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Hannele Kangas

FAMILIAL AMYLOIDOSIS OF THE FINNISH TYPE (FAF) - CONSEQUENCES OF AMYLOIDOSIS-ASSOCIATED MUTATION FOR GELSOLIN PROCESSING AND FUNCTION

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Academic dissertation

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals. In addition some unpublished data are presented.

I Paunio, T., Kangas, H., Kalkkinen, N., Haltia, M., Palo, J., Peltonen, L. (1994) Toward understanding the pathogenic mechanisms in gelsolin-related amyloidosis: *in vitro* expression reveals an abnormal gelsolin fragment. Human Molecular Genetics 3:2223-2229.

II Kangas, H., Paunio, T., Kalkkinen, N., Jalanko, A., Peltonen, L. (1996) *In vitro* expression analysis shows that the secretory form of gelsolin is the sole source of amyloid in gelsolin-related amyloidosis. Human Molecular Genetics 5:1237-1243.

III *Paunio, T., *Kangas, H., Heinonen, O., Buc-Caron, M.-H., Robert, J.-J., Kaasinen, S., Julkunen, I., Mallet, J., Peltonen, L. (1998) Cells of the neuronal lineage play a major role in the generation of amyloid precursor fragments in gelsolin-related amyloidosis. Journal of Biological Chemistry 273:16319-16324.

IV Kangas, H., Ulmanen, I., Paunio, T., Kwiatkowski, D. J., Lehtovirta, M., Jalanko, A., Peltonen, L. (1999) Functional consequences of amyloidosis mutation for gelsolin polypeptide – analysis of gelsolin-actin interaction and gelsolin processing in gelsolin knock-out fibroblasts. FEBS Letters 454:233-239.

* These authors contributed equally to this work.

(**I** has previously appeared in Dr. Tiina Paunio's PhD thesis, 1995)

ABBREVIATIONS

A549-cells	human lung carcinoma cell line
Aβ	β-amyloid
AEF	amyloid enhancing factor
AGA	aspartylglucosaminidase
AGA$^-$EF cells	embryonic fibroblasts derived from AGA knock-out mice
ApoE	apolipoprotein E
APP	β-amyloid precursor protein
Ca^{2+}	calcium
cDNA	complementary deoxyribonucleic acid
COS-1 cells	African green monkey kidney cell line
CREBAG2 cells	mouse NIH fibroblast derived cell line
ΨCRIP cells	retrovirus packaging cell line derived from NIH3T3 fibroblasts
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
ER	endoplasmic reticulum
F-actin	filamentous actin
FCS	fetal calf serum
FAF	familial amyloidosis of the Finnish type
FAF gelsolin	gelsolin carrying the FAF mutation (Asp_{187}Asn/Tyr)
G-actin	monomeric actin
GAG	glycosaminoglycan
GSN	gelsolin
GSN-c68	a 68 kDa carboxyterminal cleavage fragment of gelsolin
GSN$^-$EF cells	embryonic fibroblasts derived from gelsolin knock-out mice
GSN-NH_2	a 15-25 kDa aminoterminal cleavage fragment of gelsolin
Hep2c cells	hepatocarcinoma cell line
Ig	immunoglobulin
kDa	kilodalton
MDCK cells	Madin-Darby canine kidney cell line
mRNA	messenger ribonucleic acid
NIH3T3 cells	mouse fibroblast cell line
PAGE	polyacrylamide gel electrophoresis
PC12 cells	rat pheochromocytoma cell line
PCR	polymerase chain reaction
PIP	phosphatidylinositol 4-monophosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PPI	polyphosphoinositide
q	long arm of a chromosome
S1- S6	segments 1-6 of gelsolin
SDS	sodium dodecyl sulfate
293 cells	human kidney cell line

In addition, standard three- and one-letter abbreviations of amino acids and one-letter codes of nucleotides are used.

SUMMARY

Amyloidoses are a group of diseases in which abnormal fibrillar proteins accumulate as amyloid in the patients' tissues. Familial amyloidosis of the Finnish type, FAF, a dominantly inherited amyloid disease, is characterized by corneal lattice dystrophy, polyneuropathy, and skin changes. FAF is caused by a point mutation in gelsolin, an actin-modulating protein, which exists in intracellular and secreted forms. The FAF amyloid protein consists of internal peptides of gelsolin with the disease-causing mutation at Asp_{187}.

In the studies on which this thesis is based, the first initial step in the pathogenesis of FAF at the cellular level was elucidated. We showed that expression of secretory mutant FAF gelsolin in different cell types results in the generation of an aberrant polypeptide, which most likely represents the precursor protein for FAF amyloid. Secretory mutant FAF gelsolin was cleaved to FAF amyloid precursor in most of the cell types studied, even in the absence of gelsolin; only adult human fibroblasts and monocytes could not process the mutant FAF gelsolin. Most of the mutant FAF gelsolin was cleaved to the FAF amyloid precursor by neuronal cells, which might suggest that these cells could have a special role in the tissue pathogenesis of FAF. We suggest that lack of the disulfide bond normally present in the wild-type secretory gelsolin leads to initial abnormal folding of the mutant gelsolin polypeptide and its aberrant proteolytic processing to the FAF amyloid precursor. In the cell lines studied, intracellular FAF gelsolin was not proteolytically cleaved either to the FAF amyloid precursor or the FAF amyloid, which suggests that the FAF mutant secretory form of gelsolin is the source of the amyloid in FAF. We also showed that the FAF mutation does not disturb the normal actin-modulating function of intracellular gelsolin. This suggests that, in patients with FAF the symptoms are apparently caused by accumulation in the tissues of amyloid derived from the secreted gelsolin, and not by the disturbed function of the mutant intracellular gelsolin.

