

# Gelsolin-related Amyloidosis

## Identification of the Amyloid Protein in Finnish Hereditary Amyloidosis as a Fragment of Variant Gelsolin

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### Abstract

The Finnish type of familial amyloidosis is a systemic disease characterized by progressive cranial neuropathy, corneal lattice dystrophy, and distal sensorimotor neuropathy. Amyloid fibrils were isolated from the kidney and heart of a patient with Finnish amyloidosis. After solubilization, the amyloid proteins were fractionated by gel filtration and purified by reverse-phase HPLC. Complete amino acid sequence analyses show that the two amyloid components obtained are fragments of gelsolin, an actin-modulating protein occurring in plasma and the cytoskeleton. The larger component represents residues 173–243 and the minor component residues 173–225, respectively, of mature gelsolin. When compared with the predicted primary structure of human gelsolin a single amino acid substitution is present in amyloid: at position 15 of the amyloid proteins an asparagine is found instead of an aspartic acid residue at the corresponding position (187) in gelsolin. Antibodies to a dodecapeptide of the amyloidogenic region of gelsolin specifically stain the tissue amyloid deposits in Finnish hereditary amyloidosis. The results show that the amyloid subunit protein in Finnish hereditary amyloidosis represents a new type of amyloid that is derived from an actin filament-binding region of a variant gelsolin molecule by limited proteolysis. (*J. Clin. Invest.* 1991; 87:1195–1199.) Key words: familial amyloid polyneuropathy type IV • corneal lattice dystrophy • amyloidogenesis •  $\beta$ -fibrillogenesis • anti-gelsolin antibodies

### Introduction

Familial amyloid polyneuropathy (FAP)<sup>1</sup> syndromes are autosomal dominant disorders characterized by extracellular deposits of fibrillar protein with crossed  $\beta$ -pleated sheet conformation and a clinical syndrome of polyneuropathy. The FAP syndromes differ from each other with respect to distribution and degree of involvement of affected nerves and organs, age of onset, and ethnic origin. The Finnish type of familial amyloidosis (FAP type IV or Meretoja amyloidosis; McKusick No. 105120) is a systemic disease characterized by a distinct clinical

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1. Abbreviation used in this paper: FAP, Familial amyloid polyneuropathy.

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picture involving progressive cranial neuropathy, corneal lattice dystrophy, and distal sensorimotor neuropathy (1). In heterozygous patients, the disease has a late onset and is slowly progressive. By the age of 20 yr, corneal dystrophy is usually manifested, and by the age of 40 yr most patients have developed cranial neuropathy. Skin, renal, and cardiac manifestations also occur. In the rare, presumably homozygous patients the disease has a more severe course characterized by renal insufficiency (2). By the end of the 1970s more than 300 cases had been recorded in Finland (3). The disease is, however, not limited to Finland. Cases have also been described from the United States (4–6), Denmark (7), and the Netherlands (8).

The clinically different expression of the Finnish FAP, as compared with the FAP types I, II, and III and related syndromes (9), has raised the possibility of a unique amyloid fibril protein in this disease. Indeed, we showed that the amyloid fibril protein in Finnish FAP is different from all amyloid proteins identified so far, showing amino acid homology with gelsolin, an actin-modulating protein (10). The gelsolin nature of the amyloid in Finnish FAP was subsequently confirmed (11). The amyloid proteins accumulating in the kidney and heart have now been isolated, purified, and subjected to extensive structural studies. It is shown that the two major accumulating amyloid proteins are fragments of gelsolin in the regions of amino acids 173–243 and 173–225, respectively. At position 15 of the amyloid proteins an asparagine was found instead of the predicted aspartic acid residue in gelsolin (position 187; reference 12). A point mutation in the gelsolin gene causing the expression of the mutant asparagine-187 gelsolin molecule in Finnish FAP was recently reported from this laboratory (13).

