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# The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced A $\beta$ protofibril formation

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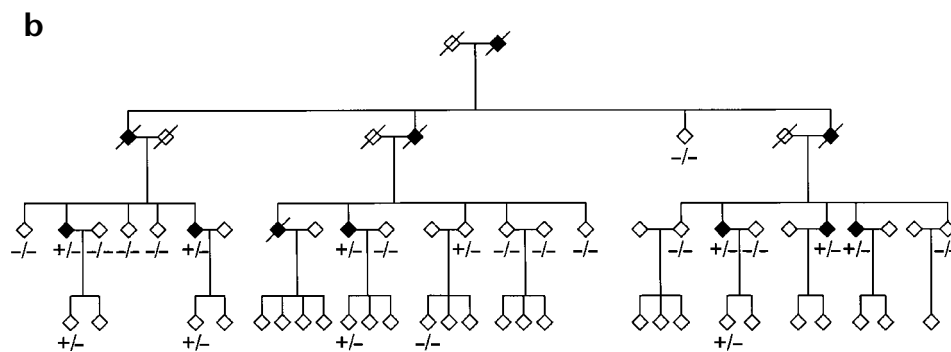
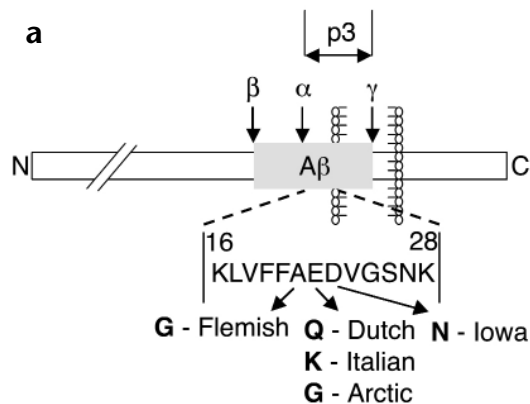
Several pathogenic Alzheimer's disease (AD) mutations have been described, all of which cause increased amyloid  $\beta$ -protein (A $\beta$ ) levels. Here we present studies of a pathogenic amyloid precursor protein (APP) mutation, located within the A $\beta$  sequence at codon 693 (E693G), that causes AD in a Swedish family. Carriers of this 'Arctic' mutation showed decreased A $\beta$ 42 and A $\beta$ 40 levels in plasma. Additionally, low levels of A $\beta$ 42 were detected in conditioned media from cells transfected with APP<sub>E693G</sub>. Fibrillization studies demonstrated no difference in fibrillization rate, but A $\beta$  with the Arctic mutation formed protofibrils at a much higher rate and in larger quantities than wild-type (wt) A $\beta$ . The finding of increased protofibril formation and decreased A $\beta$  plasma levels in the Arctic AD may reflect an alternative pathogenic mechanism for AD involving rapid A $\beta$  protofibril formation leading to accelerated buildup of insoluble A $\beta$  intra- and/or extracellularly.

Alzheimer's disease (AD) is characterized neuropathologically by progressive deposition of the 40–42 residue amyloid  $\beta$ -protein (A $\beta$ ) in brain parenchyma and cerebral blood vessels. Several pathogenic mutations have been identified in the amyloid precursor protein (APP) gene, all located close to the major APP processing sites (for reviews, see refs. 1, 2). These processing sites are located either adjacent to the A $\beta$  domain in APP (the  $\beta$ - and  $\gamma$ -secretase sites) or within the A $\beta$  sequence itself (the  $\alpha$ -secretase site). Increased production of the more amyloidogenic A $\beta$ 42 is seen with mutations positioned in the vicinity of the  $\gamma$ -secretase cleavage site. The only known AD mutation close to the  $\beta$ -secretase site, the Swedish mutation<sup>3</sup>, results in elevation of both A $\beta$ 42 and A $\beta$ 40 in plasma and fibroblasts from mutation carriers<sup>4–6</sup>.

Pathogenic mutations within the A $\beta$  sequence (Fig. 1a) generally result in a phenotype different from AD, with massive amyloid accumulation in cerebral blood vessel walls in addition to parenchymal amyloid plaques. The Dutch mutation carriers (E693Q) are clinically characterized by the occurrence of intracerebral hemorrhages<sup>7</sup>. Carriers of the Flemish mutation (A692G), another intra-A $\beta$  mutation, frequently suffer from intracerebral hemorrhage, and individuals who survive develop a progressive dementia with features of AD<sup>8</sup>. Different pathogenic mechanisms have been proposed for the Dutch and Flemish mutations. It has been observed that the Flemish mutation leads to increased A $\beta$  levels, whereas a reduced ratio of A $\beta$ 42/40 was seen in media from cells transfected with the Dutch mutation<sup>9</sup>. In addition, the Flemish mutation resulted in an increased A $\beta$ /p3 ratio, indicating effects on  $\alpha$ -secretase

