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Examination of the role of endopeptidase 3.4.24.15 in Aβ secretion by human transfected cells

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1 We have taken advantage of our recent development of highly potent and specific phosphinic inhibitors of endopeptidase 3.4.24.15 to examine the putative contribution of the enzyme in the secretion of Aβ by HK293 transfected cells overexpressing the wild type and the Swedish (Sw) double mutated form of βAPP_{751}.

2 First, we showed that HK293 cells contain a peptidase activity, the inhibition profile of which fully matches that of purified endopeptidase 3.4.24.15. Second, we established that the treatment of HK293 cells with specific phosphinic inhibitors leads to about 80% inhibition of intracellular endopeptidase 3.4.24.15 activity, indicating that these inhibitors penetrate the cells.

3 Metabolic labelling of wild type and $Sw\beta APP_{751}$-expressing cells, followed by immunoprecipitation of Aβ-containing peptides, revealed the secretion of Aβ and the intracellular formation of an Aβ-containing 12 kDa product.

4 Aβ secretion by $Sw\beta APP_{751}$ transfected cells was drastically enhanced when compared to cells expressing wild type βAPP_{751}. This production was not affected by endopeptidase 3.4.24.15 inhibitors in either cell type. This correlates well with the observation that endopeptidase 3.4.24.15 does not cleave recombinant baculoviral $Sw\beta APP_{751}$, *in vitro*.

5 Our previous data indicated that endopeptidase 3.4.24.15 activity was reduced in the parietal cortex of Alzheimer's disease affected brains and that the enzyme probably participated, in this brain area, to the catabolism of somatostatin 1-14. However, the present work indicates that endopeptidase 3.4.24.15 does not seem to behave as a β-secretase in HK293 transfected cells. Therefore, it is suggested that endopeptidase 3.4.24.15 could participate in the symptomatology, but probably not in the aetiology of Alzheimer's disease.

Keywords: Peptidase; endopeptidase 3.4.24.15; β-secretase; Aβ secretion; HK293 cells; transfection; Alzheimer's disease

Introduction

Alzheimer's disease is characterized by neurochemical deficits and neuropathological lesions that ultimately lead to severe cognitive impairments. Among various neurotransmitter or neuromodulator alterations observed in this syndrome, a drastic reduction in the content or activity of several components involved in cholinergic neurotransmission such as muscarinic and nicotinic receptors and acetylcholinetransferase has been found (for reviews see Perry, 1986; Nordberg, 1992). In addition, a consistent deficit in cortical somatostatin (SRIF) 1–28 and 1–14 immunoreactivities was detected in *post mortem* tissues from affected brains (Schettini *et al*., 1988; Bissette & Myers, 1992). The latter observation is of importance since experimental lowering of SRIF content in the rat was shown to be associated with dramatic memory impairment (Vecsei *et al*., 1984; Vecsei & Widerlöv, 1988), a feature corresponding to one of the main cognitive alterations observed in Alzheimer's disease. Nothing is clear concerning the mechanisms by which the SRIF content is reduced in affected brains but one could envisage that alterations of the proteolytic activities participating in the biosynthetic or catabolic pathways may contribute to this situation. Besides the possible implication of catabolic activities in the neuropeptide-related symptomatology of Alzheimer's disease, proteolytic enzymes probably contribute to the aetiology of the syndrome. Thus, one of the main histopathological hallmarks observed in Alzheimer's disease is the senile plaque, the main component of which corresponds to Aβ, a peptide 39–43 amino acids long (Masters *et al*., 1985; Hardy & Allsop, 1991; Gentleman, 1992; Joachim & Selkoe, 1992; Yankner & Mesulam, 1992). Aβ derives from the proteolytic attack of its precursor βAPP, by several activities called β- and γ-secretases that release the N- and C-terminal end of Aβ, respectively (for reviews see Selkoe, 1991; Checler, 1995). Several familial forms of Alzheimer's disease have been attributed to the presence of missense mutations located on the βAPP (for reviews see Tanzi *et al*., 1991; Mullan & Crawford, 1993; Schellenberg, 1995). Among them, a double substitution located adjacently to the N-terminus of Aβ and referred to as Swedish mutation (Sw, Mullan *et al*., 1992) has been shown to trigger increased recovery of secreted Aβ in transfected cells overexpressing the Sw mutated βAPP (Citron *et al*., 1992; Cai *et al*., 1993; Felsenstein *et al*., 1994). Therefore, a direct link seems to exist between severe and rapid evolution of the disease and exacerbated susceptibility of βAPP to proteolysis by β-secretase.

