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# Aminopeptidase A contributes to the N-terminal truncation of amyloid β-peptide

Jean Sevalle,* Audrey Amoyel,* Philippe Robert,† Marie-Claude Fournié-Zaluski,‡ Bernard Roques§ and Frédéric Checler*

*Institut de Pharmacologie Moléculaire et Cellulaire and Institut de NeuroMédecine Moléculaire, UMR6097 CNRS/UNSA, Équipe labellisée Fondation pour la Recherche Médicale, Sophia-Antipolis, Valbonne, France

†Centre Mémoire de Ressource et de Recherche, CHU de Nice, Université Nice-Sophia-Antipolis, Hôpital de Cimiez, 4 avenue Victoria, Nice, France

‡Pharmaleads, Paris, France

§Université Paris-Descartes (Paris V), 6 avenue de l'Observatoire, Paris, France

**Abstract**

Several lines of data previously indicated that N-terminally truncated forms of amyloid-β (Aβ) peptides are likely the earliest and more abundant species immunohistochemically detectable in Alzheimer's disease-affected brains. It is noteworthy that the free N-terminal residue of full-length Aβ (fl-Aβ) is an aspartyl residue, suggesting that Aβ could be susceptible to exopeptidasic attack by aminopeptidase A (APA)-like proteases. In this context, we have examined whether APA could target Aβ peptides in both cell-free and cellular models. We first show that the general aminopeptidase inhibitor amastatin as well as two distinct aminopeptidase A inhibitors EC33 and pl302 both significantly increase the recovery of genuine fl-Aβ peptides generated by cells over-expressing Swedish-mutated β amyloid precursor protein (APP) while the aminopeptidase N blocker pl250 did not modify fl-Aβ recovery. In agreement with this observation, we establish that over-expressed APA drastically reduces, in a calcium dependent manner, fl-Aβ but not APP IntraCellular Domain in a cell-free model of Aβ production. In agreement with the above data, we show that recombinant APA degrades fl-Aβ in a pl302-sensitive manner. Interestingly, we also show that EC33 and pl302 lower staurosporine-stimulated activation of caspase-3 in wild-type fibroblasts but not in βAPP/β-amyloid precursor protein-like protein 2 (APLP2) double knockout fibroblasts, suggesting that protecting endogenous fl-Aβ physiological production triggers neuroprotective phenotype. By contrast, EC33 does not modify staurosporine-induced caspase-3 activation in wild-type and Swedish-mutated βAPP-HEK293 expressing cells that display exacerbated production of Aβ. Overall, our data establish that APA contributes to the N-terminal truncation of Aβ and suggest that this cleavage is likely abrogating a protective function associated with physiological but not supraphysiological levels of genuine fl-Aβ peptides.

**Keywords:** aminopeptidase A, amyloid-β degradation, cell death, cell free amyloid-β production, inhibitors, N-terminally truncated amyloid-β fragments.

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Alzheimer's disease (AD) is the most common form of dementia in the elderly. At the histopathological level, AD is characterized by neurofibrillary tangles and by senile plaques which are extracellular deposits mainly composed of an aggregable peptide called Aβ (Selkoe 1991). Whether this peptide is directly and totally responsible for the neurodegeneration taking place in the brain of AD-affected patient is not yet established. However, although the exact etiology of AD is still a matter of discussion, there are few doubts concerning the fact that the disease is likely related to a modification of production of Aβ-like peptides. Thus, all

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Address correspondence and reprint requests to F. Checler, Institut de Pharmacologie Moléculaire et Cellulaire, UMR6097 CNRS/UNSA, Équipe labellisée Fondation pour la Recherche Médicale, 660 Route des Lucioles, 06560, Sophia-Antipolis, Valbonne, France.
E-mail: checler@ipmc.cnrs.fr

*Abbreviations used*: AD, Alzheimer's disease; AICD, APP IntraCellular Domain; APA, aminopeptidase A; APLP2, β-amyloid precursor protein-like protein 2; APN, aminopeptidase N; APP, amyloid precursor protein; Aβ, amyloid-β; Glu-7-AMC, glutamyl-7-amido-4-methyl-coumarin; STS, staurosporine; SwAPP, Swedish-mutated βAPP.

 

mutations responsible for familial AD modulate Aβ. Familial forms of AD are because of mutations on β amyloid precursor protein (APP), the precursor of the amyloid peptide or presenilins, two homologous proteins responsible for most of the genetic cases of AD (Van Broeckhoven 1995; Tanzi and Bertram 2001). Most of the mutations on these proteins were shown to trigger either increased production of total Aβ load (as is the case for the Swedish mutation) or specifically increase Aβ42, the longer and more aggregable Aβ species (Checler 1995, 1999). In agreement with the so-called 'amyloidogenic cascade hypothesis' (Hardy and Higgins 1992), numerous reports indicated that Aβ could trigger cell toxicity and apoptosis, the extent of which appeared exacerbated by the two amino-acids C-terminal extension harbored by Aβ42 (Burdick *et al.* 1997).

