Genetic influence on the structural variations of the abnormal prion protein

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Prion diseases are characterized by the presence of the abnormal prion protein PrP^{Sc}, which is believed to be generated by the conversion of the α-helical structure that predominates in the normal PrP isoform into a β-sheet structure resistant to proteinase K (PK). In human prion diseases, two major types of PrP^{Sc}, type 1 and 2, can be distinguished based on the difference in electrophoretic migration of the PK-resistant core fragment. In this study, protein sequencing was used to identify the PK cleavage sites of PrP^{Sc} in 36 cases of prion diseases. We demonstrated two primary cleavage sites at residue 82 and residue 97 for type 1 and type 2 PrP^{Sc}, respectively, and numerous secondary cleavages distributed along the region spanning residues 74–102. Accordingly, we identify three regions in PrP^{Sc}: one N-terminal (residues 23–73) that is invariably PK-sensitive, one C-terminal (residues 103–231) that is invariably PK-resistant, and a third variable region (residues 74–102) where the site of the PK cleavage, likely reflecting the extent of the β-sheet structure, varies mostly as a function of the PrP genotype at codon 129.

Prion diseases are neurodegenerative disorders that affect humans and animals (1, 2). In humans, they include Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome, fatal familial insomnia (FFI), and kuru. They can occur sporadically, be linked to mutations in the PrP gene, or be acquired by infection either from medical procedures, as in the iatrogenic CJD, or from the consumption of contaminated food. The last group includes kuru, transmitted through cannibalism, and the recently emerged new variant of CJD, which is believed to be acquired from cattle affected by bovine spongiform encephalopathy (3). Evidence indicates that diversity of the "prion strains," a central issue in prion diseases (4), is mediated by variations in the PrP^{Sc} structure rather than by mutations in an agent-specific nucleic acid (5–8). In human prion diseases, we have identified two major types of PrP^{Sc}, which are distinguished by a difference in size of the proteinase K (PK)-resistant core fragment (9, 10). After gel electrophoresis, type 1 migrates at 21 kDa, and type 2 migrates at 19 kDa. The difference in size results from the cleavage of PrP^{Sc} at different sites, which, in turn, is likely to reflect the distinct conformation or a different ligand interaction of the two PrP^{Sc} types. The type 1 and type 2 PrP^{Sc} core fragments generated by PK have been shown recently to be reliable markers of disease phenotype and to be in large part functions of the genotype at codon 129 of the PrP gene, the site of a common methionine/valine (M/V) polymorphism (10–12). These data suggest that PrP^{Sc} conformation may play a role in mediating the effect of the PrP genotype on the phenotypic expression. Although the PrP^{Sc} typing based on electrophoretic mobility is simple and effective in the routine diagnosis of prion diseases, it has a low resolution and provides limited information on the precise size and possible variety of the PrP^{Sc} fragments resistant to proteases.

In this study, we have determined the precise sites of PK cleavage by using N-terminal sequencing and mass spectrometry in 36 cases representing 14 groups of human prion diseases in the forms of sporadic, familial, and iatrogenic cases, as well as kuru and the new variant CJD.

Materials and Methods

Tissues. Brain tissues were obtained at autopsy from patients with prion diseases and were kept frozen at −80°C until use; 36 cases were phenotypically characterized for clinical and histopathological features, PrP^{Sc} immunohistochemistry, and the specific brain PrP^{Sc} types by immunoblot analysis as described (9, 10, 12, 13). The cases were subdivided according to the sporadic, familial, and acquired etiology. Because the methionine/valine polymorphism at codon 129 of the PrP gene has been shown to influence disease phenotype in human prion diseases (10, 13–18), efforts were made to include cases with different zygosity at codon 129.

Molecular Genetics. Genomic DNA was extracted from blood or frozen brain tissue. Genotyping of the *PRNP* coding region was performed as described (10, 15).

Immunoblot Analysis of Brain Extracts. Brain homogenate [10% (wt/vol)] was prepared in lysis buffer (100 mM NaCl/10 mM EDTA/0.5% Nonidet P-40/0.5% sodium deoxycholate/10 mM Tris, pH 7.4), and treated with PK at 50 μg/ml for 1 h at 37°C. The digestion was terminated by addition of 3 mM phenylmethylsulfonyl fluoride. Proteins were then denatured and incubated in the absence or presence of recombinant peptide *N*-glycosidase F (PNGase F) according to the manufacturer's protocol (Roche Molecular Biochemicals). Samples were separated by SDS/PAGE (12% gel) and analyzed on immunoblots with 3F4 mAb (19) recognizing an epitope of PrP109–112, as described (9, 20).