Several findings indicate that a proteolytic activity called endopeptidase 3.4.24.15 could contribute to both the symptomatology and aetiology of Alzheimer's disease. Thus, rat brain purified endopeptidase 3.4.24.15 has been shown to hydrolyse SRIF 1-14 (Chu & Orlowski, 1985; Barelli *et al*., 1991; Dahms & Mentlein, 1992) and to participate in the catabolism of the peptide by various enzymatic sources including normal and pathological human brain tissues (Ichai *et al*., 1994) and rat cortical astrocytes and neurones (Lucius & Mentlein, 1991; Mentlein & Dahms, 1994). Several studies have demonstrated the ability of endopeptidase 3.4.25.15 to cleave synthetic chromogenic or fluorimetric substrates mimicking the se-

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quence of βAPP encompassing the peptide bond targeted by β-secretase (McDermott *et al*., 1992; Papastoitsis *et al*., 1994). Furthermore, it was of interest to note that the human gene encoding endopeptidase 3.4.24.15 was identified on the chromosome 19, near a locus associated with late onset forms of the disease (Meckelein *et al*., 1996) and that monoclonal antibodies directed towards the human counterpart of the enzyme label neurofibrillary tangles, another histological lesion observed in brains of patients with Alzheimer's disease (Conn *et al*., 1996).

In the present study, we took advantage of our recent development of highly potent, selective and cell permeant inhibitors of endopeptidase 3.4.24.15 to assess the putative contribution of the enzyme in the secretion of Aβ by human cells overexpressing the wild type and Sw mutated form of βAPP. Altogether, our data indicate that endopeptidase 3.4.24.15 probably does not fulfil a role of β-secretase in these human cells.

Methods

Cell culture and transfection

HK293 cells were grown in 5% CO_2 in F12/DMEM medium (vol/vol) supplemented with 10% foetal calf serum containing penicillin (100 u ml^{-1}) and streptomycin (50 μg/ml^{-1}). Stable transfectants overexpressing the wild type and Sw$βAPP_{751}$ were obtained by the calcium phosphate precipitation technique with 2 μg of $pcDNA_3$ encoding the two cDNAs, and positive clones were identified by means of a polyclonal antibody (BR188) recognizing the C-terminal end of $βAPP_{751}$.

Preparation of cell homogenates

Confluent cells (35 mm wells) were rinsed with phosphate buffer saline (PBS), pH 7.4 containing (mM): NaCl140, Na_2HPO_4 8.5, KCl 2.7, KH_2PO_4 1.5 than scrapped and homogenized with a syringe in 500 μl of 5 mM Tris-HCl, pH 7.5.

Fluorimetric assay of endopeptidase 3.4.24.15 activity

Rat brain endopeptidase 3.4.24.15 was purified as described previously (Barelli *et al*., 1991). Purified enzyme (10 μl) or HK293 cell homogenates (15 μl) were incubated for 1 h at 37°C, in a final volume of 100 μl of 5 mM Tris-HCl, pH 7.5 containing 100 μM dithiothreitol, with 50 μM Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (QFS), in the absence and presence of various concentrations of peptidase inhibitors (preincubated for 5 h at 37°C). All incubations performed with cell homogenate membranes were carried out in the presence of 5 mM of Pro-Ile in order to prevent a putative contribution of endopeptidase 3.4.24.16. Samples were stopped by acidification and endopeptidase 3.4.24.15 activity was fluorimetrically recorded as described previously (Checler, 1993)

Baculoviruses expression and purification of recombinant Sw$βAPP_{751}$

Recombinant viruses containing the Sw$βAPP_{751}$ cDNA were obtained by transfection of the transfer plasmid together with wild-type baculovirus into Sf9 cells by use of a baculo-gold transfection kit according to the protocol supplied by the manufacturer (Pharmingen, San Diego). Supernatants of this transfection were harvested 6 days later (primary virus stock). The titre of the primary virus stock was amplified twice by passage through Sf9 cells at a low multiplicity of infection (usually 1 : 200 dilution). Supernatants recovered after two rounds of amplification were used to infect Sf9 cells at a 1 : 10 dilution to produce recombinant βAPP proteins. Sf9 cells were harvested by centrifugation 72 h after infection. The pellet was resuspended in 40 ml of 10 mM Tris, pH 7.4 containing 0.5 M NaCl, 1% Nonidet P-40 (NP-40) and 5 mM EDTA. Cells were disrupted by 15 strokes in a dounce homogenizer and centrifuged for 20 min in a SS-34 rotor at 15000 × r.p.m. The pellet was homogenized again in 40 ml of the above buffer and centrifuged. The two supernatants, referred to as cell lysate, were combined. The cell lysate was diluted four fold with 10 mM Tris, pH 7.4 containing 1% NP-40 and 5 mM EDTA (buffer A) then SwβAPP was purified by various chromatographic steps, as described previously (Chevallier *et al*., 1997), and identified by means of the BR188 antibody.

