amino acids from the termini of polypeptides precludes our ability to discriminate whether initial APP processing occurs between AβP(Gln[15]–Lys[16]) or AβP(Lys[16]–Leu[17]).

These data show that a constitutive processing event, mediated by a protease we have called the APP secretase, results in (i) secretion of the ~100-kD soluble NH_2-terminal APP fragment terminating at AβP(Gln[15]) and (ii) the generation of a 9-kD membrane-bound COOH-terminal APP fragment whose NH_2-terminal sequence begins at AβP(Leu[17]). Thus, the APP secretase cleaves APP in the interior of the amyloid peptide sequence, thereby precluding AβP formation and deposition by this pathway in 293 cells.

Hypothetical pathogenic mechanisms linking aberrant APP processing to amyloid deposition have tended to emphasize the importance of inappropriate APP cleavage, either extracellularly or intracellularly, at the NH_2-terminus of AβP. However, one can now speculate that such inappropriate cleavage may lead to AβP deposition only in the absence of efficient constitutive APP processing. Thus, the key etiological event in AD may be inefficient secretase cleavage within the AβP sequence. The resulting pathogenic accumulation of some membrane-bound form of APP that cannot be effectively cleared from the cell may thus be the source of amyloid plaque formation.

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16. The secreted forms of APP_{695} and APP_{751} from APP-transfected 293 cells were purified essentially as described (*8*), with the addition of a final RPLC purification step. Briefly, the partially purified protein eluted from DEAE-Sepharose was loaded onto a Vydac C4 column (0.46 by 15 cm, 5 μm) in buffer A (0.18 M triethylammonium phosphate, pH 6.5) and then equilibrated to 20% buffer B (20% buffer A + 80% acetonitrile). The protein was eluted with a linear gradient from 20 to 100% buffer B over 80 min at a rate of 0.2 ml/min. The NH_2-terminal APP fragment eluted at approximately 59% buffer B (F. S. Esch *et al.*, unpublished data).
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Mutation of the Alzheimer's Disease Amyloid Gene in Hereditary Cerebral Hemorrhage, Dutch Type

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An amyloid protein that precipitates in the cerebral vessel walls of Dutch patients with hereditary cerebral hemorrhage with amyloidosis is similar to the amyloid protein in vessel walls and senile plaques in brains of patients with Alzheimer's disease, Down syndrome, and sporadic cerebral amyloid angiopathy. Cloning and sequencing of the two exons that encode the amyloid protein from two patients with this amyloidosis revealed a cytosine-to-guanine transversion, a mutation that caused a single amino acid substitution (glutamine instead of glutamic acid) at position 22 of the amyloid protein. The mutation may account for the deposition of this amyloid protein in the cerebral vessel walls of these patients, leading to cerebral hemorrhages and premature death.

HEREDITARY CEREBRAL HEMORRHAGE with amyloidosis of Dutch type (HCHWA-D) is an autosomal dominant form of amyloidosis. Patients with HCHWA-D develop recurrent intracerebral hemorrhages leading to death by the sixth decade of life (*1*). The disease has been described in four families from two coastal villages in the Netherlands. Three families (136 patients) were from Katwijk and one family (14 patients) was from Scheveningen (*1*). HCHWA-D is characterized by extensive amyloid deposition in the vessel walls of the leptomeninges and cerebral cortex. Parenchymal amyloid accumulations have also been found in some patients in plaques that resemble the immature senile plaques of Alzheimer's disease (AD) and sporadic cerebral amyloid angiopathy (CAA) (*2, 3*). The amyloid fibrils in HCHWA-D are formed by a 39–amino acid amyloid that is similar to the β protein in the amyloid deposits of AD and Down syndrome (DS) (*2, 4–7*).

Sequence analysis of complementary DNA (cDNA) clones that encode the amyloid β protein reveals that it is part of a larger precursor protein proposed to be a cell surface receptor (*8, 9*). Multiple forms of mRNA are derived by alternate splicing (*10*) of a transcript from a single gene located on chromosome 21 (*8, 9*). The primary structure of the 751–amino acid splicing variant of the amyloid β protein precursor is analogous to protease nexin II, a secreted protease inhibitor (*11*).

