Two-dimensional gel mapping of the processing of the human amyloid precursor protein in rat hippocampal neurons

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Abstract The proteolytic fragments derived from the amyloid precursor protein (APP) in primary cultures of rat hippocampal neurons were analyzed by two-dimensional gel electrophoresis. The Semliki Forest Virus expression vector was used to express human APP695 and a mutant form associated with familial Alzheimer's disease (APP-FAD670/671). Hippocampal neurons expressing wtAPP695 or APP-FAD670/671 secrete at least six APP fragments of 100–110 kDa with isoelectric focusing points ranging from 4.5 to 4.0. The heterogeneity of the secreted APP forms is shown to be in part due to differences in glycosylation. In contrast to wtAPP695, neurons producing the APP-FAD670/671 variant did not secrete detectable amounts of secretory APP derived from cleavage within the amyloid βA4 domain. This result suggests that there is little α-secretase cleavage in neurons expressing the APP-FAD670/671 mutant.

Key words: APP; Secretion; βA4 amyloid; Hippocampal neuron; Alzheimer's disease (familial)

1. Introduction

The amyloid precursor protein (APP), a widely expressed membrane-spanning glycoprotein, is the precursor of a series of soluble proteins and peptides, including the βA4 amyloid peptide [1]. Several lines of evidence suggest that this βA4 peptide is involved in the pathogenesis of Alzheimer disease (AD). First, the βA4 peptide is the principal component of extracellular amyloid depositions in the form of plaques and vascular amyloid in the brains patients with AD [2,3]. Second, several missense mutations which occur within or immediately bordering to the βA4 region of the APP gene are linked to early forms of AD [4]. Third, transgenic mice overexpressing human APP/FAD 717 show the AD associated neuropathology [5].

APP is proteolytically cleaved by a putative α-secretase within the region spanned by the βA4 peptide to generate a large soluble ectodomain which is secreted [6,7]. This cleavage occurs with mature N and O glycosylated APP, probably during transport from the Golgi complex to the cell surface [8,9] and at the plasma membrane [10,11]. Alternatively, APP can be proteolytically processed within endosomes and lysosomes after internalisation of the holo-protein from the cell surface to produce the βA4 peptide [10,12].

Although pathological manifestations of AD such as plaque and vascular amyloid formation, neurofibrillary tangles and neuronal dysfunction occur in the central nervous system, APP metabolism has mainly been studied in non-neuronal cells. The available information of APP expression and metabolism in neuronal cells, however, suggest that differences exist. First, neurons express high amounts of an APP isoform (APP695), while longer APPs (751 and 770) containing a Kunitz Protease Inhibitor (KPI) insert and those lacking exon 15 (L-APPs) are more abundant in peripheral tissue [13,14]. Second, neurons have an intrinsic tendency to catabolise APP in an amyloidogenic fashion, because high amounts of βA4 and βA4 containing carboxyl-terminal remnants are produced [15]. Third, APP follows a complicated intracellular pathway in polarized neurons being initially routed to the axon followed by transport to the dendrites most likely by a mechanism similar to transcytosis in epithelial cells [16,17].

In this study, we have expressed APP in primary cultures of rat hippocampal neurons. Hippocampal neurons are among the most vulnerable nerve cells in AD. Cultured hippocampal neurons have been studied extensively and display the characteristics of hippocampal neurons in situ [18]. They undergo a sequence of differentiation steps that lead to maturation into fully polarized neurons with axon and dendritis forming stable synaptic connectivities on the culture dish [19]. The difficulty to obtain sufficient amount of material for biochemical analysis was circumvented by using the Semliki Forest Virus (SFV) expression system [20]. The neurons were infected with recombinant SFV containing human APP695 or APP/FAD670/671 and the proteolytic fragments were analyzed by two-dimensional gel electrophoresis.

