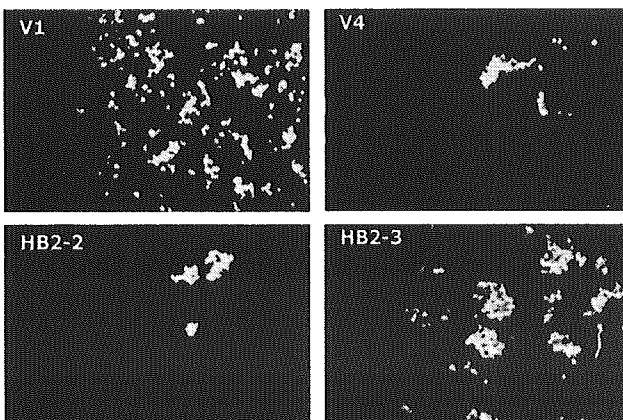


Fig. 5. Immunocytochemical analysis of PGP 9.5 expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

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

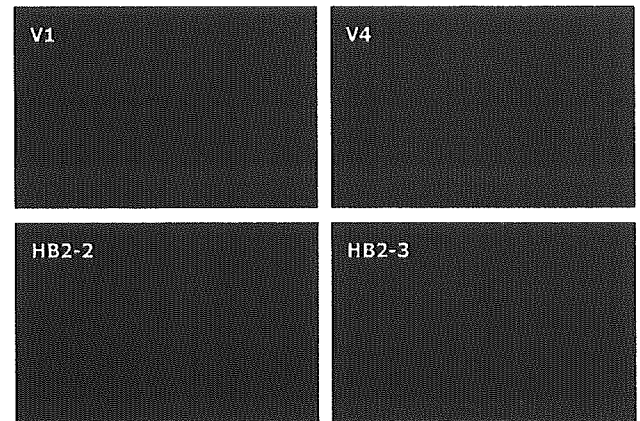


Fig. 6. Immunocytochemical analysis of PP expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

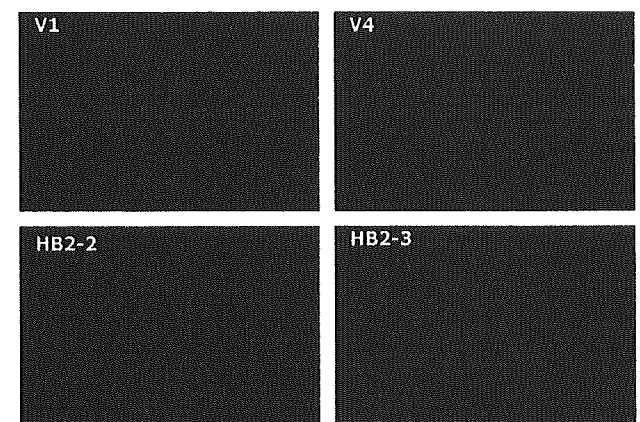


Fig. 7. Immunocytochemical analysis of IP expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

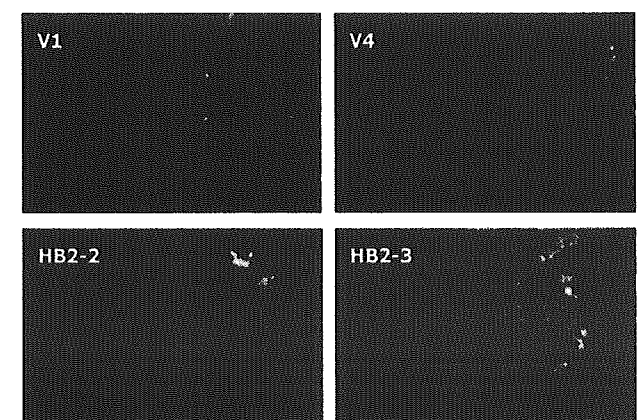


Fig. 8. Immunocytochemical analysis of VST-1 expression in control (V1/V4) and Bcl-2 over-expressing (HB2-2/HB2-3) cell lines.

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 pheochromocytomas [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.