### Methods

*Isolation and fractionation of amyloid.* Amyloid was isolated from autopsy samples of the kidney and the heart of a 71-yr-old man with Finnish hereditary amyloidosis. In the kidney the amyloid was primarily localized to the glomeruli; small amounts were also present in the larger blood vessels and the interstitium. In the cardiac tissue the amyloid was found in the arterial walls and to some extent in the perivascular tissue. For control purposes, Congo-red negative renal and splenic autopsy tissues were subjected to the same extraction procedure as the amyloid tissue. The renal amyloid was isolated by water extraction as described previously (10). For the isolation of cardiac amyloid formic acid extraction was used (14); after 11 saline washings, the sediment was sonicated in 75% formic acid (final concentration) for 1 h followed by centrifugation at 12,000 rpm for 1 h. The supernatants were lyophilized and dissolved in 6 M guanidine-HCl buffered to pH 8.5 with 0.5 M Tris-HCl containing 0.002 M EDTA and 20 mM dithiothreitol and incubated at 37°C for 3 h and then at room temperature overnight. After centrifugation, the supernatants were chromatographed on a Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (1.5 × 98 cm) using 5 M guanidine-HCl with 1 M acetic acid as eluent. The fractions were studied by gel electrophoresis (15–17% SDS-PAGE) and immunoblot (Western) techniques. The amyloid protein

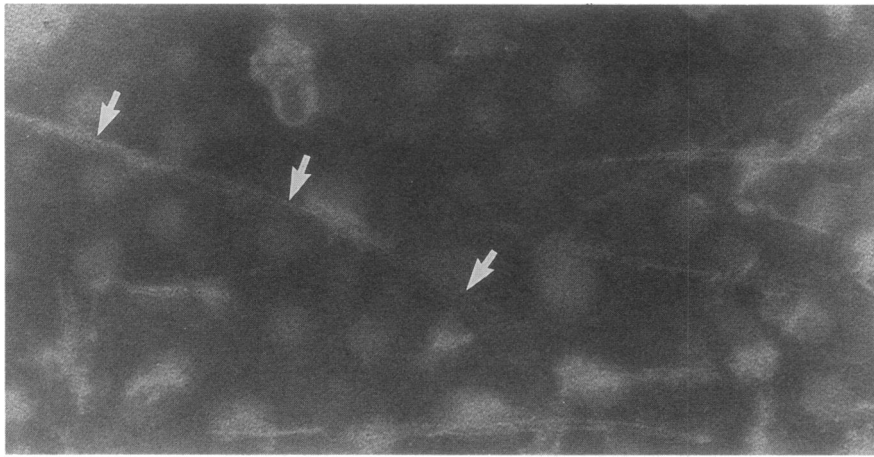


Figure 1. Electron micrograph of the amyloid fibril preparation isolated from Finnish hereditary amyloidosis kidney. Arrows indicate one of the fibrils. Negative staining. Original magnification, 130,000.

fraction was purified on reverse-phase HPLC (Beckman Instruments Inc., Fullerton, CA, System Gold, Programmable Solvent Module 126, SP 8450, UV/VIS detector, Spectra Physics Merck Hitachi D-2500 Chromato-Integration) using a Vydac  $C_{18}$  column and a 10–80% acetonitrile gradient in 0.1% trifluoroacetic acid.

