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Biochemistry

Synthetic peptides corresponding to different mutated regions of the amyloid gene in familial Creutzfeldt–Jakob disease show enhanced *in vitro* formation of morphologically different amyloid fibrils

(spongiform encephalopathy/nucleation/fibrils/crystals/prion protein)

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ABSTRACT **We synthesized polypeptides corresponding to sequences encoded by normal and mutant alleles in the regions of codon 178 (Asp → Asn) and codon 200 (Glu → Lys) of the chromosome 20 amyloid gene that have been linked to familial Creutzfeldt–Jakob disease. Peptide suspensions from both regions spontaneously formed amyloid fibrils with different morphological characteristics and aggregation tendencies. Fibrillar arrays were denser and more profuse in mutant than in normal peptide suspensions and were even more marked when the homologous mutant and normal peptides were mixed together. Preparations from the region of codon 200 were in all cases more fibrillogenic than corresponding peptides from the region of codon 178. These *in vitro* observations support the hypothesis that amino acid changes from pathogenic single-allele point mutations in Creutzfeldt–Jakob disease may nucleate the *in vivo* folding behavior of the normal host protein to favor formation of insoluble amyloid fibrils.**

The human spongiform encephalopathies, of which the most prevalent example is Creutzfeldt–Jakob disease, are multisystem neurological degenerative disorders characterized histopathologically by widespread neuronal vacuolar changes and by the accumulation in brain tissue of an abnormal β-pleated amyloid form of a host-encoded protein that in some cases is deposited as microscopically visible amyloid plaques.

The familial occurrence of Creutzfeldt–Jakob disease, as well as other types of familial spongiform encephalopathy (Gerstmann–Sträussler–Scheinker syndrome and fatal familial insomnia), has been invariably associated with one of several different mutations in the gene that encodes the precursor protein, of which the two most important are point mutations at codons 178 (Asp → Asn) and 200 (Glu → Lys) (1). The resulting amino acid substitutions presumably alter the folding pattern of the full-length precursor protein to produce a β-pleated configuration that spontaneously polymerizes into amyloid fibrils. Also, the allelic disposition of polymorphic codon 129 (Met → Val) has been shown to influence the tempo and phenotypic expression of disease in families with pathogenic mutations in other codons (2).

These observations place the spongiform encephalopathies squarely within the broad pathogenetic framework of both cerebral and systemic amyloidoses, in which different host-encoded proteins accumulate as amyloid deposits and in which mutations in the encoding gene have been linked to familial forms of illness (3). *In vitro* experiments with synthetic peptides homologous to various regions of the native proteins have revealed spontaneous formation of amyloid fibrils (4–14), and for three of the proteins (β-protein in hereditary cerebral hemorrhage, Dutch type (15, 16); gelsolin in familial amyloidosis, Finnish type (17); and transthyretin in familial amyloidotic polyneuropathy (18), polypeptides homologous to mutated regions exhibit modified or enhanced fibrillogenic properties.

Table 1. Synthetic peptides used for *in vitro* amyloid fibril formation

Peptide	Amino acid sequence	Position	Solubility	
			Water	Saline
200glu	GENFTETDVKMMERVVEQM	195–213	++	+
200lys	GENFTKTDVKMMERVVEQM	195–213	++	++
178asp	YSNQNNFVHDCVNITIK	169–185	+	±
178asn	YSNQNNFVHNCVNITIK	169–185	+	±
129met	GAVVGGLGGYMLGSAMSRPI	119–137	±	±
129val	GAVVGGLGGYVLGSAMSRPI	119–137	±	±

In this paper, we show that synthetic peptides corresponding to the mutant sequences at codons 178 and 200 in Creutzfeldt–Jakob disease are more fibrillogenic than their normal counterparts, that the morphology of the codon-178 fibrils differs from that of the codon-200 fibrils, that the mutant peptides augment and accelerate amyloidogenesis of the normal peptides, and, finally, that peptides homologous to the alternative sequences at polymorphic codon 129 neither form fibrils spontaneously nor influence the formation of fibrils by the other peptides.

METHODS

Peptide Synthesis and Purification. Four peptides—200glu, 200lys, 178asp, and 178asn (Table 1)—were synthesized by using solid-phase techniques at the Center for the Analysis and Synthesis of Macromolecules, State University of New York, Stony Brook. Crude peptides were purified by HPLC with a 20-μm Aquapore C_8 column (250 × 10 mm) (Brownlee Lab) and a linear gradient of 0–66% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 2 ml/min. Purity was corroborated by amino acid sequence analysis on a model 477 protein sequencer, and the resulting phenylthiohydantoin derivatives were identified by using its on-line 120A phenylthiohydantoin analyzer (Applied Biosystems).

