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Permalink
https://escholarship.org/uc/item/40c2m1zw

Journal
The Biochemical journal, 340 ( Pt 3)(3)

ISSN
0264-6021

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Publication Date
1999-06-01

DOI
10.1042/bj3400703

Peer reviewed
Effects of the amyloid precursor protein Glu\(^{693} \rightarrow \) Gln ‘Dutch’ mutation on the production and stability of amyloid β-protein

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INTRODUCTION

The predominant protein constituent of the cortical and cerebrovascular amyloid deposits of Alzheimer’s disease (AD) is a 40–42-residue peptide termed the amyloid β-protein (A\(β\)) [1]. Aggregated, unbranched fibrils of A\(β\), along with a variety of other proteinaceous and non-proteinaceous macromolecules, are found in the amyloid deposits [1]. Accumulating evidence suggests that A\(β\) production, formation into fibrils and deposition are key aetiologic events in AD [2]. For this reason, therapeutic strategies are being developed that target critical steps in these processes.

A\(β\) is formed by cleavage from the amyloid precursor protein (APP), a type I integral membrane protein with a large extracellular domain and a short C-terminal tail [3]. A\(β\) encompasses the 28 residues immediately N-terminal to the transmembrane domain plus the first 12–14 residues of the transmembrane domain itself. Three unidentified endoproteolytic activities, termed secretases, act on APP. \(\alpha\)-Secretase cleaves APP within the A\(β\) sequence after Lys\(^{660}\), releasing the large ectodomain. This \(\alpha\)-Secretase site. An Ala\(^{692}\) at Val\(^{14}\). The biological relevance of these various peptides is suggested by their presence in human biological fluids [8–10,16] and by the observation that A\(β\) peptides with truncated N-termini are among those found in the earliest, diffuse A\(β\) deposits [17–21].

Alterations in the quantities and/or ratios of the various A\(β\) peptides are associated with familial forms of AD and with related \(β\)-amyloidoses caused by mis-sense mutations in the APP gene on chromosome 21. Notably, these mutations cluster around the cleavage sites for the three secretase activities. The double (Swedish) mutation Lys\(^{670}\)-Met\(^{671}\) \(\rightarrow\) Asn-Leu at the \(\beta\)-secretase site causes an increase in the overall quantity of A\(β\) detectable in the plasma and in the medium of cultured fibroblasts from carriers of the Swedish APP mutation [11,22]. Increased A\(β\) secretion is also observed in the culture media of transfected cells overexpressing Swedish APP [23,24]. Mis-sense mutations near the \(γ\)-secretase site increase the proportion of secreted A\(β\) ending at Ala\(^{16}\) [25–28], a species that aggregates faster \(\text{in vitro}\) than A\(β\) ending at Val\(^{19}\) [29] and is found in diffuse A\(β\) deposits of AD and Down syndrome brain [19,21,30]. Two additional APP mutations cluster near the \(\alpha\)-secretase site. An Ala\(^{692}\) \(\rightarrow\) Gly mutation, found in a Flemish family, results in cerebral amyloid...
angiopathy and AD [31,32]. This mutation alters the normal secretory cleavage of APP, resulting in an increased overall ratio of 4 kDa peptides to 3 kDa peptides and increased quantities of N-terminally truncated Aβ peptides such as Aβ1–38 [13]. A Glu693 → Gln mutation, found in a number of large Dutch families, causes hereditary cerebral haemorrhage with amyloidosis, Dutch type (HCHWA-D) [33,34]. HCHWA-D is characterized by severe meningocortical vascular Aβ deposition resulting in fatal cerebral haemorrhages. The Glu22 → Gln substitution in Aβ accelerates its aggregation in vitro [35–38]. However, the pathogenesis of HCHWA-D seems to involve more than simply the rapid aggregation of full-length Aβ. The abundance of diffuse plaques in HCHWA-D brain parenchyma [34] suggests that N-terminally truncated Aβ peptides could have an important role in parenchymal Aβ deposition in this disease, as they do in Down syndrome [21,39,40]. In addition, studies of the effects of Dutch and wild-type peptides on neuronal, smooth-muscle and perivascular cells suggest that the Glu22 → Gln substitution produces cytotoxicity only when present in Aβ molecules of appropriate length and aggregation state [41–43]. Here we report studies to assess the effects of the Dutch amino acid substitution on the proteolytic processing of Aβ by transfected cells in culture and on the stability of the Aβ peptides secreted by these cells. The results show that the earliest pathogenetic effect of the Dutch mutation is an alteration in the proteolytic processing of Aβ.

