Baculovirus-infected cells do not produce the amyloid peptide of Alzheimer's disease from its precursor

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Abstract The amyloid peptide (Aβ) of Alzheimer's disease (AD) is produced by proteolytic cleavage of a larger precursor, the amyloid peptide precursor or APP. The discovery of pathogenic mutations in the APP gene provides strong evidence for the hypothesis that APP metabolism is involved in the etiology of AD. To study the metabolism of the protein, human APP has been expressed in several mammalian cell types. Insect cells, infected by a recombinant baculovirus carrying the human APP sequence, also provide an interesting expression system because these cells do not produce endogenous APP. Baculovirus-infected cells synthesize very high amounts of extracellular soluble APP, after cleavage of the transmembrane protein, as described for mammalian cells. However, we demonstrate here that insect cells do not produce Aβ from APP. These results suggest that while the enzymatic activity needed for the production of soluble APP is conserved between insect and mammalian cells, the enzymes required for the production of Aβ from APP are only expressed in mammalian cells.

Key words: Alzheimer's disease; Amyloid peptide precursor (APP); Aβ; Baculovirus

1. Introduction

Alzheimer's disease (AD) is characterized by severe dementia related to a serious loss of neurons. The pathology is typified by deposits of fibrilar amyloid peptide in the brain parenchyma and cerebrovasculature. The 39–43 amino acid amyloid peptide, also named Aβ, results from the proteolytic cleavage of a larger precursor, the amyloid peptide precursor (APP) [1]. Different gene mutations have been reported to cause familial AD [2–8]. The discovery of pathogenic mutations in the APP gene [2–6], which modify the cellular metabolism of the protein [9,10], provides strong evidence for the hypothesis that APP metabolism leading to altered Aβ production or deposition is an early event in the etiology of AD. The constitutive secretory pathway of APP delivers some of the precursor to the cell surface; proteolytic cleavage of the transmembrane protein leads to the secretion of a large soluble ectodomain of the protein and the retention of a small 10 kDa C-terminal fragment within the membrane. APP is cleaved in the secretory pathway by a proteolytic activity termed α-secretase [11]. Since the α-secretase cleaves within the Aβ peptide, this secretion process precludes the production of Aβ, and is known as the non-amyloidogenic catabolic pathway of APP. Only some of the APP molecules are actually processed by this secretory pathway. Recent reports show that soluble Aβ can be detected in vitro in the extracellular medium from a variety of cultured mammalian cells [12–14] as well as in vivo in the human cerebrospinal fluid [15]. The Aβ peptide is therefore produced by normal cellular metabolism using an amyloidogenic catabolic pathway. The two catabolic pathways of APP have been studied in several mammalian cell types, including polarized cells like Madin-Darby Canine Kidney cells [16–18] or neurons [19]. Baculovirus-infected cells also provide an interesting expression system in which a lot of human APP can be produced and transformed into an extracellular soluble protein following α-secretase cleavage [20]. Although human APP is processed in baculovirus-infected cells by the non-amyloidogenic catabolic pathway, we demonstrate herein that insect cells are not able to produce the amyloid peptide from its precursor.

2. Materials and methods

The cDNA encoding the entire human APP695, or the same sequence carrying missense mutations associated with familial Alzheimer's disease in Sweden [4,21], was cloned in the pAcCL29 vector [22]. Insect cells, *Spodoptera frugiperda* Sf21 or Sf9, at a density of 3×10^6 cells per 60 mm dish were cotransfected with 1 µg linear *Autographa californica* viral DNA (Invitrogen, San Diego, CA) and 5 µg of the recombinant plasmid, using the DOTMA transfection reagent (Boehringer Mannheim). Recombinant viruses were harvested 5 days post-transfection, purified and titered as described [23]. Sf9 cells culture and infection was subsequently carried out in IPL41 medium (Gibco) supplemented with 10% fetal calf serum. Different multiplicities of infection, from 1 to 10, were tested.