Two peptides, 129met and 129val, were synthesized by using solid-phase techniques at the Separation Science Unit, Massey University, Palmerston North, New Zealand (19). Crude peptides were purified by HPLC with a Synchroprep 30-μm C_4 column (250 × 10 mm) (SynChrom, Lafayette, IN). A gradient

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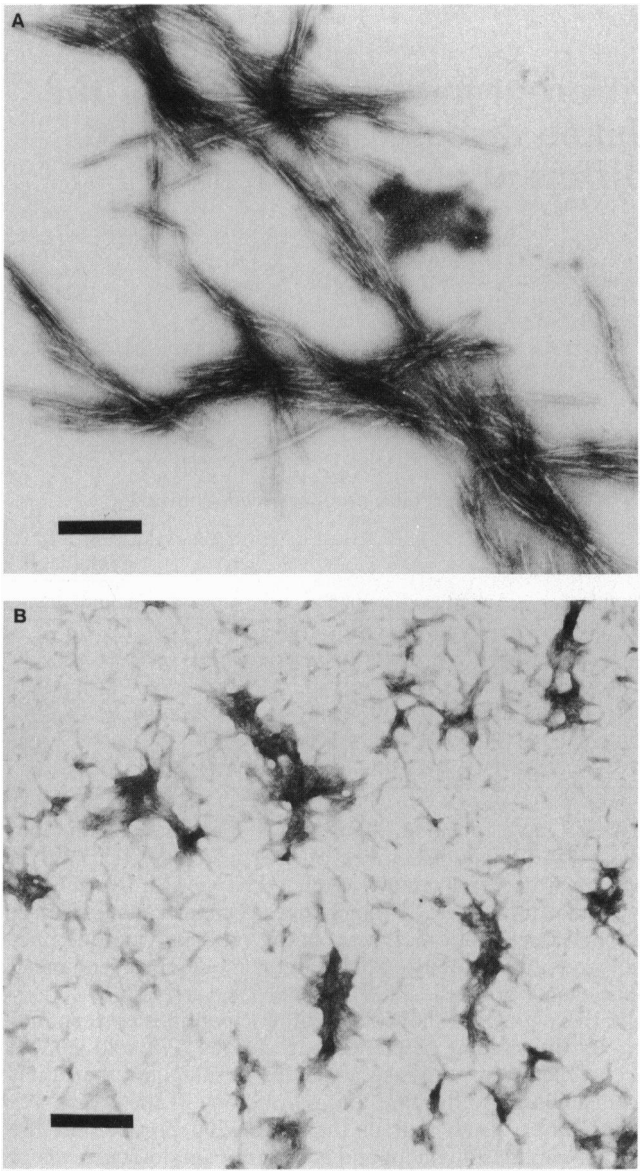

FIG. 1. Electron photomicrographs of aggregates of distinct rod-like fibrils 8–12 nm in width and several hundred nm in length, formed by mutant 200lys peptide (*A*) and scattered short, ill-defined fibrils 6–8 nm in thickness and <100 nm in length and clumps of indistinctly fibrillar material, formed by mutant 178asn peptide (*B*). (×52,500, bar = 200 nm; 2% uranyl acetate stain.)

of 10–30% acetonitrile in 0.1% trifluoroacetic acid was run for 2 hr at a flow rate of 2 ml/min to isolate the target peptides. Peptide composition was verified by amino acid analysis using an Alpha Plus 4151 instrument (Pharmacia LKB).

Sp20 (KMQQNGYENPTYKFFEQMQN), a 20-residue peptide homologous to the C terminal of all isoforms of the β-amyloid precursor protein (positions 676–695, APP_{695} numbering), was used as a positive control for spontaneous fibril formation under the conditions of the present experiment (unpublished observations).

Fibril Formation. A solution of each peptide at 10 mg/ml was prepared in either distilled water or 150 mM NaCl. After incubation at room temperature for 2 or 18 hr, a drop of each peptide solution was placed for 1 min on freshly glow-discharged 400-mesh Ni grids coated with either carbon or Formvar, then rinsed, and negatively stained with 2% uranyl acetate for 2 min. The grids were air-dried and evaluated by using a Zeiss EM10 electron microscope. The results with this series of specimens were confirmed in a second set of experiments in which solutions of peptides at 5 mg/ml were incubated for 18 hr.

Congo Red Staining. A drop of each peptide solution was placed on slides coated with bovine serum albumin, air-dried, fixed with 95% ethanol for 5 min, and stained with a solution of 1% Congo red/1% NaOH/80% (vol/vol) ethanol for 5 min. The slides were examined under bright-field and polarized light.

