

Amyloid precursor protein secretion and β A4 amyloid generation are not mutually exclusive

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Abstract

The cellular factors regulating the generation of β A4 from the amyloid precursor protein (APP) are unknown. Protein phosphorylation by protein kinase C (PKC) has been found to influence the processing and metabolism of APP. In this report, we show that in the human neuroblastoma cell line SY5Y, β A4 generation from full-length APP is not changed by PKC activation whereas production of the non-amyloidogenic secretory fragment (APPsec) and of the C-terminal fragment of β A4 (p3) are stimulated. In addition, β A4 generation from the membrane inserted C-terminal 100 residues (SPA4CT) of APP is stimulated by PKC activation. Accordingly attempts to divert APP processing from the amyloidogenic, β A4-generating, to the non-amyloidogenic, secretory, pathway, have to address the nature and regulation of the two pathways and/or of the process leading to the cleavage of APP at the C-terminus of the β A4 domain. The data reported here suggest that these mechanisms are cell-type specific.

Key words: APP; A4CT; β A4; Processing; SY5Y; Alzheimer's disease

1. Introduction

The β -amyloid protein (β A4) is a cleavage product of the amyloid precursor protein (APP) and accumulates at high levels in the brains of Alzheimer's disease (AD) patients. While a large proportion of normal secretory processing of APP involves cleavage within the β -amyloid domain and thus precludes β A4 generation [1,2,3], recent reports have shown that β -amyloid related peptides are also normally secreted by cultured cells and can be detected in human cerebrospinal fluid [4,5,6,7]. In addition to the 4-kDa β -amyloid peptide (β A4) a 3-kDa peptide (p3) corresponding to a truncated fragment of β A4 at residue 16 by APP secretase is also produced [8]. It has been reported that activation of PKC in some non-neuronal cell culture systems increased the generation of secreted forms of APP (APPsec) and p3 but markedly decrease β A4 release from these cell lines [9,10].

To analyse further the regulatory mechanisms involved in β A4 generation, especially the relationship between the two protease activities generating the N- and C-terminus of β A4 (termed protease 1 = β -secretase and protease 2 = γ -secretase, respectively) and the β A4 cleaving activity (termed APP secretase; α -secretase), we have expressed, in addition to full-length APP695, also the C-terminal 100 residues of APP, fused to the APP signal sequence (termed SPA4CT), in the human neuroblastoma cell line SY5Y. Since β A4 release from SPA4CT-type APP fragments which carry the β A4 sequence at the N-terminus, depends only on the protease activity generating the C-terminus of β A4 (i.e. protease 2, γ -secretase),

and was shown not to be influenced by APP-secretase [11], we were able to analyse β A4 generation independent of the processes generating the N-terminus of β A4 or of APP secretion.

2. Experimental procedures

2.1. Cloning procedures

Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation and bacterial transformation were carried out as described by Sambrook et al. [12].

2.2. Plasmid construction

For construction of CEP/SPA4CT the *Sma*/HindIII fragment from SP65/SPA4CT [11] was cloned into pCEP4, digested with *Pvu*II/HindIII. The resulting plasmid CEP/SPA4CT encodes the APP signal sequence and two additional residues from APP695 in frame with the amyloid β A4 sequence and the entire C-terminal domain of the APP. CEP/APP695 was obtained by cloning the *Sma*/HindIII fragment into pCEP, digested with *Pvu*II/HindIII.

2.3. Tissue culture and transfection

The subclone neuroblastoma cell line SH-SY5Y was grown in equal parts of Minimum Essential Medium (MEM, with Earle's Salts and L-glutamine) and Ham's F-12 supplemented with non-essential amino acids (Eagle's formulation), penicillin (50 U/ml), streptomycin (40 μ g/ml) and 10% (v/v) fetal calf serum (FCS) (all Gibco/BRL). For stable transfection 5×10^5 cells were seeded onto 60-mm plates, and incubated overnight at 37°C. After reaching ca. 80% confluence, cells were washed three times with Opti-MEM I (Gibco) prior to addition of DNA/Lipofectin mixture. The DNA/Lipofectin mixture was produced by combining the DNA solution (10–20 μ g DNA in 1.5 ml Opti-MEM) with the Lipofectin solution (30–50 μ g Lipofectin (BRL, Eggenstein, Germany) in 1.5 ml Opti-MEM). After incubation for 8 h at 37°C, cells were grown for a further 24–48 h in cell-culture medium and stable lines were selected by exposure to hygromycin B (300 μ g/ml).