**Peptide fragmentation and separation on reverse-phase HPLC.** The amyloid protein was alkylated and desalted and repurified on HPLC. Tryptic digestion (trypsin-TPCK, Sigma Chemical Co., St. Louis, MO) was carried out in 1% ammonium bicarbonate; incubation with 3% (wt/wt) of trypsin for 2 h at 37°C was followed by another addition (3%) of the protease and continued incubation overnight at 37°C. The released peptides were separated by reverse-phase HPLC using a Vydac  $C_{18}$  column equilibrated with 0.1% trifluoroacetic acid in water, with a linear gradient of acetonitrile (0–60% in 60 min). Digestion with endoproteinase Lys-C (sequencing grade, Boehringer-Mannheim, Mannheim, Germany) was carried out in 25 mM Tris-HCl buffer, pH 8.5; incubation with 3% (wt/wt) of enzyme for 2 h at 37°C was followed by another addition of the protease (3%) and continued incubation overnight at 20°C. Digestion with endoproteinase Asp-N (Boehringer-Mannheim) was carried out in 50 mM sodium phosphate buffer, pH 8.0 for 16 h at 37°C using 10% (wt/wt) of enzyme and digestion with endoproteinase Glu-C (proteinase V8, Boehringer-Mannheim) was carried out in 25 mM ammonium carbonate buffer, pH 7.8 at 25°C overnight using 5% (wt/wt) of enzyme. The released peptides were separated by reverse-phase HPLC using a Vydac  $C_{18}$  column equilibrated with 0.1% trifluoroacetic acid in water, with a linear gradient of acetonitrile (0–70%) in 60 min.

**Amino acid sequence analysis.** The sequence analysis was performed by automated Edman degradation using a modified Applied Biosystems 477A/120A on-line pulsed liquid phase/gas sequencer in the gas phase mode. The NBRF Swiss-PROT sequence database was used for computer search of sequence homologies.

**Electron microscopy.** Amyloid specimens were placed on cupric grids and negatively stained by 1% phosphotungstic acid, pH 6.5, and examined by a JEOL JEM 100 CX electron microscope.

**Immunoperoxidase studies.** Tissue samples from two patients with Finnish hereditary amyloidosis, two patients with secondary (amyloid A) amyloidosis, and two patients with myeloma-associated AL-lambda amyloidosis, as well as from four control subjects without amyloid, were studied. The samples included sections from the kidney (two Finnish hereditary amyloidosis, two amyloid A amyloidosis cases, and three cases with histopathologically normal renal tissue), heart (two Finnish hereditary amyloidosis cases, one myeloma-associated amyloidosis case, and one case with histopathologically normal cardiac tissue), liver (one amyloid A and one myeloma-associated amyloidosis case). Paraffin sections, 3  $\mu$ m thick, were deparaffinized, and endogenous peroxidase activity destroyed by incubating the sections with 0.5%  $H_2O_2$  in methanol for 30 min after which the sections were

washed three times in 0.05 M Tris/HCl buffer diluted to 1:10 with physiologic saline (TBS) followed by incubation with normal swine serum, diluted 1:5, in TBS for 10 min. The test sections were then incubated in moist chambers with the rabbit antisera diluted 1:5–1:500 for 30 min followed by three washings with 0.05 M TBS. As a second layer swine immunoglobulin to rabbit immunoglobulin (Dakopatts, Glostrup, Denmark) diluted 1:30, were added and incubated for 30 min. After three washings with 0.05 M TBS, rabbit peroxidase-antiperoxidase (Dakopatts), diluted 1:80, was added and incubated for 30 min.

After three washings in 0.05 M TBS, the sections were incubated for 5 min with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) in 0.01%  $H_2O_2$ . Control sections were treated similarly but the first antiserum was replaced with normal rabbit serum, anti-transthyretin, anti-amyloid P, anti-immunoglobulin light chain, anti- $\beta_2$ -microglobulin (Dakopatts) and anti-amyloid A (Calbiochem-Behring Corp., La Jolla, CA) or the anti-gelsolin antibodies absorbed with the synthetic peptide used for immunization.

Antigelsolin antibodies were raised in rabbits using a synthetic decapeptide corresponding to gelsolin residues 231–242 (> 95% pure, Multiple Peptide Systems, San Diego, CA) as antigen. The peptide was conjugated to keyhole limpet hemocyanin and administered (200  $\mu$ g of peptide/injection) in Freund's complete adjuvant subcutaneously. Booster injections were given after 3 and 6 wk. The antibody titres were studied by enzyme immunoassay. In the absorption experiments 500  $\mu$ g of uncoupled antigen were added to 200  $\mu$ l of antiserum and kept at room temperature for 2 h and then at 4°C overnight.