2. Materials and methods

2.1. Cell culture

Hippocampal neuron cultures were prepared as described by Goslin and Banker [21]. Briefly, the hippocampi of 18-day-old rat embryos were dissected, trypsinized, and further dissociated by repeated passages through a constricted Pasteur pipette. The cells were plated on poly-lysine coated plastic dishes (at a density of 4×10^5/6 cm dish) and kept in minimum essential medium (MEM) supplemented with 10% horse-serum. After attachment, cells were maintained in 5.0% CO^2 at 36.5°C in MEM with N^2 supplement ('maintenance medium'). For all experiments, cells were cultured for 8–12 days in vitro before use.

2.2. DNA constructs

A Sma I site was used to isolate the complete coding region of the cDNA encoding the human APP695 and subcloned into pSP65. The cDNA coding for human APP695 was cloned as a *Hin*dIII fragment in the expression vector pSG5 (Stratagene). The APP K670N/M671L mutations were generated by site-directed mutagenesis in the pSG5 plasmid. The construct was analyzed by restriction analysis and by sequencing of the mutated sites. A Spe I site in the non-coding region was removed by using Klenow fragment. APP695 and APP K670N/M671L were subcloned into the Sma I site of pSFV1.

2.3. Preparation of recombinant SFV

pSFV1-APP695, pSFV-APP-FAD670/671 and pSFV1-Helper1 were linerarized with Spe I, and run-off transcription was performed with

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SP6 RNA polymerase. Each APP transcription mix was cotransfected with the Helper-transcription mix to BHK cells using electroporation as described by Olkkonen et al. [22]. The culture supernatant was collected after 20–24 h incubation (5% CO_2, 37°C). Titration of the viral stocks were performed as described by Olkkonen et al. [22].

2.4. Infection of neurons, metabolic labelling, enzymatic deglycosylation and immunoprecipitation

Recombinant SFV diluted in maintenance medium was allowed to adsorb to the cells for 1 h at 36.5°C in 5% CO_2. The virus solution was replaced by maintenance medium and the infection continued for 2 to 3 h. The medium was exchanged by methionine free N^2 medium and metabolic labelling performed for 3 h with 200–300 μCi/ml [^{35}S] methionine. After metabolic labelling extracellular medium was harvested and cells extracts prepared in 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS with 10 μg/ml chymostatin, leupeptin, antipain and pepstatin.

Immunoprecipitations were performed with 3 μl of goat antiserum R217 against soluble APP [23], 10 μl of anti-Fd APP [6], 10 μl of Ab692 prepared against synthetic βA4 peptide, or 20 μl of B13 prepared against synthetic βA4 (1–16). Immune complexes were recovered with protein A-Sepharose (Pharmacia Biotech Inc.) or protein G-agarose (Immunopure, Pierce). Processing of immune complexes was as described previously [15]. For digestion with neuraminidase (Boehringer Mannheim), immunoprecipitates were made 1% SDS and 1% β-mercaptoethanol, boiled for 5 min and diluted 10-fold to final concentrations of 50 mM of sodium acetate and 4 mM calcium chloride (pH 5.5), 1% NP-40, 0.1% SDS, 10 μg/ml chymostatin, leupeptin, antipain, pepstatin and 2 U/ml neuraminidase. After incubation at 37°C for 16 h, samples were analyzed by two-dimensional gel electrophoresis.

2.5. Two-dimensional gel electrophoresis

A combination of isoelectric focusing (IEF) and SDS-PAGE was used to resolve proteins in two dimensions essentially as described previously [24]. For IEF samples were solubilized in 9.8 M urea, 4% (w/v) NP-40, 2% (v/v) ampholines, pH 7–9 (Pharmacia LKB, Bromma, Sweden), and 100 mM DTT. Two volumes of Ampholine pH 3.5–5.0 (Sigma) and one volume of Ampholine pH 3.5–10.0 were (Pharmacia LKB) used for the first dimension. IEF gels were run at 500 V for 10 min and 750 V for 3.5 h. For the second dimension 7.5%, 10% and 20% SDS-PAGE were used.