The simple view of a selective modulation of Aβ40/42 has been complicated by several studies indicating that unlike previously thought, AD is characterized by the deposition of numerous peptides related to Aβ. Thus, the 'β-amyloid'-like immunoreactivity observed *post-mortem* in the cerebral lesions or in the cerebrospinal fluid or plasma of affected patients correspond to a complex set of Aβ-related fragments, with various N- and C-termini. Iwatsubo and colleagues showed that N-terminally truncated Aβ42 deposit can be detected in diffuse plaques, i.e at an early stage of the pathology (Iwatsubo *et al.* 1996) while several other papers demonstrated the occurrence of N-terminal heterogeneity of parenchimal and cerebrovascular Aβ deposits (Tekirian *et al.* 1998; Thal *et al.* 1999; Takeda *et al.* 2004). Also interesting was the demonstration that such N-terminally truncated species occur in Down's syndrome brains (Russo *et al.* 1997) and Cotton wool plaques (Miravalle *et al.* 2005). Finally, Wiltfang and colleagues showed that Aβ2–42 was elevated in sporadic and familial AD (Wiltfang *et al.* 2001).

Besides anatomical observations, the importance of the production of N-terminally truncated fragments of Aβ in AD pathology is underlined by a series of observations. First, several newly discovered mutations on βAPP responsible for early onset AD have been shown to specifically affect the cellular production of N-terminally truncated forms of Aβ (Ancolio *et al.* 1999; Stenh *et al.* 2002). Second, another study showed that a presenilin 1 mutation responsible for AD led to the accumulation of Aβ species deleted of their first amino-acids in human brain (Russo *et al.* 2000). Finally, of most importance was the observation that in transgenic mice bearing PS mutations, intracellular species ending at the 42nd position but lacking N-terminal immunoreactivity accumulate intracellularly in neurons before any plaque formation (Chui *et al.* 2001).

AβN3(pE), that corresponds to Aβ in which the two first amino-acids have been deleted and in which glutamate at position 3 has undergone cyclisation (Saido *et al.* 1996; Russo *et al.* 1997), is a common denominator between diffuse and compact deposits (Saido *et al.* 1995; Kuo *et al.* 1997). It is interesting to note that the first N-terminal residue of Aβ is an aspartyl residue that renders the peptide particularly susceptible to aminopeptidases.

The fact that these N-terminally truncated species appear early in AD pathology could mean that they correspond to the genuine Aβ-related 'toxic' species. As a corollary, 'full length' Aβ (fl-Aβ), that is produced physiologically may have a 'non-toxic' function when occurring below its threshold of aggregation. Therefore, N-terminal truncation could be seen as an inactivation process for genuine Aβ-associated phenotype and would be responsible for the toxicity exhibited by N-terminally-truncated fragments. Such hypothesis would design proteases involved in N-terminal truncation as putative therapeutic targets. Thus, inhibitors of such enzymes, by blocking N-terminal fragments production and by preventing Aβ catabolism would slow down N-terminal fragments-associated toxicity and subsequent neurodegeneration.

Our main goals were to delineate the putative contribution of aminopeptidase A (APA) in the N-terminal truncation of Aβ and to establish the influence of N-terminal deletion on Aβ-related function. Here we show that APA participates in the release of the N-terminal aspartyl residue of Aβ peptides and apparently controls a physiological Aβ-associated protective function.

## Materials and methods

### Cell cultures and transfections

Wild-type or βAPP/β-amyloid precursor protein-like protein 2 (APLP2) double knock-out fibroblasts have been described previously (Heber *et al.* 2000). HEK293 stably transfected with pcDNA3 empty vector, stably over-expressing wild-type or Swedish mutated βAPP were obtained and cultured as described previously (Chevallier *et al.* 1997). Transient transfections of cDNA (2 μg) were carried out with Lipofectamine 2000 reagent (Invitrogen) according to previously reported procedures (Alves da Costa *et al.* 2006).

### Cell treatments and detection of secreted Aβ

Wild-type or Swedish-mutated βAPP over-expressing HEK293 cells were grown in six well-dishes and allowed to secrete for 8 h in optimen (1 mL, Gibco, Rockville, MD, USA) containing phosphoramidon (10 μM, Sigma, St Louis, MO, USA) in order to prevent Aβ degradation by neprilysin, in the absence or in the presence of amastatine, the aminopeptidase N inhibitor pl250 (Chen *et al.* 1999) or the APA specific inhibitors 3-amino-4-thio-butyl sulfonate (EC33) (Chauvel *et al.* 1994) or pl302 (David *et al.* 1999). Media were collected, completed with one-tenth of 10× radioimmunoprecipitation assay buffer (Tris-HCl pH 8.0, 100 mM) containing NaCl (1.5 M), EDTA (50 mM) and incubated overnight with a 100-fold dilution of FCA18 (Barelli *et al.* 1997; Chui *et al.* 2001) and protein A agarose beads (VWR, France). Beads were washed twice with 1× ristocetin-induced platelet agglutination and subjected to Tris/tricine 16.5% polyacrylamide gels. Proteins were transferred onto nitro cellulose and incubated overnight with the 6E10 monoclonal antibody (AbCam, Cambridge, UK) at a 1/1000 dilution. Immunological

 

complexes were detected with a goat anti-mouse peroxidase-conjugated antibody (1/2000 dilution). Chemiluminescence was recorded using a Luminescence Image Analyser LAS-3000 (Raytest, Courbevoie, France) and quantifications were performed using the AIDA analyser software.

