Gerstmann-Sträussler-Scheinker disease (GSS) is an adult-onset neurodegenerative disorder (1, 2) that is inherited as an autosomal dominant trait and segregates with variant genotypes resulting from the combination of a pathogenic mutation (P102L, P105L, A117V, F198S, D202N, Q212P, and Q217R) and a common polymorphism at codon 129 (Met/Val) in the prion protein (PrP) gene (PRNP) (3–9). The pathological hallmarks of the disease are the accumulation of altered forms of PrP, termed PrPSc, and the deposition of PrP amyloid in the central nervous system (10–15). The main physicochemical properties that distinguish PrPSc from the normal cellular isoform (PrPC) are a high content of β-sheet secondary structure, insolubility in non-denaturing detergents, and partial resistance to protease digestion (16). In the presence of detergents, the protease-resistant core of PrPSc (termed PrPres) assembles into amyloid-like fibrils (16).

In previous studies we have determined the biochemical composition of amyloid fibrils extracted from brain tissue of patients with mutations F198S and Q217R in PRNP. The smallest amyloid subunit was found to correspond to a ~7-kDa PrP fragment spanning residues ~81 to ~150 (17, 18). Although this fragment did not include the region with the amino acid substitution, it originated from mutant molecules, suggesting that the codon 198 and 217 mutations are a dominant factor for amyloidogenesis (18).

The allelic origin of the altered forms of PrP involved in the pathologic process has been investigated in other genetic prion diseases including GSS P102L, fatal familial insomnia, and Creutzfeldt-Jakob disease (CJD) associated with point or insertion mutations (19–23). These studies showed that only mutant PrP is detergent-insoluble and protease-resistant in GSS P102L (19, 20), fatal familial insomnia, and CJD D178N (21), whereas both the mutant and wild-type proteins have these properties in CJD V210I (22) and CJD with five or six extra copies of the octapeptide repeat (21). CJD E200K illustrates an intermediate state, as the wild-type PrP is insoluble but pro-
EXPERIMENTAL PROCEDURES

Immunohistochemistry—Specimens of cerebral cortex were fixed in 4% formaldehyde and embedded in paraplast. Seven μm thick serial sections were stained with hematoxylin-eosin, and sections S or S were immunostained with antibodies to the N terminus, mid-region, and C terminus of human PrP. These included a rabbit antiserum to a synthetic peptide homologous to residues 23–40 (PrP-(23–40)) (18), the monoclonal antibody 3F4 which recognizes the epitope 109–112 (26, 27), and a monoclonal antibody to a synthetic peptide spanning residues 214–231 (SP214) (28). The antibodies were used at a dilution of 1:200. Before immunostaining, the sections were treated with 98% formic acid for 60 min. The immunoreactions were revealed using the EnVision Plus-horseradish peroxidase system for rabbit or mouse immunoglobulins (Dako, Carpinteria, CA) and diaminobenzidine as chromogen (29).

Isolation of Amyloid Core Extractions—Specimens of cerebral cortex and basal ganglia were homogenized in 9 volumes of lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 2 mM MgCl2, 3 mM NaN3 at a sample to buffer ratio of 1:25 (w/v), and subjected to collagenase digestion (Collagenase type 1, Sigma) at 37 °C for 30 min. The pellet was resuspended in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and centrifuged at 100,000 × g for 10 min. The supernatant was centrifuged at 16,000 × g for 45 min. To test the protease resistance of PrP, aliquots of supernatant corresponding to 100 or 250 μg of protein were digested with proteinase K (PK) at concentrations ranging from 5 to 50 μg/ml at 37 °C for 1 h. The digestion was carried out either in lysis buffer or in Laemmli’s sample buffer containing 2% SDS. After PK digestion, aliquots were deproteinized using recombiant peptide N-glycosidase F (New England Biolabs Inc., Beverly, MA) at 37 °C for 12 h, following the manufacturer’s instructions. The samples were fractionated by 12.5 or 16.5% Tricine/SDS-polyacrylamide gel electrophoresis (Tricine/SDS-PAGE) under reducing conditions, transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA), and probed with antibodies PrP23-40 (1:1000), 3F4 (1:50,000), and SP214 (1:200). To test the solubility of PrP in non-denaturing detergents, samples of cerebral cortex were homogenized in 9 volumes of 20 mM Tris, pH 7.5, 0.32 M sucrose, 5 mM EDTA, and centrifuged at 1000 × g for 10 min. The resulting supernatant was further centrifuged at 100,000 × g for 1 h. The pellet containing cell membranes was resuspended in lysis buffer, sonicated for 1 min, and centrifuged at 100,000 × g at 4 °C for 1 h to obtain detergent-soluble and detergent-insoluble fractions. Detergent-soluble and insoluble fractions were analyzed by Western blot using the antibody 3F4.