3. Results and discussion

The SFV expression system has several features which make it a suitable expression system for studying APP metabolism in primary neuronal cultures. The SFV vector infects neurons efficiently and expresses the heterologous proteins within a few hours. The virus shuts off host cell protein synthesis and produces APP in relatively high amounts without disturbing the polarized organization and viability of the cells during the early phase of infection [15,16,22].

A dish of 4×10^5 neurons was infected by recombinant SFV carrying human APP695 and metabolic labelling was performed during 3 h. Cell homogenate was immunoprecipitated by anti-Fd APP and analyzed by two-dimensional gel electrophoresis. As shown in Fig. 1A the cellular APP forms migrated at the same apparent molecular weight of 100 kDa and at pIs ranging from 4.2 to 4.8 (theoretical Ip 4.6). The wide range of APPs isoelectric points is most likely due to different post-translational modifications.

To analyze the secreted forms of APP, culture medium from hippocampal neurons was immmunoprecipitated with either anti-Fd APP or R217. Both antibodies revealed a similar pattern on two-dimensional gel electrophoresis (Fig. 1C). Secreted APP695 appeared at least 6 separate spots (indicated from a–f, see Fig. 2B) with molecular weights between 100 and 110 kDa and isoelectric points ranging from 4.0 to 4.5 (theoretical Ip 4.5).

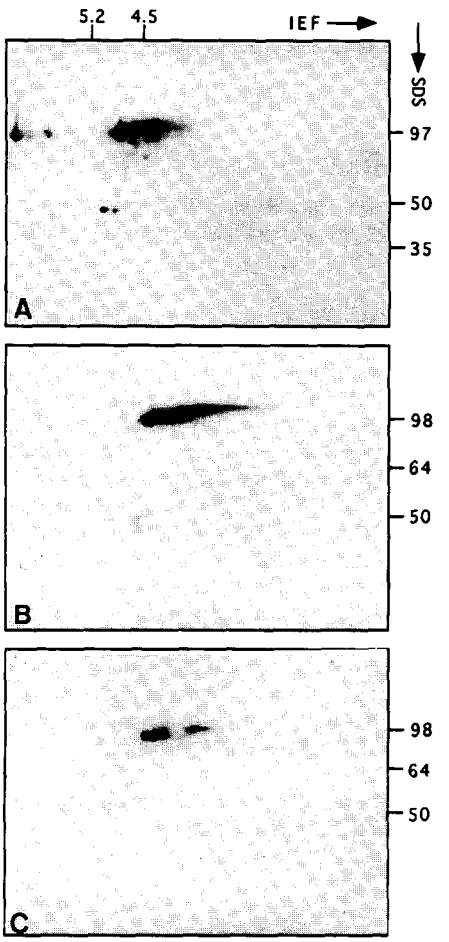

Fig. 1. Two-dimensional gel mapping of APP695 from cell-homogenate and culture medium of rat hippocampal neurons. Two-dimensional images resulted from APP immunoprecipitated from cell-homogenate (A) and from culture medium (C) of SFV/APP695 infected hippocampal neurons. Alternatively, culture medium was TCA precipitated and directly applied on two-dimensional gels (B).

Because the SFV vector efficiently produces APP, it is the main protein being secreted under these conditions. Culture medium can therefore directly be processed for two-dimensional gel electrophoresis without being immunoprecipitated as demonstrated in Fig. 1B.

To analyze the heterogeneity of secreted APP695, immunoprecipitated culture media was digested with carbohydrate cleaving enzymes. Treatment with neuraminidase resulted in the disappearance of the three most acidic spots (spots c, e and f) (Fig. 2C,D). The result demonstrates that secretory APP695 heterogeneity is partially due to different amounts of sialyl-residues in APP. To investigate if different proteolytical cleavages could be responsible for the three remaining spots on two-dimensional electrophoresis, culture media was immunoprecipitated with an antibody (B13) raised against synthetic βA4 1–16 peptide, and thus specific for α-cleaved fragments. B13 immunoprecipitates were compared to anti-Fd APP immunoprecipitates, which precipitate all secreted forms. As shown in Fig. 3A,B, no major difference were observed, indicating that all spots contained the βA4 1–16 peptide carboxyl-terminally.