2.4. Pulse-chase labelling of tissue culture cells

After removal of the culture medium, the cells were treated with 1.5 ml of MEM lacking methionine for 40 min. Then 500 μ Ci of [³⁵S]methionine were added, and cells were labelled for 60 min at 37°C. Cells were then washed once with MEM (37°C) and chased in methion-

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ine-enriched medium (MEM; 0.3 mg/ml L-methionine, 37°C) for the times indicated in results. After the chase period, the medium was harvested, and the cells were washed once with MEM and scraped from the surface of the dish. For lysis, the cells were resuspended in 0.6 ml of SOL (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF) and placed on ice. After 20 min, samples were centrifuged at $10,000 \times g$ for 5 min, and the supernatants (cell lysates) were stored at -20°C for subsequent analysis.

2.5. Immunoprecipitation [13]

For immunoprecipitation the conditioned medium (of one 6-cm cell culture dish) was first adjusted to 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 2 mM PMSF and then medium and cell lysate were incubated for 30 min with 10 μl pre-immune serum and 30 μl (3 mg) protein A-Sepharose (Pharmacia). The samples were centrifuged briefly and the supernatants (1.5 ml) were incubated with antiserum (5 μl anti-APP or 20 μl anti-2-43) for 90 min at room temperature. Following the incubation, 30 μl (3 mg) protein A-Sepharose was added for an additional 90 min at room temperature. The insoluble complexes were washed three times with wash A (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA), two times with wash B (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% Triton X-100, 2 mM EDTA) and once with wash C (10 mM Tris-HCl pH 7.5) before pellets were resuspended in $2 \times$ Laemmli sample buffer. After boiling for 5 min at 100°C , labelled proteins were separated on Tris-tricine gels (16.5% or 8–20%) and fluorographed with EN³HANCE (DuPont).

3. Results

3.1. Stimulation of βA4 generation from SPA4CT in SY5Y cells

To examine the role of protein kinase C in the regulation of βA4 production by C-terminal cleavage independent of the processes generating the N-terminus of βA4 (protease 1, γ -secretase) or the C-terminus of secretory APP (APP-secretase, α -secretase), we used SY5Y cells stably transfected with the C-terminal 100 residues of APP, fused to the APP signal sequence (termed SPA4CT). Expression of this APP-fragment in SY5Y cells resulted in a 12 kDa protein associated with the cell lysate and a βA4 -like peptide which is immediately secreted into the medium as previously shown [11]. Peptide p3, which is generated from full-length APP by the activity of βA4 -protease 2 and APP-secretase, is not generated from SPA4CT because we showed that APP secretase does not cleave this C-terminal APP-fragment.

SY5Y cells transfected with SPA4CT were pulse-labelled with [^{35}S]methionine and then chased in the absence or presence of compounds which activate PKC directly or indirectly by activation of the G-protein-coupled muscarinic receptor. Treatment of the transfected cells with carbachol (1 mM) or PMA (phorbol 12-myristate 13-acetate) (1 μM) stimulates βA4 generation by 1.6- and 2-fold, respectively, compared with control cells (Fig. 1A and B). The effect of 1 mM carbachol was blocked by the muscarinic antagonist atropine (1 μM).