## Results

The extracted amyloid fibril preparations were Congo-red positive and exhibited green dichroism when viewed under polar-

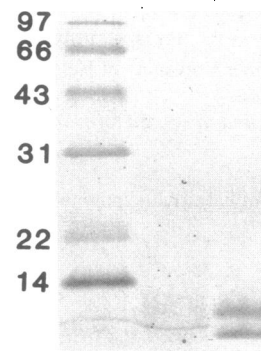


Figure 2. SDS-PAGE (15%) of the amyloid protein fraction isolated from the kidney. Molecular weight markers ( $M_r \times 10^{-3}$ ) are indicated on the left (Bio-Rad Laboratories, Richmond, CA). The amyloid proteins have apparent molecular masses of 6,000–7,000 and 8,000–9,000, respectively.

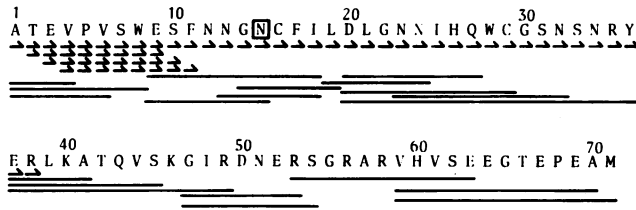


Figure 3. Primary structure of the purified larger amyloid protein of Finnish hereditary amyloidosis. The arrows below the sequence indicate automated sequence analysis from the NH<sub>2</sub>-terminal end of the intact protein, and the lines below the sequence indicate peptides derived from digestion with trypsin, endoproteinase Asp-N, Lys-C, and Glu-C. The substitution is boxed.

ized light. Electron microscopy of the fibril preparation revealed typical nonbranching amyloid fibrils of ~ 8–9 nm width (Fig. 1). Under dissociating conditions the amyloid subunit protein was eluted in gel filtration as a retarded peak, which was absent in control experiments with material extracted from nonamyloid kidney and splenic specimens. SDS-PAGE of the material eluted in the retarded peak, revealed two bands with *M<sub>r</sub>* ~ 6,000–7,000 and 8,000–9,000, respectively (Fig. 2). Final purification of the amyloid proteins was achieved by reverse-phase HPLC.

Amino acid sequence analyses were carried out on the intact protein and the alkylated derivative. Peptide fragmentation was obtained by trypsin and by endoproteinase Asp-N, Glu-C, and Lys-C treatments. The results are summarized in Fig. 3, which shows the complete primary structure of the larger amyloid protein. By direct automated sequencing 38 amino acids of the NH<sub>2</sub>-terminus were identified; the remaining sequence of the molecule was identified by analyzing the sequences of the enzymatically derived overlapping peptides. Microheterogeneity was present at the NH<sub>2</sub>-terminal end of the molecule. In addition to the major form starting with alanine (~ 60%), there were minor forms starting with either threonine (~ 30%, residue 2), glutamic acid (~ 5%, residue 3) or valine (~ 5%, residue 4). In addition to the amyloid protein ending in methionine (residue 71), there were molecules that ended in alanine (residue 70).

The amino acid sequence of the 71-residue amyloid protein is completely homologous to the predicted sequence of human plasma gelsolin (12) in the region of amino acids 173–243 of the mature protein (or residues 200–270 of the precursor pro-

tein) with one exception: at position 15 of the amyloid protein, which corresponds to residue 187 of plasma gelsolin (or 214 of the gelsolin precursor) an asparagine was found instead of an aspartic acid residue (Fig. 4). This substitution was a consistent finding in all analyses and was also present in the amyloid protein isolated from the patient's cardiac tissue. When comparing the primary structure of the amyloid protein with the other actin-modulating or -binding proteins, villin (15), severin (16), and fragmin (17), it is noted that they all have an aspartic acid residue at the position which corresponds to residue 15 (asparagine) of the amyloid protein (Fig. 4). The smaller amyloid component is a fragment of the larger component representing the NH<sub>2</sub>-terminal 53 amino acids of the molecule (Fig. 5).