Proteins from Sf9 cells or their culture medium were analyzed by SDS-PAGE and transferred to a nitrocellulose membrane for 45 min at 5 mA/cm². The filter was blocked for 1 h with 5% non-fat dry milk in TBS (50 mM Tris-HCl, 150 mM NaCl, 0.5% Tween 20, pH 8.1) and incubated overnight at room temperature with the primary antibody. Membranes were then incubated with a biotinylated goat anti-mouse antibody followed by a streptavidin-alkaline phosphate complex. The alkaline phosphate activity was detected with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Western blotting was also performed using the ECL reagents and protocols from Amersham. In this case, proteins were blotted to polyvinylidene difluoride (PVDF) membranes. The primary antibody used to detect APP was the monoclonal 3H5 antibody at a dilution of 1/500 of the ascite fluid. This antibody is raised against the extracellular anionic domain of APP [24]. For the detection of the C-terminal domain of APP, the 'Jonas' monoclonal antibody was used following the recommendations of the manufacturer (Boehringer Mannheim). This antibody is raised against the amino acids 643–695 of APP 695. The 4G8 anti-Aβ monoclonal antibody was also used at a 1/5000 dilution of the ascite fluid [25]. This antibody is raised against the amino acids 17–24 of Aβ.

For metabolic labeling, Sf9 cells were incubated overnight in 5 ml of culture medium containing 70 µCi [^{35}S]methionine (Amersham). Cells and culture media were then harvested and used for immunoprecipitation experiments, performed in a solubilisation buffer (25 mM Tris-HCl, 0.5% Triton X100, 0.5% NP 40, pH 7.5), as previously described [13]. The soluble cellular extracts or the culture media were first incubated for 1 h in the presence of 20 µl of protein A Sepharose precoated with rabbit anti-mouse IgG. After centrifugation, superna-

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tant was recovered and incubated overnight at 4°C with the 22C11 monoclonal antibody raised against the N-terminal domain of APP [26], and 30 µl of protein A sepharose (Pharmacia) precoated with rabbit anti-mouse IgG. After centrifugation, the pellet was first washed twice with 10 mM Tris-HCI, 150 mM NaCl, 0.2% NP 40, 2 mM EDTA, pH 7.5, then twice with 10 mM Tris-HCl, 500 mM NaCl, 0.2% NP 40, 2 mM EDTA, pH 7.5, and then finally once with 10 mM Tris-HCl pH 7.5. The immunoprecipitate was resuspended in Laemmli sample buffer and analyzed in SDS-PAGE. The Aβ peptide was immunoprecipitated using the SGY2134 antibody as described by Shoji et al. [13]. This rabbit polyclonal antiserum is raised against synthetic peptide Aβ 1–40. The SGY2134 antibody recognizes both full-length APP and Aβ peptide, but poorly detects soluble APP resulting from α-secretase cleavage. The immunoprecipitates were analyzed on 10–16% Tris-Tricine SDS-PAGE. For both immunoprecipitations, the gels were dried and autoradiographed.

The internalization of APP was measured using the radiolabeled 22C11 monoclonal antibody. The 22C11 monoclonal antibody was purified by immunoadsorption on a anti-mouse IgG column, and labeled by ^{125}I iodine (Amersham), to a specific radioactivity of 150 cpm/ng of protein. Baculovirus-infected cells expressing human APP were incubated for 1 h at 0°C in their culture medium containing 10 nM [^{125}I]22C11 antibody. After 6 rapid washes with the culture medium, the cells were incubated 3 times for 5 min in acidic PBS (pH 2.5). In these experimental conditions, only part of the radiolabeled 22C11 antibody was removed from the cell surface. The reincubation of the cells at 27°C before the acidic washes allowed the internalization of APP to be measured.