Genomic DNA and cDNA clones encoding the precursor amyloid β protein from AD, DS, and familial AD patients have been sequenced; the sequences were identical to those obtained from unaffected individuals (*9, 12, 13*). Although the genetic defect causing familial AD was localized to chromosome 21 (*14*), no linkage was found between familial AD and the amyloid β protein precursor gene, suggesting that this gene is not the site of the inherited defect underlying this disorder (*15*).

We hypothesized (*2*) that a defect in the amyloid β protein gene may cause the pathology associated with HCHWA-D. To sequence the amyloid β protein gene in HCHWA-D, we synthesized oligonucleotides based on known intron sequences that flank the two exons encoding the amyloid β protein (exons 14 and 15) (Fig. 1) (*12*). The oligonucleotides were used in the polymerase chain reaction (PCR) (*16*) to amplify the sequences that contain the two exons. The amplified fragments were subcloned into an M13 bacteriophage vector and sequenced by dideoxy chain termination (*17*).

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High molecular weight genomic DNA was isolated from brains of two female HCHWA-D patients from different families. One patient (DWvBO) died at the age of 48 years and the other (CVdV) at 67 years. PCR experiments with oligonucleotide primers designed to amplify exons 14 and 15 were repeated 9 and 15 times, respectively, for the two patients. The resulting sequences demonstrated that both patients had a single point mutation, cytosine (C) instead of guanine (G), at nucleotide 1852 of the amyloid β protein precursor (Pre $A4_{695}$) cDNA (*8*) (Fig. 1). In addition, both HCHWA-D patients had one normal allele. The same PCR and sequencing experiments were performed on genomic DNA isolated from a patient with AD (female of 83 years) and four unaffected individuals. All of these sequences demonstrated only the normal allele.

The single mutation we identified abolishes an Mbo II restriction site in the amyloid precursor protein gene of HCHWA-D patients. Loss of this site was detected by digestion of PCR-amplified genomic DNA with Mbo II restriction endonuclease. Four DNA fragments of 111, 92, 86, and 28 base pairs (bp) were observed after digestion of genomic DNA isolated from five unaffected individuals and one AD patient. An additional band of 203 bp was obtained from digestion of amplified DNA from the HCHWA-D patients (Fig. 2). These results indicated that the gene in both Dutch patients is polymorphic.

A second approach was taken to verify the existence of the mutation at position 22 of the amyloid β protein in Dutch patients with HCHWA and its absence in DNA isolated from unaffected individuals. An oligonucleotide that contains the mutation (5′-ACC CAC ATC TTG TGC AAA GAA CAC-3′) was hybridized to amplified DNA fragments from seven unaffected individuals, five patients with AD and two patients with DS, all of whom were not tested previously in this study. At low stringency (48°C), the oligonucleotide probe hybridized to all DNA tested. However, at high stringency (65°C) only cDNA containing the G to C base change and DNA isolated from the two HCHWA-D patients hybridized to the probe. With this technique, we recently found the same mutation in DNA isolated from blood cells of two related Dutch females who belong to a different pedigree with a history of hemorrhagic disease.

The G to C transversion results in the substitution of glutamine for glutamic acid at position 22 of the amyloid β protein. Both amino acids were found at this position in amyloid fibrils derived from leptomeninges of HCHWA-D patients (*18*).

There is a possibility that this mutation is polymorphic in certain populations, and not the cause of the specific amyloid deposition in HCHWA-D. However, polymorphism in the gene encoding the amyloid β protein has not been observed (*9, 12, 13*) and was not found in unaffected individuals in our study, suggesting that there is a relation between the presence of this mutation and cerebral hemorrhages in HCHWA-D.