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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 (PS1 Δ C) 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 PS1 Δ C 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 PS1 Δ C 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 (A β) 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 A β (for review, see Selkoe 2001). A β has two major C-

terminal variants, the A β that ends at residue 40 (A β 40) and the A β that ends at residue 42 (A β 42). Significantly, all AD-associated mutations in PS genes increase the relative production of A β 42 which is more amyloidogenic than A β 40, 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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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 (PS1 Δ C) 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 PS1 Δ C. 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 PS1 Δ C 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 (PS1 Δ C) and the

PS1 C-terminal short fragment. As shown in Fig. 1A, PS1 Δ C66 lacks PS1 C-terminal 66 amino acid residues including TM8, and PS1 Δ C37, 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. PS1 Δ C66 and PS1 Δ C37 exhibited the complete loss of A β 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 PS1 Δ C66 and PS1 cofactors restore A β generation in PS-null cells (Fig. 2A, left 5 lanes). As shown in Fig. 2A (lanes 1–5), no A β generation was observed, suggesting that PS1 Δ C66 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 PS1 Δ C66, 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 PS Δ C66, 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 PS1 Δ C66 endoproteolysis was also observed by the co-expression of C68, APH-1b and NCT (Fig. 2A, lane 8), and PS1 Δ C66 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 (C68 Δ C7) with PS cofactors also rescues the defects in γ -secretase activity and PS1 Δ C66 endoproteolysis. The result showed that the co-expression of C68 Δ C7 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

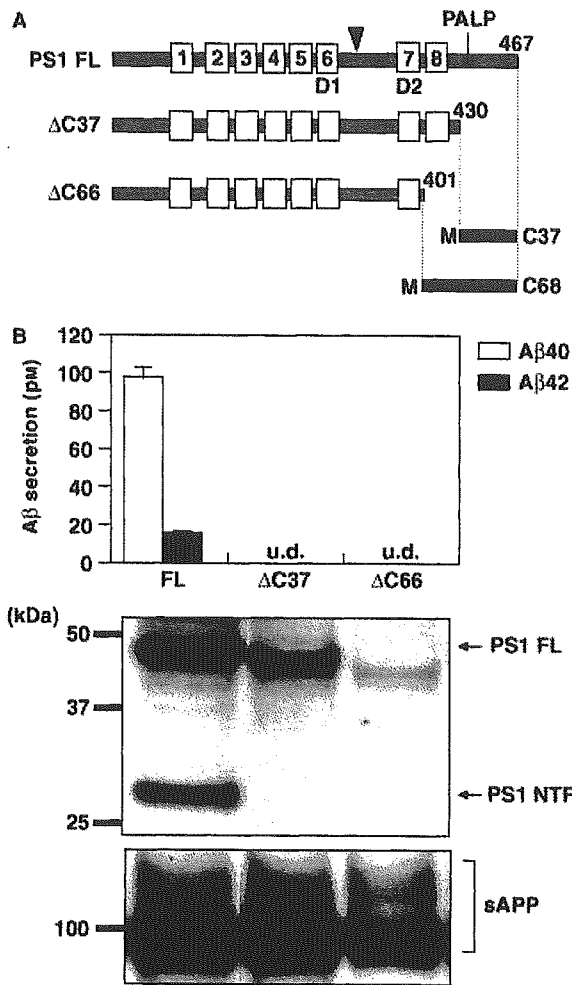


Figure 1 Schematic diagram of C-terminally truncated forms of PS1 (PS1ΔC) and PS1 C-terminal fragments expressed in (A) PS-null cells, and (B) a defect in Aβ generation and endoproteolysis of PS1ΔC. (A) A schematic diagram of C-terminally truncated forms of PS1 used in this study is shown. The last 37 (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 \pm SD of two independent dishes ($n = 2$). CHAPSO-solubilized lysates (10 μ g) 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