Degradation of recombinant Sw$βAPP_{751}$ by purified endopeptidase 3.4.24.15

Recombinant Sw$βAPP_{751}$ was incubated with 10 μl of rat brain purified enzyme in a final volume of 100 μl of 5 mM Tris-HCl, pH 7.5 containing 100 μM DTT. Incubations were stopped, dried then analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot as below.

SDS–PAGE and Western-blot analysis

Dried samples and standards were resuspended in 30 μl of 50 mM Tris, pH 6.8 containing 2% SDS, 10% glycerol and 2.5% β-mercaptoethanol (Laemmli buffer). Samples were then heated for 4 min at 95°C and electrophoresed for 45 min at room temperature at 200 V in a 8% acrylamide minislab gel (Biorad), then electrophoresed proteins were blotted onto nitrocellulose sheets (Hybond-C super, Amersham). Membranes were incubated in 20 mM Tris, pH 7.5 (Tris Buffer Saline or TBS) containing 140 mM NaCl and 5% skimmed milk, then exposed overnight to the BR188 antiserum diluted (500 fold) or mAb 22C11 (250 fold, recognizes the 60–100 N-terminal amino acids of βAPP) in TBS containing 1% skimmed milk. Sheets were rinsed in TBS (3 × 5 min) and incubated with anti-IgG coupled to alkaline phosphatase (Promega). Nitrocellulose was finally rinsed in TBS as above and the IgG-antigen complex was revealed as described by Chevallier *et al*. (1997).

Metabolic labelling of HK293 cells and immunoprecipitation of Aβ-containing peptides

HK293 cells overexpressing the wild type and Sw$βAPP_{751}$ were treated for 1 h in the absence or presence of 10 μM of peptidase inhibitors, then further incubated for 6 h in the same conditions in methionine-depleted DMEM buffer containing 0.75 mCi ml^{-1} [^{35}S]-methionine. Media were collected and diluted in 1/10 RIPA 10× buffer then incubated overnight with a 350 fold dilution of FCA18 antibody. Cells were collected, lysed in RIPA 1× buffer containing 1% NP40, 0.5% Na deoxycholate and 0.1% SDS then incubated as above with FCA18. Slurry protein A sepharose (10 mg prepared according to the manufacturer's recommendations) was then added for 4 h and samples centrifuged, washed three times with 1× RIPA, resuspended with loading buffer, electrophoresed on a 16.5% Tris/Tricine gel as below and autoradiographed.

Inhibition of intracellular endopeptidase-like 3.4.24.15 activity

HK293 cells overexpressing the Sw$βAPP_{751}$ were treated as above in absence or presence of inhibitors (preincubated for 7 h), then cell homogenates preparation and fluorimetric assays were performed as described above.

Tris-tricine SDS–PAGE

Electrophoresis was performed with a 16.5% Tris-tricine gel according to the procedure previously described (Chevallier *et al*., 1997). Briefly, samples were resuspended in 30 μl of 125 mM Tris-HCl, pH 8.45, containing 2% SDS, 20% glycerol, 2.5% β-mercaptoethanol, vortexed then spun for 10 min

at 14000 r.p.m. and heated for 4 min at 95°C. Supernatants were then electrophoresed for 2 h at room temperature at 100 V with an anode buffer at pH 8.9 and a cathode buffer containing 100 mM tricine at pH 8.45.

Antibodies

BR188 was developed against the 12 C-terminal amino-acids of the βAPP_{751}. FCA18 was raised against the first 8 N-terminal residues of Aβ. Our characterization of the IgG-purified fraction of the FCA18 antiserum indicated that it recognised only the first N-terminal aspartyl residue of native and denatured Aβ species by dot-blot, Western-blot analysis and labelled diffuse and mature plaques after an immunohistochemistry procedure.

Protein concentrations

Protein concentrations were determined by the Bradford method with egg white lysozyme as the standard.

Materials

Mcc-Pro-Leu-Gly-Pro-D Lys-Dnp (QFS) was from France Biochem. Geneticin and cell culture media were from Gibco. Penicillin and streptomycin were from Sigma. Protein A Sepharose was from Pharmacia Biotech. Methionine depleted medium and [^{35}S]-methionine were from ICN. mAb 22C11 were purchased from Bohringer.