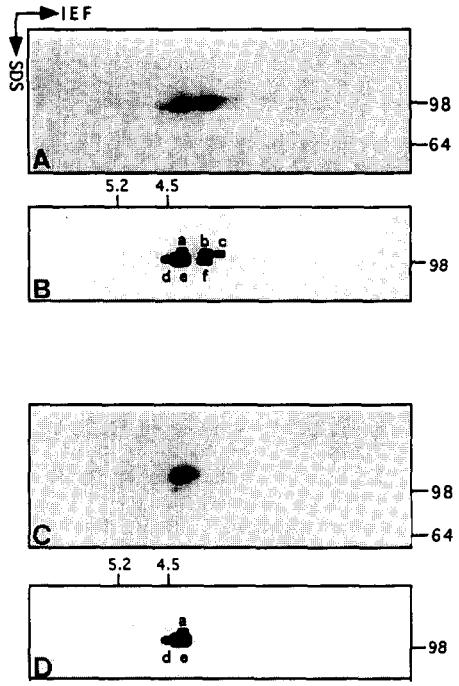

Fig. 2. Characterization of secreted APP. APP695 was immunoprecipitated from culture medium of rat hippocampal neurons infected with SFV/APP695 and enzymatically digested with neuraminidase (C). The control sample is shown in (A). Experiment is schematically summarized in (B) and (D), respectively.

Finally, we completed the two-dimensional gel mapping of secretory fragments derived from APP695 by analyzing the low molecular weight peptides in the culture medium of neurons. We have previously shown, that hippocampal neurons expressing human APP695, mainly secrete βA4 among the low molecular weight peptides [15]. Two dishes of 4×10^5 neurons were infected with SFV/APP695 and metabolically labelled during 8 h. Culture medium was pooled and immunoprecipitated with Ab692. The resulting two-dimensional gel pattern demonstrates that βA4 is efficiently produced (Fig. 4).

We extended the mapping of secretory APP to a clinical mutant associated with AD. The double mutation 670/671 was introduced into human APP695, cloned into the SFV vector and high titer viral stocks were prepared.

Secreted APP was analyzed by immunoprecipitation with anti-Fd APP and Ab B13. Anti-Fd APP revealed a similar pattern as already observed for wtAPP695 (Fig. 3A,C). While B13 clearly precipitates secreted APP from culture media of SFV/wtAPP695 infected neurons (Fig. 3B), no secreted APP could be detected from SFV/APP-FAD670/671 infected neurons. This result indicates that the APP carrying the 670/671 double mutation does not secrete any detectable α-secreted fragments in hippocampal neurons, but secrete instead a truncated APP lacking the βA4 1–16 region.

Two-dimensional PAGE is a convenient method to study APP metabolism in neurons. It is well suited for dealing with the complexity of APP derived proteolytic fragments. Due to efficient protein expression by the SFV vector, culture medium can directly be analyzed on two-dimensional gels without the use of any antibodies. Analysis of secreted APP reveals that neuronal APP is heterogeneous. This can not be appreciated on conventional SDS-PAGE. Secretory neuronal APP is resolved into at least 6 spots with a molecular weight between 100 and 110 kDa and an isoelectricfocusing point ranging from 4.0 to 4.5 on two-dimensional gel electrophoresis. The wide isoelectricfocusing range is mainly due to different amounts of sialyl-residues in secreted APP.

The characterized APP spots can now be used as a reference map to identify APP fragments in the context of neuronal cells, tissues, or subcellular fractions of patients and controls.

