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The Use of Seldi ProteinChip™ Arrays to Monitor Production of Alzheimer's β-Amyloid in Transfected Cells

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Abstract: β-Amyloid (Aβ), a 39-43 residue peptide, is the principal component of senile plaques found in the brains of patients with Alzheimer's disease (AD). There are two main lines of evidence that its deposition is the cause of neurodegeneration. First, mutations found in three genes in familial Alzheimer's cases give rise to increased production of the longest, most toxic, form, Aβ 1-42. Second, aggregated Aβ is toxic to neuronal cells in culture. Inhibitors of the proteases involved in its release from the amyloid precursor protein are, therefore, of major therapeutic interest. The best candidates for the releasing proteases are both aspartyl proteases, which are integrated into the membranes of the endoplasmic reticulum and Golgi network. A sensitive assay using Ciphergen's Seldi™ system has been developed to measure all the variants of Aβ in culture supernatants, which will be of great value in screening inhibitors of these proteases. With this assay, it has been shown that increasing intracellular cholesterol increases the activities of both β-secretase, and γ-secretase-42. Moreover, changing the intracellular targeting of amyloid precursor glycoprotein (APP) yields increased α-secretase cleavage, and increases in the amounts of oxidized/nitrated forms of Aβ.

Keywords: β-amyloid; secretases; ProteinChip™; mass spectrometry; cholesterol

INTRODUCTION

The Role of β-Amyloid (Aβ) in the Pathology of Alzheimer's Disease (AD)

AD is the most common neurodegenerative disease of the elderly that causes dementia. The disease is characterized by the neocortical accumulation in brain of Aβ in plaques and vessel walls, and by the intraneuronal accumulation of paired helical fragments composed of hyperphosphorylated tau. Wheras massive Aβ deposits in the neocortex is specific to AD, tau pathology occurs in a variety of diseases. Aβ is a 39–42-residue peptide derived from the processing of the amyloid precursor glycoprotein (APP), which consists of a polypeptide chain of 695 or 770 residues, depending on its mRNA transcription (Figure 1) [1]. Heavy, primarily vascular deposits, are seen in related disorders, such as cerebral haemorrhage with amyloidosis, which is associated with a mutation in Aβ at residue 22 (Figure 1) [2]. In plaques, Aβ is associated with α-antichymotrypsin, apolipoprotein E, apolipoprotein J, vitronectin and non-Aβ amyloid component. The central plaque core is a packed mass of amyloid fibrils, and is surrounded by astroglial and microglial processes, swollen neurites and reactive glia, indicating that the formation of these fibrils is associated with local inflammatory responses and neurodegeneration.

Genetic evidence indicates that Aβ plays a central role in the progression of AD. Individuals with Down's syndrome have an extra copy of chromosome 21, wherein the gene encoding APP is localized, and invariably develop AD pathology at an early age [3]. Mutations in the APP gene near the cleavage site for γ-secretase (Figure 1) [4–6], and in the two presenilin genes on chromosomes 1 and 14 [7,8] (see below), segregate with some forms of

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autosomal dominant familial AD. These mutations elevate the circulation of the longer form Aβ42, which is deposited preferentially in senile plaques (Figure 1) [9]. *In vitro*, Aβ peptides aggregate from buffer solutions to produce fibrils that resemble those found in natural plaques [10–12]. Aggregation is believed to involve the initial association of individual molecules of Aβ into parallel in-register β-strands, followed by formation of soluble oligomers of increasing size [12]. Further association in an ordered manner continues until the assembly exceeds the solubility limits and fibrils are deposited [10]. Aβ aggregates have been found to effect neuronal behaviour both *in vivo* and *in vitro*. For example, Aβ is neurotoxic when injected into rat brain [13], and induces apoptosis in neuronal cell lines [12,14]. Correlation between neurotoxicity, the rate at which soluble oligomers are formed, and fibril morphology have been described [12,15].

The Actions of Peroxynitrite in the AD Brain

The AD brain contains high levels of inflammatory markers, such as complement, MHC glycoproteins, *C*-reactive protein, chemokines and Il-1 and Il-6. Furthermore, high numbers of microglial cells are found in close proximity to the senile plaques. Microglial cells are major contributors to an oxidatively stressed environment, producing superoxide anions and nitric oxide. Aβ and several cytokines stimulate microglia and astrocytes to express inducible NOS [16]. Excess·NO is, in part, responsible for glutamate neurotoxicity [17], but most of the neurotoxic actions of·NO are mediated by peroxinitrite [18].