Isolation of Amyloid Cores—Amyloid plaque cores were isolated from 30 g of cerebral cortex. After removal of leptomeninges and large vessels, the tissue was homogenized with a Dounce homogenizer in buffer A (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 2 mM EGTA, 0.4 mM phenylmethylsulfonyl fluoride, 1% Triton X-100) at a sample to buffer ratio of 1:5 (w/v). The homogenate was sieved through 1-mm nylon mesh, and the filtrate was centrifuged at 10,000 × g for 20 min. The pellet was resuspended in buffer B (as for buffer A, with NaCl replaced by 0.6 M KI and the concentration of Triton X-100 reduced to 0.5%) and centrifuged at 10,000 × g for 20 min. The pellet was resuspended in buffer C (as for buffer B, with KI replaced by 1.5 M KCl) and centrifuged at 10,000 × g for 20 min. The pellet was washed three times in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and centrifuged at 70,000 × g for 30 min. The pellet was resuspended in 50 mM Tris-HCl, pH 7.5, 10 mM CaCl2, 3 mM NaN3 at a sample to buffer ratio of 1:25 (w/v), and subjected to collagenase digestion (Collagenase type 1, Sigma) at 37 °C for 18 h, using a 1:100 (w/v) ratio of enzyme to pellet. After centrifugation at 70,000 × g for 60 min, the pellet was resuspended and washed three times in 50 mM Tris-HCl, pH 7.5, loaded on a discontinuous sucrose gradient (1.0, 1.2, 1.4, 1.7, and 2.0 M sucrose in 10 mM Tris-HCl, pH 7.5), and centrifuged at 100,000 × g for 1 h. Each interface was collected, washed, and pelleted three times in 50 mM Tris-HCl, pH 7.5. Amyloid cores were identified for presence of amyloid fibrils by polarized light microscopy after Congo Red staining, fluorescence microscopy after thioflavine S treatment, and electron microscopy after negative staining with 2% aqueous phosphotungstic acid. Amyloid plaque cores were recovered in the 1.7 M sucrose fraction.

Purification and Characterization of Amyloid Proteins—The amyloid-enriched pellet was suspended in 99% formic acid and sonicated four times for 20 s. After addition of 2 volumes of distilled water, the sample was centrifuged at 10,000 × g for 10 min, and the supernatant was applied to a calibrated Sephadex G-100 column (1.2 × 120 cm) equilibrated with 3 M formic acid. Protein peaks were pooled and concentrated with a speed vacuum concentrator. The purity and molecular weight of the fractions were determined by 12.5% Tricine/SDS-PAGE under reducing conditions and by immunoblot analysis with the 3F4 antibody. The amyloid fraction was further analyzed with rabbit anti-sera to synthetic peptides homologous to residues 58–71, 90–102, 127–147, and 151–156 of human PrP (1:1000 dilution) as described previously (18). Gel filtration fraction 5 that contained the major amyloid fraction was further purified by high performance liquid chromatography (HPLC) on a reverse-phase C4 column (214TP104, Vydac) with a 0–80% linear gradient of acetonitrile containing 0.1% (v/v) trifluoroacetic acid, pH 2.5. The column eluents were monitored at 214 nm, and protein peaks were lyophilized. Following Tricine/SDS-PAGE and immunoblot analysis, aliquots of the HPLC-purified, PrP-immunoreactive peptides were dissolved in 25 mM Tris-HCl, pH 8.5, 1 mM EDTA, and incubated at 37 °C for 24 h with endoproteinase Lys-C (Roche Molecular Biochemicals) at an enzyme to substrate ratio of 1:30 (w/w). The peptides generated from enzymatic digests were separated by HPLC on a reverse-phase Delta-Pak C18 column (0.39 × 30 cm, Waters, Milford, MA) with a 0–70% linear gradient of acetoniitrile containing 0.1% (v/v) trifluoroacetic acid, pH 2.5.

