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Variant plasma gelsolin responsible for familial amyloidosis (Finnish type) has defective actin severing activity

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Familial amyloidosis, Finnish type is caused by a single base mutation in gelsolin, an actin filament severing and capping protein that is present in most tissues and in blood plasma. The mutation replaces aspartic acid with asparagine at residue 187 of the plasma sequence. This renders the gelsolin susceptible to proteolysis as a consequence of which amyloid protein is formed. Here it is shown that the mutant protein in plasma from a patient homozygous for this mutation lacks both actin severing and nucleating activities. Evidence is presented that the cleaved mutant gelsolin has dissociated under non-denaturing conditions and that the resultant 65,000 and 55,000 M_r C-terminal fragments aggregate.

Gelsolin; Amyloidosis (Finnish familial); Actin-binding protein

1. INTRODUCTION

Familial amyloidosis, Finnish type (FAF) is an autosomal dominant form of systemic amyloidosis that is characterized by corneal lattice dystrophy, cranial neuropathy, skin changes as well as renal complications, which are very severe in homozygotes [1]. The disease arises as a result of a single nucleotide substitution G^{654} to A^{654} in the genomic DNA sequence of gelsolin [2,3], which replaces Asp at residue 187 with Asn. Gelsolin is a calcium-dependent actin filament severing and capping protein that is present in a wide variety of tissues and also in blood plasma [4]. Based on the amino acid sequence [5], it is composed of six repeating segments (S1–6) [6]. Asp^{187} is located in S2, the F-actin binding domain [7]. An aspartic acid residue is conserved at this position in all six segments of gelsolin and in equivalent segments of other proteins of the gelsolin family, including villin, severin and fragmin [6], suggesting that an acidic residue at this position is important for the integrity of these proteins. The amyloid protein of 8,100 M_r contains residues Ala^{173}–Met^{243} of plasma gelsolin and there is a smaller minor component corresponding to Ala^{173}–Arg^{225} [8]. Experiments in vitro have shown that a synthetic peptide corresponding to Ala^{173}–Gly^{202} containing Asn at residue 187 has an order of magnitude greater tendency to form amyloid fibrils than the same peptide containing aspartic acid at this position [9].

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Abbreviations: S1–S6 refers to the six repeating segments of sequence in gelsolin and related proteins like villin.

The smallest part of gelsolin that retains severing activity is S1–2 [7]. S1 contains the high affinity monomer binding site [10,11] and S2 the filament binding domain [7]. The structure of S1 has recently been solved at 2.5 Å resolution in its complex with G-actin [12]. This structure shows that Asp^{66} of S1 (equivalent to Asp^{187} of S2) forms a salt bridge with a buried arginine residue, Arg^{45}. Because of the high degree of sequence homology between these two domains it seems likely that they have a similar structural fold. If this is so, Asp^{187} would be expected to form a salt bridge with Arg^{169}, the equivalent residue to Arg^{45} in S2. Mutation of the Asp to Asn would leave the arginine charge uncompensated and this would be expected to produce a local structural rearrangement to solvate the charge. A likely consequence would be increased flexibility of neighbouring residues that might make Arg^{172}, positioned at the start of a surface loop, more accessible to proteases.

Although it is clear that this single mutation is the origin of the amyloidosis, it is not clear whether proteolysis occurs immediately after the gelsolin has been synthesized or following secretion into the plasma. Nor is it known whether the intracellular form of gelsolin is cleaved in the same way as the secreted form. Because blood plasma contains many proteases, proteolysis of the secreted variant might occur more readily. It has recently been shown that circulating gelsolin in FAF patients (homozygotes) contains a major 65,000 M_r species, detected by immunoblotting with antigelsolin antibody, that is not present in normal plasma [13]. There is little staining in the region of the gel corresponding to intact gelsolin. A minor band is also seen at 55,000 M_r, which is consistent with the second cleavage at Met^{243}. Since these fragments were detected only in the

presence of SDS, this provides no information about the state of undenatured protein in the plasma.