cleavage (Fig. 1a) that were not observed for the Dutch mutation<sup>10,11</sup>. Furthermore, radiosequence analysis of the A $\beta$  and p3 proteins derived from APP with the Flemish and Dutch mutations suggests that these mutations affect both  $\alpha$ - and  $\beta$ -secretase processing<sup>10,11</sup>. Investigations of synthetic A $\beta$  peptides have indicated that the Dutch mutation accelerates protofibril formation compared to the wild-type peptide. In contrast, the Flemish A $\beta$  peptides display increased solubility and decreased fibrillogenesis rates compared to wild-type peptides<sup>12,13</sup>. Thus, distinct A $\beta$  fibrillization kinetics and effects on APP metabolism by these mutations may underlie the differences in their clinical features. A third pathogenic intra-A $\beta$  mutation was discovered in an Italian family (E693K), which had clinical manifestations similar to Dutch patients<sup>14</sup>. Finally, a mutation at codon 694 (D694N) in a family from Iowa causes progressive dementia and severe cerebral amyloid angiopathy<sup>15</sup>. There are currently no reports on effects by either of these mutations on cellular A $\beta$  formation.

The A $\beta$  protein forms amyloid fibrils that accumulate into senile plaques in AD brains. The A $\beta$  fibrillization process is a complex multi-step reaction, proceeding through a nucleation and extension phase<sup>16,17</sup>. A number of soluble fibril intermediates have been identified, including protofibrils and A $\beta$ -derived diffusible ligands (ADDLs)<sup>12,18–23</sup>. Both species are neurotoxic<sup>20,21,23</sup>. Little is known about the involvement of protofibrils or ADDLs in the pathogenesis of AD. However, findings indicate that protofibrils generated by another protein,  $\alpha$ -synuclein, are involved in early onset Parkinson's disease, and pathogenic missense mutations in  $\alpha$ -



**Fig. 1.** The intra-A $\beta$  mutations. (a) The APP molecule with localization of the A $\beta$  and p3 proteins, containing pathogenic intra-A $\beta$  mutations. (b) Pedigree showing the segregation of AD and of the E693G mutation in the Arctic family, compatible with an autosomal dominant pattern of inheritance. The pedigree has been disguised to protect the confidentiality of family members. Solid symbols, affected; open symbols, unaffected; slashed symbols, deceased; +/-, mutation carrier; -/-, non-carrier.

synuclein accelerate protofibril formation<sup>24</sup>. Thus, protofibrils may have general importance as triggers of neurodegeneration.

We identified a pathogenic mutation located at codon 693 within the A $\beta$  region of APP, at which glutamic acid is substituted for glycine (E693G). Affected subjects have clinical features of early-onset AD. We have named the mutation the 'Arctic' mutation, because the family in which it was detected is from northern Sweden. Plasma levels of both A $\beta$ 42 and A $\beta$ 40 were lower in mutation carriers compared to healthy family members, and the A $\beta$ 42 concentration was reduced in media from cells transfected with APP<sub>E693G</sub>. Furthermore, *in vitro* studies showed that the A $\beta$  peptide with the Arctic mutation (A $\beta$ 1-40Arc) had a high propensity to form protofibrils. Taken together, these data suggest that in carriers of the Arctic mutation, an alternate pathogenic mechanism for AD operates, in which increased A $\beta$  protofibril formation may be a primary event.

**RESULTS**

**Clinical description and genetic analysis**

The family carrying the Arctic mutation spans over 4 generations (Fig. 1b) and clinical information was available on 11 affected cases in 3 generations. An autosomal dominant pattern of inheritance was seen in the family with a mean age of onset at 57 ± 2.9 years (range 54–61 years). Clinical examination, neuropsychological testing, brain imaging (computed tomography or magnetic resonance imaging) and EEG were used in evaluating patients according to DSM-IV criteria. Clinical history was typical for AD, with a slow, insidious progression and decline in memory for recent events as the first presenting symptom. Signs of strokes or vascular lesions were not found on brain imaging in seven investigated patients.

The family carrying the Arctic mutation was screened for mutations in exons 16 and 17 of the APP gene by single strand conformation polymorphism analysis (SSCP). An abnormal mobility pattern was observed in exon 17. Sequencing revealed

an A→G nucleotide substitution, representing a glutamic acid to glycine substitution at APP codon 693 (E693G), corresponding to position 22 in the A $\beta$  sequence. The mutation was fully penetrant; no escapes were found. Two-point linkage analysis was performed between the mutation and affection status in the family, with an age-dependent penetrance, giving a lod score of 3.66 at recombination fraction 0.00. The E693G mutation was previously reported in one patient in the US with a Swedish origin, in whom neuropathological examination revealed neuritic plaques and neurofibrillary tangles, confirming the diagnosis of AD<sup>25</sup>.

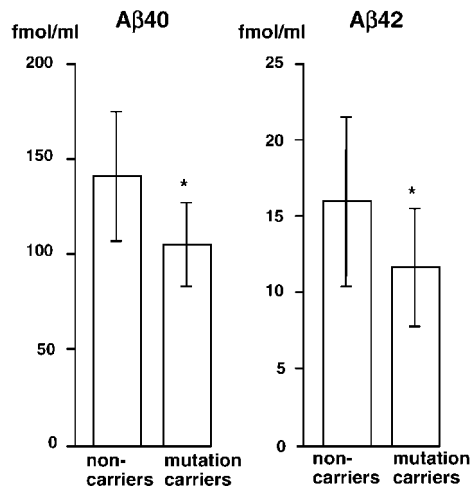
Investigations have revealed that this subject is likely to be a member of the Arctic kindred (T.D. Bird, personal communication).