Peroxynitrite ($ONOO^-$) is a highly reactive molecule, produced by the reaction of NO and superoxide [19]. Activated microglia in the vicinity of senile plaques release superoxide and hydrogen peroxide [20]. The highly reactive $ONOO^-$ is difficult to detect directly. However, at sites of inflammation, a footprint molecule, 3-nitrotyrosine, is produced in proteins and peptides, which indicates the site of $ONOO^-$ generation. In the AD brain, there is evidence of widespread $ONOO^-$ mediated damage, whereas none can be found in age-matched controls. Nitrotyrosine and dityrosine have been detected in AD brains, which indicate intense peroxynitrite oxidation [21]. The production of oxidized or nitrated forms of Aβ from cultured cells has not previously been detected, even though they are likely to be formed in the AD brain.

Proteases Involved in Processing of the Amyloid Precursor Protein

Clearly, inhibition of the biosynthesis of Aβ is a therapeutic possibility for AD. APP is subjected to a number of processing steps within the sequence that codes for Aβ. To initiate Aβ formation, β-secretase cleaves APP between Met and Asp residues at its NH_2-terminus to release APPsβ, a $\sim$100 kDa

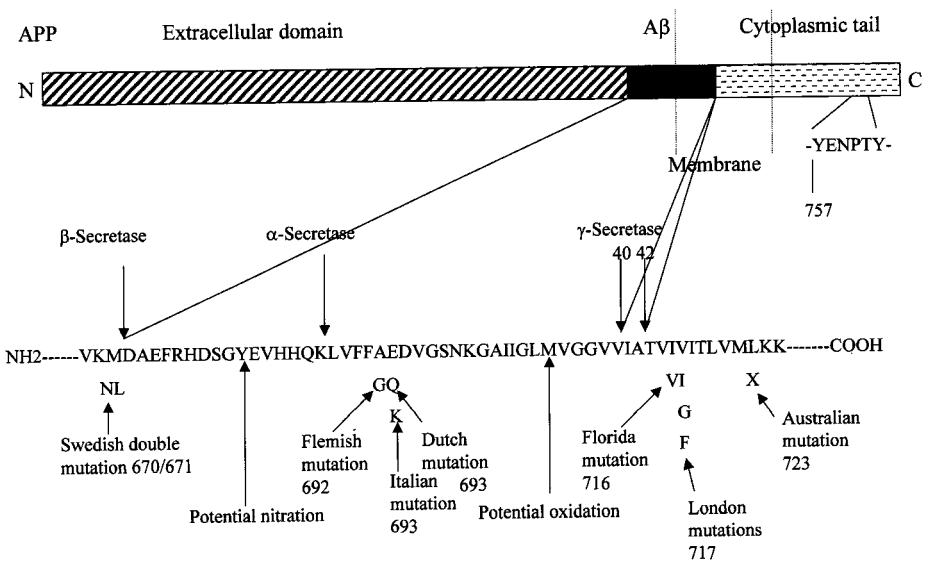

Figure 1 Sequences recognized by proteases involved in the production of Aβ. The sequence in the Aβ region of APP is shown, along with changes in the sequence that are found in some cases of familial AD. The residues cleaved by secretases are shown, along with the endocytic targeting sequence in the cytoplasmic tail of APP.

soluble NH_2-terminal fragment, and C100, a 12-kDa COOH-terminal fragment which remains membrane bound (Figure 1). Immunolocalization studies with an antibody specific for the cleaved NH_2-terminal sequence of Aβ showed that β-secretase cleavage occurs in the *trans*-Golgi network (TGN) or early endosome of human cell lines [22]. The importance of β-secretase is emphasized by the 8-fold increase of Aβ in cells transfected with APP bearing the mutations found in a Swedish family at amino acids 670 and 671 (Figure 1) [23].

An alternative metabolic pathway of APP involves cleavage by α-secretase, across the amyloid sequence, to release a large soluble NH_2-terminal fragment, APPsα, and a 10-kDa membrane-bound COOH-terminal fragment C83. Metalloproteinases, ADAM 10 and TACE are thought to be involved in the α-cleavage [24–26].