Purification of PrP^{Sc}. Brain tissues (1–10 g) were used for purification of PK-resistant PrP^{Sc} according to a published method (21) as modified (22). After the final sedimentation of PK-resistant PrP^{Sc} by ultracentrifugation, the pellet was denatured and treated with PNGase F. PrP^{Sc} was purified further by

Abbreviations: CJD, Creutzfeldt–Jakob disease; FFI, fatal familial insomnia; PK, proteinase K; PNGase F, peptide *N*-glycosidase F.

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micropreparative continuous elution SDS/PAGE (12% gel, Mini Prep Cell apparatus, Bio-Rad). Proteins were eluted at a flow rate of 70 μl/min and collected into 400-μl fractions. The fractions containing PrP^{Sc} were pooled and lyophilized. Immunoblot analysis and silver staining were used to monitor the purification throughout the procedure.

N-Terminal Protein Sequencing. Proteins were separated on SDS/12% PAGE minigels (Bio-Rad), transferred onto Problott membranes (Applied Biosystems), and visualized by Coomassie blue staining. N-terminal protein sequencing by automated Edman degradation was performed, essentially as described (23), at the ProSeq Microsequencing Facility (Boxford, MA) with an Applied Biosystems 477A Protein Sequencer. N-terminal sequencing typically proceeded for 20–30 cycles. Multiple N-terminal sequences were obtained by alignment of the experimentally determined amino acids at each cycle with the translated human PrP sequence (24) as described (25, 26). The repetitive yield of the Edman degradation was 95%. The initial yield for each sequence was derived by linear regression analysis of yields from amino acids at each cycle. The relative abundance of individual N-terminal sequence species was determined by comparison of their initial yields with the total and was rated in the order of weak (+, 5–10%), moderate (++, 10–30%), and strong (+++, >30%).

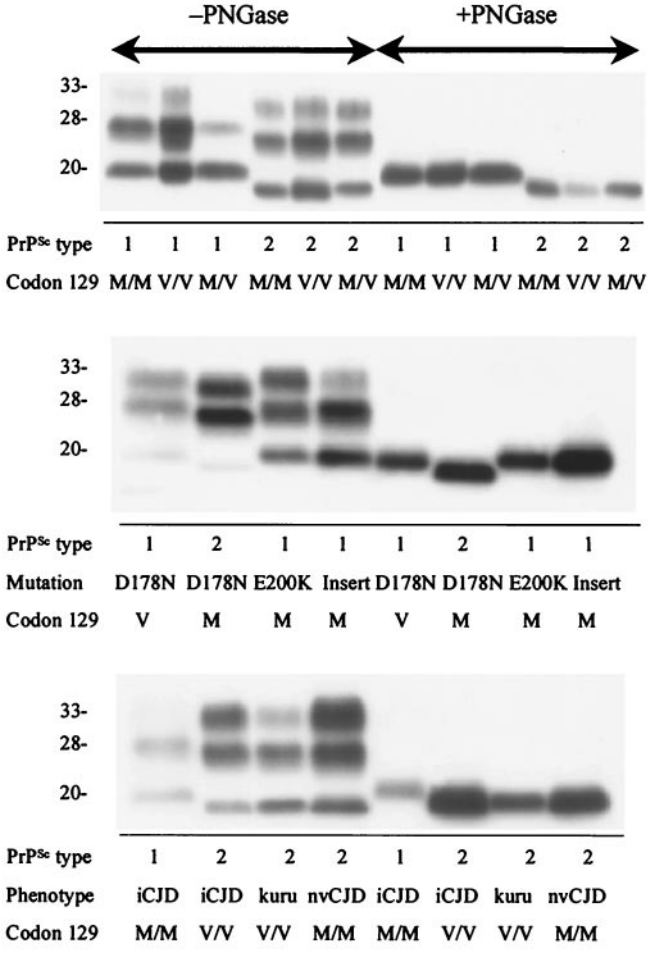

Fig. 1. Presence of type 1 and type 2 PrP^{Sc} in human prion diseases. Brain homogenate was treated with PK, followed by incubation in the absence (−) or presence (+) of PNGase F. PK-resistant PrP^{Sc} was detected on immunoblots by using 3F4 mAb. (*Top*) Sporadic cases with codon 129 genotypes on both alleles. (*Middle*) Familial cases with a point mutation at indicated codons or an insert mutation, coupled with the codon 129 on the mutant allele. (*Bottom*) Acquired cases including those of iatrogenic CJD (iCJD), kuru, and the new variant CJD (nvCJD). Their codon 129 on both alleles is also indicated. Molecular size markers are shown in kilodaltons on the left.