### Aminopeptidase A assay on plated cells

Wild-type, βAPP/APLP2 double knock-out fibroblasts, HEK293 stably transfected with pcDNA3 empty vector, stably over-expressing wild-type or Swedish mutated βAPP were grown in six well-dishes. Media were discarded and cells incubated in 1 mL of phosphate-buffered saline containing 50 μM of the glutamyl-7-amido-4-methylcoumarin (Glu-7-AMC) substrate in the absence or in the presence of various EC33 concentrations. Media (100 μL) were taken out at various time intervals then fluorimetric measurements were performed using the Fluoroscan Ascent apparatus (Thermo Fisher Scientific, Courtaboeuf, France). Once the media removed, cells were harvested, lysed and protein concentrations were measured by the Bradford's method (Bradford 1976). Values correspond to specific activities expressed in nmol/mg/min of 7-AMC released.

### Monitoring of aminopeptidase A in membrane homogenates

Cells were rinsed, gently scraped and pelleted by centrifugation. Cell pellets were homogenized in Tris 10 mM, pH 7.5 and lysates were spun at 800 *g* for 10 min at 4°C in order to eliminate cell debris. APA activity was measured in 96-well plates in which 5 μL of supernatants were added to 85 μL of assay buffer Tris 50 mM, pH 7.5 containing $CaCl_2$ (25 mM) in the presence or in the absence of EC33 (100 μM). Then Glu-7-AMC (final concentration of 50 μM) was added in each well to start the reaction. Fluorescence was recorded at 360 and 460 nm as excitation and emission wavelengths, respectively. APA specific activity was calculated from the linear part of the recorded fluorescence and expressed in nmol/min/mg of proteins.

### Production of recombinant C100 fragment

cDNA encoding the C-terminal 99 amino acids of human βAPP was sub-cloned into a pet29c vector fused to FLAG tag at the C-terminus and harboring an additional Met at the N-terminus. Recombinant protein was expressed in *Escherichia coli*. Cell pellets were solubilized with a lysis buffer [Tris 20 mM, pH 7.5 containing EDTA (0.1 mM), egg lysosyme (1 mg/mL) and *N*-lauroylsarcosyne (1% (v/v)]. After 3 h under agitation at 4°C, the lysate was spun at 5000 *g* for 120 min at 4°C. The supernatant was next spun for 75 min at 20 000 *g* at 4°C and the resulting supernatant was subjected to filtration on a 30 kDa cut-off membrane.

### Preparation of the 'solubilized' membrane fraction

Intact cell pellets were suspended in Tris 10 mM, pH 7.5 complemented with a cocktail of inhibitors (Sigma), and subjected to repeated passages through a 25G needle. Homogenates were first centrifuged at 800 *g* for 10 min at 4°C to eliminate cell debris and the resulting supernatant was subjected to an additional 20 000 *g* centrifugation for 1 h at 4°C. Pellets containing the membranes were then resuspended in a solubilization buffer {sodium citrate (150 mM, pH 6.4) containing 3-[(3-cholamydopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate [CHAPSO, 1% (v/v)] and a cocktail of inhibitors}. The samples are diluted with solubilization buffer to yield a 1 mg/mL final protein concentration and are referred to as 'solubilized membranes'. All steps were performed at 4°C.

### *In vitro* γ-secretase assay

'Solubilized membranes' (5 μL) were diluted with sodium citrate buffer, (5 μL, 150 mM, pH 6.4) and reaction buffer (10 μL, sodium citrate 150 mM, pH 6.4) containing dithiothreitol (20 mM), bovine serum albumin (0.2 mg/mL), egg phosphatidyl choline (1 mg/mL) and recombinant C100-FLAG (50 μg/mL) then either incubated over constant agitation for 16 h at 37°C or stored at 4°C (negative controls). Samples were then supplemented with 2× Tris-tricine loading buffer (20 μL), boiled for 5 min and subjected to western blot for Aβ analysis with 6E10 monoclonal antibody as described above or monitored for βAPP IntraCellular Domain (AICD) by using the anti-FLAG M2 monoclonal antibody (Sigma) at a 1/1000 dilution.

### Aβ degradation by recombinant aminopeptidase A

Synthetic Aβ (0.5 ng/μL) (Bachem) and recombinant aminopeptidase A (2 ng/μL) (R&D Systems) were mixed in 40 μL of assay buffer Tris 50 mM, pH 7.5 containing $CaCl_2$ (25 mM) and bovine serum albumin (0.1 μg/μL) in the absence or in the presence of pl302 (5 μM) for 4 h at 37°C. Samples were then diluted with 260 μL of buffer [Tris-HCl, pH 8.0 (10 mM) containing NaCl (150 mM), EDTA (5 mM)] and immunoprecipitations were performed with FCA18 as described above.