**Decreased A $\beta$  plasma levels in Arctic mutation carriers**

Pathogenic APP mutations affect APP processing, as reflected in an increase of either total A $\beta$  or A $\beta$ 42 in the plasma of affected family members<sup>6</sup>. We investigated whether the Arctic mutation also affected A $\beta$  plasma levels. Plasma from 9 mutation carriers, of whom 4 were symptomatic, and from 11 non-carriers in the family was analyzed by well-characterized sandwich enzyme-linked immunosorbent assay (ELISA) systems detecting A $\beta$ 42 (BAN50/BC05) and A $\beta$ 40 (BAN50/BA27)<sup>26</sup>. To prove that the Arctic mutation did not change any of the antibody recognition sites, A $\beta$ 1-40wt and A $\beta$ 1-40Arc peptides were tested and found to be recognized equally well. However, the antibodies BNT77 and 4G8, both raised against epitopes in the middle of the A $\beta$  protein, had reduced affinity for the Arctic peptide; thus, they were not used. The A $\beta$ 42 plasma concentration was 11.7 ± 3.9 fmol/ml in mutation carriers, 27% less than the concentration in non-carriers, 16.0 ± 5.6 fmol/ml ( $p = 0.04$ , Fig. 2). The A $\beta$ 40 plasma concentration was 105 ± 22 fmol/ml, 26% less than the concentration in non-carriers, 141 ± 34 fmol/ml ( $p = 0.01$ , Fig. 2). The A $\beta$ 42/40 ratio was calculated for each individual, but no significant difference between mutation carriers and controls was found ( $p = 0.13$ ).

**A $\beta$ 42 is reduced in media from Arctic cells**

The effect of the Arctic mutation on A $\beta$  formation was further investigated *in vitro* in transiently transfected HEK293 cells. APPwt was compared to the Arctic (APP<sub>E693G</sub>), Dutch (APP<sub>E693Q</sub>), Italian (APP<sub>E693K</sub>) and Flemish (APP<sub>A692G</sub>) mutations (Fig. 1a). Constructs containing the Swedish double mutation (APP<sub>Swe</sub>) and one APP mutation at codon 717 (APP<sub>V717F</sub>), both with well-studied APP processing characteristics<sup>1</sup>, were used as positive controls. Media were conditioned and analyzed for A $\beta$  levels by the



**Fig. 2.** Plasma Aβ levels in the family carrying the Arctic mutation. Plasma from 9 mutation carriers and 11 non-carriers in the family were analyzed by end-specific ELISAs. A significant decrease (\*) of Aβ42 ( $p = 0.04$ ) and Aβ40 ( $p = 0.01$ ) was observed in carriers of the Arctic mutation.

same Aβ42- and Aβ40-specific sandwich ELISA systems as used for human plasma (Table 1). Care was taken to obtain similar APP expression between different mutations and experiments, which resulted in almost identical APP levels (data not shown). In accordance with previous reports<sup>27</sup>, Aβ42 and Aβ40 protein concentrations in media were increased 4–5 times by the Swedish mutation. Increased Aβ42 levels were seen in media by the APP717 mutation, leading to an approximately three-fold increase in the Aβ42/40 ratio, in agreement with data previously reported<sup>26</sup>. All mutations located at codon 693 (Arctic, Dutch and Italian) showed the same effect on Aβ levels: the Aβ42 concentration was significantly lower, whereas Aβ40 levels were similar to that in APPwt-transfected cells. Consequently, the Aβ42/40 ratio showed a 22–33% reduction (Table 1). This finding was in contrast to the APP692 Flemish mutation, which caused increased levels of both Aβ42 and Aβ40 in conditioned media, producing a slight increase in Aβ42/40 ratio, in accordance with previous reports<sup>26</sup>.

### Radiosequence analysis

Radiosequence analysis was done to exclude the possibility that the Arctic mutation caused an increase in the amount of N-terminally truncated forms of Aβ, which might not be detected by the BAN50 ELISA. To increase Aβ levels, hybrid APP mutants were used containing the Swedish mutation alone or the Swedish mutation together with the Arctic mutation. The 4-kD Aβ proteins produced by each sample were almost identical, yielding peaks of radioactivity (cycles 4, 19, 20) corresponding to Aβ beginning at Asp 1 (Fig. 3a and c). Both p3 bands produced profiles of radioactivity consistent with APP cleavage events occurring near the α-secretase site (Lys<sup>16</sup>–Leu<sup>17</sup>). For example, most of the protein produced from APP-Swe starts at Val 18 but peptides starting at Leu 17 and Phe 19 could also be observed (Fig. 3b). In the p3 sample from APP-Swe/Arc, peptides started primarily at Val 18 and Phe 19. In addition, a smaller peak was observed at cycles 9 and 10, corresponding to Aβ beginning at Glu 11 (Fig. 3d). All of these species are observed commonly in samples from HEK cells<sup>10,11</sup>.