Both C100 and C83 can be cleaved further by γ-secretases at the C-terminal sequence of Aβ to release either Aβ 1-40 or Aβ 1-42, and C-terminal fragments. These are unusual cleavages, occuring within a portion of APP which is buried in the phospholipid bilayer of the membrane (Figure 1). It is known that γ-secretase activity is regulated by the presenilin genes, as the mutations in presenilins associated with familial AD alter the processing of APP and increase levels of the pathogenic long-form (1-42) of Aβ [27]. The genes coding for presenilin-1 (PS-1), a protein of 467 amino acid residues, on chromosome 14 [7], and presenilin 2, coding for 448 residues, on chromosome 1 [8], are highly homologous to each other, with 67% identity in amino acid sequence. PS-1 is 47% identical to sel 12, a protein involved in the regulation of the *Notch* developmental signalling pathway in *C. elegans*.

The presenilins and sel 12 contain 6–8 transmembrane regions, and one large cytoplasmic loop. They are normally proteolytically processed in the large cytoplasmic domain [28] by proteases which are inhibited by proteosome inhibitors, *N*-acetyl-L-leucine-L-norleucinal and lactacystin [29]. During apoptosis, presenilins are cleaved at an alternative site in the cytoplasmic loop by caspase-3 [30]. Mutation of either of two conserved transmembrane aspartates in PS-1, Asp 257 in TM6 and Asp 385 in TM7, substantially reduces Aβ production and increases production of the overlapping C-terminal fragments, and also prevents endoproteolysis of PS-1 in its cytoplasmic loop [31]. Thus, either presenilin is γ-secretase, or it regulates γ-secretase activity. PS-1 has also been shown to participate in cleavage of the cell surface Notch protein, which in similarity to γ-secretase, occurs in its trans-membrane region [32]. Presenilins have been localized primarily in the endoplasmic reticulum, but it is possible that sufficient PS-1 recycles to the cell surface to regulate Notch activity by proteolytic action. Molecular modelling has shown that many of the mutations in PS-1 found in familial AD could effect the interaction between the transmembrane catalytic aspartyl residues and the transmembrane region of APP [33]. Alternatively, presenilins may function to traffic APP and Notch to the cellular compartments in which proteolytic processing takes place.

Four research groups have recently produced evidence for identification of the same gene product, named BACE or ASP-2, as β-secretase [34–37]. Identification involved expression cloning [34], purification on a substrate analogue inhibitor [36], search of a WormPep database of *C. elegans* proteins [37], and search of a mammalian EST database [38] for D(S/T)G active site motifs. Transfection of cells with the cDNA of BACE/ASP-2 increased the production of the C100 fragment and Aβ. The BACE gene predicts an open reading frame of 501 amino acids with a signal peptide of 21 amino acids, a pro-sequence domain from residue 22–45, a lumenal domain of 414 residues with two DT/SGT/S motifs and four potential glycosylation sites, with about 30% amino acid identity to human pepsin.

Analysis of Aβ Fragments Released from Cultured Cells

The elucidation of the identity of α, β and γ-secretases should lead to the rapid design and development of inhibitors and activators as potential therapeutic agents. As a complex series of Aβ fragments are produced by metabolism *in vivo*, we have sought to develop a novel sensitive assay system with potential for screening both β and γ-secretase inhibitors, or α-secretase activators, in intact cells. The assay allows the rapid, single-step monitoring of the production and analysis of Aβ fragments from cells in culture.

We have employed Ciphergen's proprietary technique, Seldi™, to capture and analyse in parallel a mixture of truncated Aβ variants from a single microlitre of cultured cell supernatant or clinical sample. A highly selective anti-Aβ antibody, anti-NTA4 raised to the ten N-terminal residues of Aβ [22], was coupled covalently to a Preactivated ProteinChip™ Array, forming a biologically active surface for the

micropurification of immunoreactive amyloid species directly from crude biological samples. Once captured and purified by washing on the chip surface, the bound peptides are detected directly by mass using Ciphergen's PBS-I, a time-of-flight mass spectrometer.

MATERIALS AND METHODS

Cell Culture

The transfection of HEK 293 cells with a pCEP4 vector containing the cDNA coding for APP, APP constructs mutated in the cytoplasmic tail, and hygromycin resistance for stable expression of APP has been described previously [22]. Cells were cultured in DMEM with glutamax (Life Technologies, Paisley, UK), penicillin (100 Iu ml^{-1}) and streptomycin (100 ug ml^{-1}; ICN Flow, Thame, UK) and hygromycin B (200 ug ml^{-1}) (Boehringer-Mannheim, Lewes, UK). Cells transfected with the Swedish familial APP mutation in HEK 293 cells were a gift from Dr D Selkoe (Brigham & Women's Hospital, Boston, USA). To eliminate the lipoprotein contribution of serum, the transfected cells were acclimatized to growth on 2% Ultraser G (Boehringer-Mannheim) in place of foetal calf serum. Cells were split 1:20 at 80% confluence after suspension with 0.02% EDTA for 5 min at 37°C. Cells at 80% confluence in a 75 cm^2 flask were cultured for 40 h with cholesterol water-solubilized with methyl-β-cyclodextrin (Sigma, Poole, UK). Control cells (no cholesterol) were cultured for the same length of time with methyl-β-cyclodextrin alone.