Results

Our preliminary data indicated that synthetic decapeptides encompassing the sequence thought to be targeted by β-secretase in βAPP behaved as substrates of endopeptidase 3.4.24.15 but that the site of cleavage on these peptides did not correspond to that expected from a β-secretase activity (not shown). This prompted us to examine whether the natural substrate of β-secretase, i.e. βAPP_{751} could be degraded by endopeptidase 3.4.24.15. In order to exacerbate further a putative β-secretase activity of the enzyme, we introduced a double mutation (Sw) that was shown to increase drastically the β-secretase activity and Aβ secretion by transfected cells (Citron *et al*., 1992; Cai *et al*., 1993; Felsenstein *et al*., 1994). Thus, we produced and purified baculoviral $Sw\beta APP_{751}$ (Figure 1). Our data indicate that prolonged incubations of $Sw\beta APP_{751}$, even in the presence of dithiothreitol, a potent activator of endopeptidase 3.4.24.15 activity (Orlowski *et al*., 1983; Barelli *et al*., 1991; Lew *et al*., 1995), does not lead to substrate degradation or the appearance of SwβAPP catabolites (Figure 1a) although the enzyme was still active in these experimental conditions (Figure 1b).

We examined whether endopeptidase 3.4.24.15 could act as a β-secretase in human cells. In this context, we have established stable transfectants overexpressing the wild type βAPP_{751} and its Swedish mutated counterpart. Metabolic labelling and immunoprecipitation of Aβ-containing peptides by means of specific antibodies directed towards the N-terminus of Aβ, indicate that both transfectants generate an intracellular 12 kDa product and secrete Aβ (Figure 2a and b). The formation of both catabolites appears to be drastically increased by the presence of the double mutation (Figure 2a, b). This cannot be accounted for by distinct levels of βAPP expression since wild type and mutated βAPP immunoreactivities appeared to be similar in both transfectants (Figure 2c). Furthermore, an immunoreactive band with a molecular weight around 20 kDa was detected (Figure 2b), the nature of which remains to be established.

These cells were therefore used to examine whether endopeptidase 3.4.24.15 could contribute to the formation of Aβ. Homogenates of HK293 cells contain a proteolytic activity able to cleave Mcc-Pro-Leu-Gly-Pro-D Lys-Dnp, a fluorimetric substrate that was previously used to monitor endopeptidase 3.4.24.15 (Tisljar *et al*., 1990). This activity was dose-dependently and fully inhibited by two selective blockers of this enzyme, Cpp-Ala-Ala-Tyr-pAB (Orlowski *et al*., 1988) and Z(L,D)-Phe$\Psi(PO_2CH_2)$-Ala-Lys-Met (Jiràcek *et al*., 1995) (Figure 3) with K_i values identical to those obtained with purified endopeptidase 3.4.24.15 (Table 1). By