The mutation in the structural gene for the amyloid β protein may be the primary defect in HCHWA-D, rendering the protein resistant to normal turnover or, alternatively, causing increased susceptibility to proteolytic breakdown and polymerization. A change in tertiary structure of the protein or the generation of a novel binding site may

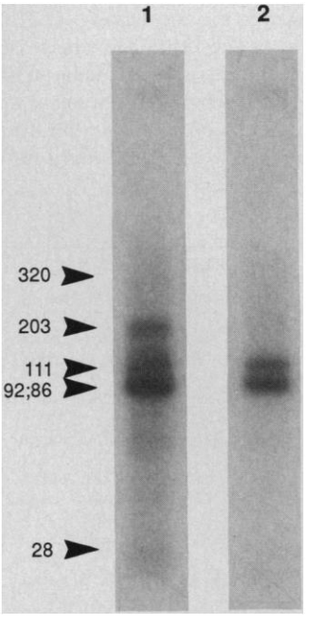

Fig. 2. Restriction patterns of DNA digested with Mbo II. Genomic DNA isolated from the brain of an HCHWA-D patient (lane 1) and an unaffected individual (lane 2) was amplified with the *Thermus aquaticus* (Taq) heat-stable DNA polymerase. Amplification reactions in a total volume of 100 μl contained 1 μg of DNA, 0.25 μM of each primer designed to amplify exon 15 (*12*), and 2.5 units of Taq polymerase in reaction buffer (Perkin-Elmer-Cetus). The samples were subjected to 25 cycles set at 94°C for 1 min to denature the DNA, 70°C for 2 min to anneal the primers, and 72°C for 3 min to extend the annealed primers. The reaction product was fractionated on a low melting point agarose gel (1%) from which a 320-bp fragment was excised. The isolated fragment was labeled with [α-^{32}P]deoxycytidine 5′-triphosphate by nick translation without deoxyribonuclease and digested to completion with Mbo II restriction endonuclease. The DNA was separated on a polyacrylamide gel (8.4%). The x-ray film was exposed for 10 min. The number preceding the arrowhead indicates base pairs; 320 ▶, position of the undigested fragment; 28 ▶, fragment seen on longer film exposures.

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caggcctagaaagaagttttgggtaggctttgtcttacagtgttattatttatgagtaa

                                                  1739
                                                   |
aactaattggttgtcctgcatactttaattatgatgtaatacag GT TCT GGG TTG
                                              Gly Ser Gly Leu
                                                 581

ACA AAT ATC AAG ACG GAG GAG ATC TCT GAA GTG AAG ATG GAT GCA
Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys Met Asp Ala

                                                          1839
                                                           |
GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT CAT CAT CAA AAA TTG
Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu
                                                         613

gtacgtaaaatAATTTACCTCTTTCCACTACTGTTTGT.....................


cctcatccaaatgtccccgtcatttaagaaatgaaattcttctaattgcgtttataaat

                                                          1840
                                                           |
tgtaaattatattgcatttagaaattaaaattcttttttcttaatttgttttcaag GTG
                                                         Val
                                                         614
            Gln
            C
TTC TTT GCA GAA GAT GTG GGT TCA AAC AAA GGT GCA ATC ATT GGA
Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly


CTC ATG GTG GGC GGT GTT GTC ATA GCG ACA GTG ATC GTC ATC ACC
Leu Met Val Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr

TTG GTG ATG CTG AAG AAG AAA CAG TAC ACA TCC ATT CAT CAT GGT
Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly

          1986
           |
GTG GTG GAG gtaggtaaacttgactgcatgtttccaagtgggaattaagactatga
Val Val Glu
        669