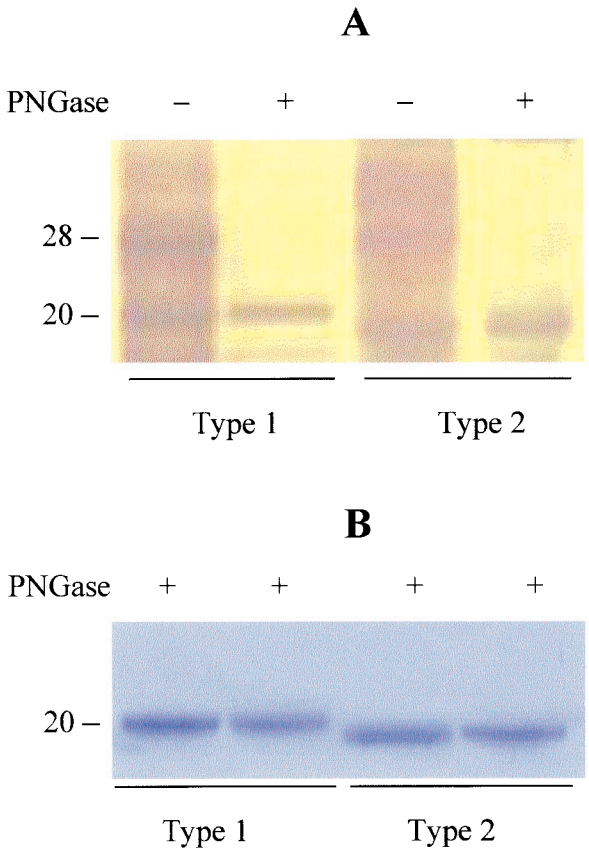

Fig. 2. Analysis of purified type 1 and type 2 PrP^{Sc}. (*A*) Silver staining of SDS/PAGE. (*B*) Coomassie blue staining of the sequencing membrane used for automated Edman degradation. Samples were either untreated (−) or treated (+) with PNGase F. Molecular size markers are shown in kilodaltons on the left.

Mass Spectrometry. The N-terminal peptides of PrP^{Sc} were generated by incubation of an aliquot of the purified PrP^{Sc} fraction with endoproteinase Lys-C and were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as described (25).

Results

Immunoblot analyses confirmed the presence of either type 1 (21 kDa) or type 2 (19 kDa) of the PK-resistant fragments of PrP^{Sc} in all cases (Fig. 1), according to the nomenclature proposed by Parchi *et al.* (10, 12). Because both the type 1 and 2 fragments had an intact C terminus but differed in the N-terminal region because of differential PK cleavage (9, 22), N-terminal sequencing analysis with automated Edman degradation was performed on the PK-resistant fragments after they were isolated and purified to apparent homogeneity (Fig. 2). A total of 36 cases divided into 14 disease groups were studied (Table 1). In selected cases (cases 3, 11, and 23), protein sequencing data were confirmed by mass spectrometry (ref. 25 and data not shown). In all but one of the cases examined, multiple signals appeared simultaneously in a given sequencing cycle from both type 1 and type 2, consistent with the presence of PrP^{Sc} variants with distinct N-terminal ends (Table 1). However, the distribution of the

Table 1. N-terminal variants of type 1 and type 2 PrP^{Sc} in human prion diseases