Seldi™ Analysis

Anti-NTA4[22] or bovine IgG control (0.4 mg ml^{-1}) (1 µl) in PBS was added to the spots of a Preactivated Seldi ProteinChip™ Array (Ciphergen Biosystems Inc., Palo Alto, CA, USA) and incubated in a humidity chamber at 4°C for 1 h at room temperature, or overnight at 4°C to allow covalent binding to the ProteinChip™ surface. Glycine (0.1 M) (1 µl per spot) at pH 8 was added to block remaining pre-activated sites, and incubation continued for 20 min. Antibody solution was removed, and spots were washed with 50 mM Tris-HCl (pH 8) (5 µl). The whole chip array was then washed with 2 × 10 ml PBS containing 0.5% (v/v) Triton X-100, 15 min each wash, followed by 2 × 10 ml PBS for 10 min. Medium (5 µl per spot) from cell cultures of HEK cells transfected with the Swedish mutation of APP were added to the spots containing the covalently bound antibody, and incubation continued for 1.5 h at room temperature in the humidity chamber. Medium was removed, and the chips washed twice with 10 ml of PBS containing 0.5% (v/v) Triton X-100 for 10 min each wash, twice with 10 ml of PBS for 10 min each wash, then twice with 10 ml water for 10 min each wash. After drying, α-cyano-4-hydroxy cinnamic acid (2 mg ml^{-1}) in 50% (v/v) acetonitrile, 0.2% (v/v) trifluoroacetic acid was added, and mass identification made by 100 averaged shots in a Ciphergen Seldi™ Protein Biology System I (PBS I). Masses were calibrated externally by recombinant chaperonin CPN10 (10674 + 1H).

RESULTS AND DISCUSSION

The profile of immunoreactive Aβ peptides secreted by human HEK cells permanently transfected with a cDNA encoding Swedish familial AD mutant, APP, were examined using a novel technique in which multiple fragments were captured and purified onto a monospecific anti-NTA4 polyclonal antibody immobilized covalently on the surface of a ProteinChip™ Array. Captured peptides were detected directly from the chip surface by time-of-flight mass spectrometry using Ciphergen Biosystem's PBS I. The whole process can be completed in 2–3 h with multiple samples analysed in parallel on the same chip array.

The anti-NTA4 antibody used in this study is raised against the first ten N-terminal amino acids of the cleaved Aβ peptide enabling the capture of multiple Aβ fragments truncated or extended *in vivo* from the C-terminus. Commercially available antibodies, such as 6E10 (anti-Aβ (1-16)) (Senetek, Maryland Heights, MO, USA), have also been used successfully in this assay (results not shown). Figure 1 shows the predominant fragment captured to be Aβ 1-40, as determined by its mass of 4329.4, flanked by peaks corresponding to Aβ peptides 1-37, 1-38, 1-39 and 1-42. A cluster of smaller peptides with masses corresponding to Aβ 1-15, 1-16, 1-17, 1-18, 1-19 and 1-20 were also detected. Table 1 shows that the measured masses of captured peptides typically matched to within less than one Dalton of the predicted masses from the known human Aβ amino acid sequence, easily enabling unambiguous identification. As expected, given its specificity for the N-terminus of the Aβ cleaved sequence, all of the detected peptides binding to the anti-NTA4 polyclonal antibody are truncated or

Table 1 Comparison of Measured Masses by Seldi™ with Calculated Masses of Human Aβ Peptide Sequences

MW measured	ID	MW calculated	Amino acid sequence
4514.1	1-42	4514.1	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
4329.4	1-40	4329.9	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
4230.9	1-39	4230.9	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGV
4131.1	1-38	4131.5	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGG
4074.4	1-37	4074.5	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVG
3673.7	1-33	3674.0	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIG
2460.3	1-20	2461.7	DAEFRHDSGYEVHHQKLVFF
2314.5	1-19	2314.5	DAEFRHDSGYEVHHQKLVF
2165.7	1-18	2167.3	DAEFRHDSGYEVHHQKLV
2068.2	1-17	2068.2	DAEFRHDSGYEVHHQKL
1955.8	1-16	1955.0	DAEFRHDSGYEVHHQK
1828.1	1-15	1826.9	DAEFRHDSGYEVHHQ