gagaattaggc
```

Fig. 1. DNA sequence of the two exons (14 and 15) that encode the amyloid β protein. Capital letters, exons; lowercase letters, flanking introns; dots, regions that have not been sequenced; underline, sequences used to synthesize the oligonucleotides; arrowheads, the cerebrovascular amyloid β protein (39 amino acids); broken line, Mbo II restriction sites in the amplified fragment that contains exon 15. Nucleotides and amino acids are numbered as for the corresponding Pre $A4_{659}$ cDNA (*8*).

facilitate the molecule's attachment to the cell's membrane. Thus, this mutation may accelerate amyloid deposition in the cerebral vessel walls.

Hereditary cerebral hemorrhage with amyloidosis described in Icelandic patients (HCHWA-I) is histopathologically and clinically similar to HCHWA-D (*19*). The brains of HCHWA-I patients demonstrate amyloid angiopathy; however, unlike HCHWA-D, the brain parenchyma is not affected, and the amyloid fibrils are derived from degradation of a variant of cystatin C, an inhibitor of cysteine proteinases (*20*). The variant cystatin C gene contains a point mutation that results in a single amino acid substitution (glutamine instead of leucine) (*21, 22*). Therefore, the amyloid precursor proteins in two inherited forms of cerebral amyloid angiopathies, HCHWA-D and HCHWA-I, are protease inhibitors that may be present in the circulation (*11, 21, 23, 24*) and have a substitution in their respective genes that gives rise to the same amino acid. The relation between the presence of glutamine in these proteins and the formation and deposition of amyloid fibrils in cerebral vessel walls is unknown.

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Platelet Coagulation Factor XI_a-Inhibitor, a Form of Alzheimer Amyloid Precursor Protein

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An inhibitor of coagulation factor XI_a was purified from serum-free conditioned medium of HepG2 liver cells. Platelets stimulated with thrombin or calcium ionophore (A23187) secrete a protein functionally and immunologically identical to the inhibitor, implying a role for this inhibitor in hemostasis. Analysis of the amino-terminal amino acid sequence and immunologic reactivity showed the inhibitor to be a truncated form of the Alzheimer's amyloid precursor protein that contains a Kunitz-type serine protease inhibitor domain and at least a portion of the amyloid β protein. It inhibits factor XI_a and trypsin with a K_i of 450 ± 50 pM and 20 ± 10 pM, respectively. Heparin (1 unit/ml) did not significantly effect inhibition of trypsin, but inhibition of XI_a was 15 times greater (K_i = 25 ± 15 pM) in the presence of heparin.

COAGULATION FACTOR XI CIRCULATES in plasma as the zymogen of a serine protease. Individuals deficient in factor XI suffer a mild to moderate bleeding diathesis characterized by mucosal and post-operative bleeding (*1*). Factor XI is activated by limited proteolytic cleavage through the action of the contact factors (factor XII_a, prekallikrein, and high molecular weight kininogen). Activated factor XI (XI_a) is a protease that consists of two identical disulfide-bonded subunits, each with a catalytic site, and functions in coagulation as an activator of factor IX. Platelets stimulated by thrombin or calcium ionophore (A23187) release a protein that reversibly inhibits XI_a but does not form XI_a-inhibitor complexes that are stable to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (*2, 3*).

Lysed platelets and the supernatant of A23187-treated platelets contain a protein of ~112,000 kD that binds [^{125}I]-XI_a (Fig. 1A). The serum-free conditioned medium of human hepatoma cells (HepG2) contains the same moiety (Fig. 1A). Approximately 1.3 mg of purified inhibitor (*4*) was recovered from 90 liters of serum-free conditioned medium. SDS-PAGE shows a predominant band at 112 kD (120 kD when reduced) with additional faint bands at 94, 66.5, and 55 kD (Fig. 1B). Antibodies to the purified factor XI_a-inhibitor (XI_aI) from HepG2 medium adsorbed the inhibitor released from platelets; thus, the HepG2 and platelet proteins are related antigenically (Fig. 2A).

The NH_2-terminal amino acid sequence of the 112-kD protein is X E V P T D G N A G L L A E P Q (*5*), which matches exactly residues 19 through 35 of the amino acid sequence predicted from the amyloid precursor protein cDNA sequence (*6–8*). When trypsin (rather than anhydrotrypsin) affinity chromatography was used in the purification, the minor bands present in Fig. 1B were considerably more abundant. The NH_2-terminal sequences determined from each band are identical to that of the 112-kD protein, suggesting that the smaller peptides resulted from proteolysis at the COOH-terminus, which may have occurred during the affinity chromatography.

A prominent protein found in the brains of patients with Alzheimer's disease is the amyloid β protein (amyloid A4 protein) (*9, 10*). The amyloid β protein represents a small part of a transmembrane amyloid precursor protein (APP) that, through alternative mRNA splicing, is produced in at least three different forms (*6–8*). The two longer proteins (APP_{751} and APP_{770}) contain a Kunitz-type serine protease inhibitor domain, whereas this domain is lacking in the shorter form containing 695 amino acids

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