3. Results

A recombinant baculovirus encoding the human APP was constructed and used to infect Sf9 cells. The time course of APP expression was determined using immunoblotting analysis of both total cellular extracts and culture medium of infected cells (Fig. 1). The APP synthesis could be observed after 24 h of infection, and was maximal after 48 h (Fig. 1). No effect of the multiplicity of infection, ranging from 1 to 10, was observed (Fig. 1). For a longer period of time after infection, a lower expression of APP was observed. This could correspond to a degradation process of APP occurring in infected cells which release proteolytic enzymes as a result of cell lysis following baculovirus infection. Thus, a 2-day infection

Fig. 2. The culture medium (A) of Sf9 cells infected by a recombinant baculovirus carrying the human APP695 cDNA sequence was analysed by immunoblotting using the 3H5 (1), the 4G8 (2), and the Jonas (3) monoclonal antibodies. The cellular extracts (B) were analysed by immunoblotting using the Jonas (1,3) or the 4G8 (2,4) antibodies. ECL detection was performed for 20 s (1,2) or 7 min (3,4).
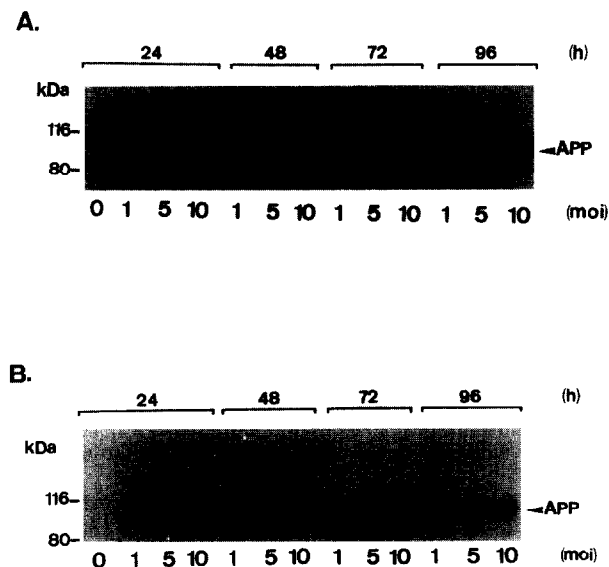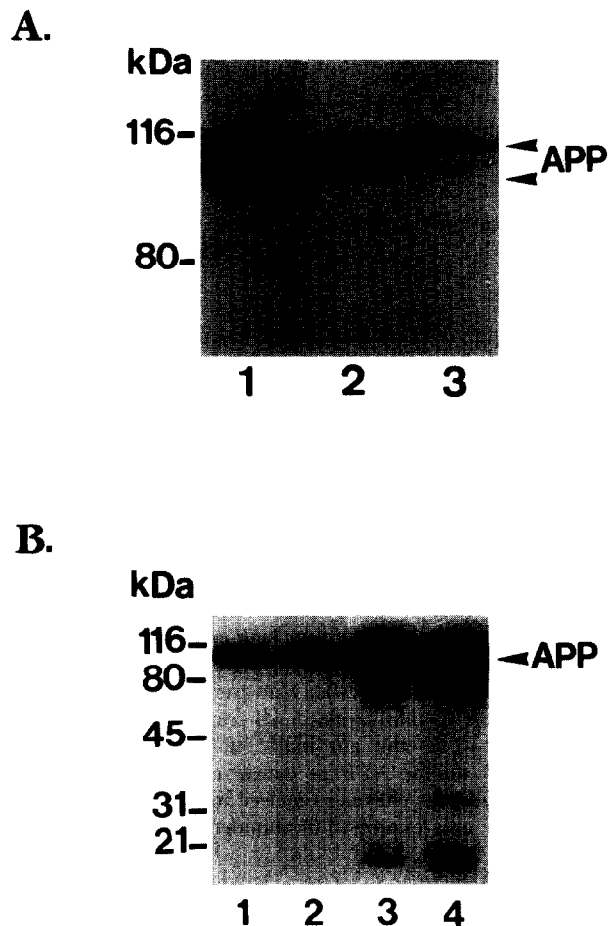
Fig. 1. Baculovirus-infected Sf9 cells (A) and their culture medium (B) were analysed by immunoblotting using the 3H5 monoclonal anti-APP antibody, different times after infection (h) at different multiplicities of infection (moi).

was performed in further experiments. In these conditions, soluble extracellular APP was produced by baculovirus-infected cells and was detected by the 3H5 monoclonal antibody (Fig. 2A, lane 1). When the same extracellular APP was analyzed by immunoblotting using the 4G8 and the Jonas antibodies, the upper band, but not the lower bands, was detected (Fig. 2A, lanes 2 and 3). This indicates that extracellular APP with the highest molecular weight contains the C-terminal domain of APP, including amino acids 17 to 24 of the Aβ peptide, and is not cleaved by α-secretase. Therefore, not all the extracellular soluble APP produced by baculovirus-infected cells results from the α-secretase cleavage.