Amino Acid Sequencing—Sequence analyses of the intact amyloid protein and peptides generated by enzymatic digestion were carried out on a 477A microsequencer, and the resulting phenylthiohydantoin amino acid derivatives were identified using the on-line 120A PTH analyzer and the standard program (PE Biosystems, Foster City, CA).

Matrix-assisted Laser Desorption Mass Spectrometry—N- and C-terminal fragments of the amyloid protein generated by endoproteinase Lys-C digestion were further purified by HPLC on a reverse-phase C4 column, and prepared for matrix-assisted laser desorption/ionization mass spectrometry using the dried droplet method (30). The matrix used was α-cyano-4-hydroxycinnamic acid (Sigma), which was purified by recrystallization. To produce the dried droplets, a saturated solution of matrix was prepared in 2:1 aqueous 0.1% trifluoroacetic acid:acetic acid at room temperature. The sample was added to this solution so that the final sample concentration was 1–10 μm. One-half microliter of the solution was placed on the probe of the mass spectrometer and allowed to dry. The sample was then ready for analysis. The mass spectrometer was a custom-built linear time-of-flight mass spectrometer with a 1-m flight tube with a wire ion guide operated at ~50 V DC. The ion source operated at +40 kV ion acceleration potential. The laser used was a PbSI-357 ND (Laser Science, Boston, MA) nitrogen laser (2 ns pulse width), which was focused onto a 200-μm diameter fiber optic, conducted down 1 m of fiber optic, and then imaged onto the matrix deposit with a doublet of positive lens (magnification = 1). The detector of the mass spectrometer was a combination of a microchannel plate (the input stage) and a discrete dynode electron multiplier (amplification stage). The custom data system uses a Lecroy 9350M oscilloscope configured for dynamic range enhancement to improve the fidelity of small signals.

Fibrillogenesis in Vitro—Peptides homologous to residues 23–41, 85–148, 191–205, and 217–228 of human PrP were synthesized by solid-phase chemistry and purified by reverse-phase HPLC as described previously (31). Purity was greater than 95%. The peptides were suspended in deionized water or in 20 mM Tris, pH 7.0, at a concentration of 1, 5, and 10 mg/ml. Following incubation at 37 °C for 1 h or at 20 °C for 1, 3, and 7 days, 50 μl of each sample were air-dried on gelatin-coated slides, stained with 1% aqueous thioflavine S, and analyzed by fluorescence microscopy. For electron microscopy, 5 μl of suspension were applied to Formvar-coated nickel grids, negatively stained with 5% (w/v) uranyl acetate, and observed in an electron microscope model 410 (Philips Electronic Instruments, Mahwah, NJ) at 80 kV.

RESULTS

The patient selected for this study was a 24-year-old man, whose clinico-pathologic and genetic profile has been reported in detail previously (24, 25). He started at age 20 with a pseudobulbar syndrome, followed by cerebellar, extrapyramidal, and pyramidal signs, and cognitive deterioration. Neuro-pathologic examination revealed massive deposition of PrP-immunoreactive deposits in the cerebral cortex, thalamus, basal ganglia, and cerebellum. Most PrP deposits exhibited the
tinctorial and optical properties of amyloid, i.e., birefringence after Congo Red staining and fluorescence following thioflavine S treatment. Genetically, the patient carried the silent A to G transition at the third position of PRNP codon 117 and a C to T transition at the second position of that codon, resulting in Ala → Val substitution. Furthermore, he was heterozygous Met/Val at codon 129, Val129 being in coupling phase with mutant Val117 (24).

To define the PrP species involved in the pathologic process, immunohistochemical and biochemical studies were undertaken. The immunohistochemical analysis of formalin-fixed, paraplast-embedded brain sections showed that the amyloid cores were immunoreactive with antibodies to the central region of the molecule, whereas antibodies to the N and C termini immunostained only the periphery of the cores (Fig. 1). Western blot analysis of homogenates of cerebral cortex and basal ganglia revealed the presence of a PK-resistant PrP fragment of ~7 kDa (Fig. 2A). This fragment was immunoreactive with antibodies to the central region while unreactive with antibodies to N- and C-terminal domains, and its electrophoretic mobility was unmodified by N-deglycosylation. The protease resistance of the 7-kDa peptide was observed under standard conditions (50 μg/ml PK in lysis buffer, 37 °C, 1 h) and was abolished by the addition of 2% SDS to the reaction buffer (data not shown). A similar 7-kDa fragment was present in brain homogenates before PK digestion, as a prominent component of a complex PrP pattern composed of −35, −33, and −28-kDa full-length PrP (i.e., di-, mono-, and nonglycosylated species), 21–22-kDa N-terminal-truncated peptides, and 16–17-kDa N- and C-terminal truncated fragments (Fig. 2A). As previously observed in the Indiana kindred of GSS with F198S mutation (32), the relative abundance of the low molecular weight fragment was not dependent upon the extent of amyloid burden. The analysis of detergent solubility showed that ~40% of full-length PrP partitioned in the insoluble fraction, whereas the 21–22-kDa N-terminal truncated peptides were mostly soluble, and the N- and C-terminal truncated fragments of 16–17 and 7 kDa were entirely insoluble (Fig. 2B). Digestion of the insoluble fraction with PK verified that only the 7-kDa peptide was protease-resistant (data not shown).