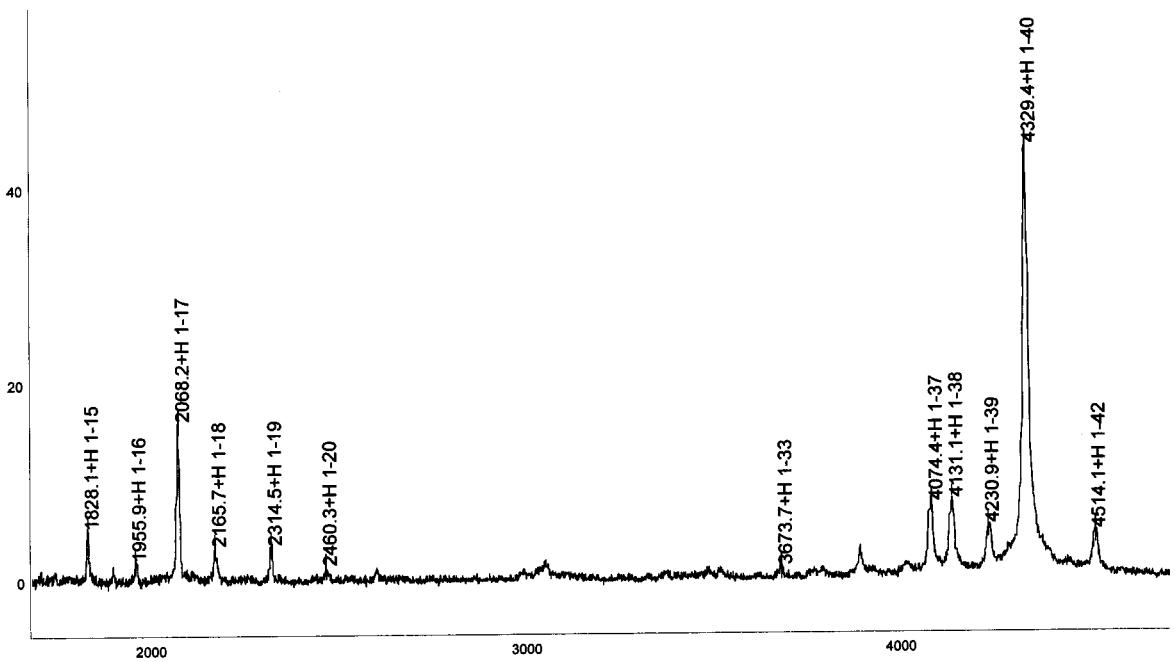

Figure 2 Seldi™ profile of Aβ fragments captured from supernatants of APP-wt transfected cells. The Aβ fragments were captured from 5 μl of supernatant on covalently-linked anti-NTA4 on a ProteinChip™ Array chip, washed in buffers containing Triton X100, and then analysed on the Seldi™ mass reader.

elongated at the C-terminus. None of the above peptides were retained on a control bovine IgG, also immobilized on the ProteinChip™ surface, indicating that the peptides are indeed specific for the anti-NTA4 polyclonal antibody (Figure 2(D)).

The role of intracellular targeting in amyloid production was investigated using Seldi™. PCR-directed site-specific mutagenesis was used to investigate the role in generation of Aβ of the consensus internalization signal -YENPTY- (Figure 1) in the cytoplasmic tail of APP in generation. Using a casette approach, and nested PCR, the essential tyrosine residues within this motif were mutated to alanine. Constructs were then permanantly transfected into HEK 293 cells on a pCEP4 vector, and the mutant cells selected and cloned in hygromycin, as described previously [38]. Cell-surface biotinylation has previously shown there was a progressive decrease in the rates of endocytosis in APP containing the mutation -YENPTA- and -AENPTA- [38].