### Caspase-3 activity assay

Stably transfected cells were pre-treated for 8 h with vehicle, EC33 or pl302 then incubated for various times without or with various concentrations of staurosporine, then caspase-3-like activity was fluorimetrically measured as extensively detailed (Alves da Costa *et al.* 2000).

### Statistical analysis

Statistical analysis was performed with Prism software (Graphpad, San Diego) using the Student-Newman-Keul's multiple comparison test for one-way analysis of variance or unpaired *t* test for pairwise comparison.

## Results

### Aminopeptidase A inhibitors increase the recovery of full-length Aβ peptides in Swedish-mutated βAPP-expressing HEK293 cells

We took advantage of the obtention of a polyclonal anti-serum [FCA18, (Barelli *et al.* 1997; Chui *et al.* 2001)] that specifically recognizes the N-terminal aspartyl residue of Aβ (Fig. 1a) to examine the contribution of APA to Aβ N-terminal truncation by intact cells. We first show that plated HEK293 cells exhibit Glu-7-AMC-hydrolysing proteases, the activity of which was drastically reduced (Fig. 1b) by amastatin (an aminopeptidase inhibitor) and by EC33, a selective blocker of APA (Chauvel *et al.* 1994), indicating that HEK293 cells display APA in its proper ectoproteasic conformation (Checler 1993), i.e with its catalytic site facing the extracellular space.

 

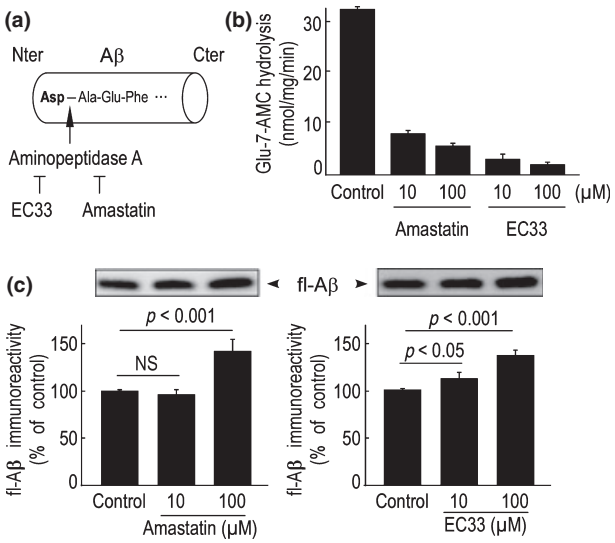


**Fig. 1** Amastatin and E33 augment fl-Aβ recovery from Swedish-mutated HEK293 cells. (a) Schematic representation of Aβ N-terminal tetrapeptide. Note that the putative release of aspartyl 1 residue by APA disrupts the FCA18-selective Aβ epitope. (b) Glu-7-AMC substrate hydrolysis by plated HEK293 cells in the absence (control) or in the presence of the indicated concentrations of amastatin or EC33. Bars are means ± SE of three independent experiments. (c,d) Swedish mutated APP-over-expressing HEK293 were allowed to secrete for 8 h in absence (control) or in the presence of the indicated concentrations of amastatin (left panel) or EC33 (right panel). fl-Aβ was monitored by combined immunoprecipitation with FCA18 and western blot with 6E10 then quantified as described in the Methods. Bars are the means ± SE of 4–7 independent experiments and are expressed as percent (taken as 100) of control untreated cells.

Interestingly, both inhibitors increase the recovery of FC18-positive Aβ-like immunoreactivity in secretate of Swedish-mutated βAPP (SwAPP) (Fig. 1c). These observations were fully confirmed by another specific APA inhibitor, pl302 (David *et al.* 1999) that dose-dependently enhanced fl-Aβ recovery while the specific aminopeptidase N (APN) blocker, pl250 (Chen *et al.* 1999) remained inactive (Fig. 2). Altogether, these data demonstrate that APA but not APN inhibitors protect fl-Aβ from N-terminal truncation by HEK293 cells. Similar results were obtained with wild-type APP-expressing cells (data not shown).

## Cell-free production of fl-Aβ peptides is lowered by APA over-expression

We have examined the influence of APA on the *in vitro* production of fl-Aβ. The membrane fraction prepared from mock-transfected HEK293 cells (see Methods and Fig. 3a) was first analyzed for the production of fl-Aβ from a C100 recombinant fragment corresponding to the sequence theoretically derived from the sole attack of βAPP by β-secretase (Vassar *et al.* 1999). Figure 3b first shows that fl-Aβ was readily produced by HEK293 membrane preparation at 37°C but not at 4°C. Second, we were also able to monitor

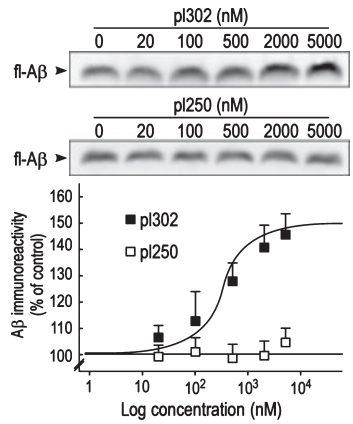


**Fig. 2** The selective APA inhibitor pl302 dose-dependently increases fl-Aβ produced by Swedish-mutated HEK293 cells. Swedish mutated APP-over-expressing HEK293 were allowed to secrete for 8 h in absence (control) or in the presence of the indicated concentrations of pl302 and pl250. fl-Aβ was monitored by combined immunoprecipitation with FCA18 and western blot with 6E10 then quantified as described in the Methods. Values are expressed as percent of control fl-Aβ immunoreactivity obtained in absence of inhibitor and are the means ± SE of three independent experiments.