Anti-gelsolin antibodies raised against a synthetic dodecapeptide, corresponding to residues 231–242 of human gelsolin, specifically stained the amyloid deposits in the diseased tissues (Fig. 6). The staining was completely abolished by absorption of the antiserum with the synthetic peptide 231–242 used for the immunization, but not by a synthetic peptide corresponding to residues 213–221. The P<sub>231–242</sub> antiserum did not stain normal glomerular or cardiac vascular tissue or the amyloid deposits found in primary amyloidosis (amyloid light chain) or secondary amyloidosis (amyloid A), demonstrating the specificity of the P<sub>231–242</sub> antiserum for Finnish hereditary amyloid.

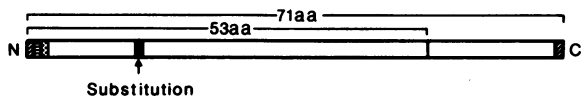
## Discussion

In the systemic forms of amyloidosis several types of amyloid proteins derived from whole or fragmented plasma precursor proteins have been identified. In primary and myeloma-associated amyloidosis, the amyloid protein is related to immunoglobulin light chains (18), in secondary amyloidosis to serum amyloid A protein (18), in haemodialysis-associated amyloidosis to β<sub>2</sub>-microglobulin (19), in FAP I, II, and some related syndromes to transthyretin (20–30), and in FAP III to apolipoprotein A-I (31). The present study provides definite evidence for the unique, gelsolin nature of the amyloid in Finnish FAP, and shows that the amyloid protein is derived from a variant gelsolin molecule.

The calculated molecular weight of the amyloid protein was 8.1 kD, which is consistent with the estimate based on SDS-PAGE. The amyloid protein thus represents a fragment of the precursor gelsolin molecule (~ 83 kD) and is obviously formed through limited proteolysis of the precursor. In accor-

Amyloid protein 1	A T E V P V S W E S F N N G <b>N</b> C F I L D L G N N I H Q W C G S N S N R	35
Gelsolin 200	A T E V P V S W E S F N N G <b>D</b> C F I L D L G N N I H Q W C G S N S N R	234
Villin 149	A G E V E M S W K S F N R G D V F L D L G K L I Q W N G P E S T R	183
Severin 174	V A E V P L A T S S L N S G D C F L L D A G L T Y D F N G S K S S P	208
Fragmin	... V H E V P K T Y K S L N S G D V F V L T A G K T V I Q W N G A K A G L	
Amyloid protein 36	Y E R L K A T Q V S K G I R D N E R S G R A R V H V S E E G T E P E A M	71
Gelsolin 235	Y E R L K A T Q V S K G I R D N E R S G R A R V H V S E E G T E P E A M	270
Villin 184	N E R L R G M T L A K E I R D Q E R G E R T Y V G V V D G E H E L A S P	219
Severin 209	Q E K N K A A E V A R A D D A E R K G L P K V E V F C E T D S D I P A	243
Fragmin	L E K V K A A E L L Q A D I E G E R E G I A S G R V A E G A D N D T E F...	

Figure 4. Comparison of the amino-acid sequence of the 71-residue amyloid protein with the deduced sequences of human plasma gelsolin precursor (12), human villin (15), severin (16), and the sequence of fragmin (17). The residues of fragmin are not numbered, because the sequence is not complete. Homologous amino acids are boxed. The amyloid protein and gelsolin sequences are identical except at position 15 of the amyloid protein; the residue is asparagine in contrast to the aspartic acid at the corresponding positions in human gelsolin. The numbering of gelsolin refers to the precursor molecule (residues 200–270 correspond to residues 173–243 of mature secreted gelsolin).