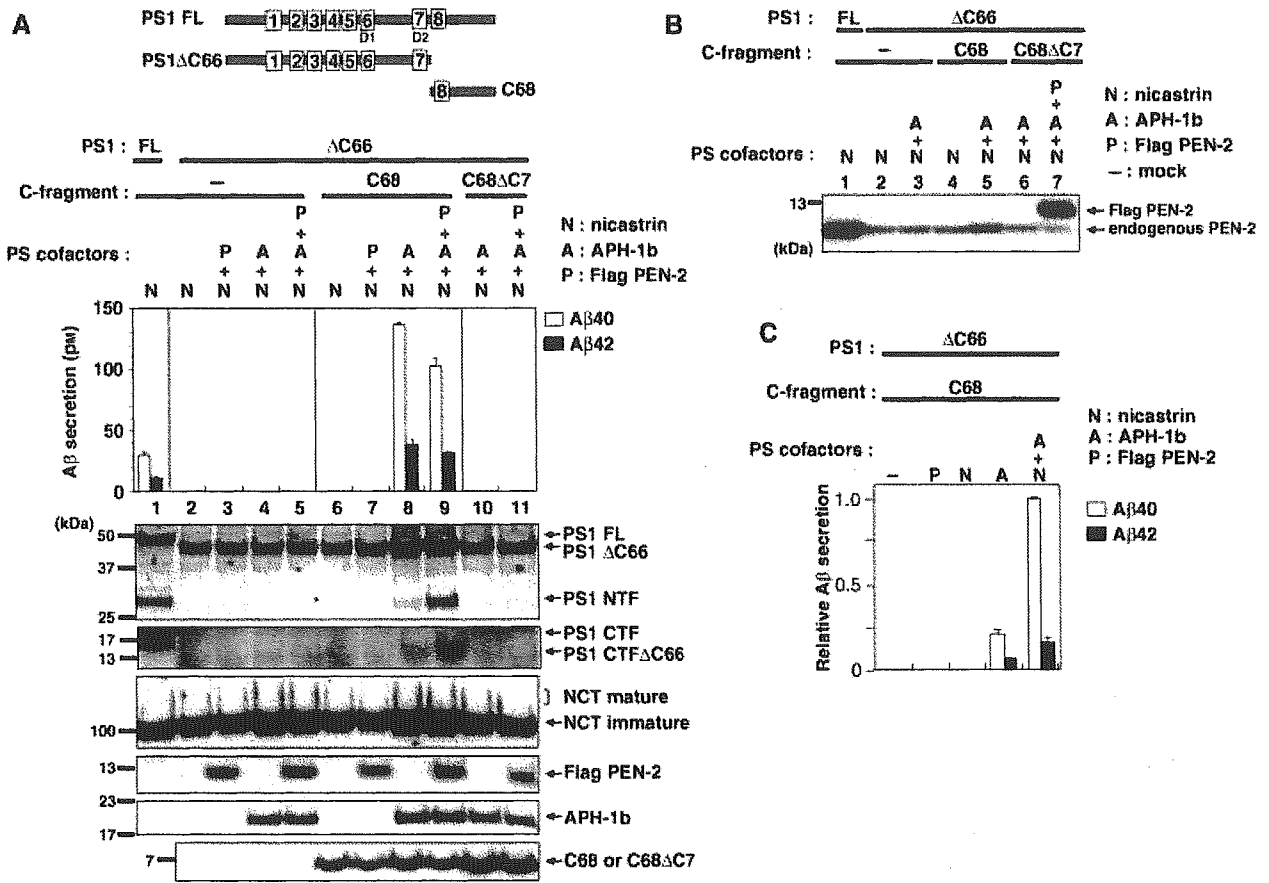


Figure 2 The expression of C68 with PS cofactors rescued defects in γ -secretase activity and endoproteolysis of PS1ΔC66. (A) After APP695 and the indicated exogenous PS cofactors were retrovirally expressed with PS1ΔC66 and C68 or C68ΔC7 in PS-null cells, A β secreted from these cells during a 48-h culture was quantified by ELISA, and CHAPSO-solubilized lysate (10 μ g) 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 \pm 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, A β secreted from these cells during a 48-h culture was quantified by ELISA. The A β levels were expressed as relative to A β 40 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-expressed, compared with that when APH-1 was not expressed (Fig. 4A, lanes 2–5 and Fig. 4B, lanes 6–9).