Amyloid cores were isolated from cerebral cortex by a procedure combining buffer extraction, sieving, collagenase digestion, and sucrose gradient fractionation. As shown by light and electron microscopic analysis, amyloid cores were the main component of the final preparations; minor contaminants included lipofuscin granules and small microvessel fragments. Proteins were extracted from amyloid plaque cores with formic acid and fractionated on a Sephadex G-100 column. Gel filtration yielded two major peaks, the void volume (fraction 1) and a low molecular weight peak (fraction 5) that was present as a broad band centered at ~7 kDa on Tricine/SDS-PAGE minigels. In addition, minor intermediary peaks (fractions 2–4) were observed in the chromatograms (data not shown). Immunoblot analysis of the fractions obtained by gel filtration showed that the 7-kDa band was immunoreactive with antibodies to PrP residues 90–102, 109–112, and 127–147 and was unreactive with antibodies to residues 58–71 and 151–165.
suggested that it corresponded to an internal fragment of the molecule (Fig. 2C).

Further purification of fraction 5 by HPLC on a reverse-phase C4 column yielded one major and several minor peaks (Fig. 2D). Immunoblot analysis showed that the major peak (Fig. 2D, peak 1) was immunoreactive with antibodies to PrP. Amino acid sequencing revealed that peak 1 was composed of a PrP fragment with a ragged N terminus, the major signals corresponding to residues 88 and 90. Peak 1 was digested with endoproteinase Lys-C; the enzymatic digest was fractionated by HPLC on a reverse-phase C18 column and the peptides subjected to microsequencing and laser desorption mass spectrometry. Only two cleavage sites (i.e. positions 106/107 and 110/111) resulting in three peptides were predicted on the basis of molecular weight, antigenic profile, N-terminal sequence analysis, and the known resistance to cleavage of Lys-Pro bonds at positions 106/107 and 104/105 of PrP. Microsequencing and mass spectrometry of the HPLC-purified peptides revealed a spectrum of N termini spanning residues 85–95, although Gly69, Gly70, and Gly72 were the predominant components (Fig. 3, A and B). Moreover, the C terminus was heterogeneous corresponding to Arg748, Glu152, or Asn153 (Fig. 3C). Although the patient was heterozygous for the Ala → Val mutation at codon 117 and the Met/Val polymorphism at codon 129, only Val was found at these positions (Fig. 4).

In addition to the ~7-kDa amyloid peptide, fraction 5 obtained by gel filtration chromatography also contained N- and C-terminal truncated PrP fragments corresponding to residues 23–41, 191–205, and 217–228. To investigate whether these fragments could contribute to amyloid fibril formation, we compared the fibrillogenic properties of homologous synthetic peptides with those of the synthetic amyloid peptide spanning residues 85–148. The peptides were incubated in deionized water or in 20 mM Tris, pH 7.0, for 1, 24, 72, and 168 h at a concentration of 1 mg/ml (Fig. 5A). Under the same conditions, peptide PrP-(191–205) also formed fibrillar structures, although morphologically different; they were more narrow and less regular in size, both in diameter (range ~4.5–10 nm) and length (100 nm to ~1–2 μm) (Fig. 5B). The PrP-(85–148) and PrP-(191–205) aggregates exhibited fluorescence after thioflavine S staining. Conversely, peptides PrP-(23–41) and PrP-(217–228) did not generate amyloid-like fibrils in vitro even at the highest concentration after 1 week.