This carbachol-stimulated βA4 secretion was mediated by the muscarinic M_3 receptor, as shown by the effects of selective muscarinic-receptor antagonists

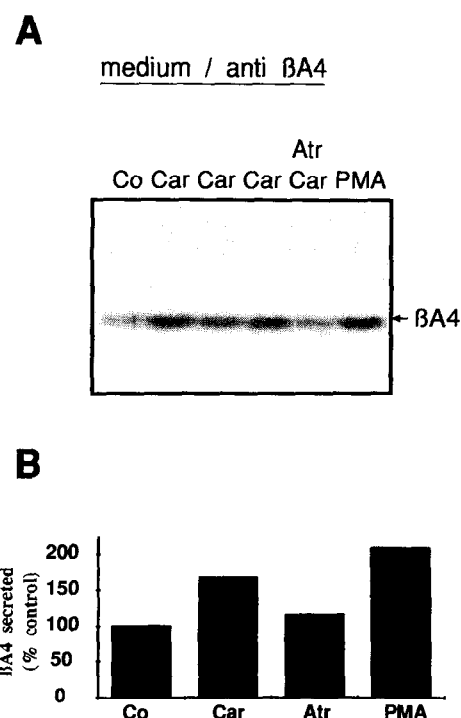


Fig. 1. Stimulation of βA4 generation from SPA4CT in SY5Y cells. SY5Y cells stably transfected with CEP/SPA4CT were labelled for 60 min with [^{35}S]methionine and chased in methionine-enriched medium for 3.5 h in the presence or absence of the compounds indicated. The conditioned medium was analysed by immunoprecipitation with anti-2-43. Standardisation of the quantity applied in each lane was done by measuring the total radioactivity incorporated into protein. Co = control; Car = 1 mM carbachol; Atr = preincubation with 1 μM atropine for 15 min; PMA = 1 μM phorbol ester. (A) Tris-tricine PAGE (16.5%). B: the autoradiography was quantified by densitometric scanning of the βA4 band of the autoradiogram (Amersham RAS System). The data represent the signal-intensity in percent of the 4 kDa signal of control cells.

(Fig. 2, lane M3). Preincubation of cells with 1 μM 4-DAMP (M_3 -receptor antagonist) abolished carbachol-induced stimulation of βA4 secretion, whereas preincubation with 1 μM Telenzepine (M_1 -receptor antagonist) had no effect (Fig. 2, lane M1).

3.2. Regulation of APP metabolism by PMA and carbachol

To confirm the role of phosphorylation in the regulation of βA4 generation also from full-length APP, the effects of the cholinergic agonist carbachol and the phorbol ester PMA were studied in SY5Y cells that had been transfected with APP695.

When these transfected cells were incubated with carbachol (1 μM) or PMA (1 μM), there was a 1.5-fold or 2.3-fold increase in the levels of secreted APP respectively, during a 60 min chase period compared with control cells (Fig. 3). Again the effect of 1 mM carbachol was blocked by the muscarinic antagonist atropine (1 μM).

To analyse further the effect of these compounds on

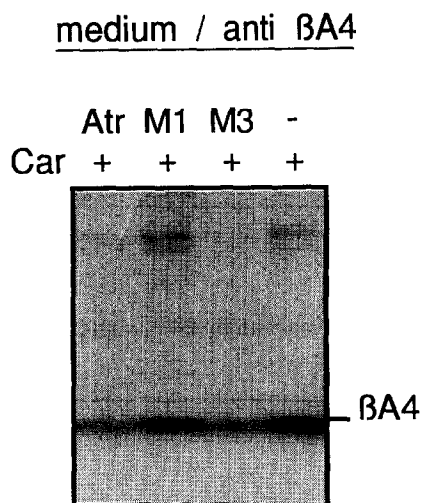


Fig. 2. Effect of muscarinic-receptor antagonists on carchol-induced β A4 secretion SY5Y cells stably transfected with CEP/SPA4CT were labelled for 30 min with [35 S]methionine in the presence or absence of 1 μ M concentrations of the muscarinic-receptor antagonists atropine (Atr), telenzepine (M1) or 4-DAMP (M3) as indicated. Thereafter 1 mM carchol (Car) was added and the cells were chased with methionine-enriched medium for 3 h. The proteins were analysed by 16% Tris-tricine-PAGE after immunoprecipitation. Standardisation of the quantity applied in each lane was done by measuring the total radioactivity incorporated into protein.

β A4 generation, the chase time was extended to 3.5 h to ensure processing and release of all labelled APP fragments. Under these conditions, analysis of conditioned medium revealed only a 1.2-fold stimulation of APP-secretion with carchol and 1.7-fold with PMA (Fig. 4A and Fig. 5). This decline of the effect on APP secretion in the 3.5 h chase experiment in comparison to the 60 min chase time period is due to the short half life time of APPsec [1].