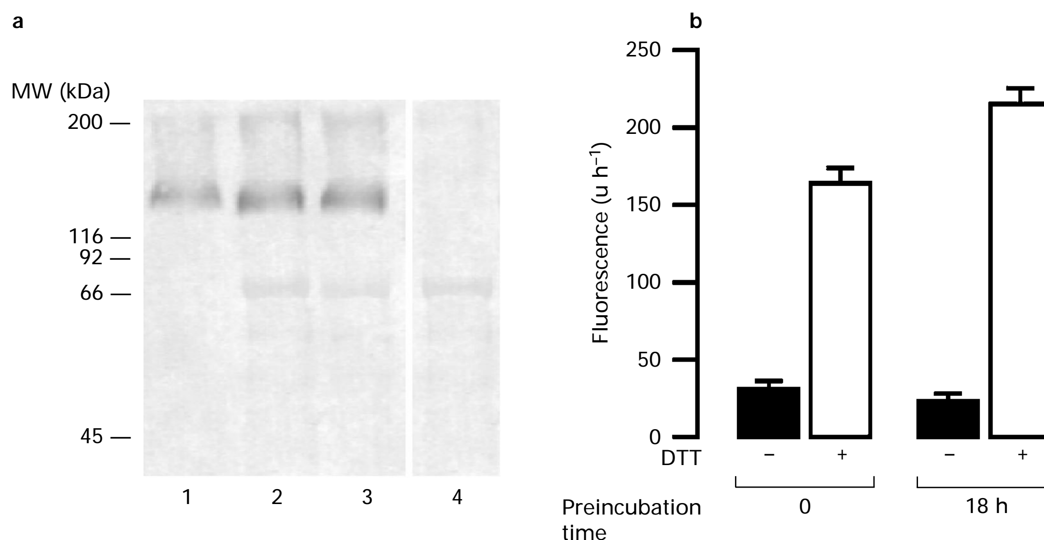

Figure 1 Western-blot analysis of $Sw\beta APP_{751}$ degradation by purified endopeptidase 3.4.24.15. Purified recombinant baculoviral $Sw\beta APP_{751}$ was obtained as described in the Methods and incubated for 1 (lane 2) or 18 h (lane 3) at 37°C with 15 μl of purified endopeptidase 3.4.24.15 in a final volume of 100 μl of 5 mM Tris-HCl pH 7.5 containing 0.5 mM dithiothreitol. Incubations were stopped at 4°C, dried and then resuspended in 30 μl of loading buffer. After being heated for 4 min at 95°C, samples were submitted to electrophoresis on a 8% acrylamide minislab gel and blotted onto nitrocellulose sheet (a). Membrane blots were immunostained with BR188 antiserum as described in Methods. Note that the low molecular weight obtained at about 75 kDa represents non-specific labelling revealed when endopeptidase 3.4.24.15 was electrophoresed alone (lane 4). Lane 1 indicates control $Sw\beta APP_{751}$ immunoreactivity obtained without enzyme. In (b), endopeptidase 3.4.24.15 activity was fluorimetrically recorded as described in Methods in the absence and presence of dithiothreitol (500 μM) without or with a 18 h preincubation time at 37°C. Columns show the mean ± s.e.mean of 3 independent experiments.

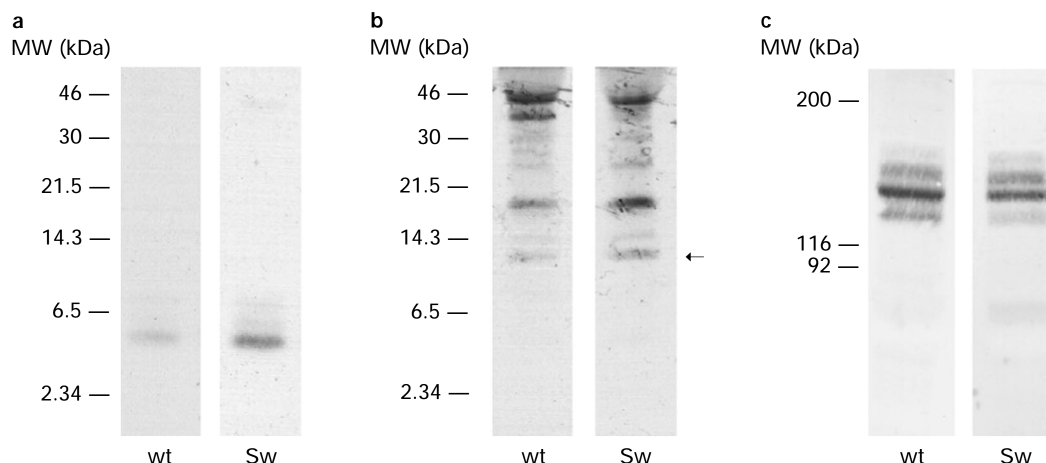

Figure 2 Wild-type (wt) and SwβAPP expression and characterization of Aβ-containing catabolites in stably transfected HK293 cells. Transfected cells overexpressing wild-type (wt) or the Swedish mutated form (Sw) of βAPP_{751} were metabolically labelled as described in the Methods. Media (a) and cell lysates (b) were immunoprecipitated with FCA18 antibody as described. After centrifugation, immunoprecipitate pellets were washed, electrophoresed on a 16.5% Tris/tricine gel and radioautographed. (C) The level of wt and $Sw\beta APP_{751}$ immunoreactivities in transfectants as revealed by Western blot analysis with 22C11 monoclonals. Arrow indicates the p12 fragment.