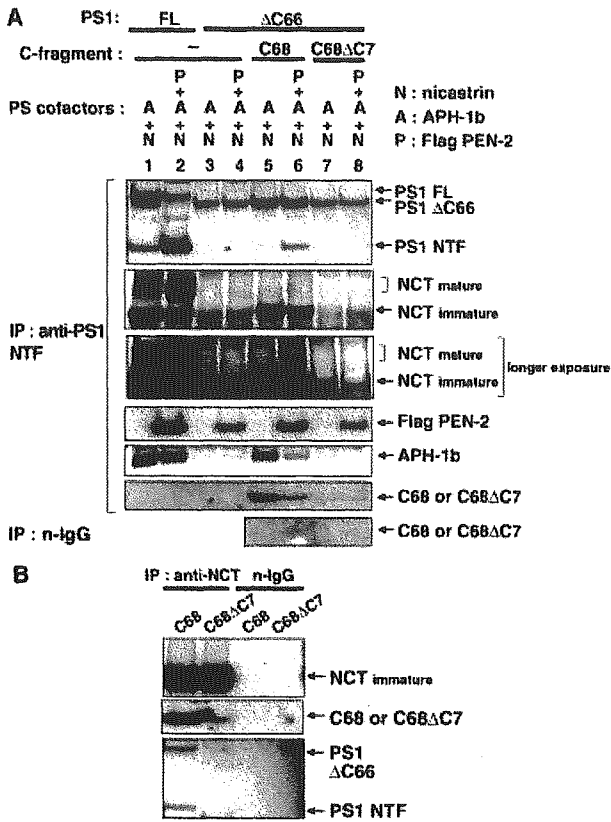


Figure 3 The expression of C68 rescued a defect in the assembly of PS1ΔC66 with APH-1 and NCT. (A) After APP695 and the indicated exogenous PS cofactors were retrovirally expressed with PS1ΔC66 and C68 or C68ΔC7 in PS-null cells, CHAPSO-solubilized lysate (1 mg) was immunoprecipitated (IP) with the anti-PS1 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-immunoprecipitation 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

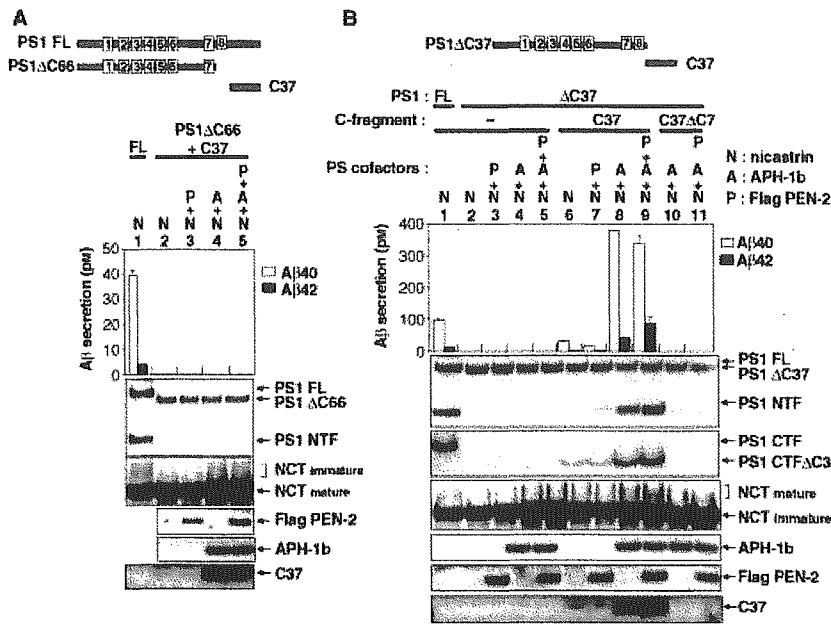


Figure 4 The rescue of a defect in γ -secretase activity of PS1 Δ C by the expression of PS1 C-terminal fragment with PS cofactors requires the PS1 TM8 region. After APP695 and the indicated exogenous PS cofactors were retrovirally expressed with (A) PS1 Δ C66 and C37 or (B) PS1 Δ C37 and C37 in PS null cells, A β secreted from these cells during a 48-h culture was quantified by ELISA, and CHAPSO-solubilized lysate (10 μ g) was immunoblotted with the anti-PS1 NTF antibody (for detection of PS1 FL and PS1 Δ C37), anti-PS1 loop antibody (for detection of PS1 CTF and PS1 CTF Δ C37), 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 C37 Δ C7). -, mock transfection (pMX). Values are means \pm SD of two independent dishes ($n = 2$). Data are representative of three independent experiments. The difference in A β 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.