### Aβ fibril formation

We investigated the effect of the Arctic amino acid substitution (Glu<sup>22</sup>→Gly) on amyloid fibrillization kinetics. Size exclusion chromatography (SEC) analysis of freshly dissolved Aβ1-40wt produced a

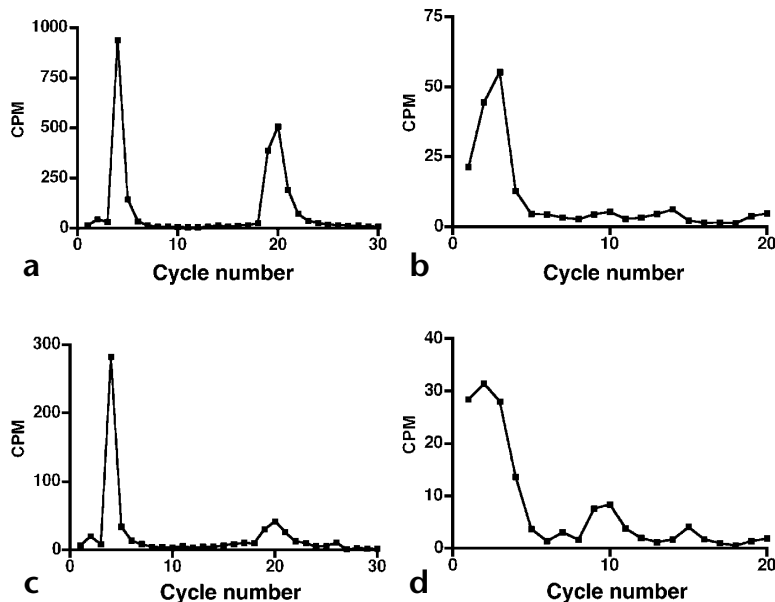
single peak at a retention time of approximately 20 minutes (Fig. 4a). This peak represented the monomeric/dimeric form of Aβ1-40wt<sup>12</sup>. With increasing incubation time, a second distinct peak appeared in the gel-excluded fraction with a retention time of about 12 minutes. This earlier peak contained protofibrils (Fig. 4b and c), as verified by ultracentrifugation and by negative stain and transmission electron microscopy (TEM) of Aβ1-40wt (data not shown), in line with previous findings<sup>12</sup>. Similar retention times were obtained for the Aβ1-40Arc peptide (Fig. 4d–f). However, Aβ1-40Arc generated protofibrils much faster and in larger quantities than Aβ1-40wt (Fig. 4). The monomeric/dimeric Aβ1-40Arc peak declined in parallel with the growth of the protofibrillar peak (Fig. 4d–f). The maximum concentration (111 μM) of Aβ1-40Arc protofibrils was observed at 6.5 hours. Kinetic studies up to 48 hours showed that Aβ1-40wt generated a small quantity of protofibrils with a maximum concentration at 25 hours (Fig. 5a). In contrast, a rapid formation of protofibrils was seen with the Aβ1-40Arc within the first five hours of incubation with a simultaneous rapid decline in the concentration of the monomeric/dimeric peptide (Fig. 5b). Despite the dramatic kinetic difference between Aβ1-40wt and Aβ1-40Arc, there was no detectable difference in fibrillization rate. Because carriers of the Arctic mutation are heterozygotes, they produce both Aβwt and AβArc. Assuming equimolar *in vivo* production, the kinetics of protofibril formation was studied in a 1:1 mixture of Aβ1-40wt and Aβ1-40Arc. This mixture of peptides showed kinetics that were intermediate to the single peptide curves (Fig. 5c).

The fibrillar and protofibrillar morphology of Aβ1-40Arc in sedimented samples from kinetic studies was confirmed by negative stain and TEM. Many Aβ1-40Arc fibrils displayed larger diameters (10–18 nm) relative to the Aβ1-40wt samples, due to intertwining of several thinner fibrils (Fig. 6b). Protofibrils could also be discerned in the sedimented samples (Fig. 6). The presence of protofibrils was confirmed in parallel from the same preparation using SEC. Many longer and straighter protofibrils were found in the Aβ1-40Arc preparation, relative to the wild-type sample, although curved variants were also found (Fig. 6b and c). Similar to the Aβ1-40wt, mesh-works of curved Aβ fibrils could be seen

**Table 1. Aβ42/40 ratio and Aβ42 and Aβ40 levels in conditioned media from transiently transfected HEK293 cells.**

APP constructs	Aβ42/40 ratio (%) ± s.d.	Aβ42 ± s.d. (fmol/ml)	Aβ40 ± s.d. (fmol/ml)
APPwt	9.6 ± 0.7	13.8 ± 1.0	144 ± 6
Arctic (E693G)	7.5 ± 0.5*	11.2 ± 0.6	149 ± 3
Dutch (E693Q)	6.6 ± 0.6*	9.6 ± 0.7	147 ± 12
Italian (E693K)	6.4 ± 0.6*	8.0 ± 0.7	126 ± 17
Flemish (A692G)	11.7 ± 1.6*	27.0 ± 2.0	232 ± 25
Swedish	8.8 ± 0.9	59.1 ± 4.9	675 ± 59
V717F	27.4 ± 1.7*	24.4 ± 2.4	89 ± 11
Mock (vector only)	7.2 ± 2.4	2.1 ± 1.0	28 ± 5

\* $p = 0.004$  in comparison to APPwt.