A further question concerns the actin binding activity of the mutant gelsolin. Here we show that the severing activity of gelsolin in plasma from homozygous FAF is less than 5% of normal values. The severing activity is also reduced (by about 50%) in plasma from heterozygotes. We further show from immunoblots of gels run under non-denaturing conditions that the abnormal gelsolin migrates in a different position from controls, consistent with its having fallen apart in the absence of denaturing agents.

2. MATERIALS AND METHODS

Plasma samples were collected from 10 patients with FAF, 5 unaffected family members and from other control subjects. The plasma was stored frozen at −20°C. Based on oligonucleotide hybridization [3], 9 of the patients were heterozygous and one homozygous for the Asn^{187} gelsolin mutation. Samples (50 μl) of plasma were tested blind in an actin severing assay as described previously [14] and the rate constant for disassembly of 200 nM actin (containing 15% pyrenyl actin) was measured. Rate constants were also measured using different volumes of individual samples to determine the concentration dependence of the severing rate. The slope of a plot of k_{obs} as a function of gelsolin concentration is a measure of the number of ends generated by severing action, i.e. the concentration of active gelsolin in the plasma [14]. Measurements were also made of the nucleating potential of the plasma gelsolin [14].

PAGE under non-denaturing conditions was carried out as described by Safer [15] and electroblotted onto nitrocellulose [16]. The gelsolin was detected with murine monoclonal anti-gelsolin (Sigma) and an amplification system using Vectastain (biotinylated anti mouse IgG, avidin and biotinylated peroxidase with diaminobenzidine as substrate (Vector Laboratories Ltd. Bretton, Peterborough PE3 8RF, UK)). Two dimensional gels were carried out using the Safer system in the first dimension and 10% SDS-PAGE in the second [17]. Gels were electroblotted as described above and the blots scanned on an Apple Colorl Scanner and images compiled using Adobe Photoshop 2.5 programme.

Actin was fluorescently labelled at Cys^{374} with *N*-pyrenyl iodoacetamide as described previously [14]. Kinetics measurement were made using a Perkin-Elmer LS-50 spectrofluorimeter and exponential reactions analysed by non-linear least squares analysis with Enzfitter (Biosoft, Cambridge).

3. RESULTS

The actin severing activity of 21 different samples of frozen plasma was measured using 50 μl volumes and compared with freshly prepared plasma from one of the authors. Based on the observed activities, the results were classified into 4 groups, two of which showed severing activity substantially below that of the fresh plasma sample and one with somewhat higher activity (Table I). Six of the samples gave a mean value similar to that of fresh plasma and there was very good agreement between 2 different samples of frozen plasma from the same individual. The origin of the samples was not known until after the analysis was completed. With a single exception, all of these 6 samples were from controls or unaffected family members; the exception was from a heterozygote who was asymptomatic for amyloidosis. The group of samples with somewhat higher severing activity were from four different unaffected family members. Eight samples showed about 50% of the activity of the main group of controls: all were from different heterozygotes. The fourth group with activity 5% that of controls were from the single homozygote, one from a plasma sample taken a year later than the other two.

Although determination of k_{obs} using a single volume of plasma from each sample should provide a reliable measure of severing activity, a more accurate method is to compare k_{obs} values using different concentrations of each sample [14]. Fig. 1 shows results for a single member from each of the four groups in Table I. The severing activity in plasma from the homozygote is less than 2% that of the control value, while that from the heterozygote is about 50%. Analysis of one of the gelsolins in the high activity class showed a severing frequency that was only 20% above the control values. This suggests that Class 2 of Table I does not represent a significantly higher activity than the controls, but we have no explanation for the higher apparent activity observed using the 50 μl volumes.