In parallel to elevated APP levels in the media, secretion of a 3-kDa truncated β A4 species (p3), the amount of which has been shown to parallel that of APPsec released from cells [4,8], was elevated 1.6-fold by carchol and 3.4-fold by PMA (Figs. 4A and 5). In contrast to the elevated levels of APPsec and p3, the level of β A4 peptide production was unchanged by PKC activation. This is surprisingly since an increase of secretory APP as a consequence of cleavage within the β A4 domain of APP is expected to reduce the pool of β A4 precursors accordingly and thus lower the amount of β A4 molecules released from the cells.

4. Discussion

It is now well established that transmembrane APP can either undergo secretion as APPsec, be processed into β A4-like peptides or degraded in the lysosomal compartment. In this regard, protein kinase C activation by

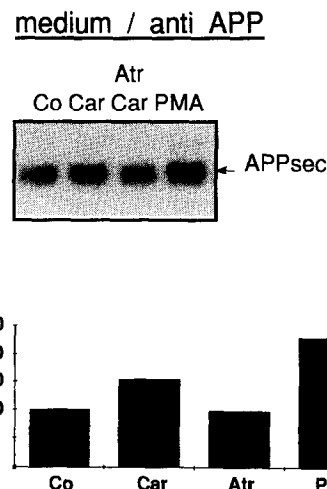


Fig. 3. Release of APPsec upon PKC stimulation SY5Y cells stably transfected with CEP/695 were labelled for 60 min with [35 S]methionine and chased in methionine-enriched medium for 60 min in the presence or absence of the compounds indicated. The conditioned medium was analysed by immunoprecipitation with anti APP. Co = control; Car = 1 mM carchol; Atr = preincubation with 1 μ M atropine; PMA = 1 μ M phorbol ester. Standardisation of the quantity applied in each lane was done by measuring the total radioactivity incorporated into protein. (Top) Tris-tricine PAGE (8–20%). (Bottom) The autoradiography was quantified by densitometric scanning of the APP band of the autoradiogram (Amersham RAS System). The data represent the signal-intensity in percent of the APP signal of control cells.

phorbol esters has been shown to stimulate secretion of APPsec and to increase the amounts of membrane-retained C-terminal fragments in PC12 pheochromocytoma cells [14,15], as well as in human embryonic kidney 293 cells [16]. In addition, cells transfected with muscarinic acetylcholine receptor subtypes m1 or m3 show increased secretion of APPsec upon addition of the cholin-

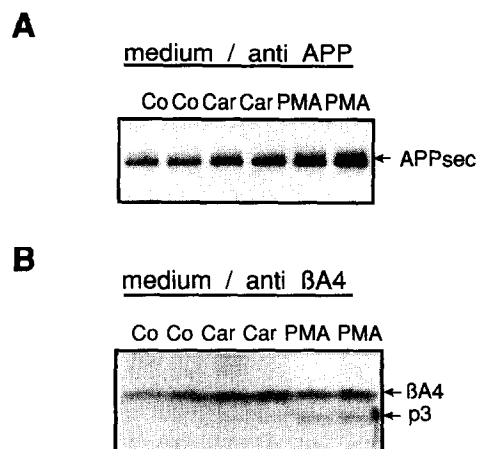


Fig. 4. Release of APPsec, p3 and β A4 upon PKC stimulation. SY5Y cells transfected with CEP/695 were labelled for 60 min with [35 S]methionine and chased in methionine-enriched medium for 3.5 h in the presence or absence of the compounds indicated. The conditioned medium was analysed by immunoprecipitation with anti-APP (A) or anti-2–43 (B) and subsequent Tris-tricine-PAGE (16.5%). Standardisation of the quantity applied in each lane was done by measuring the total radioactivity incorporated into protein.