**Fig. 3.** Radiosequencing of Aβ <sup>3</sup>H-phenylalanine-labeled Aβ and p3. We sequenced 4-kD bands from APP containing the Swedish mutation (a) or the Swedish/Arctic mutation (c) and corresponding 3-kD bands with the Swedish (b) or the Swedish/Arctic mutation (d). The peaks at cycle numbers 4, 19 and 20 in (a) and (c) correspond to the positions of phenylalanine residues in Aβ beginning at Asp 1. Differences between the Swedish and Swedish/Arctic samples in the relative heights of the peaks at cycles 4 and 19/20 are related to sequencing chemistry. The differences do not bear on the conclusions reached in the experiment. The major peaks in (b) and (d) also originate from the two phenylalanines at positions 19 and 20 in Aβ. In these cases, however, the proteins start primarily at Val 18, Phe 19 or Phe 20.

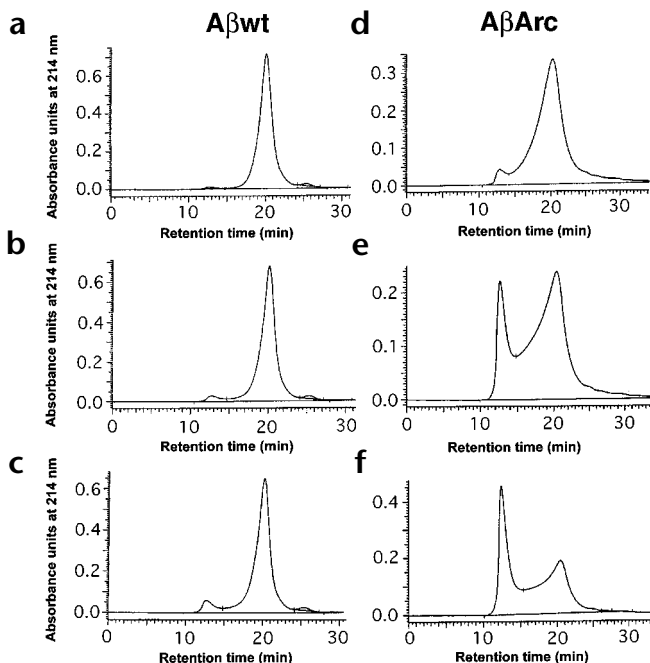
in the Aβ1-40Arc preparations. These are likely to be assemblies formed through intermolecular association of many protofibrils.

**DISCUSSION**

We identified the first pathogenic mutation, designated the Arctic mutation, located within the Aβ protein domain of the APP gene, which produces a classic AD phenotype. Carriers of this mutation develop progressive dementia with clinical features typical of AD, but without the severe cerebral amyloid angiopathy that characterizes other mutations in the Aβ region of APP<sup>7,8,14,15</sup>. Aβ42 is elevated in plasma from subjects with APP717 mutations as well as from those with presenilin (PS) 1 and PS 2 mutations. In carriers of the Swedish mutation (APP KM670/671NL), both Aβ42 and Aβ40 were elevated in plasma<sup>6,28</sup>. On the basis of these findings, it has been suggested that overproduction of Aβ42 is a central event in the pathogenesis of AD<sup>1</sup>. However, inconclusive results on Aβ plasma levels in sporadic AD have been reported<sup>6,29-31</sup>. In the present study, Aβ42 and Aβ40 plasma levels were shown to be significantly decreased in carriers of the Arctic mutation. Low levels of Aβ were also observed in the youngest mutation carriers investigated, 20–30 years before the expected onset of the disease, suggesting a long period of biochemical abnormality before clinical onset. The other four intra-Aβ mutations identified (Dutch, Flemish, Iowa and Italian) give rise to a clinical phenotype different from AD. To our knowledge, there is no information on Aβ plasma levels available for these families.

*In vitro* studies of the effects of APP and PS mutations on APP metabolism correlate well with *in vivo* findings (for review, see ref. 32). Thus, the metabolic effects of the Arctic mutation were compared to those of the other intra-Aβ mutations in transiently transfected cells by ELISA measurements. Strikingly, all three

mutations at codon 693 (Arctic, Dutch and Italian) led to decreased Aβ42 concentrations in conditioned media, whereas increased levels of both Aβ42 and Aβ40 in media were found for the Flemish APP692 mutation. These results were verified by a second independent ELISA using monoclonal antibody 6E10 as capture antibody and end-specific polyclonal antibodies for detection (unpublished observations). This is consistent with previously reported *in vitro* effects of the Flemish and Dutch mutations<sup>9</sup>. Increased Aβ levels in conditioned media were reported for the Flemish mutation and reduced Aβ42/40 ratios were observed in cells transfected with the Dutch mutation. The reduced levels of Aβ could occur as a result of the BAN50 ELISA used, because this method does not capture truncated forms of Aβ. However, both Aβ40 and Aβ42 levels would be reduced in equal amounts if this were the case, whereas our results showed that only Aβ42 was reduced in the media. In addition, radiosequence analysis revealed that most of the Arctic Aβ is not N-terminally truncated. When the p3 band was sequenced, the Swedish/Arctic mutation produced increased amounts of protein beginning at Glu 11 compared to the amount produced by the Swedish mutation alone. Even though this protein does not contribute greatly to the total amount of protein