Nucleation experiments were carried out with individual samples from control and FAF patients. Control plasma samples eliminated the lag phase in actin polymerization in a manner similar to pure gelsolin (Fig. 2). Comparing the exponential rate constant $\{= k^+[N]$, where k^+ is the association rate constant at the pointed end and [N] the gelsolin concentration [14]$\}$ with a gelsolin standard suggested a concentration of gelsolin in the plasma of 1.8 μM. Plasma from the heterozygote also eliminated the lag phase of polymerization and gave a nucleating activity at 3 different concentrations that was 53% of the control value. Plasma from the homozygote, used at 5 times the concentration of control and heterozygote samples, did not reduce the lag phase of actin polymerization. There was a substantial reduction in the end point of polymerization with this sample, consistent with a reduced concentration of polymerizable actin (see below). This can be seen by comparing arrow A with B. The other plasma samples also reduced the extent of polymerization as determined

Table I

Severing activity in 50 μl of blood plasma

Class	Number of samples	$k_{obs} \times 1000/s/50\ \mu l$	FAF
I	6	10.1 ± 1.31	No
II	4	17.7 ± 2.03	No
III	8	5.55 ± 1.11	Heterozygote
IV	3	0.551 ± 0.11	Homozygote

The activity obtained for 50 μl of fresh plasma was 10.4×10^{-3}/s. 20 nM gelsolin gave an activity of 3.77×10^{-3}/s, suggesting that the concentration of gelsolin in this plasma sample is 1.7 μM.

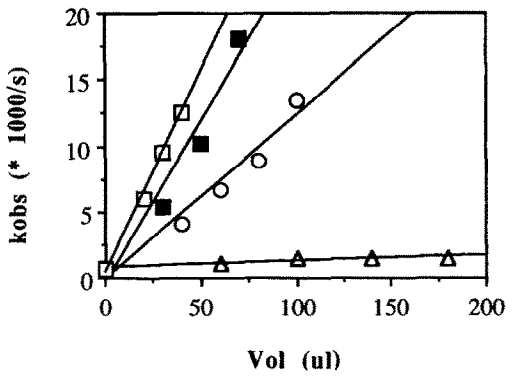

Fig. 1. Severing activity of plasma samples from Class I, controls or unaffected family members (solid squares, 0.243); Class 2, unaffected family members (open squares, 0.299); Class 3, heterozygotes (open circles, 0.124) and Class 4, homozygote (open triangles, 0.0046). The severing activities (given in parentheses) are the slopes of the lines expressed as $s^{-1} \cdot ml^{-1}$.

from the final fluorescence value after several hours, but the effect was less marked because samples were smaller. This reduction is probably due to the presence of vitamin D binding protein, which sequesters actin monomers [18].

The reduced severing and nucleating activities of the gelsolin might be caused by a lower concentration of gelsolin in the plasma or by defective actin binding activity of the mutant protein. Plasma samples were analysed by gel electrophoresis under non-dissociating conditions and the gelsolin was detected by immunoblotting. Fig. 3 shows that control gelsolin migrated as a group of closely spaced bands, the mobility of which were identical to those obtained using bacterially expressed gelsolin (not shown). Immunostaining of plasma samples from the homozygote showed a series of bands of much lower mobility, although there was also very weak staining in the same position as control samples. Samples from heterozygotes showed both sets of bands, at roughly equal intensity (not shown). The difference in mobility suggests that the mutant gelsolin in non-denatured form has a significantly different size or net charge from the control. Two-dimensional gel electrophoresis confirmed that the lower mobility bands of the homozygote corresponded to the 65,000 and 55,000 M_r components of cleaved mutant gelsolin. The lower part of Fig. 3 shows a composite 2-dimensional gel containing both forms of gelsolin.

4. DISCUSSION

Our results show that mutant gelsolin in the plasma of a patient homozygous for the Asn^{187} mutation is almost completely inactive in its severing and nucleating properties. Plasma from patients heterozygous for the mutation showed significantly lower activities, about 50% of control values. We were unable to show whether the actin monomer binding activity of the mutant gelsolin is also reduced, because plasma contains high concentrations of vitamin D binding protein, which also binds monomeric actin [18]. This can be seen in Fig. 2 which shows about 50% reduction in the concentration of polymerizable actin in the presence of 50 μl of plasma from the homozygote, even though the mutant gelsolin was inactive both in its severing and nucleating activities.