REVIEW OF THE LITERATURE

1 AMYLOIDOSIS AND AMYLOID

1.1 Amyloidoses

The amyloidoses are a heterogeneous group of disorders characterized by deposition of insoluble abnormal protein fibrils as amyloid in the extracellular spaces of tissues, causing organ damage (Glenner 1980). Amyloidoses include many disorders such as cancer, Alzheimer's disease, and familial amyloid polyneuropathy. The diseases may be hereditary or acquired. The systemic amyloid deposits involve multiple organs of the body, whereas in other amyloid diseases the amyloid is localized to a single organ (reviewed by (Sipe 1994)). The different amyloid diseases in humans are summarized in Table 1.

The amyloidoses are classified according to the disease-specific fibril protein. The current nomenclature for amyloid fibril proteins was established in 1990 (Husby et al. 1990). To date, if lactoferrin and the amyloid protein in Familial British dementia (ABri) are also accepted as amyloid proteins, at least 20 different amyloid proteins have been identified in humans (Table 1) (Westermark 1999; Vidal et al. 1999). The first amyloid fibril protein to be identified, immunoglobulin light chain (AL), was purified from tissues of patients with AL amyloidosis associated with multiple myeloma and plasma cell disorders (Glenner et al. 1971; Glenner 1980). The amyloid A protein (AA) is a proteolytic cleavage product of the circulating acute-phase reactant serum amyloid A (SAA) (Benditt et al. 1971; Levin et al. 1973; Levin et al. 1972). AA amyloidosis is caused by an inflammatory stimulus, for example in rheumatoid arthritis (Table 1). Many localized amyloid deposits in endocrine tissues are derived from polypeptide hormones: calcitonin, islet amyloid polypeptide (IAPP) or amylin, atrial natriuretic factor derived amyloid (ANF), insulin and prolactin. The majority of these polypeptide hormone-derived amyloids consist of full-length proteins, although fragments of the full-length precursor have been found, for example in the case of prolactin amyloid (Table 1) (reviewed by (Westermark 1994)), (Hinton et al. 1997). Beta-2 microglobulin amyloid deposition occurs in patients with chronic dialysis. The deposition of amyloid results from decreased excretion of beta-2 microglobulin and the amyloid protein is deposited typically in the intact full-length form (Table 1). However, in a few cases, truncated forms of beta-2 microglobulin have been found in the amyloid deposits (Stoppini et al. 2000).

In some cases, a mutation in the precursor protein for amyloid may lead to an amyloid disease. Such mutations have been found in hereditary forms of amyloidoses such as transthyretin, gelsolin, apolipoprotein A1, cystatin C, the amyloid precursor protein (APP), lysozyme, fibrinogen and prion protein associated amyloidoses. Non-mutated forms of some of these precursors (for example transthyretin and APP) can also form amyloid (Table 1).

1.2 Amyloid (deposits)

The term amyloid meaning "cellulose-like", was introduced by Rudolf Virchow in the 1850s. It is still in use, despite the fact that it soon became clear that the major component of amyloid deposits is protein ((Virchow 1851; Friedreich and Kekule 1859) referred by (Sipe 1994)). A specific feature of all amyloid deposits is their affinity for Congo red stain and the typical green birefringence of amyloid when viewed under polarized light. This histologic test for the identification of amyloid is still widely used, reviewed in (Sipe 1994).

In addition to their tinctorial properties, the various amyloid proteins also share structural similarities. Electron microscopic studies have shown the organized fibrillar ultrastructure of amyloid (Cohen and Calkins 1959). The cross-beta-pleated sheet conformation of amyloid proteins was determined by X-ray diffraction analyses (Eanes and Glenner 1968). In this structure, the adjacent polypeptide chains are folded with antiparallel orientation and perpendicularly to the axis of the fibril (Glenner 1980; Sipe 1994) (Fig. 1).