**Figure 5.** Schematic presentation of the renal amyloid proteins in Finnish hereditary amyloidosis. The 53-residue component is a fragment of the larger 71-amino acid (aa) long component. The amino acid deletions at the NH<sub>2</sub>- and COOH-terminal ends are indicated by hatched areas.

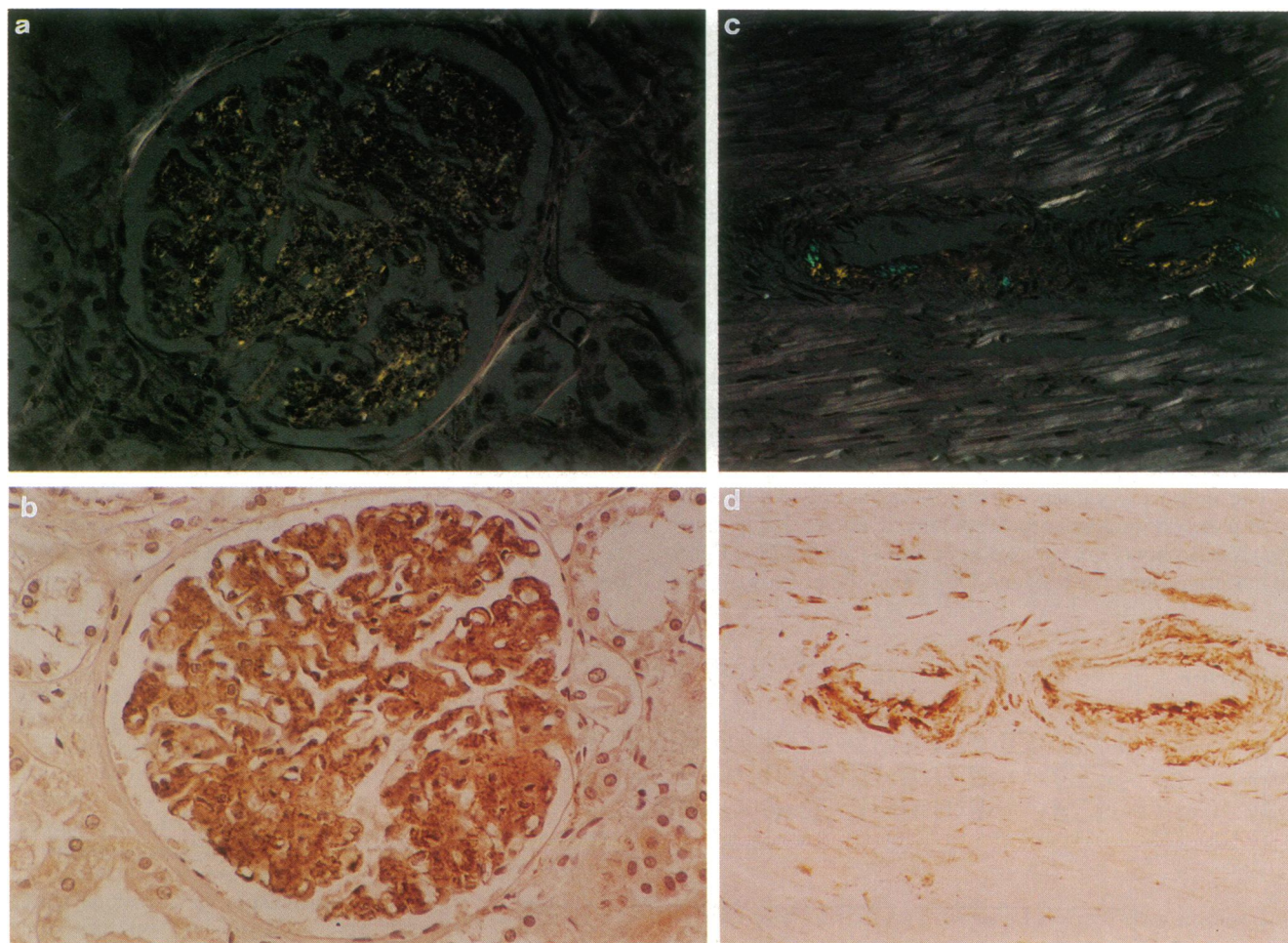
dance with previous data (11) the NH<sub>2</sub>-terminus showed microheterogeneity, which has also been described in transthyretin-related amyloid proteins of Swedish (23), Japanese (24, 25), and Appalachian (29) types of FAP and may be a reflection of the amyloid disease process.