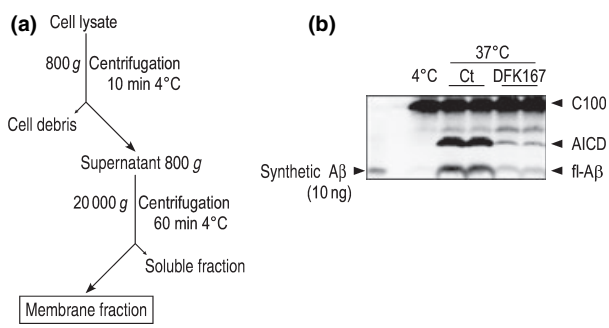


**Fig. 3** Cell-free Aβ and AICD productions from recombinant C100 substrate. (a) Schematic representation of the procedure used to obtain the 'solubilized' membranes fractions. (b) Cell-free assay of C100-FLAG hydrolysis was performed as described in the Methods at 4°C or 37°C in the absence (control) or in the presence of DFK167 (50 μM). C100, AICD and fl-Aβ expressions were monitored as described in the Methods.

a temperature-sensitive generation of the AICD [that corresponds to the C-terminal stub released by the cleavage of βAPP by γ-secretase (Passer *et al.* 2000)]. As expected, both AICD and Aβ production were drastically decreased by the γ-secretase inhibitor DFK167 (Wolfe *et al.* 1998). It should be noted that a DFK167-insensitive fraction of fl-Aβ remained detectable (Fig. 3b). The latter fl-Aβ fraction could derive from the presenilin-independent γ-secretase-like activities previously reported (Armogida *et al.* 2001; Wilson *et al.* 2002, 2003; Lai *et al.* 2006; Yagishita *et al.* 2008). Overall, the above data indicate that our membrane preparation of HEK293 cells harbors all the enzymatic machinery necessary to produce fl-Aβ and AICD. We therefore

 

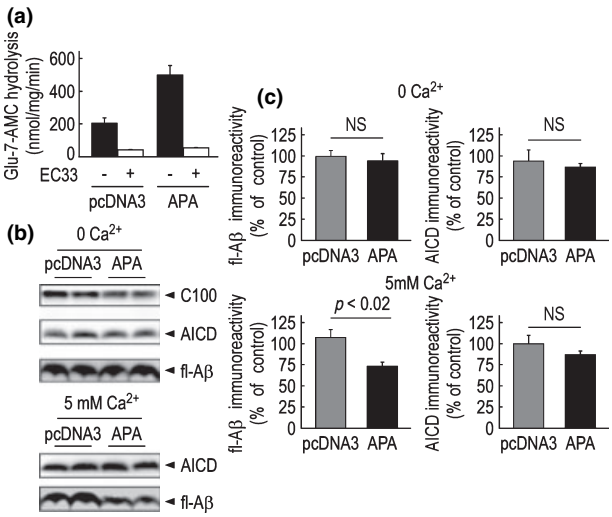


**Fig. 4** APA over-expression decreases recovery of cell-free-produced Aβ without affecting AICD immunoreactivity. (a) Glu-7-AMC hydrolysis by solubilized membranes prepared from pcDNA3- or APA-transiently transfected HEK293 cells in the absence (black bars) or in the presence (white bars) of EC33 (100 μM). Bars are means ± SE of three independent experiments. (b,c) Recombinant C100-FLAG hydrolysis by the solubilized membrane fractions in the absence (upper panel) or in the presence (lower panel) of $CaCl_2$ (5 mM). AICD and fl-Aβ were monitored and quantified as described in the Methods. Bars in (c) correspond to the means ± SE of six independent experiments. Values are expressed as percent of control mock-transfected cells (taken as 100).

examined the influence of APA over-expression on fl-Aβ and AICD production by HEK293 membranes. First, we confirmed that APA-expressing cells display enhanced Glu-7-AMC-hydrolysing activity (Fig. 4a). Clearly, APA expression drastically reduces fl-Aβ but did not affect AICD (Fig. 4b, lower panel and c). It is noteworthy that fl-Aβ reduction was only observed in the presence of $Ca2^{+}$ (Fig. 4b and c), in agreement with the strict requirement and dependency of APA for calcium (Kugler 1982).