**Fig. 4.** Size exclusion chromatograms of Aβ. SEC profiles illustrating time-dependent growth of protofibril peak (left peak) and concomitant decline of monomeric/dimeric peak (right peak) for Aβ1-40wt (a–c) versus Aβ1-40Arc (d–f) at 5 (a, d), 45 (b, e) and 125 (c, f) minutes incubation. Initial peptide concentrations were 143 μM (Aβ1-40wt) and 138 μM (Aβ1-40Arc). Each SEC analysis was run for 35 min (x-axis). Chromatograms are from one experiment representative of four.

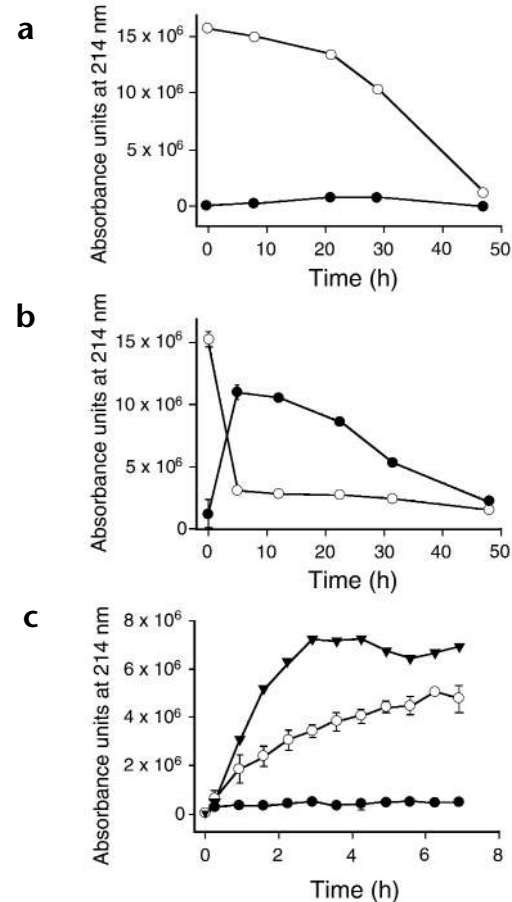


**Fig. 5.** Kinetics of A $\beta$ . Kinetics of A $\beta$ 1-40wt (88  $\mu$ M) (a) and A $\beta$ 1-40Arc (92  $\mu$ M) (b) monitoring decline in monomeric/dimeric peak area (○) and concomitant increase and subsequent decline of protofibrillar peak area (●). Each data point is the mean  $\pm$  s.d. of three injections in one experiment representative of three. Protofibril formation monitored for 7 h using 50- $\mu$ M peptide concentrations. A $\beta$ 1-40wt (●) and A $\beta$ 1-40Arc (▼) were compared to a 1:1 mixture of A $\beta$ 1-40wt:A $\beta$ 1-40Arc (○) (c). Maximum protofibrillar concentrations were 1.3  $\mu$ M (A $\beta$ 1-40wt), 14  $\mu$ M (A $\beta$ 1-40wt + Arc) and 21  $\mu$ M (A $\beta$ 1-40Arc). Each curve is the mean of three separate experiments (A $\beta$ 1-40Arc, one experiment)  $\pm$  s.d. (with 2–3 injections/point).

produced, it could be important as a nidus for A $\beta$  oligomerization. Proteins beginning at Glu 11 are more prone to aggregate and may be important in the initial events of AD pathogenesis<sup>13,33</sup>.

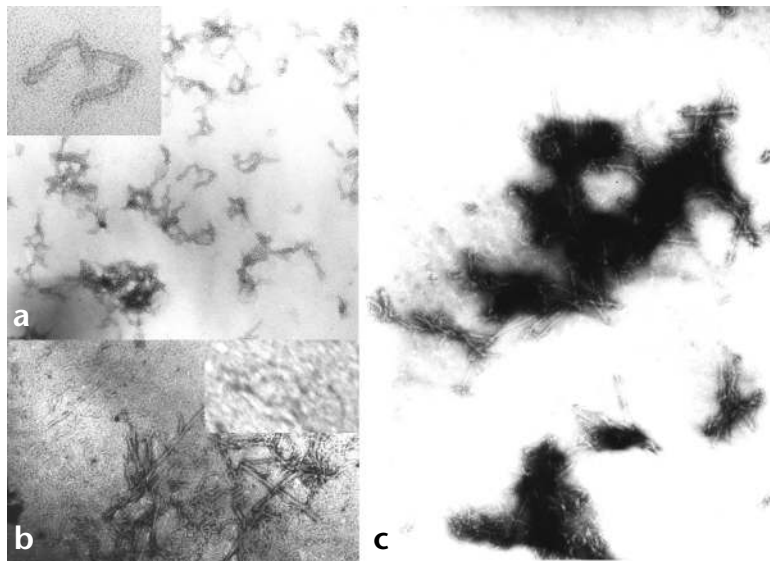
It is intriguing that mutations located at the same Glu693 codon of APP lead to such different phenotypes, whereas *in vitro* studies indicate a similar effect on A $\beta$  concentration in conditioned media. It is evident that A $\beta$  deposition is a central event in the pathological cascade, but why does the E693G Arctic mutation lead to AD, whereas the E693Q, E693K, A692G and D694N mutations lead to a different clinical phenotype with predominately vascular symptoms? The answer may lie within the A $\beta$  sequence. The KLVFF motif at position 16–20 in the A $\beta$  protein is central in the fibrillization process<sup>34,35</sup>. Mutations at position 21–23 in A $\beta$  are located close to the KLVFF region and could therefore affect the conformation of the peptide and its fibrillization process. Indeed, *in vitro* studies on the Dutch peptide have demonstrated that it polymerizes into protofibrils and then into fibrils significantly faster than the wild-type peptide<sup>12</sup>. However, at present, the understanding is limited concerning the differences in clinical expression by the pathogenic intra-A $\beta$  mutations.