The most dramatic result of this study was the comparison of secreted APP from SFV infected hippocampal neurons expressing wild-type or FAD 670/671 APP. Secreted APP containing βA4 1–16, and thus most likely a result of α-cleavage, was readily detected in neurons expressing wtAPP695, but was virtually absent from neuronal cultures expressing APP-FAD670/671. This result suggests that hippocampal neurons containing APP-FAD670/671 do not secrete any appreciable amounts of α-cleaved APP, but secrete mainly a shorter APP form. We do not know why this mutant escapes α-cleavage in neurons. One possible explanation would be that proteolytic cleavage responsible for secretion of APP in case of the FAD-

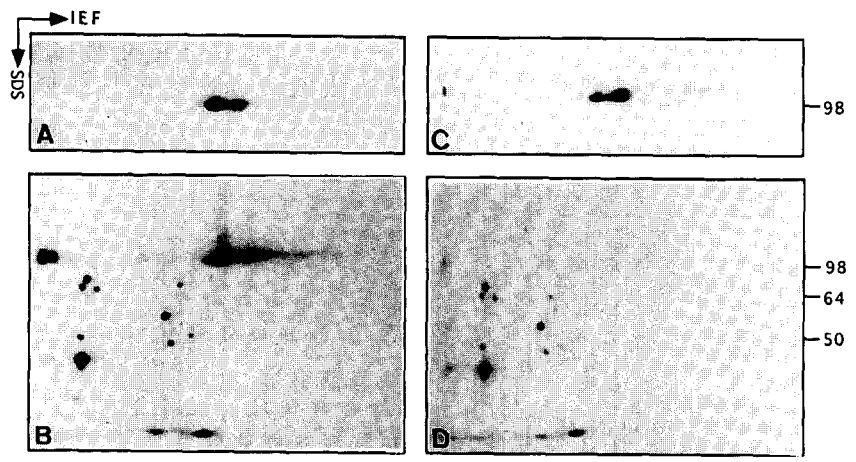

Fig. 3. Comparison of secreted APP from hippocampal neurons expressing APP695 (A,B) or APP-FAD670/671 (C,D). Culture medium was immunoprecipitated with anti-Fd APP (A,C) or B13 raised against synthetic βA4 1–16 (B,D). While B13 clearly immunoprecipitates APP from culture medium of cells infected with SFV/APP695, it does not recognize any APP in culture medium of cells infected with SFV/APP-FAD670/671.

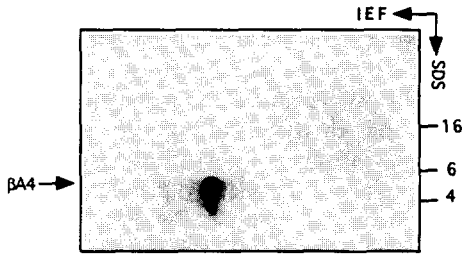

Fig. 4. βA4 secretion of hippocampal neurons infected with SFV/APP695. Culture medium was immunoprecipitated with Ab692 and separated with two-dimensional gel electrophoresis.

670/671 mutant occurs before α-secretase cleavage in the biosynthetic pathway. An altered intracellular routing of the APP mutant leading to transport to different processing compartments must also be considered. The double mutation 670/671 could also directly inhibit α-cleavage and thus favour β-cleavage. However, this is unlikely because the mutations are located proximally to the β-cleavage site [25].

Several cell-lines have been transfected with constructs containing the double mutation 670/671 and increased amounts of βA4 have been detected [26,27]. The increase of βA4 production has so far not been associated with changes in APP secretion. This study now demonstrates that increased amounts of βA4 production is accompanied by the secretion of APP forms not containing the carboxyl-terminal region resulting from α-secretase cleavage. This suggests that in case of FAD670/671, βA4 production starts before the mutant protein has reached the cell surface and is not simply a degradative process of full-length APP in endosomes/lysosomes.

Neurons are not only the cells mostly affected during the pathogenesis of AD, but also a prominent source of βA4, therefore it will be important in the future to study mechanism of βA4 production from mutant APP and wtAPP in these cells. The experimental strategies used in this study will be useful for pursuing these goals.