The full-length APP sequence was also detected in the cellular extracts from baculovirus infected cells analysed in Western blotting using the Jonas and the 4G8 antibodies (Fig. 2B, lanes 1 and 2). When the ECL detection time was increased, both antibodies were able to detect C-terminal fragments of APP, as previously described [20,27]. A 15 kDa fragment (Fig. 2B, lanes 3 and 4) has been previously demonstrated to correspond to the C-terminal domain of APP resulting from the α-secretase cleavage [20]. Fragments with higher molecular weights are also detected, although in lower amounts, and are recognized by the 4G8 monoclonal antibody. These results
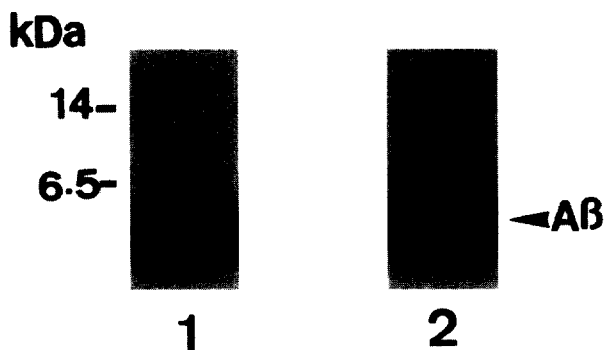
F.g. 3. Radiolabeled Aβ secreted by transfected CHO cells expressing the human APP695 was incubated for 16 h at 37°C in the culture medium conditioned by CHO cells (1) or baculovirus-infected ce ls (2), before immunoprecipitation with the SGY2134 anti-Aβ ai tibody.

c. nfirm that baculovirus-infected cells are able to produce C-terminal fragments of APP which are recognized by an anti-A β antibody.

Since baculovirus-infected cells produce a lot of extracellular human APP as well as amyloidogenic C-terminal fragments, the production of soluble extracellular Aβ peptide w is also investigated. After metabolic labeling of insect cells 24 h after infection and immunoprecipitation with the SGY2134 antibody, no Aβ peptide was detected in the culture medium (not shown). Because the replication of the baculovirus induces cell lysis, with a concomitant release of proteolytic activities in the extracellular medium, the absence of extracellular Aβ peptide could result from the extracellular degradation of the amyloid peptide. Such a degradation of A β peptide produced in vitro by proteases released by cultured cells has been previously described [28]. To test this hypothesis, radiolabelled Aβ peptide secreted by transfected CHO cells was incubated for 16 h at 37°C in the culture medium conditioned by either CHO cells or baculovirus-infected cells. After this incubation, the Aβ peptide was immunoprecipitated using the SGY2134 antibody. The results presented in Fig. 3 indicate that Aβ peptide was recovered from the culture medium of both CHO cells and baculovirus-infected cells. Therefore, the absence of Aβ in the culture medium of baculovirus-infected cells does not result from the degradation of the amyloid peptide by insect cells.

It has been previously demonstrated that the deletion of the C terminal intracellular region of APP very significantly inhibits APP internalization and precludes Aβ production in cultured cells [21,29,30]. The absence of Aβ in the culture medium of baculovirus-infected cells could therefore result from an alteration of the internalization process related to baculovirus infection. To test this hypothesis, we have measured the endocytic process in Sf9 cells, 24 h after baculovirus infection. The baculovirus itself was used as a marker of receptor-mediated endocytosis, since it is well established that a baculovirus must be taken up by receptor-mediated endocytosis in order to infect a host cell [31]. A wild-type baculovirus was therefore used to infect Sf9 cells at a multiplicity of infection of 50, which allows infection of 100% of the cells. 24 h later, the recombinant baculovirus carrying the human APP cDNA sequence was added to Sf9 cells previously infected by the wild-type virus, and the synthesis of APP was analyzed by immunoblotting analysis of the secreted proteins. The results