					N-terminal species‡								
Group	Case	Disease*	Codon 129†	PrP^{Sc} type	G74	G78	**G82**	G86	G90	G92	**S97**	W99	S103
A	1	Sporadic CJD	M/M	1		+	+++						
	2	Sporadic CJD	M/M	1		++	+++						
	3	Sporadic CJD	M/M	1		++	+++						
B	4	Sporadic CJD	M/V	1		++	+++		+		+		
	5	Sporadic CJD	M/V	1		++	+++				+		
C	6	Sporadic CJD	V/V	1			+++	++					
	7	Sporadic CJD	V/V	1			+++	++	+				
D	8	Iatrogenic CJD	M/M	1		++	+++						
	9	Iatrogenic CJD	M/M	1		++	+++						
E	10	CJD (D178N)	V/M	1		++	+++	++					
	11	CJD (D178N)	V/V	1		++	+++	+					
F	12	CJD (E200K)	M/M	1		+++	+++						
	13	CJD (E200K)	M/M	1	+	+++	+++						
	14	CJD (E200K)	M/M	1	+	+++	+++						
G	15	CJD (Ins. 4)	M/M	1		+++	+++						
	16	CJD (Ins. 5)	M/M	1		+++	+++						
H	17	Sporadic CJD	M/M	2						+	+++		+
	18	Sporadic CJD	M/M	2						+	+++	+	+
I	19	Sporadic CJD	M/V	2			+++				+++		
	20	Sporadic CJD	M/V	2						++	+++	++	+
	21	Sporadic CJD	M/V	2			++	++		+++	+++	+	
	22	Sporadic CJD	M/V	2			++			++	+++	++	++
	23	Sporadic CJD	M/V	2			++	++		++	+++	+	
J	24	Sporadic CJD	V/V	2				+	+	+++	+++	+	+
	25	Sporadic CJD	V/V	2				++	++	+++	+++	+	
	26	Sporadic CJD	V/V	2				+	++	++	+++	++	
K	27	Iatrogenic CJD	V/V	2				++	++	+++	+++		
	28	Iatrogenic CJD	V/V	2					+	+++	+++		
	29	Iatrogenic CJD	V/V	2					+	+++	+++		
L	30	Kuru	V/V	2				+	+	+++	+++		
M	31	New variant CJD	M/M	2						++	+++	++	+
	32	New variant CJD	M/M	2						++	+++	++	
	33	New variant CJD	M/M	2						+	+++	++	+
	34	New variant CJD	M/M	2						+	+++	+	
N	35	FFI (D178N)	M/V	2							+++		
	36	FFI (D178N)	M/V	2						+	+++	+	

*Familial cases are distinguished by the type of mutation in parentheses (normal residue followed by codon number and mutated residue); Ins. 4, insertion of four octapeptide repeats; Ins. 5, insertion of five octapeptide repeats.

†The residues specified by codon 129 on two alleles. For familial cases, they correspond to the residues on the mutant and normal alleles, respectively.

‡Only the first N-terminal residue is shown for each individual N-terminal sequence as determined by 20–30 cycles of Edman degradation. The N-terminal residues found in all type 1 or type 2 cases are indicated in bold. Relative signal abundance for the N-terminal sequences present is rated as follows: +, weak; ++, moderate; +++, strong.

variant PrP^{Sc} sequences among the cases examined was not random; it seemed to be related to the PrP^{Sc} type as well as to the genotype at codon 129 of the individual subjects. The N-terminal species starting at the Gly-82 residue (G82) was found in all cases associated with type 1 PrP^{Sc} (groups A–G), whereas the species beginning at Ser-97 (S97) was present in all cases linked to type 2 PrP^{Sc} (groups H–N). The additional PrP^{Sc} variants were present in different quantities among the various disease groups. Overall, the N terminus was more ragged in PrP^{Sc} type 2 than in type 1. Furthermore, in type 2, the N termini of the variant forms were farther away from and more often located at both sides of the common S97 N terminus (Table 1).

The influence of the polymorphism at codon 129 on the variations of the PrP^{Sc} N terminus was best demonstrated in sporadic CJD, probably because of the larger size of this group. The sporadic cases with the PrP^{Sc} type 1 and the 129 M/M genotype (Table 1, group A) had only one additional PrP^{Sc} variant, which begins at G78, at the N-terminal side of G82. In contrast, the subjects with the 129 V/V genotype (group C) had variants that started at residues G86 and G90, which are located at the C-terminal side of G82. The additional PrP^{Sc} variants associated with the 129 M/V genotype (group B) flanked G82 mainly at the N-terminal side, which begins at G78, and to a lesser extent at the C-terminal side extending to residue S97. Therefore, the methionine homozygosity favors a PK secondary cleavage that is more N-terminal than the principal cleavage at G82, whereas the presence of the valine codon shifts the secondary cleavages toward the C-terminal side of G82. All of the CJD sporadic cases associated with type 2 PrP^{Sc} had both elongated and truncated PrP^{Sc} variants flanking the invariable S97 cleavage site, regardless of the 129 genotype (groups H–J). However, in the subjects carrying the 129 M/M genotype (group H), the G92 was the only elongated variant. In contrast, the 129 M/V and 129 V/V subjects showed additional N-terminally elongated species that extended to residues G90 and G86 in the 129 V/V homozygous subjects and up to residue G82, thereby