Seldi™ analysis of the Aβ fragments secreted into cell supernatants of the wild-type APP and mutant-APP transfected HEK 293 cells cultured for 2 days under identical conditions showed significant differences induced by the mutations (Figure 3(A)–(C)). The double mutation -AENPTA- induced the formation of a fragment 62 mass units larger than Aβ 1-40. (Figure 3(B)). The mass of this unit would be in keeping with a species in which the single Met residue is oxidized to sulphoxide, and its single Tyr nitrated. No other common post-translational modification would give an increase in mass of 62. The -YENPTA- single cytoplasmic tail mutation also produced a nitrated-oxidized species (Figure 3(C)), but the ratio of its peak area to that of Aβ 1-40 was less than that produced by the double mutant. The results suggest that decrease of endocytosis at the cell surface of the mutated APP, or rerouting through different intracellular pathways, resulted in increased exposure of APP to superoxide and peroxynitrite, and subsequent modification of the amyloid produced. The mutations also increased the production of shorter N-terminal Aβ fragments, Aβ 1-17, 1-15, 1-14 and 1-13 (Figure 2(B)). The profiles suggest that intracellular re-routing caused by the tail mutations lead to increased exposure to α-secretase. The properties of nitrated/oxidized β-amyloid have not been examined, but the possibility remains that the action of this peptide in stimulating oxidants and inflammatory mediators from microglia could lead to an increased cascade of neurodegeneration.

The assay has recently been used to monitor the effects of changing levels of intracellular cholesterol on the levels of secretion of different Aβ species into cultured cell media. Cells were incubated in media contained exogenous cholesterol added with methyl-β-cyclodextrin carrier, compared with control cells incubated for 40 h with the carrier alone. Seldi™ analysis revealed that secreted Aβ fragments with affinity for anti-NTA4 were all increased by an increase in cholesterol relative to an internal control peak at 6093 mass units. Peak areas of other peaks were measured following normalization against the peak area of Aβ 1-40. The ratio of Aβ 1-17, which requires cleavage by two amyloid processing enzymes β and α-secretase for its release (Table 2), to Aβ 1-40 was not significantly altered by increased levels of cholesterol. Instead, the relative amounts of Aβ 1-42, the more toxic form of Aβ, relative to Aβ 1-40, was changed by cholesterol (Table 1; Figure 4), indicating increased cleavage at residue 42 by γ-secretase. These subtle changes in amyloid peptide cleavage and secretion would be difficult to measure with other conventional assay methods. However, it is possible that some Aβ 1-42 present at concentrations higher than those required to initiate fibril formation [1] may be lost from biological fluids by precipitation prior to analysis by Seldi™.

Table 2 Normalized Peak Areas Obtained from Cells Treated with Cholesterol (50 μg ml^{-1}) and Carrier, or with Carrier Alone

Cells treated with cholesterol		Cells treated with carrier	
Peak	Peak area	Peak	Peak area
1-40	736.4	1-40	735.3
1-42	132.0	1-42	71.5
1-17	104.4	1-17	113.7

The relative increase in β-amyloidogenic fragments compared the total APP biosynthesized under changed conditions of intracellular cholesterol has been confirmed by immunoprecipitation with anti NTA4 and a C-terminal antibody to APP in extracts of metabolically-labelled transfected cells and reported elsewhere [39]. Moreover, reducing the cells intracellular content of cholesterol by previous incubation with the cholesterol biosynthesis inhibitor lovastatin was shown to reduce the relative amounts of Aβ-genic fragments, compared with the total amount of APP biosynthesized.

The mechanism of upregulation of β-secretase action on APP by choleseterol is, at present, unknown. Axonal APP is concentrated in caveolae-like vesicles in differentiating corticol neurones, which, although lacking caveolin, are enriched in cholesterol. In the brain, Aβ is localized to a detergent-insoluble membrane compartment (DIG) enriched in glycolipids and cholesterol, which is thought to be a subcompartment of the TGN [40]. Thus, increases in intracellular cholesterol would be expected to enhance the formation of DIG, which in turn could increase the extent of β-secretase cleavage, which has been shown to occur in the TGN in transfected 293 cells [22]. Transcription of enzymes controlling intracellular cholesterol are regulated by cleavage of transmembrane domains of sterol regulatory element binding proteins (SREBP), which have similar topography to APP [41]. However, it is not known how the proteases that activate SREBPs are related to the APP secretases.