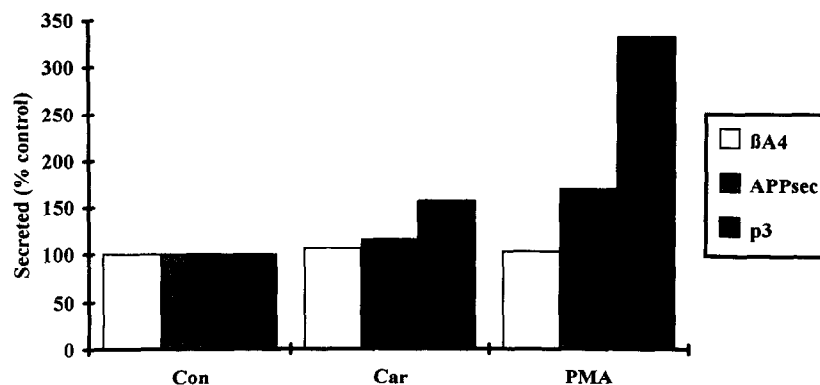


Fig. 5. Quantification of secreted APP-fragments upon PKC stimulation. APP, p3 and β A4 were quantified by cutting out the precipitated proteins from the dried gel (Fig. 4) and measurement of radioactivity in the slices in 5 ml of scintillation fluid. Each column represents the mean of two independent experiments. Co = control; Car = 1 mM carbachol; PMA = 1 μ M phorbol ester.

ergic agonist carbachol, a process which is blocked by the PKC inhibitor staurosporine [17,18]. Moreover, some groups have recently reported that activation of PKC, either directly by phorbol esters or indirectly by cholinergic agonists, results in a marked inhibition of β A4 release [9,19].

To examine further the role of protein kinase C in the regulation of β A4 production, we used human neuroblastoma cells stably transfected with both APP and the C-terminal 100 residues of APP (SPA4CT). The expression strategy allowed membrane insertion and signal peptide removal of SPA4CT and thus mimics the natural situation of this C-terminal APP fragment. Using these stable cell lines permits a more detailed analysis of the regulation of the three cleavages involved in β A4-generation and APP secretion. Analysis of cell lysates and conditioned medium of the stable cell lines showed that both SPA4CT and APP are processed into β A4-like peptides which are immediately released into the medium [11]. Using the stable SY5Y cells expressing SPA4CT we have been able to demonstrate for the first time that β A4 generation from the C-terminal 100 residues of APP, which may be an intermediate in the process of β A4 generation [20,21,22], is stimulated by PKC activation. In SY5Y cells the carbachol-stimulated β A4 secretion was mediated by the muscarinic M_3 receptor as demonstrated by inhibition with the selective M_3 -receptor antagonist 4-DAMP.

By analysing the effect of PKC stimulation on the metabolism of full-length APP in SY5Y cells we have shown that whereas activation of PKC increased the generation of APPsec and P3 it did not influence β A4 generation, an observation in contrast to other results obtained with other cell types. These experiments demonstrate for the first time that in some cellular systems, production of β A4 is not inhibited by PKC. Because in our cell system stimulation of APPsec did not lead to an inhibition of β A4 production, we conclude that in this

system these two APP processing events, APP secretion and β A4 generation, are not mutually exclusive. Our results would suggest the existence of a surplus of full-length APP which either gives rise to APPsec, β A4, or lysosomal degradation products, depending on the enzymatic activities and regulatory mechanisms present. Thus, in SY5Y cells the simultaneous activation of APP secretase (4-fold), and protease 2 (2-fold), as indicated by experiments with the APP- and SPA4CT-transfected cells, in total results in two-fold increase in APP secretion and no change in the quantity of β A4 generation. Accordingly the consequences of PKC stimulation in different tissue – of cellular systems depends on the kinds of enzymatic activities and regulatory mechanisms present. While in SY5Y cells both β A4 protease 2 and APP-secretase appear to be stimulated by PKC activation, other cell types may contain different regulatory mechanisms for these two proteolytic activities. This is in agreement with the observation that in some cell types, inhibition of β A4 production can occur without increased secretion of APPsec [10].

The conclusion of our results is that different cell types seem to exercise different fine-tuning of the APP processing machinery. Accordingly, results concerning APP regulation and β A4 generation in special cell culture systems, and therapeutic strategies which are based on these results, have to be treated with caution.