presented in Fig. 4 indicate that the second infection of Sf9 cells by the recombinant baculovirus leads to APP synthesis. The amount of APP recovered in the extracellular medium was actually very similar to that found when the two viruses were used in a co-infection experiment. APP internalization was more directly demonstrated by internalization of the 22C11 anti-APP monoclonal antibody. 24 h after infection, baculovirus-infected cells expressing APP were incubated for 1 h at 0°C in the presence of 10 nM of the [^{125}I]22C11 anti-APP monoclonal antibody. Following six washes with culture medium, the cells were dissolved in sodium deoxycholate, counted, and the protein content was measured using Bradford's method. The non-specific binding of the 22C11 antibody, measured on Sf9 cells infected by the wild-type baculovirus, was subtracted. The specific binding of [^{125}I]22C11 antibody corresponded to 3367 ± 401 cpm/mg protein ($n = 2$). After 1 h incubation at 0°C, $42.6 \pm 3.5\%$ ($n = 2$) of the radioactivity was resistant to acidic washes, suggesting that internalization can occur in insect cells at 0°C. When baculovirus-infected cells expressing human APP were rewarmed for 1 h at 27°C, $68.9 \pm 5.6\%$ ($n = 2$) of the radioactivity became resistant to acidic washes, indicating a significant increase ($P = 0.0298$) of APP internalization. Therefore, the absence of Aβ production by Sf9 cells expressing APP does not result from the absence of internalization of cell surface APP.

It has been recently demonstrated that the production of Aβ from APP carrying a double mutation associated with familial Alzheimer's disease in Sweden is independent of the internalization of APP [21,32]. These results indicate that the cellular compartments and mechanisms involved in the production of Aβ from Swedish APP are different from those involved in the production of Aβ from the wild-type sequence. In order to study whether baculovirus-infected cells are able to produce Aβ from Swedish APP, a recombinant baculovirus carrying the double mutation associated with familial Alzheimer's disease in Sweden was used to infect Sf9 cells. After metabolic labeling, the SGY2134 antibody was able to immunoprecipitate full-length APP following expression of both wild-type (Fig. 5 lane 2) and mutated (Fig. 5 lane 3) APP sequences. However, the anti-Aβ antibody was unable to immunoprecipitate the amyloid peptide from the culture medium of baculovirus-infected cells. The same SGY2134 serum was able to demonstrate the production of Aβ by transfected
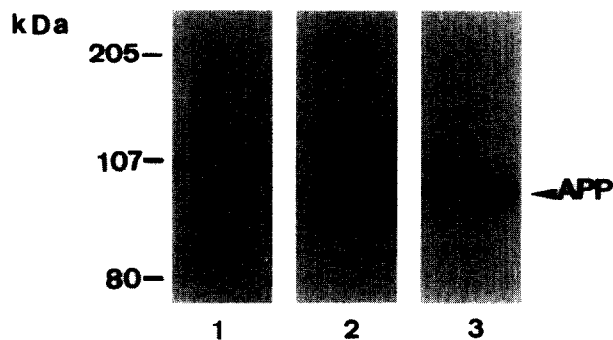

Fig. 4. The culture medium of Sf9 cells infected by a wild-type baculovirus (1), co-infected by both a wild-type and the recombinant baculovirus carrying the human APP695 cDNA sequence (2), or infected by the same recombinant virus 24 h after a first infection by the wild-type virus (3) was analysed by immunoblotting using the 3H5 monoclonal antibody.
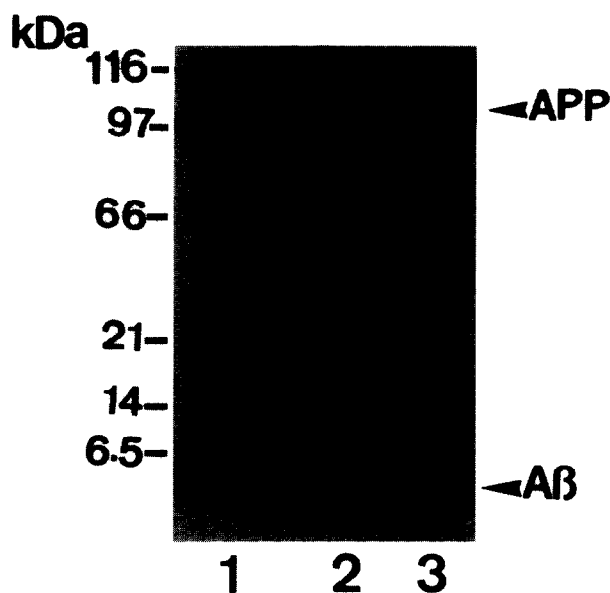
Fig. 5. The culture medium of CHO cells expressing human APP695 (1) or Sf9 cells infected by a recombinant baculovirus carrying the wild-type human APP695 cDNA sequence (2) or the same sequence with a double mutation associated with familial Alzheimer's disease in Sweden (3) was immunoprecipitated after metabolic labeling, using the SGY2134 anti-Aβ antibody.