The amino acid sequence of the amyloid protein showed identity with the predicted sequence of human gelsolin representing residues 173–243 of the mature secreted plasma gelsolin (12) with one exception: at position 15 of the amyloid protein on asparagine was found in contrast to the predicted aspartic acid at the corresponding position (187) in normal secreted gelsolin. The asparagine-for-aspartic acid substitution was found both in the amyloid protein extracted from the kidney

and the heart and was a consistent finding in the sequence analyses.

Whether the asparagine-for-aspartic acid substitution in the precursor gelsolin molecule explains a defective proteolytic processing in Finnish FAP and a subsequent polymerization of the accumulating fragments to tissue amyloid cannot be settled at present. However, this possibility seems likely, because previous studies have shown that single amino acid substitutions in the amyloid precursor proteins, transthyretin in FAP type I and II and some related syndromes (21–30), apolipoprotein A-I in FAP type III (31) and cystatin C in Icelandic hereditary amyloidosis (32), are all associated with the development of amyloid in these diseases. Moreover, we have recently demonstrated a point mutation in the gelsolin gene that is responsible for the expression of the mutant asparagine-187 gelsolin molecule in patients with Finnish FAP (13).

Gelsolin is a calcium- and polyphosphoinositide-regulated actin-modulating protein that occurs in a cytoplasmic and a secreted form (12, 33). Both types are derived by alternative transcriptional initiation sites and message processing from a single gene located on chromosome 9 (34). The function of circulating gelsolin has not been defined, but it may be essential



**Figure 6.** Immunoperoxidase staining of tissue amyloid deposits in Finnish hereditary amyloidosis with antibodies to residues 231–242 of gelsolin. (a) Dichroism of glomerular amyloid in a renal section. Congo-red staining and polarized light. (b) Adjacent section stained with the P<sub>231-242</sub> anti-gelsolin antibodies. Magnification, 300. (c) Dichroism of vascular amyloid in the myocardium. (d) Adjacent section stained with the P<sub>231-242</sub> anti-gelsolin antibodies. Magnification, 150.

for the clearance of actin filaments released into the circulation during tissue injury and cell senescence.

Recent studies (35–38) have suggested that calcium-binding to the COOH-terminal half of gelsolin leads to a conformational change which permits the NH<sub>2</sub>-terminal half to sever actin filaments. Severing by the NH<sub>2</sub>-terminal half of gelsolin is calcium-independent, but requires the participation of two actin-binding domains located within residues 1–149 and residues 150–406. The latter peptide, which contains the amyloid protein sequence, binds to the sides of actin filaments in a polyphosphoinositide-sensitive manner. Data have also been presented that the related gelsolin fragment 150–373 promotes the aggregation of G-actin into large complexes and filament formation. The sequence of the amyloid protein is thus part of a functional domain of the gelsolin molecule, and it may be speculated that a disturbed proteolytic degradation of this region of gelsolin and the consequent accumulation of the undegraded fragments into amyloid during fibrillogenesis may lead to disturbance in gelsolin-actin interactions.

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