## Recombinant APA degrades fl-Aβ

We examined whether APA could directly degrade Aβ40. Figure 5 shows that APA drastically reduced FCA18-precipitable fl-Aβ immunoreactivity, indicating that this aminopeptidase removed the N-terminus of fl-Aβ that corresponds to the only residue recognized by FCA18 (Barelli *et al.* 1997; Chui *et al.* 2001). As expected, pI302 fully restored FAC18-labelled fl-Aβ expression (Fig. 5). This data fully confirmed the ability of recombinant and over-expressed APA to truncate the N-terminus of Aβ.

## The aminopeptidase A inhibitors EC33 and pI302 reduce staurosporine-induced caspase-3 activation in wild-type but not in APP/APLP2-knockout fibroblasts

We examined whether the blockade of APA could have Aβ-related functional consequences. Previous studies have

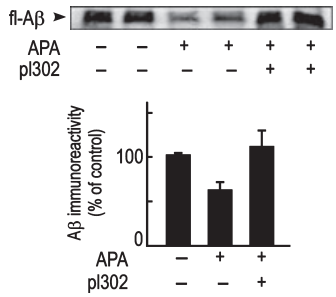


**Fig. 5** PI302-sensitive reduction of fl-Aβ immunoreactivity by recombinant APA. Synthetic Aβ (0.5 ng/μL) was incubated with recombinant aminopeptidase A (2 ng/μL) for 4 h at 37°C in a final volume of 40 μL of assay buffer Tris 50 mM, pH 7.5 containing $CaCl_2$ (25 mM) containing bovine serum albumin (0.1 μg/μL), in the absence or in the presence of pI302 (5 μM). Samples were then diluted with 260 μL of buffer [Tris-HCl, pH8.0 (10 mM) containing NaCl (150 mM) and EDTA (5 mM)] then analyzed for fl-Aβ immunoreactivity by FCA18 immunoprecipitations as described in the Methods.

indicated a link between Aβ and cell death (Loo *et al.* 1993; Blanc *et al.* 1997; Troy *et al.* 2000). We therefore measured staurosporine-stimulated caspase-3 activation as a functional read out of Aβ-related function. We first established that mock-transfected HEK293 cells display an EC33-sensitive Glu-7-AMC-hydrolyzing activity (Fig. 6a). Interestingly,

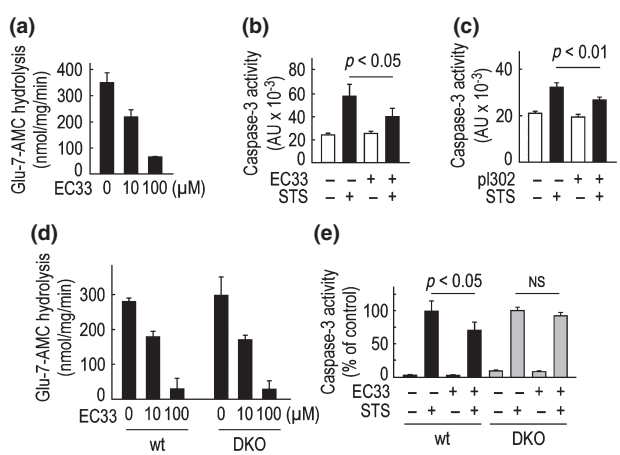


**Fig. 6** EC33 and pI302 reduction of STS-induced caspase-3 activation is abrogated by βAPP-deficiency. (a,d) Glu-7-AMC substrate hydrolysis by membrane homogenates prepared from mock-transfected HEK293 cells (a) wild-type or knockout (DKO) fibroblasts (d), in the absence or in the presence of the indicated concentration of EC33. Bars are means ± SE of three independent experiments. (b,c) Mock transfected HEK293 cells were treated for 8 h in the absence or in the presence of EC33 (100 μM, b) or pI302 (5 μM, c) then incubated with STS (2 μM, black bars) or DMSO (white bars) for 16 h and then caspase-3 activity was measured as described in the Methods. Bars are means ± SE of three independent experiments. (e) Wild-type or DKO fibroblasts were treated for 16 h in the absence or in the presence of EC33 (100 μM) then treated for 2 h with STS (1 μM) or DMSO. Caspase-3 activity is expressed in percent of STS-induced control conditions. Bars are means ± SE of 3–7 independent experiments.

 

EC33 (Fig. 6b) and pl302 (Fig. 6c) reduced staurosporine (STS)-induced caspase-3 activation in these cells. This indicates that APA contributes to the modulation of cell death and it was tempting to speculate on the possibility that APA-associated control of endogenous fl-Aβ could account for this phenotype. In order to strengthen this view, we examined the effect of βAPP depletion on APA-induced modulation of caspase-3 activity. If our hypothesis were right, one would expect to observe a full abolishment the APA-associated effect on caspase-3 activity in βAPP/APLP2 knockout cells that cannot produce endogenous fl-Aβ. First, we verified that wild-type and knockout fibroblasts display similar EC33-sensitive Glu-7-AMC-hydrolysing activity (Fig. 6d). Interestingly, EC33 reduced STS-induced caspase-3 activation in wild-type but not in knockout fibroblasts (Fig. 6e). Three lines of conclusion can be drawn from the above data. First it shows that EC33 effect on caspase-3 was not cell specific. Second, it demonstrates that APA-associated control of endogenous fl-Aβ has functional consequence on caspase-3 activity. Third, that physiological production of endogenous fl-Aβ likely triggers neuroprotection rather than toxicity, in cellular models.