EXPERIMENTAL

Nomenclature

For ease of comparison with other studies, numerical references to antibody epitope and mutations (e.g. Val177 → Ile) are based on the 770-residue isoform of APP (APP770). Specific amino acid residues or mutations within Aβ are noted as, for example, Phe30 or Glu22 → Gln respectively.

Cell lines

Human embryonic kidney 293 (HEK293) cells were stably transfected with wild-type APP695 cDNA or with an APP695 cDNA containing the HCHWA-D mutation [44], essentially as described [45]. The pCMV695 plasmid encoding the HCHWA-D mutant APP was generously provided by Dr. Eddie Koo (University of California, San Diego, CA, U.S.A.). In the cell lines used in this study, expression levels of Dutch APP were approximately double those of wild-type APP (results not shown). The generation of HEK293 cells stably expressing an APP695 cDNA containing the Swedish double mutation (Lys670-Met671 → Asn-Leu) has been described previously [23]. Transfected cells were maintained in Dulbecco’s modified Eagle’s medium containing 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 0.2 mg/ml G418 sulphate/Geneticin® (all from Gibco, Gaithersburg, MD, U.S.A.) and 10% (v/v) fetal bovine serum (HyClone, Logan, UT, U.S.A.), at 37 °C, in an atmosphere of 5% CO2 in air.

Metabolic labelling and immunoprecipitation

For radiosequencing, round 10 cm diameter dishes of 80–90% confluent transfected cells were labelled for 12–15 h with 5 μCi of [1-14C][2,3,4,5-3H]phenylalanine (124 Ci/mmol; Amersham, Arlington Heights, IL, U.S.A.) in 5 ml of phenylalanine-free medium (Select-Amine Kit; Gibco). Soluble Aβ, p3 and related peptides were immunoprecipitated from labelled, conditioned medium with the use of the polyclonal antisera R1280 or R1282, specific for synthetic Aβ1–46 peptides [46,47]. Immunoprecipitated species were separated by SDS/PAGE on 10–20% Tris/tricine gels (Novex, San Diego, CA, U.S.A.). For measurement of the stability of secreted Aβ species, cells were radiolabelled with [35S]Met Express protein labelling mix (1175 Ci/mmol; DuPont-NEC, Newtown, CT, U.S.A.) in methionine-free medium for 3 h, then the conditioned media were collected, pooled and redistributed to unlabelled transfected cells for chase times of 0–12 h. Unlabelled methionine in PBS was then added to the labelled medium to produce a final concentration of 10 mM, a 2 × 10-fold molar excess relative to [35S]Met. Culture medium was collected and cleared of cells and cell debris by centrifugation (2000 g, 30 min, 4 °C), then Aβ, p3 and related peptides were immunoprecipitated from the medium with R1282 and separated by SDS/PAGE, as described above.

Quantitative immunoprecipitation and phosphorimage analysis

Stably transfected cells, grown in 10 cm diameter dishes to 80–90% confluence, were labelled for 18 h with 300 μCi of [35S]Met Express in methionine-free medium. An aliquot of this medium (3 ml) was incubated with antisera R1282 (diluted 1:100, v/v) and 25 μl of 100 μg/ml Protein A-Sepharose CL-4B (Sigma, St. Louis, MO, U.S.A.). After incubation overnight at 4 °C, the Protein A-Sepharose beads were collected by centrifugation (3000 g, 10 min, 25 °C), and the remaining medium was immunoprecipitated with R1282 twice more. Protein A-Sepharose beads were washed sequentially in 0.5 × STEN [25 mM Tris/HCl (pH 7.6)/75 mM NaCl/1 mM EDTA/0.1% (v/v) Nonidet P40], 1 × STEN + 0.1 % SDS, then 1 × STEN. Immunoprecipitated species were separated by SDS/PAGE as described above. Each immunoprecipitate was loaded into a separate lane of the gel. Phosphorimages of fixed and dried immunoprecipitated gels were obtained on a Molecular Dynamics PhosphorImager 400A. Quantification was performed with IMAGEQUANT software (Molecular Dynamics, Sunnyvale, CA, U.S.A.). For calculation of Aβ/p3 ratios, the signals from all three R1282 immunoprecipitations were added by using the IMAGEQUANT software.