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References

[1] Selkoe, D.J. (1994) Annu. Rev. Neurosci. 17, 489–517.
[2] Glenner, C.G. and Wong, C.W. (1984) Biochem. Biophys. Res. Commun. 120, 885–890.
[3] Masters, C.L., Multhaupt, G., Simms, G., Pottgiesser, J., Martins, R.N. and Beyreuther, K. (1985) Proc. Natl. Acad. Sci. USA 82, 4245–4249.
[4] Mullan, M. and Crawford, F. (1993) Trends Neurosci. 16, 398–403.
[5] Games, D., Adams, D., Allessandrini, R. et al. (1995) Nature 373, 523–527.
[6] Weidemann, A., Koenig, G., Bunke, D., Fischer, P., Salbaum, J.M., Masters, C.L. and Beyreuther, K. (1989) Cell 57, 115–126.
[7] Esch, F.S., Keim, P.S., Beattie, E.C., Blacher, R.W., Culwell, A.R., Oltersdorf, T., McClure, D. and Ward, P.J. (1990) Science 248, 1122–1124.
[8] De Strooper, B., Umans, L., Van Leuven, F. and Van den Berghe, H. (1993) J. Cell Biol. 121, 295–304.
[9] Sambamurti, K., Shioi, J., Anderson, J.P., Pappolla, M.A. and Robakis, N.K. (1992) J. Neurosci. Res. 33, 319–329.
[10] Haass, C., Koo, E.H., Mellon, A., Hung, A.Y. and Selkoe, D.J. (1992) Nature 357, 500–503.
[11] Sisodia, S.S., Koo, E.H., Hoffmann, P.N., Perry, G. and Price, D.L. (1993) J. Neurosci. 13, 3136–3142.
[12] Golde, T.E., Estus, S., Younkin, L.H., Selkoe, D.J. and Younkin, S.G. (1992) Science 255, 728–730.
[13] Sandbrink, R., Masters, C.L. and Beyreuther, K. (1994) J. Biol. Chem. 269, 1510–1517.
[14] Sisodia, S.S. (1992) Proc. Natl. Acad. Sci. USA 89, 6075–6079.
[15] Simons, M., De Strooper, B., Multhaup, G., Tienari, P.J., Dotti, C.G. and Beyreuther, K. (submitted).
[16] Simons, M., Ikonen, E., Tienari, P.J., Cid-Arregui, A., Moenning, U., Beyreuther, K. and Dotti, C.G. (1995) J. Neurosci. Res. 41, 121–128.
[17] Yamazaki, T., Selkoe, D.J. and Koo, E.H. (1995) J. Cell Biol. 129, 431–443.
[18] Craig, A.M. and Banker, G.A. (1994) Annu. Rev. Neurosci. 17, 267–310.
[19] Dotti, C.G., Sullivan, C.A. and Banker, G.A. (1988) J. Neurosci. 8, 1454–1468.
[20] Liljestroem, P. and Garoff, H. (1991) Bio/Technology 9, 1356–1361.
[21] Goslin, K. and Banker, G. (1991) in: Culturing Nerve Cells (Banker, G., Goslin, K., Eds.) pp. 251–281, MIT Press, Cambridge.
[22] Olkkonen, V.M., Liljestroem, P., Garoff, H., Simons, K. and Dotti, C.G. (1993) J. Neurosci. Res. 35, 445–451.
[23] Lowery, D.E., Pasternsck, J.M., Gonzales-De Whitt, P.A. et al. (1991) J. Biol. Chem. 266, 19842–19850.
[24] Celis, J.E., Gesser, B., Rasmussen, H.H. et al. (1990) Electrophoresis 11, 989–1071.
[25] Mullan, M., Crawford, F., Axelman, R., Houlden, H., Lilius, L., Winblad, B. and Lannfelt, L. (1992) Nature Genet. 1, 345–347.
[26] Citron, M., Oltersdorf, T., Haass, C. et al. (1992) Nature 360, 672–674.
[27] Cai, X.D., Golde, T.E. and Younkin, G.S. (1993) Science 259, 514–516.