### The aminopeptidase A inhibitor EC33 does not reduce staurosporine-induced caspase-3 activation in wild-type and Swedish-mutated APP-expressing cells

Several studies proposed that Aβ could be protective (Wu *et al.* 1995; Luo *et al.* 1996) while on the other hand, others suggest that Aβ could be toxic [for review see (Small *et al.* 2001)]. This apparent discrepancy could likely be explained by the intrinsic levels and the nature of the Aβ considered (truncated, oligomeric, etc…). In this context, it was of interest to assess whether the modulation of fl-Aβ-associated function by APA occurred independently of Aβ levels or if, alternatively, APA could more selectively regulate the function linked to physiological levels of fl-Aβ. We therefore examined the putative effect of EC33 on STS-induced caspase-3 activation in HEK293 cells expressing either wt-APP or SwAPP. As could have been anticipated, SwAPP-expressing cells produce more fl-Aβ than wt-APP cells (Fig. 7a). Interestingly, in agreement with previous studies, we showed that wtAPP-expressing cells display anti-apoptotic phenotype (Fig. 7b) as underlined by the statistically significant reduction of STS-stimulated caspase-3 activation (Fig. 7b, see insert). Conversely, SwAPP cells display a

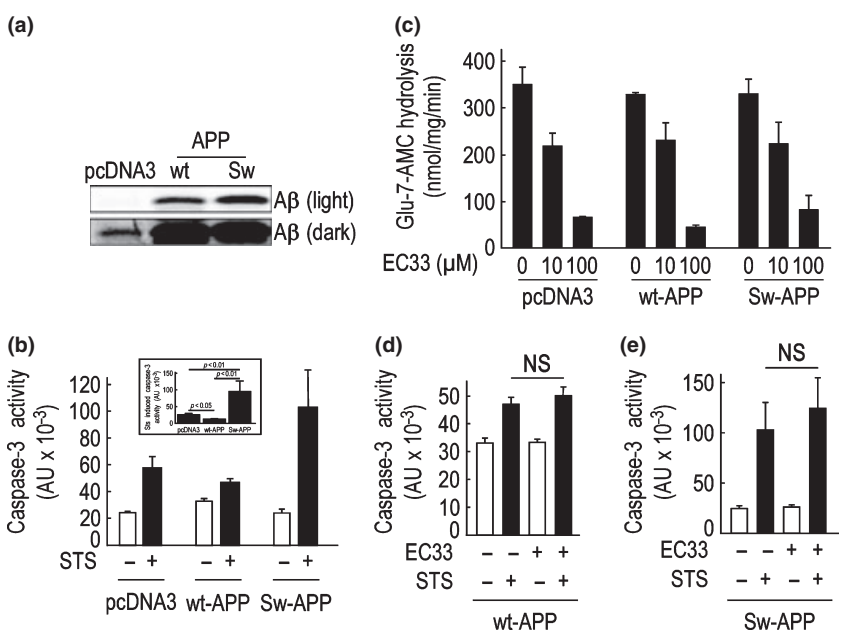


**Fig. 7** EC33 does not affect STS-induced caspase-3 activation in wild-type or swedish mutated APP transfected cells. (a) Stably transfected HEK293 cells expressing empty pcDNA3 vector (DNA3) or either wild-type (wt) or Swedish mutated (Sw) βAPP cells were grown in six well-dishes and allowed to secrete for 16 h in 1 mL optimen containing phosphoramidon (10 μM). fl-Aβ was immunoprecipitated and detected as described in the Methods. (b) Indicated cell lines were treated for 16 h without (–) or with (+) staurosporine (sts, 2 μM). Insert, bars represent the STS-stimulated caspase-3 activity. Bars are means ± SE of three independent experiments. (c) Glu-7-AMC substrate hydrolysis by homogenates prepared from the indicated transfected HEK293 cells, in the absence or in the presence of the indicated concentrations of EC33. Bars are means ± SE of three independent experiments. (d) Indicated HEK293 cells were treated for 8 h in the absence (–) or in the presence (+) of EC33 (100 μM) then treated for 16 h with STS (2 μM, black bars). Bars are means ± SE of three independent experiments.

proapoptotic phenotype (Fig. 7b). Therefore, wtAPP and SwAPP cells were particularly useful to examine the influence of APA on their associated phenotype. wt-APP and SwAPP display similar EC33-dependent Glu-7-AMC hydrolyzing activities when compared to their parent cell line (Fig. 7c). However EC33 did not modify wt-APP and SwAPP-associated modulation of caspase-3 activity (Fig. 7d).