RESULTS

Peptides from the Area of Codon 200. Normal (200glu) peptide produced long, rod-like fibrils 8–12 nm in thickness and several hundred nm in length; only these fibrils showed moderate aggregation. Mutant (200lys) peptide (Fig. 1*A*) produced similar fibrils, but these fibrils had a stronger tendency to aggregate and covered noticeably more grid area than did the 200glu preparations. A mixture of both peptides produced very abundant and large fibrillar aggregates. The appearance of the fibrils in each preparation was similar at 2 hr and 18 hr, except that in the peptide mixture, the density of aggregated fibrils was greater at 18 hr.

Peptides from the Area of Codon 178. Normal (178asp) peptide produced shorter, somewhat narrower fibrils than did the codon-200 peptides, usually 6–8 nm in width and <100 nm in length, that formed dense roundish clumps of indistinctly fibrillar material. The fibrils in the mutant (178asn) peptide solution (Fig. 1*B*) were morphologically similar to 178asp, but their overall quantity was noticeably greater. The normal/mutant peptide mixture showed a pattern similar to the mutant peptide but displayed even larger amounts of fibrillar material.

Peptides from the Area of Polymorphic Codon 129. Neither of the peptides 129met or 129val, alone or mixed together, produced any fibrils. Also, when mixed with the codon-178 or codon-200 peptides, neither 129-codon peptide changed the morphologic patterns of the codon-178 or codon-200 fibrils.

Congo Red Birefringence. Codon 200 peptides showed distinct green birefringence under polarized light; the birefringence was more intense in preparations containing the mutant peptide or the normal/mutant peptide mixture than in the normal peptide solution (Fig. 2*A*). Codon 178 peptides were all less intensely birefringent than their codon-200 peptide analogs (Fig. 2*B*), but the normal/mutant mixture was brighter than either individual peptide solution. No birefringence was seen in either codon-129 peptide preparation.

A semiquantitative appreciation of the EM appearance and Congo red birefringency of each peptide preparation is summarized in Table 2.

DISCUSSION

We and others have previously shown that peptides homologous to various regions of amyloidogenic proteins form distinctive amyloid fibrils *in vitro* and that mutant peptides may be more fibrillogenic than their normal counterparts. The potentially important observation in this study is that mixtures of normal and mutant peptides from regions of amyloid protein corresponding to two different pathogenic mutations linked to Creutzfeldt–Jakob disease are more fibrillogenic than either of the component peptides alone. The significance of this observation lies in the fact that the familial forms of transmissible cerebral amyloidosis are invariably associated with heterozygous mutations in this gene, resulting in heterozygosity of the encoded protein species.

Although neither of the codon-129 peptides used formed fibrils, small shifts in peptide end points or lengths can spell the difference between failure and success: for example, peptides composed of amino acids 121–136 or 129–140 do not form fibrils (13), whereas a peptide composed of amino acids 127–147 forms excellent fibrils (14). More importantly, in view of the influence of codon 129 on the phenotype of patients with familial Creutzfeldt–Jakob disease associated