Radiosequencing analysis

[3H]Phe-labelled proteins isolated by immunoprecipitation and SDS/PAGE were electroblotted to Hyperbond PVDF membranes (Porton Instruments, Tarzana, CA, U.S.A.) at 400 mA for 4 h at 4 °C in 25 mM Tris/HCl, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol. After autoradiography, labelled protein bands were excised and radiosequenced as described [13]. Each experiment was done a minimum of five times. To compare the relative quantities of each peptide present, initial yields were calculated from the formula Yn = Y0 / (Yn)n, where Y0 is the measured radioactivity (c.p.m.) at cycle n and Yn is the repetitive yield. For each sample, Yn was determined experimentally by log regression analysis of 4 kDa band signals occurring at cycles 4, 19 and 20, which derived from Aβ beginning at Asp3. These values for Yn were used in analyses of the corresponding 3 kDa bands, each of which was sequenced immediately after its 4 kDa partner. [3H]Phe peaks from the 3 kDa band occurring in cycles 9 and 10 resulted from a p3-related peptide beginning at Glu41. In sequencing other 3 kDa peptides, the radioactive signals at cycle 1 could have come from p3 beginning at Phe16 or Phe19, signals at cycle 2 could have come from p3 beginning at Val19 or Phe19, and signals at cycle 3 could have come from p3 beginning at Leu19 or Val19. To make correct assignments, the graph of radioactivity was modelled mathematically by summing the predicted signals contributed by each peptide species at each cycle. The predictions used the known
repetitive yields from each experiment to calculate the lag for one cycle after the occurrence of each radiolabelled phenylalanine residue. Then, by systematically varying the relative percentages of each peptide in the population of molecules being sequenced, a precise fit to the experimentally observed signals could be made and the relative quantities of each peptide determined. These quantities were then used to calculate the initial yields of each peptide, according to the formula above.

RESULTS

N-terminal heterogeneity of secreted $\alpha$ and p3 peptides

In conditioned media of APP-transfected cells and in amyloid deposits of AD brain, both $\alpha$ and p3 exist as peptide families with variable N- and C-termini. To determine whether the Dutch mutation altered the secretase cleavage patterns of APP and/or the relative quantities of cleavage products, cells stably transfected with either wild-type or Dutch APP were metabolically labelled for 18 h with $[\text{H}]$phenylalanine. After immunoprecipitation, SDS/PAGE and electroblotting, peptides of approx. molecular mass 2–5 kDa were radiosequenced from strips corresponding to the 4 kDa (A $\alpha$) and 3 kDa (p3) gel bands. The identity of each species was deduced from the patterns of $[\text{H}]$-containing peaks (representative traces are shown in Figure 1). The initial yield of each peptide was calculated (see the Experimental section) and used to determine the quantity of each peptide as a percentage of the total 4 kDa or 3 kDa signal (Table 1). The results show that the Dutch mutation, relative to the wild-type APP sequence, produced significant changes in the relative quantities of various A$\beta$- and p3-related peptides within each gel band.

In the medium of cells expressing Dutch APP, the quantity of A$\beta$ beginning at Asp$^3$ increased from 68% to 78%. Coordinate decreases in the levels of peptides beginning at Ile$^4$ and Arg$^5$ were observed. Within the 3 kDa band, a significant decrease in the quantity of the Leu$^{17}$ species, the normal $\alpha$-secretase-generated peptide, was accompanied by significant increases in the levels of peptides beginning at Val$^{14}$ and Phe$^{19}$. A consistent observation in the Dutch 4 kDa samples was that of a small peak at cycle 2 (Figure 1C). A peptide beginning at Glu$^3$ would produce such a signal and, in addition, signals at cycles 17 and 18. However, these latter signals were not present. We therefore note this difference between the Dutch and wild-type 4 kDa samples but cannot explain it at present. Finally, insignificant changes were noted in the levels of p3-related peptides beginning at Glu$^{11}$ and Phe$^{19}$. Together, these results are consistent with a physiological effect of the Dutch mutation on APP processing that, although not blocking the recognition of the APP substrate by $\alpha$, $\beta$- or $\gamma$-secretases, is characterized by altered cleavage kinetics.