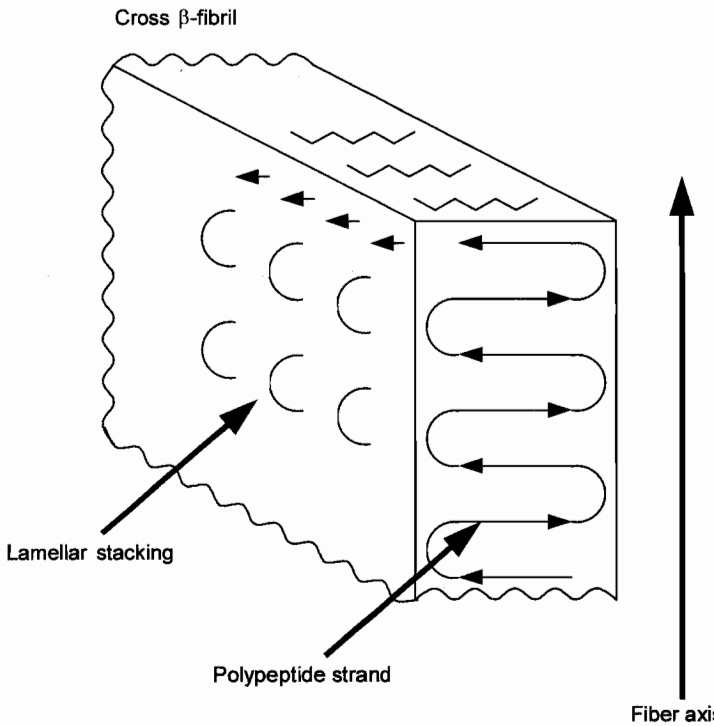

Figure 1. Cross-beta pleated structure of amyloid. The polypeptides of the amyloid fibrils are arranged in an anti-parallel orientation perpendicularly to the axis of the fibrils. Lamellar stacking allows for the fibril breadth (7-10 nm). Adapted from (Kelly and Lansbury Jr 1994).

The detailed structure of the amyloid fibrils is still unknown because amyloid is non-crystalline and insoluble in aqueous solution. Solid state NMR, X-ray diffraction, electron

Table 1. Summary of amyloid fibril proteins, their precursors, associated clinical syndromes, likely amyloidogenic mechanisms and the fibril components of the amyloid proteins in humans.

Amyloid protein	Precursor	Clinical syndrome	Suggested mechanism of amyloidogenesis	Fibril component of amyloid
AL	Immunoglobulin light chain	Idiopathic (primary), myeloma-or macroglobulinemia-associated	Clonal expansion, primary structure [1]	Intact light chain or light chain fragments [2]
AH	Immunoglobulin heavy chain	Primary, myeloma-associated	Clonal expansion, mutation [1]	Heavy chain
ATTR	Transthyretin	Familial amyloid polyneuropathy Familial amyloid cardiomyopathy	Mutation [1,3]	Transthyretin or transthyretin fragments [2]
	Transthyretin	Systemic senile amyloidosis	Age-related deposition of transthyretin	Normal transthyretin or transthyretin fragments [2]
AA	ApoSAA	Reactive (secondary) Familial Mediterranean fever Muckle-Wells' syndrome	Overproduction [1]	Amyloid A, 76 amino acid fragment of ApoSAA [2]
$A\beta_2M$	β_2–microglobulin	Associated with chronic dialysis	Decreased excretion [1]	β_2-microglobulin [2] Fragments of β_2–microglobulin [4]
AApoAI	ApoAI	Familial amyloid polyneuropathy	Mutation [1]	Fragments of ApoAI [2]
AGel	Gelsolin	Familial amyloidosis, Finnish type	Mutation [1]	71 amino acid internal fragment of gelsolin [2]
ALys	Lysozyme	Familial renal amyloidosis	Mutation [1]	Lysozyme or lysozyme fragments [2]
AFib	Fibrinogen α-chain	Familial renal amyloidosis	Mutation [1]	Fragment of fibrinogen [2] Hybrid protein peptide: internal fragment of fibrinogen and novel carboxyterminal sequence [5]
ACys	Cystatin C	Hereditary cerebral hemorrhage with amyloidosis, Icelandic type	Mutation [1]	Cystatin C minus 10 aminoterminal amino acids [2]

Continues in the next page

Table 1 (continued). Summary of amyloid fibril proteins, their precursors, associated clinical syndromes, likely amyloidogenic

with amyloidosis, Icelandic type — aminoterminal amino acids

Continues in the next page

Table 1 (continued). Summary of amyloid fibril proteins, their precursors, associated clinical syndromes, likely amyloidogenic mechanisms and the fibril components of the amyloid proteins in humans.

Amyloid protein	Precursor	Clinical syndrome	Suggested mechanism of amyloidogenesis	Fibril component
Aβ	Aβ protein precursor (APP)	Alzheimer's disease	Mutation [1], increased production of long Aβ (Aβ42/43)	Fragments of APP [2], mainly Aβ 1-42 and Aβ 1-43
		Down's syndrome	Overproduction [6]	
		Hereditary cerebral hemorrhage with amyloidosis, Dutch	Mutation	
APrP^{sc}	Prion protein	Creutzfeldt-Jacob disease, Gerstmann-Sträussler-Sheinker syndrome	Conformational change [1]	Prion or prion fragments [2]
ACal	(Pro)calcitonin	In medullary carcinomas of the thyroid	Local overproduction [1]	Calcitonin or calcitonin fragments [2, 7]
AIAPP	Islet amyloid polypeptide (amylin)	In islets of Langerhans, Diabetes type II, insulinoma	Local overproduction [1]	IAPP or IAPP fragments [2, 7]
AANF	Atrial natriuretic factor (ANF)	Isolated atrial amyloid	Local overproduction [1]	ANF [2]
APro	Prolactin	Amyloidosis of the pituitary gland		Prolactin [8]
		Pituitary adenoma		Fragment of prolactin [9]
AIns	Insulin	Iatrogenic amyloid	Local injection of insulin [10]	Intact pig insulin [10]
ALac*	Lactoferrin	Familial subepithelial corneal amyloidosis		Lactoferrin [11]
ABri** [12]	BRI [12]	Familial British dementia [12]	Mutation [12]	Carboxyterminal peptide of mutant BRI [12]