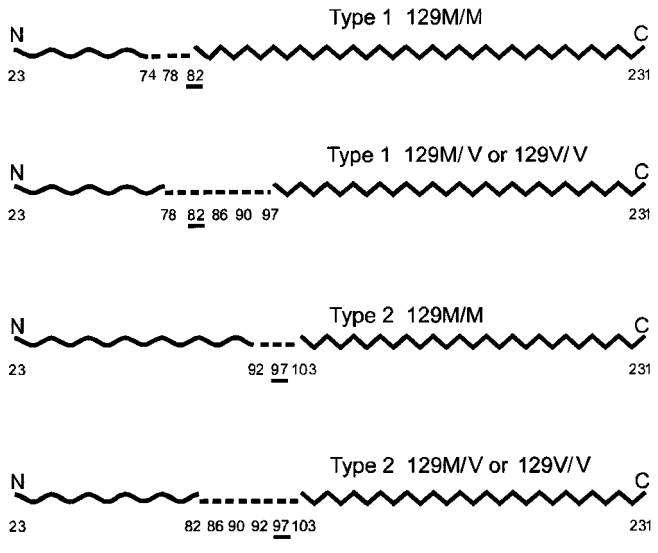

Fig. 3. Diagram of the structural variations of human PrP^{Sc} related to the PK cleavage patterns. Three distinct regions are represented along the full-length uncleaved PrP^{Sc} (residues 23–231): (*i*) a region that is PK-sensitive (wavy line); (*ii*) the ''variable'' region (broken line), which carries the multiple PK cleavage sites [residues 74–102; the primary (underlined) and secondary cleavages are indicated]; and (*iii*) a region that is invariably PK-resistant (residues 103–231, zigzagged line). The PK cleavage site, reflecting the size of the PK-resistant region and thus the conformation of PrP^{Sc}, is a function of PrP^{Sc} type and codon 129 genotype (indicated on the top and the right side of each line).

overlapping with type 1 PrP^{Sc}, in the 129 M/V heterozygous subjects (group I). Therefore, in sporadic CJD with type 2 PrP^{Sc}, the presence of the 129 V allele favors secondary cleavages toward the N terminus rather than the C terminus as in the corresponding cases with type 1 PrP^{Sc}. However, the variant forms seem to extend farther away from the main cleavage site in the subjects that are heterozygous (group I) than in those that are homozygous at codon 129 (group J). Therefore, the presence of one or two 129 V alleles results in a more ragged N terminus of PrP^{Sc} in both type groups. Furthermore, the 129 V alleles seem to move the secondary PK cleavage sites in a way that is opposite but partially symmetrical in the PrP^{Sc} types 1 and 2 associated with the sporadic form of CJD. This effect results in the overlap of the secondary fragments generated by the PK cleavage.

Similar findings were observed in the forms acquired by infection (compare group A with D, and group J with K and L). Of note, the new variant CJD, which is characterized by PrP^{Sc} type 2 and the 129 M/M genotype, showed PrP^{Sc} variant forms that are indistinguishable from those of the sporadic CJD with corresponding PrP^{Sc} type and 129 genotype. Also, in the familial form (groups E–G and N), the general pattern of primary and secondary cleavage was comparable to that of sporadic CJD with some exceptions. First, the presence of the 129 V allele seemed to cause less heterogeneity in the cleavage sites. Second, the heterogeneity seemed to be caused only by the codon 129 coupled with the mutation, whereas the codon 129 located on the normal allele seemed to have little or no effect (groups E and N). In addition, in two cases with E200K mutation, a longer variant beginning at residue G74 was also observed (group F).