Relative levels of secretion of A$\beta$ and p3

Quantitative immunoprecipitation was used to determine whether the Dutch mutation affected the ratio of A$\beta$ to p3 in the media of APP-transfected cells. Because a single antiserum was

![Figure 1](https://example.com/figure1.png)

**Figure 1** Radiosequencing of A$\beta$ and p3 peptides

(A) Wild-type 4 kDa band; (B) wild-type 3 kDa band; (C) Dutch 4 kDa band; (D) Dutch 3 kDa band. Anilinothiazolinone amino acid derivatives were collected after each cleavage cycle of Edman chemistry and the level of $[\text{H}]$phenylalanine present was determined by scintillation counting. For each sample, the deduced peptide sequences (shown on the top of each panel) are aligned with the profile of radioactivity, and the respective N-termini are noted to the right.

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used to immunoprecipitate several Aβ- and p3-related peptides, control experiments were performed to ensure the quantitative collection of all molecules. First, the quantities of labelled media and antiserum R1282 were each optimized to maximize the precipitation efficiency (results not shown). Next, sequential immunoprecipitations were done to determine whether quantitative collection of the Aβ and p3 peptides had been achieved. On average, 97% of the 4 kDa signal and 95% of the 3 kDa signal were collected in two sequential rounds of immunoprecipitation (results not shown). However, to obtain as close to quantitative amounts of Aβ and p3 as possible, three immunoprecipitations were used for all experiments.

Cells stably expressing either wild-type or Dutch APP were metabolically labelled overnight with [35S]methionine. Aβ, p3 and related species from the conditioned media were then quantitatively immunoprecipitated, separated by SDS/PAGE and subjected to phosphorimage analysis (Figure 2). Because these peptides each contained one methionine residue, Met35, the radioactive signal from each band provided a direct measurement of the quantity of each species. For each cell line, media from a total of 20–35 dishes, from six separate platings, were each assayed independently. As a positive control, cells expressing APP containing the Swedish double mutation (Lys670→Met→Asn-Leu) were labelled as above, and the Aβ and p3 families were immunoprecipitated. In this case, an approx. 5-fold increase of the ratio of Aβ to p3 was observed (Figure 2), which is consistent with previous results demonstrating the increased production of Aβ by HEK293 cells and fibroblasts expressing Swedish mutant APP [22–24]. In contrast, the ratio of Aβ to p3 for both wild-type and Dutch transfectants was found to be approx. 0.9 (Figure 2). The Dutch mutation, unlike the Swedish, did not therefore facilitate the preferential processing of APP into Aβ.

### Stability of secreted peptides

In addition to altering the relative quantities of Aβ and p3 peptides produced, the Glu22→Gln substitution could also affect peptide catabolism. To explore this issue, HEK293 cells stably transfected with either wild-type or Dutch APP were pulsed for 3 h with [35S]methionine, after which the culture media were collected, pooled and redistributed into fresh dishes of unlabelled transfected cells. Because the conditioned medium still contained unincorporated [35S]methionine, a 2×10^−3-fold molar excess of unlabelled methionine was added to prevent the significant production of labelled, nascent Aβ and p3 during the chase period. After chase times of up to 12 h, the media were collected, and Aβ and p3 peptides were recovered by immunoprecipitation and separated by SDS/PAGE. The amounts of the peptides were then quantified by phosphorimaging of the dried gels. This method monitors alterations in the concentrations of soluble Aβ and p3 resulting from degradation by cell-surface or secreted proteases, epitope masking due to complex formation with components of the medium, re-internalization, and sticking to cell surfaces or the tissue culture dish itself.