Table 1 adapted from (Husby et al. 1990) and (Westermark 1999). [1](Teng and Buxbaum 1996), [2](Kelly 1996), [3](for transthyretin mutations, see http://www.ibmc.up.pt/~mjsaraiv/home.htm), [4](Stoppini et al. 2000), [5](Hamidi et al. 1997), [6](Rumble et al. 1989), [7](Westermark 1994), [8](Westermark et al. 1997), [9](Hinton et al. 1997), [10](Dische et al. 1998), [11](Klintworth et al. 1997), [12](Vidal et al. 1999). * Preliminary, waiting for confirmation that it is an amyloid fibril protein (Westermark 1999). ** Not included in the list of amyloid proteins (Westermark 1999).

microscopy and computational studies have been used to analyze the molecular structure of amyloid (Kelly 1996). However, the crystal structures of several wild-type forms of precursor proteins for amyloid, for example transthyretin, beta-2-microglobulin, lysozyme, plasma gelsolin, and transthyretin- and lysozyme variants causing amyloidosis have been solved (Blake et al. 1978; Becker and Reeke Jr 1985; Artymiuk and Blake 1981; Burtnick et al. 1997; Hamilton et al. 1993; Terry et al. 1993; Funahashi et al. 1996; Booth et al. 1997).

The tissue pathology varies between the different amyloid diseases. In familial amyloid polyneuropathy, amyloid deposits have been found, for example, in the spleen, the heart, the vitreous of the eyes, the thyroid and the adrenal glands, whereas in Alzheimer's disease, for example, localized cerebral amyloid deposits are involved (Gillmore et al. 1997). The "amyloid hypothesis" – the putative cause-and-effect relationship between amyloid deposition and the onset of the amyloid disease – although strongly supported by data of different studies, is not yet proven (Kelly 1998).

In addition to the individual amyloid protein (see Table 1), the amyloid deposits in tissues also contain apolipoprotein E, glycosaminoglycans (GAG) and the amyloid P component (SAP). These and other common elements found associated with amyloid deposits are discussed in Section 1.3.2.

1.3 Pathogenic mechanisms in amyloidogenesis

1.3.1 Fibrillogenesis

Amyloid fibril formation is an *in vivo* process in which a normally soluble human amyloidogenic protein is converted into an insoluble amyloid (Kelly 1996). Thus, the amyloidoses can also be viewed as a group of "protein folding diseases" (Westermark 1998). The exact mechanisms underlying the fibril formation are unknown. It has been proposed that polymerization of amyloid fibrils requires the formation of a nucleus or a nidus, after which the process occurs rapidly (Jarret and Lansbury 1993). Assembly of an amyloidogenic protein into amyloid fibrils involves several fibrillogenic intermediates, including amyloid protofibrils, which grow slowly. For fibril formation to occur, an adequate concentration of the intermediate is needed. When the appropriate concentration is reached, the fibrils are rapidly converted into amyloid fibrils (Lansbury 1999). For example, point mutations can predispose the precursor protein for amyloid toward formation of the protofilaments by destabilizing the normal structure of the protein, as has been shown for transthyretin and for lysozyme mutants (Lashuel et al. 1998; Booth et al. 1997) (Fig. 2).