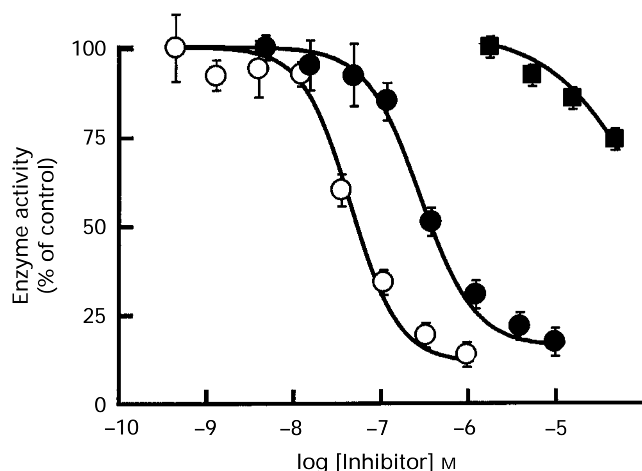

Figure 3 Effect of inhibitors on Mcc-Pro-Leu-Gly-Pro-D Lys-Dnp-degrading activity present in HK293 cell homogenates. HK293 cell homogenate membranes (25 μl) were preincubated for 5 h at 37°C in the presence of various concentrations of Z(L,D)-PheΨ(PO_2CH_2)-Ala-Lys-Met (○), Cpp-Ala-Ala-Tyr-pAB (●) or Pro-PheΨ(PO_2CH_2)-Gly-Pro (■) in a final volume of 100 μl of 5 mM Tris-HCl, pH 7.5, containing 0.5 mM dithiothreitol. Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (5 nmol, 50 μM) was then added for 1 additional hour at 37°C. Incubations were stopped and fluorimetrically analysed as described in Methods. Points indicate the mean value of 3 independent determinations; vertical lines show s.e.mean.

contrast, the activity was not prevented by Pro-PheΨ-(PO_2CH_2)-Gly-Pro, a potent and selective inhibitor of the closely related peptidase, endopeptidase 3.4.24.16 (Jiràcek *et al.*, 1996). Altogether, these data indicate that HK293 cells contain a proteolytic activity, the pharmacological spectrum of which corresponds to that expected for genuine endopeptidase 3.4.24.15. Interestingly, Z(L,D)-PheΨ(PO_2CH_2)-Ala-Lys-Met appears to be capable of blocking intracellular endopeptidase 3.4.24.15, in a dose-dependent manner while another unrelated inhibitor Pro-PheΨ(PO_2CH_2)-Gly-Gly does not (Figure 4). The apparent rightward shift in the EC_{50} (between 0.1 and 1 μM, see Figure 4) seems to indicate that the rate of penetration of the inhibitor inside HK293 cells remains marginal. However, a 10 μM concentration of the inhibitor induced about 80% of inhibition of intracellular endopeptidase 3.4.24.15 activity and therefore this

Table 1 Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis by purified endopeptidase 3.4.24.15 and HK293 cell homogenates: effect of inhibtors

Inhibitor	*K_i values (nM)* *HK293 cell homogenate*	*Endopeptidase 3.4.24.15*
Cpp-Ala-Ala-Tyr-pAB	45	35
Z(L,D)-PheΨ(PO_2CH_2)-Ala-Lys-Met	7	7
Pro-PheΨ(PO_2CH_2)-Gly-Pro	>100000	>100000

Mcc-Pro-Leu-Gly-Pro-D Lys-Dnp (5 nmol, 50 μM) was incubated in the absence (control) or presence of various concentrations of the indicated inhibitors with 25 μl of HK293 cell homogenate membranes or with 10 μl of purified endopeptidase 3.2.24.15. Incubations were stopped and fluorimetrically analysed as described in Methods. Values are expressed as % of control activity which allowed us to estimate IC_{50}s and to derive the K_i value according to the formula $IC_{50} = K_i\ (1 + S/K_m)$ where $K_m = 8$ μM (Checler *et al.* 1995).

concentration was chosen to examine any putative effect of the inhibitor on the secretion of Aβ by transfected cells expressing the wild type and $Sw\beta APP_{751}$. Z(L,D)-PheΨ-(PO_2CH_2)-Ala-Lys-Met was totally unable to affect the secretion of Aβ by both transfected cells expressing wild type and Swedish mutated βAPP (Figure 5). Furthermore, the intracellular formation of the 12 kDa fragments derived by a unique cleavage by β-secretase without subsequent γ-secretase breakdown was not affected (not shown).

Discussion

Increasing evidence suggests that several early onset-clinically diagnosed familial forms of Alzheimer's disease are due to missense mutations taking place on the βAPP, thereby leading to an exacerbation of a β-secretase activity-derived formation of Aβ. Among them, a double mutation (Swedish mutation, Sw) located adjacently to the N-terminus of Aβ was shown to trigger about a six fold increase in the secretion of Aβ by transfected cells overexpressing the SwβAPP (see Introduction). It is therefore expected that a genuine β-secretase candidate would display enhanced affinity and/or catalytic