Loss of actin severing and nucleating activity of the mutant gelsolin might reflect a lower concentration of mutant gelsolin in the plasma. We do not believe that this is the case. The staining intensities of the mutant and control gelsolin in the plasma samples on immunoblots (Fig. 3) suggest that the concentrations are not significantly different, assuming that the extent of transfer to nitrocellulose is similar for all samples. These immunoblots were run under conditions in which gelsolin was the limiting component; assuming the plasma concentration of gelsolin is 1.7–1.8 μM (from nucleation assays and Table I; this is consistent with previous estimates using the DNase I inhibition assay [17]), each slot on the gel contains 3.5 pmol gelsolin and the antibody is added at 66 nM.

Comparison of mutant and control gelsolins by electrophoresis under non-denaturing conditions shows that they have very different mobilities (Fig. 3). The presence of multiple gelsolin bands in electrophoresis under native conditions is not unexpected, because gelsolin is resolved into 3 components by isoelectric focussing in urea [19–21]. The markedly different mobility of the mutant gelsolin at pH 8.6 cannot be explained by the loss of a single negative charge in a protein with an isoelectric point about 6.2 [19,20,22], containing 98

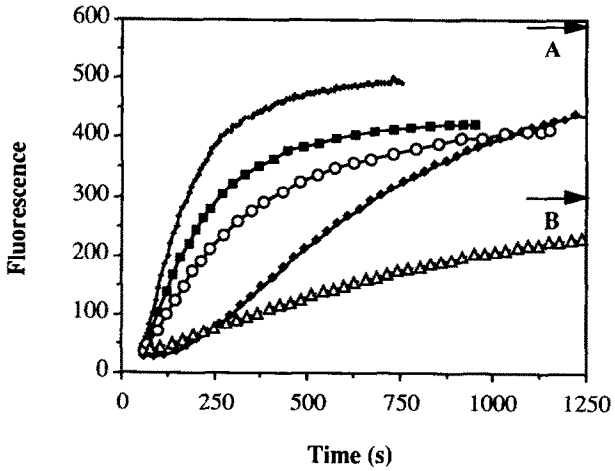

Fig. 2. Nucleating activity of plasma samples in the polymerization of 6 μM actin, containing 15% pyrenyl-actin. Actin polymerized in the absence of gelsolin or plasma (◆ with end point marked by arrow A); actin polymerized in the presence of 60 nM gelsolin (no symbols, $k_{obs} = 7.29 \times 10^{-3}$/s); 10 μl control plasma (■ $k_{obs} = 5.49 \times 10^{-3}$/s); 10 μl plasma from heterozygote (○ $k_{obs} = 3.38 \times 10^{-3}$/s); 50 μl plasma from homozygote (△ $k_{obs} = 1.01 \times 10^{-3}$/s, with end point marked by arrow B). Fluorescence in arbitrary units.

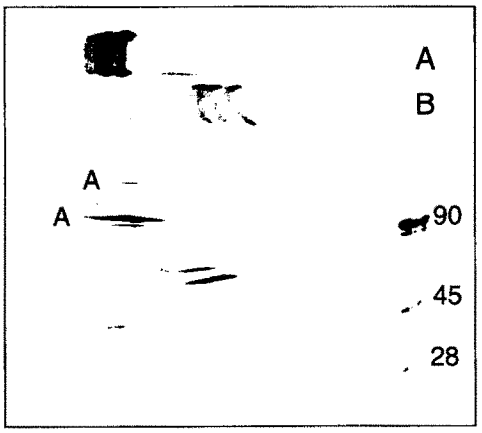

Fig. 3. The upper part shows native gel electrophoresis of plasma samples containing control (A) and mutant (B) forms of gelsolin, stained after transfer to nitrocellulose with anti-gelsolin antibodies. The proteins have migrated from right to left towards the anode. The lower part is a composite two dimensional gel obtained by superposition of gels of control and mutant proteins. Bands labelled A are from the control gel A above and the minor upper band is material that has not penetrated the gel in the second dimension. The four lower bands are from the mutant gelsolin in B. At the right is a molecular weight calibration showing the positions in the SDS-PAGE dimension of gelsolin and two of its fragmentation products.