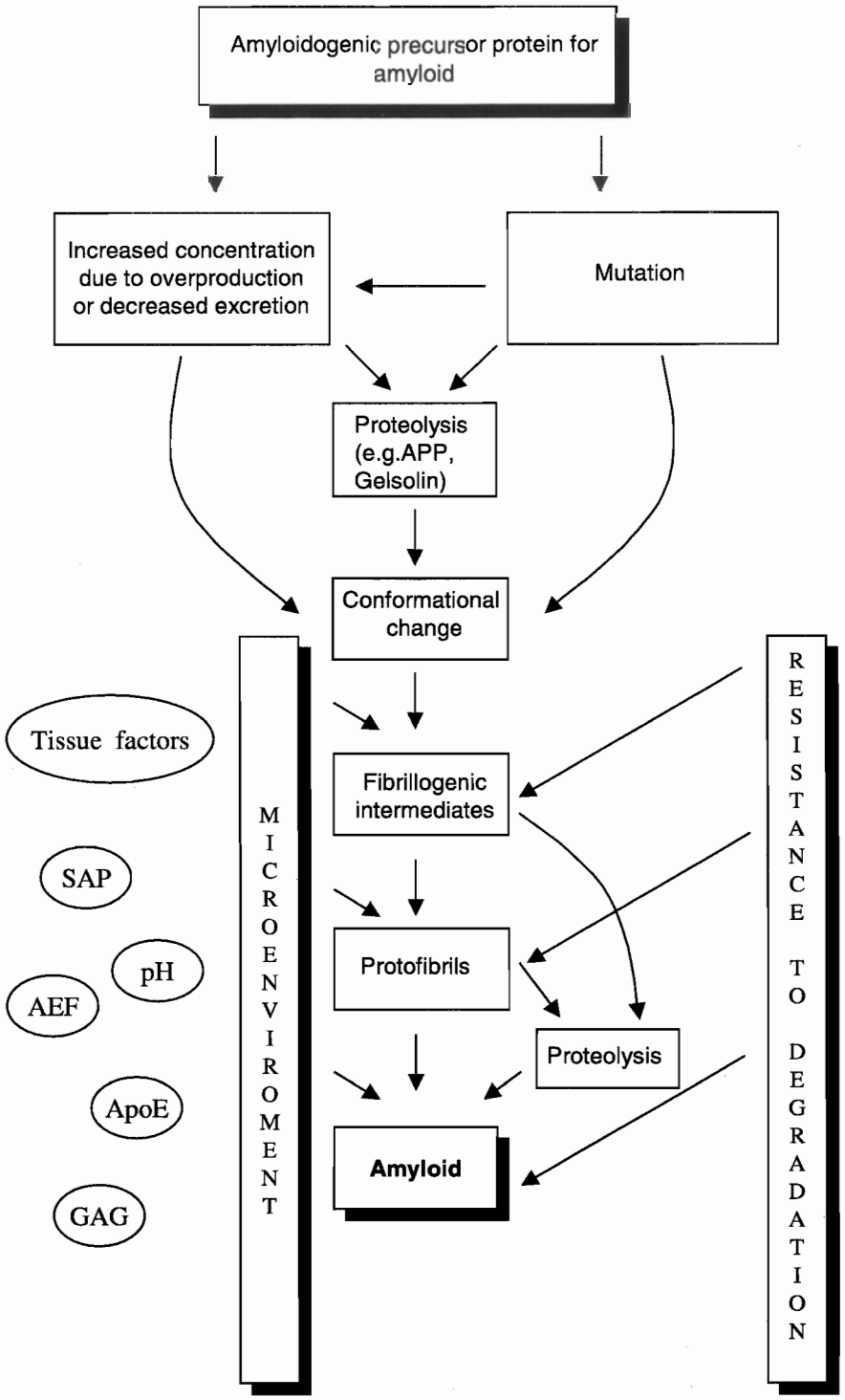

Figure 2. Schematic representation of the major factors contributing to amyloidogenesis. SAP = serum amyloid P component, AEF = amyloid enhancing factor, ApoE = apolipoprotein E, GAG = glycosaminoglycans. This figure is based on the references discussed in the text.

Amyloid deposits are, by definition, extracellular. However, some cytoplasmic inclusions like the intracellular neurofibrillary tangles found in the brains of patients with Alzheimer's disease, have several of the properties of extracellular amyloid (Westermark 1999). An interesting, but so far unsolved, question in amyloid research is whether in systemic amyloidosis amyloid fibril formation begins intra- or extracellularly (Westermark 1994). It has been suggested that in experimental AA-amyloidosis the first fibrils are formed in lysosomes (Shirahama and Cohen 1975). In addition, intracellular amyloid formation has been described in some polypeptide-hormone-derived amyloidoses, reviewed in (Westermark 1994). Intracellular amyloid deposits, released into the extracellular space by exocytosis or cell death, have been found in human insulinomas (O'Brien et al. 1994). Overexpression of human IAPP or amylin in COS-1 cells results in intracellular amyloid deposits that induce apoptosis (O'Brien et al. 1995; Hiddinga and Eberhardt 1999). Intracellular amyloid has also been found in neurons infected with Semliki Forest viruses encoding for the human wild-type or amyloidosis-associated mutant APP (Tienari et al. 1997).

1.3.2 Factors affecting fibrillogenesis

Pathogenesis of amyloidosis is influenced by several factors (Fig. 2). It appears that an amyloidogenic amino acid sequence is necessary for amyloid formation to occur. However, no uniform amyloidogenic amino acid sequence has been characterized. Alterations in the amino acid sequence of a protein by mutation can make the protein more susceptible to proteolysis or a non-amyloidogenic protein more amyloidogenic (Kisilevsky and Fraser 1997). For example, a single amino acid substitution has been shown to accelerate fibril formation by the mutant Aβ peptide carrying the mutation found in the Dutch type of hereditary cerebral hemorrhage with amyloidosis (Wisniewski et al. 1991). A mutation could also affect the protein's tissue- or cell-specific affinity or function (Kisilevsky and Young 1994). Another important factor in fibrillogenesis, as shown in *in vitro* experiments, is the local concentration of the fibril precursor (Jarret and Lansbury 1993). A number of precursors show increased synthesis, while decreased clearance of β-2 microglobulin can also lead to amyloidosis (Table 1). However, other factors, in addition to the amyloidogenic amino acid sequence and the concentration of the fibril precursor, are needed for amyloid formation to occur (Kisilevsky and Fraser 1997).