The quantities of each peptide secreted during the 3 h pulse-labeling (0 h chase time) were used to normalize results collected over chase times from 1 to 12 h (Figure 3A). The means and S.D. for six experiments for each transfected cell line were determined (Figures 3B and 3C). No statistically significant differences in the temporal change in concentration of either the 4 kDa or 3 kDa peptides were observed. In control experiments, cells metabolically labelled for 12 h in the presence of a 2×10^−3-fold excess of unlabelled methionine produced no detectable radiolabelled Aβ or p3 (Figure 3A, lane CTRL). To exclude differential immunoreactivity as an explanation for a potential lack of recognition of the Dutch peptides, synthetic Aβ peptides were applied to a nitrocellulose filter and detected with R1282. The
effects of the Dutch mutation have been addressed through a systematic, quantitative analysis of the production and stability of Aβ in cell culture. This analysis revealed that the Dutch mutation increased the relative levels of full-length Aβ beginning at Asp1, and of p3 beginning at Val18 and Phe19. Co-ordinate decreases in the levels of Aβ starting at Ile8 and Arg3, and of p3 starting at Leu17, were found. The mutation did not alter the ratio of secreted Aβ to p3, induce alternative APP cleavages leading to the secretion of novel Aβ species, or alter the stability of the secreted Aβ and p3 peptides.

Recently, De Jonghe et al. [48] used ELISA methods to quantify the levels of full-length Aβ peptides secreted by cultured stably transfected Chinese hamster ovary K1 cells and by transiently transfected HEK293 and human H4 neuroglioma cells. They found trends towards lower secretion, and a decreased ratio of Aβ(1–41) to Aβ(1–40) in Dutch-transfected cells compared with wild-type transfectants, but the differences were mainly insignificant. It should be noted that these studies used the monoclonal antibody BAN50, selected for its reactivity to Aβ1–16, to capture Aβ. Therefore differences in secretion of N-terminally truncated Aβ species might not have been detectable. In addition, BAN50 has recently been shown not to react with SDS-stable Aβ dimers [49]. Other studies have used immunoblot analysis to quantify N-terminal and C-terminal APP fragments arising from secretase action [50–52]. In H4 cells, glioblastoma U-87 MG and COS-1 cells expressing wild-type or Dutch APP, no differences in levels of APP fragments were observed. These results are consistent with those from our studies of relative levels of Aβ and p3 secretion. Existing results therefore indicate that the endoproteolytic processing of Dutch APP into Aβ follows the same overall pathway as the processing of wild-type APP but with alterations in the efficiency of cleavage at specific sites.

The consistency among data derived from studies with five different cell types (H4, U-87 MG, Chinese hamster ovary K1, COS-1 and HEK293) suggests that the alteration in APP processing observed in our experiments probably also occurs in cells in vitro. This is certainly true of the Swedish mutation, in which increased Aβ levels have been observed in the plasma and in the conditioned media of primary fibroblasts from presymptomatic and symptomatic individuals [11,22] and in the conditioned media from Swedish APP-transfected HEK293 and human neuroblastoma M17 cells [23,24]. In this regard, it would be of interest to examine whether the effects of the Dutch mutation on APP processing in HEK293 cells are also observed in cultured vascular endothelial and smooth-muscle cells.

The location of the Dutch mutation near the z-secretase cleavage site raised the possibility that the normal secretory processing of APP might be altered. In fact, we found that the Glu22 → Gln mutation shifted the distribution of N-terminal cleavage products in favour of peptides beginning at Asp1, Val18 and Phe19. In studies of transfected cells expressing APP containing the adjacent Flemish (Ala21 → Gly) mutation [13], immunoprecipitation and radiosequencing of 4 kDa and 3 kDa Aβ-related peptides demonstrated relative increases in the levels of Aβ peptides beginning not only at Asp1 but also at Arg3 and Glu11. Here we found a significant decrease in the production of peptides beginning at Arg2 and no change in the secretion of peptides beginning at Glu11. Because the tertiary conformation of APP is unknown, a structure-based explanation of the similarities and differences in processing induced by these mutations is not possible. However, it is reasonable to postulate that the glycine substitution, owing to its potential for destabilizing the ordered structure of the polypeptide, could facilitate endoproteolytic access to normally less available cleavage sites (namely Arg3 and Glu11). In contrast, the Dutch substitution might

results of this dot-blot assay demonstrated that even at very low peptide levels (150 fmol), wild-type and mutant peptides were recognized equally well by the immunoprecipitating antiserum (results not shown). Taken together, these results provide strong evidence that the observed changes in the levels of the various Aβ and p3 peptides secreted by cells expressing Dutch APP (Table 1) arose from differences in processing APP owing to the Glu22 → Gln substitution.