The Glu to Gly single amino acid substitution at position 22 in the A $\beta$ 1-40Arc molecule caused a dramatic increase in rate and capacity to form protofibrils compared to the A $\beta$ 1-40wt peptide, even when protofibril formation was measured at equimolar amounts of wild-type:Arc. We propose that when A $\beta$ 42Arc and A $\beta$ 40Arc are formed *in situ* in the brain, they are more prone to be retained by cellular systems due to the accelerated drive to form protofibrils. Protofibril formation will significantly enhance the bulk and insolubility of A $\beta$ . This, in turn, could effectively accelerate disease initiation and progression. Recent cell biolog-



ical studies suggest that A $\beta$  is generated intracellularly (for review, see ref. 36). In addition, it has been reported that human neurons preferably accumulate A $\beta$ 42 over A $\beta$ 40<sup>37</sup>, consistent with studies showing that C-terminally extended proteins have a much higher tendency to form amyloid fibrils<sup>38</sup>. This offers an explanation to our finding of decreased A $\beta$ 42 concentration in media from cells transfected with mutations at APP codon 693. The effect of the Arctic mutation on A $\beta$ 42 fibrillization was not studied here because of technical problems associated with A $\beta$ 42 proteins, although it may be visualized indirectly by intracellular quantification of both A $\beta$ 40 and A $\beta$ 42 (ongoing studies). However, previous studies have shown that A $\beta$ 42 also forms protofibrils<sup>12</sup>; thus, we anticipate that A $\beta$ 42Arc

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**Fig. 6.** Electron micrographs of A $\beta$  protofibrils. High-magnification transmission electron micrographs, 104,000 $\times$  (insets, 630,000 $\times$ ), of sedimented negatively stained A $\beta$ 1-40wt (a) and A $\beta$ 1-40Arc (b, c) samples. A $\beta$ 1-40wt protofibrils are seen with their typical curved appearance and with lengths of  $\sim$ 30–60 nm (a). Both curved (arrows; b, c) and straight A $\beta$ 1-40Arc protofibrils can be discerned (c) in this preparation. The A $\beta$ 1-40Arc protofibrils appear longer than the A $\beta$ 1-40wt protofibrils, and a larger percent of straight, short protofibrils were noted. The diameters of the two protofibril types were similar ( $\sim$ 4–6 nm; insets). A $\beta$ 1-40Arc fibrils were also observed that exhibited large diameters ( $\sim$ 10–18 nm) resulting from intertwining of two or more thinner fibrils (b). Scale bars, 100 nm, 20 nm (insets).



will be more prone to form protofibrils than both the A $\beta$ 42wt and A $\beta$ 40Arc.

The Arctic form of AD may be an example of a rare disease variant that helps explain molecular mechanisms that may underlie more common forms of the disorder. Clinical, genetic and biochemical data support the hypothesis that the Arctic mutation produces AD through an alternative pathogenic mechanism. We propose that this mechanism involves rapid A $\beta$  protofibril formation leading to accelerated buildup of insoluble A $\beta$  intra- and/or extracellularly. (The vast majority of sporadic AD cases do not show increased A $\beta$  plasma levels<sup>6,30</sup>.) Thus, factors promoting protofibril formation should be considered in the pathogenesis of sporadic AD. Increased protofibril formation may also be operating in these more common forms of the disease. Indeed, our findings open new avenues for possible therapeutic intervention using drugs targeted at preventing protofibril formation<sup>24,39</sup>.

## METHODS

**PCR amplification and sequencing.** Venous blood was drawn into tubes containing EDTA, and DNA was prepared according to standard procedures. SSCP was done as described<sup>40</sup>. To sequence exon 17 of the APP gene, a 319-bp fragment was amplified with the following primers: 5'-CCTCATCCAAATGTCCCGTCATT-3' and 5'-GCCTAATCTCTCATAGTCTTAATCCAC-3'. Direct sequencing was done in both 3' and 5' directions using the same primers. The Arctic mutation was seen in one family and not in 56 controls or 254 cases with dementia.