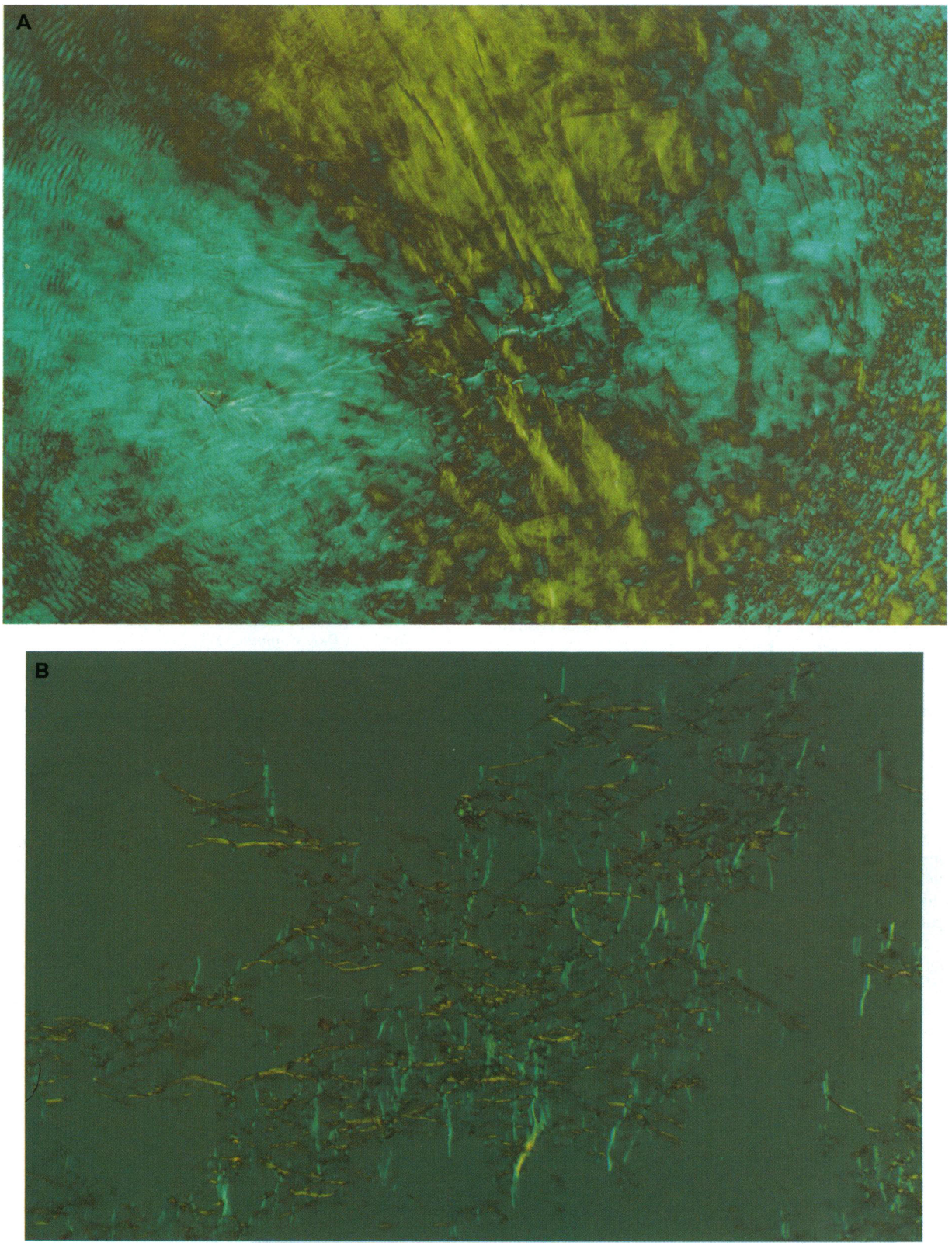

FIG. 2. Polarized light photomicrographs of fibrils formed by mutant 200lys peptide (*A*) and mutant 178asn peptide (*B*). (×1000; 1% Congo red stain.)

with the codon-178 mutation (2), neither peptide influenced either the morphology or speed of fibril formation in mixtures with the codon-178 peptides.

All fibril polymerization requires an initial nucleation, or pattern-setting association with the nucleant, followed by a phase of fibril growth, which is susceptible to steric interference by a large nucleant (an oligomer of the precursor or a heterologous or homologous microfibril). The demonstration of normal precursor molecules within amyloid deposits of mutant molecules in the genetically determined amyloidoses of familial amyloidotic polyneuropathy (20), hereditary cerebral hemorrhage (Dutch type) (21) and the Gerstmann–

Table 2. Fibril formation and characteristics of the synthetic peptides homologous to different normal and mutant regions of amyloid precursor protein

Peptide	Fibril formation		Birefringence
	Disperse	Aggregates	
200glu (normal)	+	+	++
200lys (mutant)	+	+++	+++
200glu + 200lys	+	++++	+++
178asp (normal)	+	+	±
178asn (mutant)	+	++	+
178asp + 178asn	+	+++	++
129met	−	−	−
129val	−	−	−

Sträussler syndrome (22) suggests that mutant precursor molecules are endowed with an increased amyloidogenic potential that can facilitate or augment the conversion to amyloid of the sluggishly amyloidogenic normal precursor. These molecules are either copolymerized with or absorbed onto amyloid fibrils of mutant protein.

A single protein molecule can be heterologously nucleated to form different crystalline morphologies and diverse patterns of growth by different minerals (23–25). The homologous nucleation using baby crystals of each specific morphology continues to nucleate distinct patterns of crystal growth—i.e., they breed true! Thus, we expect that our mutant polymer fibrils act *in vitro* as heterologous nucleants for fibril formation of the normal synthetic polymers and that the *in vivo* finding of normal/mutant mixtures in deposited amyloids results from the same phenomenon (3).

These *in vitro* peptide experiments point the way to the exploration of fibrillogenesis in cell cultures and experimental animals, which may eventually define the pathogenesis of amyloid formation in both spongiform and nonspongiform encephalopathies. However, it should not be forgotten that, in spite of their many pathogenetic similarities, the spongiform encephalopathies show one feature thus far absent from these other amyloidoses: transmissibility. Numerous primates and laboratory rodents inoculated with peptide suspensions and mixtures remain well 3 mo after inoculation, but in view of the potentially very long incubation periods in these diseases, it will be several years before a final assessment can be made.

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