Multiple tissue components may contribute to amyloid formation. All the amyloid deposits studied, for example, consist of apolipoprotein E, which might act as a pathological chaperone in fibrillogenesis by inducing a β-pleated sheet conformation (Wisniewski and Frangione 1992). During the initial stages of fibrillogenesis, SAP and glycosaminoglycans may be important (Kisilevsky and Young 1994) (Fig. 2). In addition, environmental changes, such as a drop in local pH, may be important for amyloid fibril formation, as has been shown for gelsolin *in vitro* (Ratnaswamy et al. 1999) (Fig. 2).

Amyloid P component (AP), which is present in all types of amyloid, is a glycoprotein that is identical to the normal circulating plasma protein, serum amyloid P component

MATERIALS AND METHODS

More detailed descriptions of the materials and methods used are given in the original publications (**I-IV**).

1 CONSTRUCTION OF EXPRESSION VECTORS FOR SECRETORY GELSOLIN

Secretory gelsolin cDNA (a generous gift from Professor Helen Yin) was subcloned into a M13mp18 phage and mutagenized, using Taylor's phosphorothioate DNA selection method to change the nucleotide G_{654} to A or to T ($Asp_{187}Asn/Tyr$) (Taylor et al. 1985). The mutant clones were identified, using the solid-phase minisequencing test for gelsolin, and the correct nucleotide sequence of the clones was confirmed by dideoxynucleotide sequencing (Paunio et al. 1992; Sanger et al. 1977). The wild-type (G_{654}) and mutant ($G_{654}A/T$) secretory gelsolin cDNAs were further subcloned to a pCD-X expression vector with the SV-40 early promoter (Okayama and Berg 1983), a pAdPsy-LTRSVpolyA vector with Rous sarcoma virus promoter containing the adenoviral sequences needed for homologous recombination (Strafford-Perricaudet et al. 1992), and a Moloney murine leukemia virus-based retrovirus vector pM48 with phosphoglycerate kinase promoter (Moullier et al. 1993) (**I-IV**).

The change of nucleotide G_{658} to C ($Cys_{188}Ser$) was performed with a Chameleon^{TM} double-stranded, site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions on pCD-X expression vectors containing either the wild-type (G_{654}) or the FAF mutant ($G_{654}A$) secretory gelsolin cDNA (**III**).

2 CONSTRUCTION OF EXPRESSION VECTORS FOR INTRACELLULAR GELSOLIN

To obtain the intracellular gelsolin cDNAs, the pCD-X vector constructs encoding for the wild-type (G_{654}) or the FAF mutant secretory gelsolin ($G_{654}A/T$) were digested with *NarI* to disrupt the initiation site for secretory gelsolin, removed from the vector with *EcoRI*, and subcloned either to the pCD-X expression vector or to the pM48 retrovirus vector (**II, IV**).

3 PRODUCTION OF RECOMBINANT ADENOVIRUSES

Linearized pAdPsy-LTRSVpolyA containing the wild-type (G_{654}) or FAF mutant ($G_{654}A$) gelsolin cDNA and the large *ClaI* fragment of Ad-5 (adenovirus) DNA (with a deletion in the E3 region) were co-transfected into the human kidney 293 cell line (ATCC CRL 1573). After homologous recombination, the replication deficient recombinant E1/E3 deleted adenoviruses were plaque purified and expanded in 293 cells (Strafford-Perricaudet et al. 1992). The final virus stocks were purified by CsCl (cesium chloride) density gradient centrifugation and Sephadex PPD column (Pharmacia) in PBS. The correct structures of the recombinant viral DNA constructs were confirmed by restriction enzyme digestion, Southern blotting, and minisequencing (**III**).

4 PRODUCTION OF RECOMBINANT RETROVIRUSES

The gelsolin pM48 constructs were cotransfected into the mouse NIH3T3 fibroblast-derived φCRIP packaging cells (Danos and Mulligan 1988) with pSV2neo vector by lipofectin transfection (Felgner et al. 1987) and the transfected cells were selected with 500 μg/ml G418. Clones producing recombinant retroviruses encoding for the wild-type or mutant forms of intracellular or secretory gelsolin were identified by PCR and Western blotting, after which the best producer clones were expanded (**IV**).