PS1 Δ C66 has two aspartate residues in TM6 and TM7, which are essential for γ -secretase activity, but PS1 Δ C66 lacks the entire C-terminal region, including TM8 and the region immediately downstream from TM8. PS1 Δ C66 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 PS1 Δ C66. Indeed, this interpretation was supported by our result showing that PS1 Δ C66 had a marked defect in the binding of NCT and APH-1, but PS1 Δ C66 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 PS1 Δ C66 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 PS1 Δ C66, 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 C68 Δ C7 with PS1 Δ C66 and NCT was lower than that of C68 (Fig. 3B). Therefore, the failure of C68 lacking the C-terminal seven-amino acid residues (C68 Δ C7) to rescue the formation of the active γ -secretase complex was likely to be caused by the lower association of C68 Δ C7 with PS1 Δ C66 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 Δ exon 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

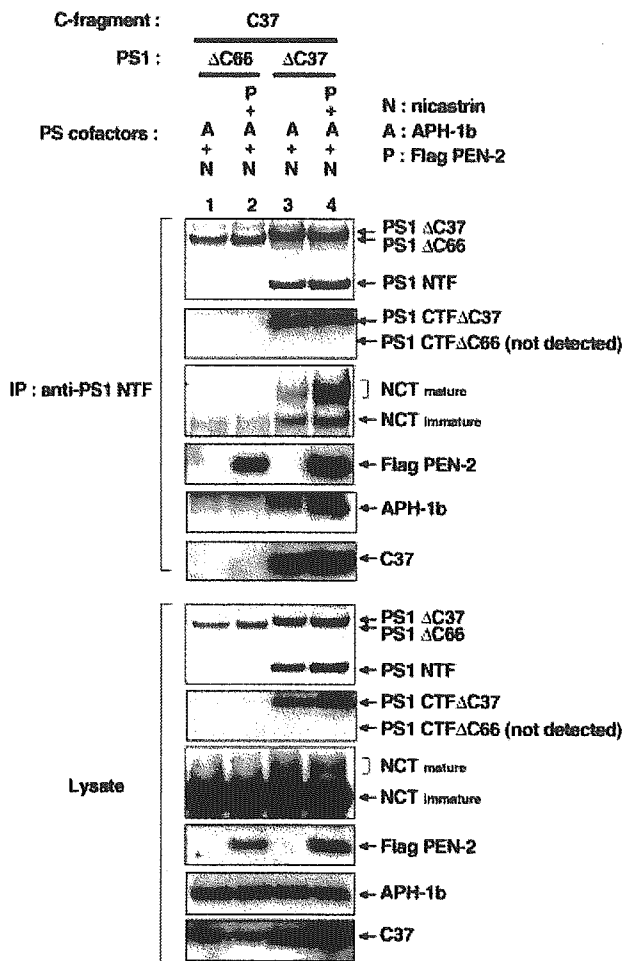


Figure 5 The assembly of the γ -secretase complex requires the PS1 TM8 region. After APP695 and the indicated exogenous PS cofactors were retrovirally expressed with PS1 Δ C66 and C37 or with PS1 Δ C37 and C37 in PS-null cells, CHAPSO-solubilized lysate (1 mg) was immunoprecipitated (IP) with the anti-PS1 NTF antibody (H-70) and then immunoblotted (IB) with the anti-PS1 NTF antibody (Chemicon International, Inc.; for detection of PS1 Δ C37, PS1 Δ C66 and PS1 NTF), anti-PS1 loop monoclonal antibody (for detection of PS1 CTF Δ C37), anti-APH-1b antibody, anti-FLAG antibody, anti-NCT antibody and PS-C3 antibody (for detection of C37). CHAPSO-solubilized lysate (10 μ g) was also immunoblotted with the same antibodies (the bottom four panels). Data are representative of two independent experiments. Note: PS1 NTF and PS1 CTF Δ C66 in lanes 1 and 2 were not detected, because the expression of PS1 Δ C66 with C37 and PS cofactors did not cause the endoproteolysis of PS1 Δ C66.