**DISCUSSION**

A clear mechanistic explanation for the pathology of HCHWA-D would facilitate efforts to treat both this disease and the amyloidotic component of AD. Here, certain of the possible

**Figure 3 Stability of Aβ and p3 peptide families**

(A) In a pulse–chase experiment, cells stably expressing wild-type or Dutch APP were labelled with [35S]methionine for 3 h, then the media were collected, pooled, combined with excess unlabelled methionine and redistributed to unlabelled cells for 0–12 h. Aβ and p3 peptides were immunoprecipitated from the medium and separated by SDS/PAGE. The peptide levels at each time point were quantified by phosphorimaging with a Molecular Dynamics PhosphorImager (ImageQuant software). A separate dish of cells was incubated for 12 h with [35S]methionine for 3 h, then the media were collected, pooled, combined with excess

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stabilize a conformation of APP with a more subtle structural alteration, resulting in the relative inaccessibility of the normal Leu$^{17}$ $\alpha$-secretase site and improved access to the alternative sites at Val$^{18}$ and Phe$^{19}$. The production of truncated A$\beta$ peptides in particular could be relevant to the disease process because these shorter peptides have been found to form fibrils more rapidly than A$\beta_{1-40}$ (D. M. Walsh and D. B. Teplow, unpublished work) and to be neurotoxic in vitro [53]. Because small changes in A$\beta$ structure can markedly accelerate fibrillogenesis kinetics [54], modest alterations in the relative quantities of different A$\beta$ peptides produced could be of significance in vivo. This feature of APP processing must be considered in the development of therapeutic strategies involving $\beta$-secretase inhibitors, where inhibition of the cleavage of the Met$^{671}$-Asp$^{672}$ peptide bond could result in the production of highly toxic truncated molecules.

The pathogenetic effects of the Dutch mutation could result from a combination of APP processing effects and the biophysical properties intrinsic to the Dutch A$\beta$ from a combination of APP processing effects and the biophysical effects of the mutation could facilitate fibril–fibril interactions, leading to accelerated plaque formation and stability [36,37]. Relevant to this change in A$\beta$ aggregation rates in vitro [35–37,55–57]. If the loss of a glutamic acid residue (or the glutamic acid substitution itself) were indeed the primary cause of the accelerated deposition of amyloid in HCHWA-D, this would suggest that Dutch A$\beta$ would be the primary constituent of amyloid deposits in patients heterozygous for the mutation. However, studies by Prelli et al. [58] have shown that both wild-type and Dutch A$\beta$ can be isolated from cerebrovascular amyloid deposits of HCHWA-D patients, although the relative quantities of each peptide were not determined. The presence of both peptides contrasts with the peptide composition of renal amyloid deposits found in patients suffering from hereditary systemic amyloidosis, where only the mutant form of human lysozyme accumulates [59]. In HCHWA-D, therefore, the Dutch mutation might not only cause quantitative differences in the kinetics of mutant A$\beta$ fibrillogenesis and aggregation [54], but could also influence the assembly of wild-type A$\beta$ peptides by providing Dutch fibril nuclei from which wild-type or mixed fibrils could elongate. In addition, the mutation could facilitate fibril–fibril interactions, leading to accelerated plaque formation and stability [36,37]. Relevant to these issues is the recent finding that a Glu$^{671}$ $\rightarrow$ Lys mutation in two Italian families apparently produces clinical and pathological findings highly similar to those of the Dutch disease [60]. This observation is consistent with the notion that the clinical manifestations of the Dutch mutation arise in part from the biophysical consequences of the replacement of Glu$^{672}$.

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