Discussion

After our initial proposal (10) of two major types of PrP^{Sc} in sporadic CJD, Collinge *et al.* (11) have reported a classification of four types of PrP^{Sc} in which the type 3 variant is associated with the cases of iatrogenic CJD with at least one 129 V allele and type 4 is associated with the new variant CJD. According to Collinge *et al.* (11), type 4 and type 3 differ only in the glycosylation pattern but have the same gel mobility that is lower than that of the PrP^{Sc} type 1 and type 2 associated with sporadic CJD. However, in a subsequent study, we extended our analysis to the new variant CJD and iatrogenic CJD with 129 V/V and found that in both of these forms, the gel mobility of PrP^{Sc} was indistinguishable from that of the type 2 associated with sporadic CJD (12). Based on these data, we suggested that Collinge *et al.* had incorrectly matched our type 1 and type 2 with theirs and that our type 2 actually corresponds to their type 3, whereas their type 1 and 2 likely represent an artifactual subdivision of our type 1. More recently, Collinge and coworkers (27) have reported that the PrP^{Sc} identified by them as type 1 may result from a conformation acquired by PrP^{Sc} in the presence of copper.

The identification of the PK cleavage sites carried out in the present study offers a valuable approach to the study of PrP^{Sc} structural features, because it allows more accurate mapping of the PrP^{Sc} fragments than the previous assays based on gel migration. Nevertheless, only two primary PK cleavage sites, at G82 in type 1 and at S97 in type 2 PrP^{Sc} (Table 1), were present in all cases. These two cleavages generate PrP^{Sc} fragments of 149 and 134 residues containing the GPI anchor consistent with the molecular masses of 21 kDa and 19 kDa of type 1 and 2, respectively. Therefore, using a different methodology, we confirm that under standard conditions two major types of PrP^{Sc} are present in a series of 36 cases, which include the sporadic, genetic, and acquired forms of human prion diseases. Remarkably, the PK-resistant fragments of the PrP^{Sc} associated with the new variant CJD were indistinguishable from those of the PrP^{Sc} type 2 associated with sporadic CJD.

In addition to the two major cleavages at G82 and S97, there are a number of other cleavage sites clustered around them showing that both PrP^{Sc} types have ragged N termini. Such data reveal the complexity in the structural variations of PrP^{Sc}. However, these ''secondary'' cleavages are not randomly distributed. They are related to the PrP^{Sc} type and the genotype at codon 129, and follow a fairly defined pattern.

Although the limited number of cases available for the present study dictates caution, the finding that the familial cases and those acquired by infection have a PK cleavage pattern similar to that of the sporadic form suggests that the apparent etiology of the diseases has no major or direct effect on the conformation of human PrP^{Sc}. This finding is especially striking for the new variant CJD in which the secondary cleavages mirror those of the sporadic CJD with the same PrP^{Sc} type and 129 genotype. The lower N-terminal heterogeneity observed in familial cases (groups E and N) may be related to the findings that, in CJD (E200K), CJD (D178N), and FFI, PrP^{Sc} is derived exclusively from the mutant allele rather than from both alleles (22, 28) and therefore may lack the variability influenced by the second codon 129.

By analyzing the PK cleavage pattern, the present study allows for the identification of three main structural regions in human PrP^{Sc} (Fig. 3): an N-terminal region from residue 23 to residue 73 that is invariably sensitive to PK and most likely unstructured as in PrP^{C}; a C-terminal region comprising residues 103–231 that is consistently PK-resistant and is likely to have a high β-sheet content; and a third variable region encompassing residue 74 and residue 102 where the conversion from an unstructured conformation to the β-sheet conformation may occur at various sites, mostly as a function of the residue at position 129. The methionine and valine residues at position 129 seem to regulate the size of this variable region and, most likely, the extent of the β-sheet transformation in PrP^{Sc}. This finding provides a rationale for the striking effect of codon 129 polymorphism on phenotypic variability observed in all human prion diseases (10, 13–18).

Findings that show some similarity to those of this study also begin to emerge in Alzheimer's disease (AD). In familial AD variants characterized by the early clinical onset and short disease duration, N-terminally truncated forms of the pathogenic amyloid β-peptide (Aβ), the hallmark of AD, are overrepresented (29). These data suggest that as for the PK-resistant PrP^{Sc} fragment in prion diseases, Aβ species of different lengths are associated with different disease phenotypes.

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