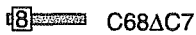
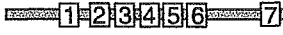





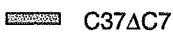
although the stimulation of PSAC endoproteolysis by the expression of exogenous PEN-2 was observed in this reconstitution system (Figs 2A, 3A, and 4B). In addition, the reconstituted γ -secretase activity by the truncated PS

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 PS1 Δ C66 and C37 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 glycosylation 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 PS1 Δ C66, 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 PS1 Δ C in PS-null cells

	Transgene (+ exogenous three PS cofactors)	γ -secretase activity	Assembly of PS1 Δ C with		
			NCT	APH-1	PEN-2
1	PS1 FL 	+	+	+	+
2	PS1 Δ C66 	-	-	-	+
3	PS1 Δ C66  C68 	+	+	+	+
		PS1 Δ C66  C68 Δ C7 	-	-	-
4	PS1 Δ C66  C37 	-	-	-	+
5	PS1 Δ C37 	-	-	-	+
6	PS1 Δ C37  C37 	+	+	+	+
		PS1 Δ C37  C37 Δ C7 	-	* -	* -

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 PS1 Δ C66. 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 PS1 Δ C37 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 PS1 Δ C66 with NCT and APH-1, but it significantly rescued a defect in the assembly of PS1 Δ C37 with NCT and APH-1, indicating that TM8 is involved in the assembly of PS1 Δ C37 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 A β 1-17 was purchased from Senetek (St. Louis, MO, USA). The other A β 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 PS1 Δ C were generated from pMX-PS1 by the PCR method. The primer sequences used for the PCR were as

follows: a sense primer: 5'-TGCAGAATTCATGACAGAGT-TACCTGCA-3'; and anti-sense primers: 5'-CATGCTCGAGT-CATTTCTTGAAAATGGCAAGGAG-3' (PS1 Δ C37: the last 37 amino acids deletion), and 5'-CATGCTCGAGTCAACT-GGCTGTTGCTGAGGCTTT-3' (PS1 Δ C66: the last 66 amino acids deletion). The PCR products were digested with *Eco*R1 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*R1 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'-TGCA-GAATTCACCATGGCATTGCCAGCTCTTCCA-3' (C37) and 5'-TGCAGAATTCACCATGGCCAGTGGAGACTGGAAC-3' (C68); and an anti-sense primer, 5'-CATGCTCGAGCTAGATATAAAATTGATGG-3'. The resultant cDNAs were inserted into pMX at *Eco*R1 and *Xho*I. cDNAs encoding C37 Δ C7 and C68 Δ C7 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*R1 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, GCGAATTCCTTCCGCGGTGGCCATGACT and anti-sense primer, GCAGATCTGAAGTGCTGGTTCCCTGAGG. The PCR product was digested with *Eco*R1 and inserted into pMX at the *Eco*R1 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).

A β detection and co-immunoprecipitation techniques

A β 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 A β 40) and HRP-conjugated BC05 (for A β 42). ELISA data were statistically analyzed by ANOVA using StatView-J.4.11 (Abacus Concepts, Inc., Berkeley, CA, USA). Cultured cells were lysed 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 μ L 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.

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Supplementary material

The following supplementary material is available for this article online:

Figure S1 Interaction of C68 with NCT and APH-1

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

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 (A β) 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 A β , GRP78, and β 2 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 A β , 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

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Abbreviations used: A β , amyloid- β ; A β PP, 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 Ca²⁺ 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 (A β) (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 A β , 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 A β 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.

A β 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 A β 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 A β , suggesting that they may play a role in A β folding and/or attempted disposal of A β 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 A β , ER chaperone BiP/GRP78 (immunoglobulin heavy chain-binding protein/glucose-regulated protein 78), 20S β 2 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- μ m-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)