**DISCUSSION**

Previous studies showed that a common feature of GSS patients with different PRNP mutations is the presence of low molecular weight N- and C-terminal truncated PrP fragments (9, 32, 33). These fragments can be detected in unprocessed brain homogenates, although they are more prominent after PK digestion, and their relative abundance is not dependent upon the amyloid burden (32). This suggests that they are generated in vivo by a distinct proteolytic pathway of PrP species associated with mutant GSS genotypes. Noteworthy, GSS patients with different PRNP mutations show some variation in the molecular mass of these internal PrP fragments, ranging from ~7 to ~15 kDa (9). In our patient we found a ~7-kDa peptide that had physicochemical properties of PrP75 species found in prion diseases, i.e. insolubility in non-denaturing detergents and resistance to PK digestion (16). This is at variance with a previous report that brain tissue of GSS A117V patients contains little if any protease-resistant PrP (34). Notably, the degree of protease resistance of this peptide was lower than that of other GSS mutant proteins since, unlike analogous fragments from patients with F198S and Q217R PrP variants, it was completely degraded in the presence of SDS.

The analysis of PrP extracted from amyloid plaque cores showed that the amyloid protein corresponded to an ~7-kDa fragment spanning residues 85–153. Similar to previous studies on GSS F198S and Q217R, this fragment was derived from the mutant allele since only Val was found at position 117 and 129 (18). This finding is consistent with the observation that the detergent-soluble fraction from total brain homogenates
contained ~60% of full-length PrP which was protease-sensitive, whereas the remainder of full-length molecules as well as the N- and C-terminal truncated fragments of 16–17 and 7 kDa partitioned in the insoluble fraction. Mass spectrometric analysis revealed that the amyloid peptide had ragged N and C termini; in particular, the N terminus was remarkably heterogeneous, starting at each position between residue 85 and 95. The amyloid fraction also contained peptides corresponding to the 19 N-terminal amino acids of full-length PrP and two C-terminal fragments comprising residues 191–205 and 217–228. This finding was consistent with the observation that the periphery of the amyloid plaque cores was strongly immunoreactive with antibodies to N- and C-terminal domains. The copurification of these small peptides with the larger amyloid protein was likely due to insufficient resolution of the gel filtration column within this molecular weight range; alternatively, these peptides could have been bound to the amyloid protein. Collectively, these data suggest that in GSS A117V, mutant PrP is at least partially degraded by proteases in the extracellular compartment, with formation of a major amyloid peptide whose size and sequence is similar to that found in patients with different mutations. Other fragments generated by partial degradation of the amyloid precursor protein contribute to plaque formation and may also participate in amyloidogenesis, since we found that the peptide comprising residues 191–205 was able to assemble into amyloid-like fibrils in vitro.

Studies with cell-free translation systems containing endoplasmic reticulum-derived microsomal membranes have revealed that PrP may exist in multiple topological forms, including a secretory form that is fully translocated into the endoplasmic reticulum lumen and two transmembrane forms with opposite membrane orientation (35–37). A specific transmembrane form was found to be increased in transgenic mice expressing the A117V mutation and was detected in brain tissue of patients with GSS A117V (34). This form could be recognized by limited proteolysis of brain homogenates using PK at low temperature in the absence of ionic detergents; conversely, no protease-resistant PrP peptides were found under conditions used to detect PrP\textsuperscript{res} in other prion diseases. Based on these observations it has been proposed that the transmembrane form of PrP rather than PrP\textsuperscript{res} plays a central role in neuropathological changes observed in GSS A117V (34) as well as in other prion diseases (38). In the present study we found a remarkable accumulation of PrP amyloid in brain tissue by immunohistochemistry and substantial amounts of a PK-resistant, low molecular weight PrP peptide both in brain tissue homogenates and in purified amyloid fractions by West-
expressing low levels of the P101L mutation resulted in neurologic dysfunction and neuropathological changes consistent with GSS. Conversely, larger doses of a non-β-form of the same peptide failed to induce these changes, emphasizing the importance of protein conformation in disease initiation and propagation. Indeed, although the 7-kDa PrP amyloid fragment is a component of the minimal PrP sequence required for disease transmissibility, it is unlikely that this peptide per se might support an efficient interaction with PrP\(^{C}\) and PrP\(^{Sc}\) conversion due to its insoluble β-sheet secondary structure and aggregation state.

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A 7-kDa Prion Protein (PrP) Fragment, an Integral Component of the PrP Region Required for Infectivity, Is the Major Amyloid Protein in Gerstmann-Sträussler-Scheinker Disease A117V

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