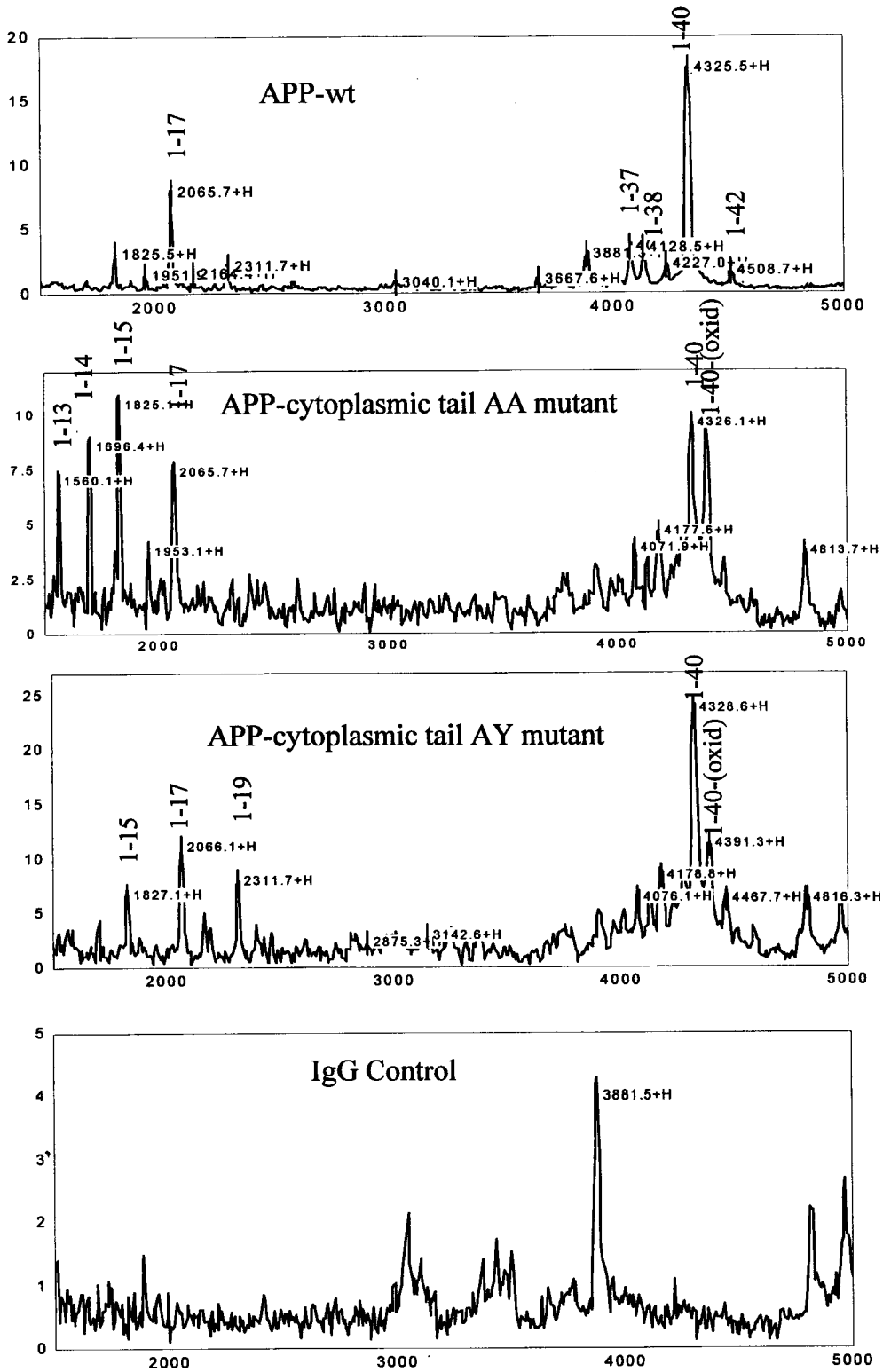

Figure 3 Seldi™ profiles obtained from supernatants of APP-wt and cytoplasmic-tail mutants of APP. The Aβ-fragments were captured from 5 μl of supernatants on (A–C) immobilized anti-NTA4, (D) or bovine IgG.