## Discussion

Several lines of histochemical and genetic data have indicated that the generic term of Aβ peptides indeed refer to a set of N- and C-terminally truncated species that are even more abundantly produced than the parent peptides Aβ1–40 and Aβ1–42 (Kumar-Singh *et al.* 2000) and that often harbor exacerbated toxicity (Pike *et al.* 1995; Kar *et al.* 1996; Dahlgren *et al.* 2002; Russo *et al.* 2002; Wiltfang *et al.* 2002 Funamoto, 2004 #5070). The genesis of N-terminal truncation is partly understood. Direct endoproteolytic cleavages of βAPP by α- and β-secretases can lead to the production of Aβ17–40/42 and Aβ11–40/42, respectively (Checler 1995). Conversely, the bulk of 3–40/42 likely derives from the exoproteolysis of Aβ that liberates two N-terminal residues. This exopeptidasic attack is reminiscent of that triggered by aminopeptidases (Checler 1993). The first N-terminal amino-acid of Aβ is an aspartyl residue. Four lines of data indicate that APA contributes to the N-terminal truncation of Aβ. First, two distinct inhibitors of APA (EC33 and pl302) increased the recovery of fl-Aβ. Second, over-expression of APA drastically lowers fl-Aβ production in a cell free system. Third, fl-Aβ truncation by APA-expressing membranes was strictly dependent upon the presence of $Ca^{2+}$. Fourth, recombinant APA reduces fl-Aβ immunoreactivity, in a pl302-sensitive manner. Overall, these data indicate that both endogenous and over-expressed APA could contribute to the N-terminal proteolysis of Aβ. It is noteworthy that AβN3(pE), that corresponds to Aβ in which the two first amino-acids have been deleted and in which glutamate at position 3 has undergone cyclisation (Saido *et al.* 1996; Russo *et al.* 1997) is likely the main final product of N-terminal truncation. However, APA displays very poor specificity for aliphatic residue and likely does not contribute to the release of the alanyl residue in position two that is probably because of proteolytic attack by aminopeptidase N-like activities. However, APN clearly does not contribute to the truncation of fl-Aβ as demonstrated by the lack of effect of its specific inhibitor pl250. Therefore, APA can be seen as the limiting enzyme triggering the initial N-terminal residue of Aβ and giving access to additional aminopeptidasic proteolysis ultimately leading to AβN3(pE) production. It should also be noted that APA is a metallo-ectopeptidase, i.e an enzyme which catalytic site of is facing the extracellular space. This has been confirmed here by the effect of APA inhibitors on fl-Aβ recovery from intact plated cells. Thus, APA likely truncates secreted fl-Aβ rather than contributes to the catabolism of intracellular pool of Aβ. This does not preclude the possibility that extracellular truncated Aβ could be translocated into the cell and trigger intracellular toxicity.

Amyloid-β is a physiological product of βAPP processing (Shoji *et al.* 1992; Haass *et al.* 1993). Several works previously suggested that Aβ could display neuroprotection (Plant *et al.* 2003; Zou *et al.* 2003). This could appear paradoxical with respect to the huge amount of studies reporting on a toxic role of 'Aβ' [for review see (Small *et al.* 2001)]. However, close inspection of the data indicates that Aβ refers to a mix of Aβ-related species, often used at high concentrations where the final biophysical state of the peptides is often poorly controlled. In fact, few studies could indeed delineate the physiological function of genuine full length Aβ. The possibility to prevent N-terminal truncation of endogenously produced Aβ provided this opportunity and allowed examining whether APA could be seen as an enzyme triggering inactivation of Aβ physiological function. We show here that the selective APA inhibitors EC33 and pl302 lowers STS-associated caspase-3 activation in both mock-transfected HEK293 and wild-type fibroblasts that both display endogenous contents of Aβ. The fact that APA-associated modulation of caspase-3 could be directly because of its ability to control fl-Aβ levels was demonstrated by the abolishment of APA-mediated phenotype by βAPP deletion. This set of data indicates that fl-Aβ could display neuroprotective function and that APA could be seen as an inactivating enzyme for Aβ function. Very interestingly, when Aβ is produced at supraphysiological levels in cells expressing either wild-type or mutated βAPP, EC33 remained biologically inert. This observation could reconcile the view of a beneficial protective role of physiologically produced Aβ while exacerbated production of Aβ would abolish this function and triggers concentration- and species-dependent toxicity. This possibility fits well with our recent demonstration that β-site APP cleaving enzyme promoter transactivation could be promoted by exogenous or transfected Aβ42 as well as in cells over-expressing βAPP while physiological production of Aβ did not modify BACE1 promoter transactivation (Buggia-Prévot *et al.* 2008).

The lack of effect of APA inhibitors when Aβ production is enhanced could be either because of the fact that Aβ aggregates are poorly targeted by APA or, more likely, that both full-length- and truncated- Aβ species display similar toxicity when produced at high concentrations. The corollary of the above statements is that APA inhibitor treatment could be likely useful only at early stages of AD pathology when Aβ production is not yet too exacerbated. Obviously, the use of transgenic animals treated with systemically active APA inhibitors (Chauvel *et al.* 1994; David *et al.* 1999) should prove useful to examine this hypothesis and is currently examined in our laboratory.

 

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