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References

- [1] Weidemann, A., König, G., Bunke, D., Fischer, P., Salbaum, J.-M., Master, C.L. and Beyreuther, K. (1989) *Cell* 57, 115–126.
- [2] Sisodia, S.S., Koo, E.H., Beyreuther, K., Unterbeck, A. and Price, D.L. (1990) *Science* 248, 492–495.

- [3] Esch, F.S., Keim, P.L., Beattie, E.C., Blacher, R.W., Culwell, A.R., Oltersdorf, T., McClure, D. and Ward, P.J. (1990) *Science* 248, 1122–1124.
- [4] Haass, C., Schlossmacher, M.G., Hung, A.Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B.L., Liebergurg, I., Koo, E.H., Schenk, D., Teplow, D.B. and Selkoe, D.J. (1992) *Nature* 359, 322–325.
- [5] Seuber, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, S., Sinha, S., Schlossmacher, M., Whaley, J., Swindlehurst, C., McCormack, R., Wolfert, R., Selkoe, D., Lieberburg, I. and Schenk, D. (1992) *Nature* 359, 325–327.
- [6] Shoji, M., Golde, T.E., Ghiso, J., Cheung, T.T., Estus, S., Shaffer, L.M., Cai, X.-D., McKay, D.M., Tintner, R., Frangione, B. and Younkin, S.G. (1992) *Science* 258, 126–129.
- [7] Busciglio, J., Gabuzda, D.H., Matsudaira, P. and Yankner, B.A. (1993) *Proc. Natl. Acad. Sci. USA* 90, 2092–2096.
- [8] Haass, C., Hung, A.Y., Schlossmacher, M.G., Teplow, D.B. and Selkoe, D.J. (1993) *J. Biol. Chem.* 268, 3021–3024.
- [9] Hung, A.Y., Haass, C., Nitsch, R.M., Qiu, W.Q., Citron, M., Wurtman, R.J., Growdon, J.H. and Selkoe, D.J. (1993) *J. Biol. Chem.* 268, 22959–22962.
- [10] Gabuzda, D., Busciglio, J., and Yankner, B.A. (1993) *J. Neurochem.* 61, 2326–2329.
- [11] Dyrks, T., Dyrks, E., Mönning, U., Urmoneit, B., Turner, J. and Beyreuther, K. (1993) *FEBS Lett.* 335, 89–93.
- [12] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [13] Anderson, D.J. and Blobel, G., *Methods Enzymol.* 96, 110–120.
- [14] Buxbaum, J.D., Gandy, S.E., Cicchetti, P., Ehrlich, M.E., Czernik, A.J., Fracasso, R.P., Ramabhadran, R.V., Unterbeck, A.J. and Greengard P. (1990) *Proc. Natl. Acad. Sci. USA* 87, 6003–6006.
- [15] Caporaso, G.L., Gandy, S.E., Buxbaum, J.D., Ramabhadran, R.V. and Greengard, P. (1992) *Proc. Natl. Acad. Sci. USA* 89, 3055–3059.
- [16] Gillespie, S.L., Golde, T.E. and Younkin, S.G. (1992) *Biochem. Biophys. Res. Commun.* 187, 1285–1290.
- [17] Buxbaum, J.D., Oishi, M., Chem, H.I., Pinkas-Kramarski, P., Jaffe, E.A., Gandy, S.E. and Greengard P. (1992) *Proc. Natl. Acad. Sci. USA* 89, 10075–10078.
- [18] Nitsch, R.M., Slack, B.E., Wurtman, R.J. and Growdon, J.H. (1992) *Science* 258, 304–307.
- [19] Buxbaum, J.D., Koo, E.H. and Greengard, P. (1993) *Proc. Natl. Acad. Sci. USA* 90, 9195–9198.
- [20] Golde, T.E., Estus, S., Younkin, L.H., Selkoe, D.J. and Younkin, S.G. (1992) *Science* 255, 728–730.
- [21] Haass, C.H., Koo, E.H., Mellon, A., Hung A.Y. and Selkoe, K.J. (1992) *Nature* 357, 500–503.
- [22] Felsenstein, K.M., Hunihan, L.W. and Roberts, S.B. (1994) *Nature Genet.* 6, 251–256.