**Linkage analysis.** Two-point lod score was calculated using Mlink from the Linkage software package (version 5.1) at each of the following recombination fractions 0.00, 0.10, 0.20, 0.30 and 0.40 ( $q$  males =  $q$  females). A single-locus model with an autosomal dominant inheritance was assumed, which was compatible with the inheritance as it appeared in the pedigree. A cumulative age-dependent penetrance was assigned from the known ages of onset in the family. Individuals were put into different liability classes depending on the age at onset (affected) or age at last examination (unaffected). The disease gene frequency and the marker allele frequency were estimated to be 0.001 and the phenocopy rate was set to 0.0001.

**A $\beta$ 42 and A $\beta$ 40 measured by ELISA.** The concentration of A $\beta$  in plasma and in the conditioned media was measured by the end-specific ELISAs, as described previously<sup>26</sup>. A $\beta$  was captured with BAN50. A $\beta$ 40 and A $\beta$ 42 were subsequently detected with BA27 and BC05, respectively. Plasma was spiked with synthetic peptides, revealing that both A $\beta$ 1-40Arc and A $\beta$ 1-40wt peptides were recovered by ELISA to the same extent. The data obtained was analyzed by non-parametric Mann-Whitney analysis.

**In vitro mutagenesis of APP cDNA and transfection of HEK293 cells.** The mutations were introduced to APP695 cDNA in pcDNA3 using QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, La Jolla, California). The mutated constructs were verified by sequencing. For the ELISA measurements, HEK293 cells were transfected with the different constructs using FuGENE 6 Transfection Reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were conditioned for 48 h in OptiMEM containing 5% newborn calf serum. The APP expression in the cells were investigated by western blot using monoclonal antibody 22C11 (Roche Diagnostics).

**Radiosequence analysis.** HEK293 cells were transiently transfected with APP containing the Swedish mutation alone or APP with both the Swedish and the Arctic mutation. Twenty-four hours after transfection, the cells were metabolically labeled for 48 h with 360  $\mu$ Ci/ml L-[2, 3, 4, 5, 6-<sup>3</sup>H]phenylalanine (Amersham Pharmacia Biotech, Buckinghamshire, UK). The media were collected and the A $\beta$  protein was isolated by immunoprecipitation using a polyclonal A $\beta$ 40-end specific antibody, K12Ger, followed by SDS-PAGE and electroblotting. The bands corre-

sponding to A $\beta$  and p3 were excised from the membrane and radiosequenced as described<sup>10</sup>.

**Synthetic peptides.** A $\beta$ 1-40wt was purchased from Bachem, Bübendorf, Switzerland or Biosource International/QCB (Camarillo, California) and A $\beta$ 1-40Arc from Biosource International/QCB. The peptides were trifluoroacetic salts; they were stored at -20°C. All other chemicals were of the highest purity available.

**A $\beta$  fibrillization studies.** Samples of each peptide were incubated without agitation at 30°C in 50 mM Na<sub>2</sub>HPO<sub>4</sub>·NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4) containing 0.1 M NaCl, for various time points. Initial peptide concentrations were 88–143  $\mu$ M, similar for both peptides in each experiment. After centrifugation (17,900 g for 5 min at 16°C) A $\beta$ 1-40 species, sampled from the supernatant, were separated using SEC. Chromatographic separation and analysis were done with a Merck Hitachi D-7000 LaChrom HPLC system, having a diode array detector model L-7455, a L-7200 model autosampler and a model L-7100 pump, coupled to a Superdex 75 PC3.2/30 column (Amersham Pharmacia Biotech). Samples were eluted at a flow rate of 0.08 ml/min (ambient temperature) using 50 mM Na<sub>2</sub>HPO<sub>4</sub> NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4) and 0.15 M NaCl. Chromatograms were obtained by measuring ultraviolet absorbance at 214 nm. Peak areas for monomeric/dimeric and protofibrillar A $\beta$  were integrated using Merck-Hitachi Model D-7000 Chromatography Data Station Software (Amersham Pharmacia Biotech). The mean of triplicate integrated peak values from the SEC measurements were used to generate each data point. In addition, a standard curve was produced by correlating integrated peak areas with peptide concentrations as determined by quantitative amino acid analysis. The concentrations of total (at  $t = 0$  h) and soluble peptides remaining in solution after centrifugation were calculated from the standard curve.

**Transmission electron microscopy.** A $\beta$  peptide samples were prepared and incubated as indicated for the kinetic studies, using higher peptide concentrations (617  $\mu$ M). After eight days, aggregated A $\beta$  species were sedimented using the same centrifugation parameters as described above. Buffer was removed and pelleted material was suspended in 50  $\mu$ l water using gentle sonication (2  $\times$  6 s). We applied 8- $\mu$ l samples to carbon-stabilized Formvar film grids (Ted Pella, Redding, California). Samples were negatively stained with 8  $\mu$ l uranyl acetate (1%; E. Merck, Darmstadt, Germany). Four grids were prepared for each sample and examined using a Philips CM10 TEM. We also examined samples from pellets sedimented during the kinetic experiments.

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