5 CELL CULTURE, TRANSFECTION AND TRANSDUCTION

African green monkey kidney cell line COS-1 (ATCC CRL 1650), the mouse fibroblast cell line NIH3T3 (ATCC CRL 1658), the primary human fibroblast cell line, the mouse NIH fibroblast-derived CREBAG2 (Danos and Mulligan 1988) and hepatocarcinoma cell lines (Hep2c) were cultrured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal calf serum (FCS). The Madin-Darby canine kidney cell line (MDCK) (ATCC CCL 34) and the human lung carcinoma cell line (A549) (ATCC CCL 185) were cultured in modified Eagle's medium supplemented with 5% or 10% FCS. φCRIP cells were cultured in DMEM supplemented with 10% newborn calf serum. $GSN^{-/-}$ mouse embryonic fibroblasts (GSN^{-}EF) were derived from embryos of gelsolin knock-out mice (Witke et al. 1995) and cultured on dishes coated with 50 μg/ml poly-D-lysine. $AGA^{-/-}$ mouse embryonic fibroblasts (AGA^{-}EF) were derived from E14 embryos of mice with homozygous disruption of the aspartylglucosaminidase gene (Jalanko et al. 1998) and cultured on dishes coated with 0.1% gelatin. GSN^{-}EF and AGA^{-}EF cells were maintained in DMEM supplemented with 15% FCS. Undifferentiated rat pheochromocytoma PC12 cells (ATCC CRL 1721) were cultured on dishes coated with a 1:40 dilution of Matrigel and in RPM1 1640 medium (GIBCO) supplemented with 10% normal horse serum and 5% FCS. For differentiation, a medium with 1% horse serum and 50 ng/ml nerve growth factor was used. All media were supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, and 50 mg/ml streptomycin (**I-IV**).

Primary human schwannoma cell cultures were derived from the trigeminal schwannoma of a 15-year-old male and from the vestibular schwannoma of a 59-year old female, as published earlier (Kaasinen et al. 1995). Human macrophages and monocytes were obtained as described by (Ronni et al. 1997). Human neuronal progenitor cells were obtained from a legal abortion of an 8-week-old fetus and cultivated as described by (Buc-Caron 1995) with addition of dibutyryl cyclic AMP (1mM) to the culture medium (**III**).

COS-1 cells were transfected with the gelsolin-pCD-X constructs (G_{654}, G_{654}A/T) with lipofectin or DEAE-Dextran and chloroquine methods (Felgner et al. 1987; Luthman and Magnusson 1983). Two days after transfection, a serum-free medium was added, and after incubation for 6-20 h, the medium was collected and the cells were harvested by trypsinization (**I-III**). To study the enzymatic processing of gelsolin, transfected cells were incubated for 2-10 h in a serum-free medium supplemented with EDTA (1.2-10

mg/l), 1.10 phenantroline (1 mM), phosphoramidon (200 µg/ml), pepstatin (0.7 mg/,l), E-64c (0.1 mM), TLCK (100 µg/ml), chymostatin (100 µg/ml), antipain (50 µg/ml) or leupeptin (0.5 µg/ml). The culture media were supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, and 50 mg/ml streptomycin (**I**).

Cells were infected with recombinant adenoviruses for 1-2 h in a serum-free medium. After infection, medium supplemented with 2% FCS was added. For PC12 cells, 1% FCS and 1% horse serum was added. The medium of the human neuronal progenitor cells contained no FCS. A serum free medium was added 8-24 h prior to collection. The cells and the medium were collected 36-72 hours after infection. All media were supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, and 50 mg/ml streptomycin (**III**).

For retroviral infections, cells were infected 10 times at half-day or daily intervals with 3 ml of the recombinant retrovirus supernatant and 1.5 ml of cell growth medium in the presence of 8 µg/ml of polybrene. The transduced cells were maintained in the growth medium for several passages. One day prior to collection, a serum-free medium was added. All media were supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, and 50 mg/ml streptomycin (**IV**).

For exogenous delivery of mutant gelsolin polypeptides, culture media derived from MDCK cells expressing FAF mutant secretory gelsolin were incubated with non transduced fibroblast or monocyte cell cultures for 8 - 20 hours.

To inhibit proteases, EDTA, PMSF, aprotinin, or E-64c was used when collecting of the samples after transfection or transduction (**I-IV**).

6 PATIENT AND CONTROL SAMPLES

Patient and control samples of CSF (cerebrospinal fluid) and plasma were obtained from Dr. Sari Kiuru and Dr. Seppo Kaakkola as described in (Paunio et al. 1994) (**I, II**).

7 GELSOLIN SPECIFIC ANTIBODIES

To obtain the NH951 antibody, the cDNA coding for the aminoterminal part of gelsolin (coding for amino acids 1-172) was amplified by PCR and subcloned to the pGEX-2T-glutathione –S transferase fusion vector (Amersham Life Sciences) for expression in *E.coli*. To obtain the COOH961 antibody, the gelsolin cDNA (coding for amiro acids 420-755) in a pCD-X vector was digested with *BglII* and *EcoRI* and subcloned into the pGEX2T+1 glutathione –S transferase fusion vector (Amersham Life Sciences, modified and donated by Dr. Krister Melén) for expression in *E.coli*. After induction for 3 hours with IPTG (isopropylthio-ß-D-galactoside), the cells were harvested and lysed. The fusion proteins were purified from bacterial lysate, using preparative SDS-PAGE. Rabbits were immunized by subcutaneous injection with 200-500 µg of the fusion protein in Freund's complete adjuvant. The immunizations were repeated after 2 and 6 weeks. Blood was collected 1 week after the last immunization and the antibody titers and specificity were estimated by Western blotting (**II-IV**).