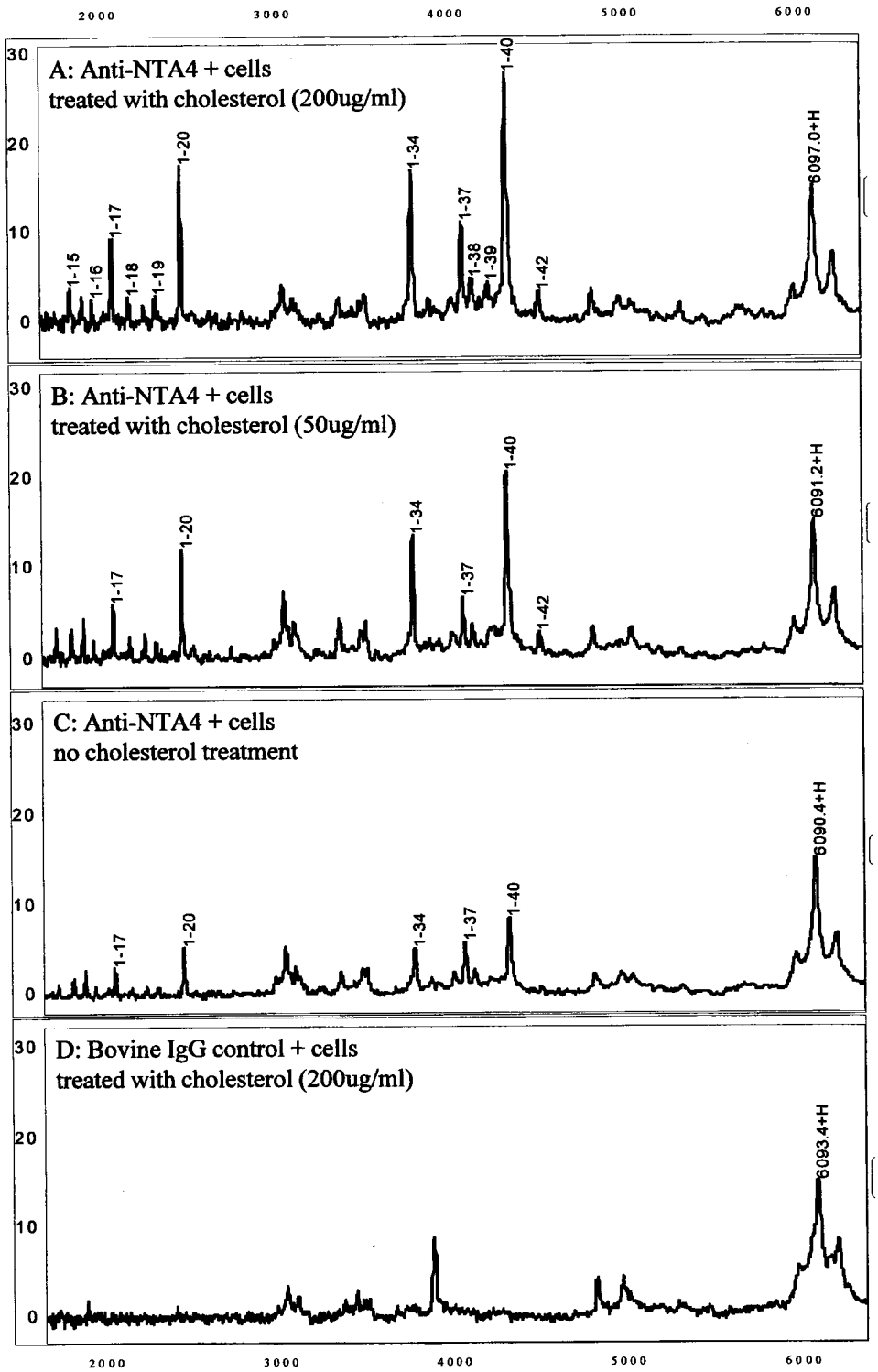

Figure 4 Seldi™ profiles changed by addition of exogenous cholesterol to APP Swedish mutant transfected cells. The Aβ-fragments were captured by anti-NTA4 from 5 µl of supernatnts of cells treated with added cholesterol in (A, B) methyl-β-cyclodextrin carrier or (C) carrier alone. Supernatants from A were also captured on (D) bovine IgG.

This assay offers a rapid method for the capture and analysis of multiple Aβ species directly from small volumes of unfractionated biological samples. The ability to easily measure the ratio of truncated forms of Aβ using a single antibody makes this assay particularly useful for studying amyloid processing *in vivo*.

Seldi™ offers significant advantages over existing techniques, such as enzyme-linked immunoabsorbant assays (ELISA) that rely on indirect chemical or radioactive methods of detection. Accurate and sensitive detection of captured analytes directly by native mass means that the identity of multiple immunoreactive Aβ fragments can be easily determined using the same antibody in a single assay. Different samples can be assayed in parallel on a ProteinChip™ Array to rapidly measure changes in the relative concentration of different amyloid species over time or in response to changes in biological conditions. Alternatively, a panel of antibodies can be screened on the same chip surface to determine antibody specificities or expand further the range of peptides to be analysed. Typically, a single microlitre or less of unfractionated biological sample is used on each spot of the ProteinChip™ Array. This small volume sample handling makes the assay particularly useful for screening Aβ variants in scarce amounts.

Seldi™ has also been used to capture different Aβ fragments from clinical samples, such as brain-tissue extract (data not shown) and plasma The small volumes required and the sensitivity of this assay make it ideal for studying relative peptide levels in clinical samples taken from AD patients and control individuals.

Acknowledgements

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