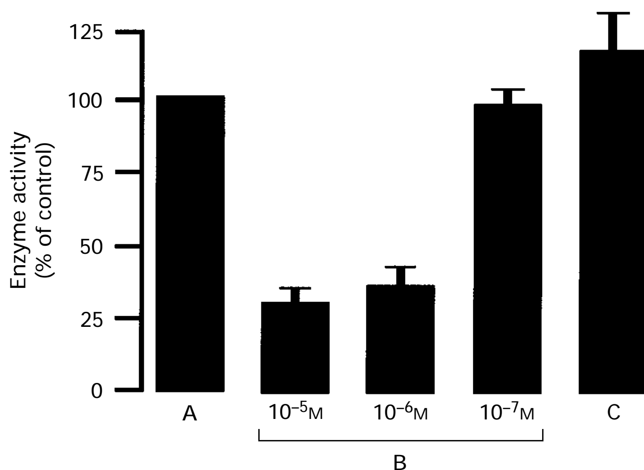

Figure 4 Effect of peptidase inhibitors on intracellular Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp-degrading activity in HK293 cells. Confluent cells were preincubated for 7 h at 37°C in the absence (A) or in the presence of the indicated concentrations of Z(L,D)-PheΨ(PO_2CH_2)-Ala-Lys-Met (B) or 10 μM of Pro-PheΨ(PO_2CH_2)-Gly-Gly (C). Cells were then rinsed twice with PBS^-, scrapped and homogenized as described in Methods. Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (5 nmol, 50 μM) was then incubated for 1 h at 37°C with 25 μl of cell homogenate membranes in 100 μl of 5 mM Tris-HCl, pH 7.5, containing 0.5 mM dithiothreitol and 5 mM Pro-Ile (in order to prevent any contribution of endopeptidase 24.16). Incubations were stopped and fluorimetrically analysed as described in Methods. Columns represent the mean value $\pm$s.e.mean of 6 independent expriments. Activity in (A) is 155.9$\pm$23.7 u h^{-1} mg^{-1}.

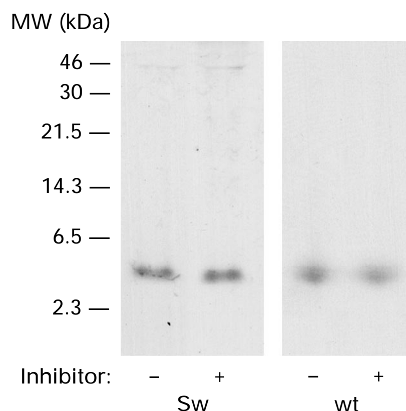

Figure 5 Effect of Z(L,D)-PheΨ(PO_2CH_2)-Ala-Lys-Met on Aβ secretion by wild-type (wt) and SwβAPP-expressing HK293 cells. Transfected cells overexpressing wt or SwβAPP_{751} were incubated in the absence (−) or presence (+) of 10 μM Z(L,D)-PheΨ(PO_2CH_2)-Ala-Lys-Met and metabolically labelled as described in Methods. Media was then collected and immunoprecipitated with FCA18 as described in Methods, then centrifuged. Immunoprecipitated pellets were washed, electrophoresed on a 16.5% Tris/tricine gel and radioautographed.

constants towards peptides or protein substrates in which the Sw double mutation would have been incorporated. Another possibility that cannot be excluded would be the occurrence of a β-secretase only acting on the mutated βAPP and unable to process the wild type counterpart.

Interestingly, previous studies have indicated that endopeptidase 3.4.24.15 immunoreactivity appears to be associated with neurofibrillary tangles (Conn *et al*., 1996) that correspond to one of the major histological lesions occurring in Alzheimer's disease (Goedert, 1993). Furthermore, the identification of the human gene encoding endopeptidase 3.4.24.15 on chromosome 19 was of interest, since late onset forms of the disease have been linked with a locus present on this chromosome (Corder *et al*., 1993). With regard to these observations, several studies have examined whether endopeptidase 3.4.24.15 could behave as a β-secretase. By means of synthetic peptides mimicking the Aβ sequence encompassing the theoretical β-secretase cleavage site, two studies have documented the ability of the enzyme to act as a β-secretase (McDermott *et al*., 1992; Papastoitsis *et al*., 1994) while another one excluded such a possibility (Brown *et al*., 1996). Our unpublished data established that although endopeptidase 3.4.24.15 could cleave peptides mimicking the β-secretase-targeted sequence, it was not very potent. Subsequent h.p.l.c. analysis, sequencing and mass spectrometry of the catabolites did not allow the adequate identification of the catabolites (data not shown). Furthermore, endopeptidase 3.4.24.15 did not display preferred affinity/catalysis for peptides exhibiting the Sw mutation (not shown). It should be noted that such a peptide screening strategy led us to establish, recently, that the acidic protease cathepsin D cleaved these synthetic substrates at the expected peptide bond, and displayed preferred kinetic parameters for those bearing the Sw mutation (Chevallier *et al*., 1997).