CHO cells which do not secrete the full-length APP (Fig. 5 lane 1).

4. Discussion

Insect cells infected by a recombinant baculovirus carrying the APP cDNA sequence synthesize human APP found both in cells and in their culture medium, as previously described [20,27,33–36]. Part of the extracellular soluble APP results from the cleavage of the transmembrane protein by α-secretase [20,27]. The amino acid sequence analysis of extracellular APP has demonstrated that, in both insect and mammalian cells, the cleavage of the precursor occurs at the same position [20]. In addition to a truncated APP obtained by α-secretase cleavage, APP containing the C-terminal domain of APP, including amino acids 17 to 24 of the amyloid peptide, is also detected in the extracellular medium of baculovirus-infected cells. The presence of the full-length APP in the extracellular medium could result from cell lysis induced by infection.

In the cellular extract of baculovirus-infected cells producing human APP, several C-terminal fragments of APP have been previously described [27]. In addition to a 15 kDa fragment corresponding to the C-terminal domain of APP resulting from α-secretase cleavage, fragments are labeled by anti-Aβ antibodies and are long enough to contain the full-length sequence of Aβ. These fragments are therefore amyloidogenic.

Although the baculovirus expression system produces APP and amyloidogenic fragments thereof, the Aβ peptide can not be detected in either infected cells (not shown) or their culture medium. The absence of Aβ in the culture medium of baculovirus-infected cells does not result from degradation of the peptide, since the amyloid peptide produced by CHO cells was not degraded by a further incubation at 37°C in the presence of medium conditioned by baculovirus-infected cells. During the late phase of Sf9 cell infection by a baculovirus, extensive

viral DNA replication and baculovirus production occur. Cellular modifications observed during infection [31] could alter the internalization process. A decrease of internalization during the late phase of infection could explain the absence of Aβ production by Sf9 cells, since production of Aβ from human APP requires endocytosis [21,29,30]. However, we demonstrate here that 24 h after a first baculovirus infection, insect cells are still able to be infected by a second virus. In addition, internalization of APP is demonstrated by the use of a radiolabeled anti-APP antibody. A significant internalization is observed even at 0°C, suggesting that, in insect cells, the internalization process is not completely inhibited at this temperature. In the same way, a surface labeling by fluorescent neurotensin of Sf9 cells expressing the neurotensin receptor was observed only if the cells were incubated at −5°C [37]. Finally, baculovirus-infected cells are not able to produce Aβ from Swedish APP, although this production has been demonstrated to be independent of the internalization process [21,32].

All together, these results demonstrate that baculovirus-infected cells produce high amounts of soluble APP, part of it being cleaved by α-secretase. Although these results could indicate that α-secretase is conserved between insects and mammals, they probably confirm that α-secretase is highly non-specific. On the contrary, the N-terminal cleavage generating the Aβ peptide appears to be mediated by a sequence-dependent protease [38]. Our results demonstrate that while baculovirus-infected cells are able to produce amyloidogenic C-terminal fragments of APP, they are not able to process these fragments into amyloid peptide. Therefore, insect cells could represent an interesting cellular model for the further identification of enzymatic activities involved in the generation of Aβ from its precursor.

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