acidic and 99 basic residues. The mobility difference most likely reflects the electrophoretic behaviour of the proteolytically cleaved mutant gelsolin. The second dimension in SDS confirms the presence of the expected components of 65,000 and 55,000 M_r (see composite gel in Fig. 3). The antibody used to detect the gelsolin recognises only epitopes in the C-terminal half of the protein [23]. It therefore stains intact gelsolin and the 65,000 and 55,000 M_r bands of the mutant protein, but not any of the smaller fragments from the N-terminal end of the protein.

The lower mobility of the mutant protein on non-denaturing gels is not consistent with expectation, based on both the smaller sizes and 35% higher net negative charge calculated for the 65,000 and 55,000 M_r components. One explanation is that dissociation of the N-terminal fragments from the cleaved protein promotes aggregation. This conclusion is supported from experiments on N-terminal truncation of bacterially expressed S1, which showed progressively increased aggregation as N-terminal residues were removed [24].

The non-denaturing gel of mutant gelsolin also showed weak bands in the position corresponding to control samples (Fig. 3B) However, the second dimension in SDS revealed only trace amounts of 90,000 M_r protein (not shown). Instead there were two bands of about 45,000 M_r, suggesting that limited further fragmentation has occurred, possibly during preparation of the samples for gel electrophoresis. Storage at 4°C overnight or longer of samples containing the glycerol buffer for the native gels showed greatly increased fragmentation of this kind.

Loss of both severing and nucleating activities by the mutant gelsolin can be explained by dissociation of the N-terminal fragment following proteolysis at Arg^{172}. We have previously shown that a truncated form of gelsolin (S2–6), lacking S1, has no severing activity [14]. By contrast, nucleation by gelsolin does not require S1, since S2–6 has nucleating activity equal to that of intact gelsolin. Since S1–3 does not nucleate, the two actin-binding domains required for this activity are those in S2 and S4–6 [7,14]. Since the bulk of the mutant gelsolin migrates with an M_r of 65,000 [13], loss of nucleating activity by the mutant suggests that removal of only the first 23 N-terminal residues from S2 is sufficient to destroy nucleating potential. This is consistent with the localization of an F-actin binding site to a 16 residue peptide of villin, located within the first 23 residues of the corresponding S2 segment of that protein [25].

The demonstration that plasma gelsolin is inactive in a patient homozygous for FAF and has reduced severing activity in heterozygotes may not present a serious problem for clearing any F-actin that is released into the bloodstream as a result of tissue damage because other actin binding proteins are present. In addition to gelsolin, plasma contains high concentrations of vitamin-D binding protein [18], which sequesters monomeric actin; thus F-actin would be expected to be depolymerized, albeit more slowly [26]. Loss of activity by cytoplasmic gelsolin is likely to be much more serious if gelsolin plays an essential role in cell locomotion and motility [27]. However, fragmentation of the mutant secreted protein in plasma does not mean that the cytoplasmic variant is similarly fragmented, even though the two isoforms arise from the same single gene [28]. Plasma is rich in proteases and the secreted variant may therefore be much more susceptible to breakdown than the cytoplasmic form. More experiments will be needed to analyse the properties of mutant cytoplasmic gelsolin and to explore the cause of proteolysis in plasma. Because insufficient amounts of the mutant are available from natural sources, this can only be done by expressing it in bacteria.

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