Although it appears crucial to establish that such sequences were also targeted when embedded in the whole structure of the putative natural substrate, i.e. βAPP, very few studies were concerned with the ability of endopeptidase 3.4.24.15 to cleave βAPP itself. Papastoitsis *et al*. (1994) indicated that this enzyme hydrolyzed human recombinant βAPP, even if this seemed to be with apparently poor affinity. In the present study, we expressed and purified baculoviral recombinant SwβAPP_{751} and we failed to detect any catabolites of SwβAPP_{751} upon prolonged exposure to purified endopeptidase 3.4.24.15 (Figure 1), even in the presence of dithiothreitol, a drastic activator of the enzyme. In agreement with our data, a recent study by Brown *et al*. (1996) indicated that purified endopeptidase 3.4.24.15 failed to hydrolyse SwβAPP when used as a substrate in its natural membrane-embedded configuration. Once again, such an approach allowed us to show that cathepsin D preferentially hydrolysed SwβAPP_{751} and released a C-terminal product, the immunological characterization of which unambiguously indicated that it derived from a cleavage liberating the N-terminus of the Asp1 residue of the Aβ sequence (Chevallier *et al*., 1997). Altogether, some doubts were cast about the potential of endopeptidase 3.4.24.15 to act as a β-secretase.

A straightforward approach to decide definitely between the two possibilities is to assess the effect of enzyme blockers on the Aβ secretion by cells overexpressing βAPP. In this context, we took advantage of the recent development of highly potent and fully selective inhibitors of endopeptidase 3.4.24.15 (Jiràcek *et al*., 1995). One of these phosphinic peptides Z(L,D)-PheΨ(PO_2CH_2)-Ala-Lys-Met displayed a subnanomolar affinity for the enzyme ($K_i = 0.16$ nM) and did not affect a series of other peptidases belonging to the same metalloenzyme family (Jiràcek *et al*., 1995). It was therefore used to examine the putative β-secretase role of endopeptidase 3.4.24.15 in human HK293 cell line, the relevance of which as a model of Alzheimer's disease pathology has been documented in numerous studies (for review see Checler, 1995). In agreement with previous studies, we established here that transfected HK293 cells overexpressing the βAPP_{751} secrete Aβ and generate an intracellular Aβ-bearing fragment of 12 kDa. Both products appear to be drastically exacerbated by the presence of the double Sw mutation (Figure 2a, b). These cells contain an endogenous endopeptidase 3.4.24.15-like activity. Thus, the pharmacological spectrum of a quenched fluorimetric substrate-hydrolysing activity present in HK293 cells appears to be closely reminiscent of that exhibited by purified endopeptidase 3.4.24.15 (see Table 1 and Figure 3). Interestingly, the phosphinic endopeptidase 3.4.24.15 inhibitor appears to be partly cell-permeant since 80% blockade of intracellular activity could be achieved after pretreatment of the transfectants with this agent (Figure 4). However, an

identical procedure did not affect the secretion of Aβ by wild type and SwβAPP$_{751}$-expressing cells (Figure 5) or 12 kDa product formation, indicating that endopeptidase 3.4.24.15 does not participate in production of Aβ.

Several studies indicate that endopeptidase 3.4.24.15 is an oligopeptidase (Orlowski *et al*., 1983; Barelli *et al*., 1991; Lew *et al*., 1995). This concept implies strong requirements of the substrate size that are usually ranging between 5 to 20 amino acid residues. In support of these observations, to our knowledge, there are no examples of high molecular weight proteins behaving as substrates of endopeptidase 3.4.24.15. This agrees well with the inability of the enzyme to cleave recombinant baculoviral SwβAPP (present study). However, although our data seem to exclude endopeptidase 3.4.24.15 from the aetiology of Alzheimer's disease, its capability to cleave small peptides could be of importance in the symptomatology of the disease. Thus, several studies, have indicated that the enzyme purified from various brain sources hydrolyzed somatostatin (Chu & Orlowski, 1985; Barelli *et al*., 1991; Dahms & Mentlein, 1992) and that it contributes to the catabolism of this peptide in several tissue and cell systems (Lucius & Mentlein, 1991; Ichai *et al*., 1994; Mentlein & Dahms, 1994). This is of particular interest since experimental lowering of somatostatin content has been shown to trigger severe memory impairment, a cognitive dysfunction reminiscent of the main intellectual alteration occurring during the degenerative process of Alzheimer's disease. Since we established previously that the concentration of endopeptidase 3.4.24.15 is selectively affected in discrete regions of the pathological human brain and that this apparently led to an altered rate of hydrolysis of exogenously applied somatostatin (Ichai *et al*., 1994), one could postulate that endopeptidase 3.4.24.15 participates in the post-translational events responsible for the control of endogenous somatostatin concentration in human brain. Altogether, our work suggests a possible role of endopeptidase 3.4.24.15 in the symptomatology but probably not the aetiology of Alzheimer's disease.

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