Polyclonal AM904 (anti-am) rabbit antiserum was raised against gelsolin fragments extracted from amyloid in the tissues of a patient with FAF (Haltia et al. 1990a). Rabbit antiserum K572 was raised against a synthetic peptide of amino acid residues 231-242 of gelsolin. Monoclonal anti-gelsolin (GS-2C4) (Sigma) antibody recognizes a 47 kDa peptide derived from a chymotryptic cleavage of gelsolin extending to the carboxyterminus of the protein (Chaponnier et al. 1986). Figure 10 in the results section shows the target regions of the gelsolin-specific antibodies (**I-IV**).

8 METABOLIC LABELING, IMMUNOPRECIPITATION, AND WESTERN BLOTTING

For metabolic labeling of proteins, the transfected or infected cell cultures were incubated in cysteine- and methionine-free medium for 30 min, labeled with a mixture of 100 μCi/ml of ^{35}S cysteine and 100 μCi/ml of ^{35}S methionine (labeling media) for 30 min–2 h, and chased for different time periods in a serum-free medium. In some experiments the cell cultures were incubated for 2-4 h at 20°C to inhibit the transport of proteins from the trans-Golgi network (Saraste and Kuismanen 1984). The cells and media were collected, the cells were lysed by freeze-thawing, and the media were concentrated by Centricon 3-30 (Amicon). Immunoprecipitations were carried out either with monoclonal anti-gelsolin antibody or with a mixture of polyclonal AM904 (anti-am) and COOH961 antibodies, using protein G- or A-Sepharose, respectively. The immunoprecipitated polypeptides were separated on a 9 or 14% SDS-PAGE and visualized by fluorography using Amplify reagent (Amersham) (**I-III**).

For Western blotting, the cell lysates or cell culture media from the transfected or infected cells were analyzed on a 7, 9, 14 or 15% SDS-PAGE and blotted onto Hybond-C cellulose membranes (Amersham). For immunoblotting analyses, monoclonal anti-gelsolin or different polyclonal gelsolin specific antibodies (see Fig. 10) were used. Enhanced chemiluminescence (ECL) or the ProtoBlot Western Blot AP System (Promega) was used for the final detection of the polypeptides (**I-IV**).

9 GELSOLIN PEPTIDE DELIVERY TO PC12 CELLS

PC12 cells were infected with recombinant adenoviruses encoding for the wild-type or FAF mutant secretory gelsolin. Two days after infection, the cells were starved for 30 min in unlabeled cysteine- and methionine-free media, after which 20 or 50 μM gelsolin peptide (GSN 150-169) was added to the labeling media (described in section 8) and incubated with the cells for 15 min. Addition of the 20 or 50 μM peptide was repeated for another 15 min, after which the polypeptides in the cell lysates and culture media were immunoprecipitated as described above in section 8.

10 IMMUNOFLUORESCENCE- AND IMMUNOCYTOCHEMICAL STAININGS

For immunofluorescence or immunocytochemical stainings, the cells were grown on either uncoated, poly-D-lysine-coated (GSN-EF cells), or Matrigel-coated (PC12 cells)

coverslips, fixed with 3 or 4% paraformaldehyde, and permeabilized with saponin. Monoclonal anti-gelsolin antibody or polyclonal COOH961 antibody were used as the primary antibodies. Rhodamine- or fluorescein-conjugated goat anti-mouse IgGs or goat anti-rabbit IgGs were used as secondary antibodies in immunofluorescence analysis (**I-IV**).

Immunocytochemical stainings of gelsolin with anti-gelsolin antibody were performed, using the Vectastain ABC-standard kit (Vector Laboratories). The secondary antibody was peroxidase-conjugated anti-mouse antibody. When the cells were double-labeled with two monoclonal antibodies, the staining and development reactions were carried out separately. The actin filaments were stained with Oregon green 488 phalloidin (Molecular Probes). The cells were viewed with either a Zeiss Axiophot microscope or a Leica DMR confocal microscope, using a 40 or 63 x objective (**I-IV**).

11 AMINOTERMINAL SEQUENCING

Forty milliliters of cell culture medium, obtained from COS-1 cells transfected with FAF mutant gelsolin pCD-X construct ($G_{654}A$), was concentrated and subjected to gel filtration in a Superdex HR 10/30 colum. The gelsolin-containing sample was further purified by reversed-phase chromatography and the fractions containing the 83 kDa full-length gelsolin and the 68 kDa gelsolin fragment were subjected to SDS-PAGE and blotted to PVDF membrane. The 83 kDa and 68 kDa bands were individually cut out and subjected to aminoterminal sequence analysis on Applied Biosystems Procise 494 A sequencer. The results were analyzed, using an Applied Biosystems 610A Data Analysis system (Perkin Elmer Corporation) (**II**).

RESULTS

1 THE SECRETORY FORM OF GELSOLIN IS PROCESSED TO FAF AMYLOID PRECURSOR IN COS-1 CELLS (I, II)

To analyze the processing of secretory gelsolin in mammalian cells, African green monkey kidney cells (COS-1) were transfected with pCD-X expression vectors containing the cDNA encoding for the wild-type or FAF mutant forms ($Asp_{187}Asn/Tyr$) of secretory gelsolin. The cell lysates and the corresponding media were analyzed by Western blotting or immunoprecipitation analysis, using different gelsolin-specific antisera (**I, II**) (Fig. 10).

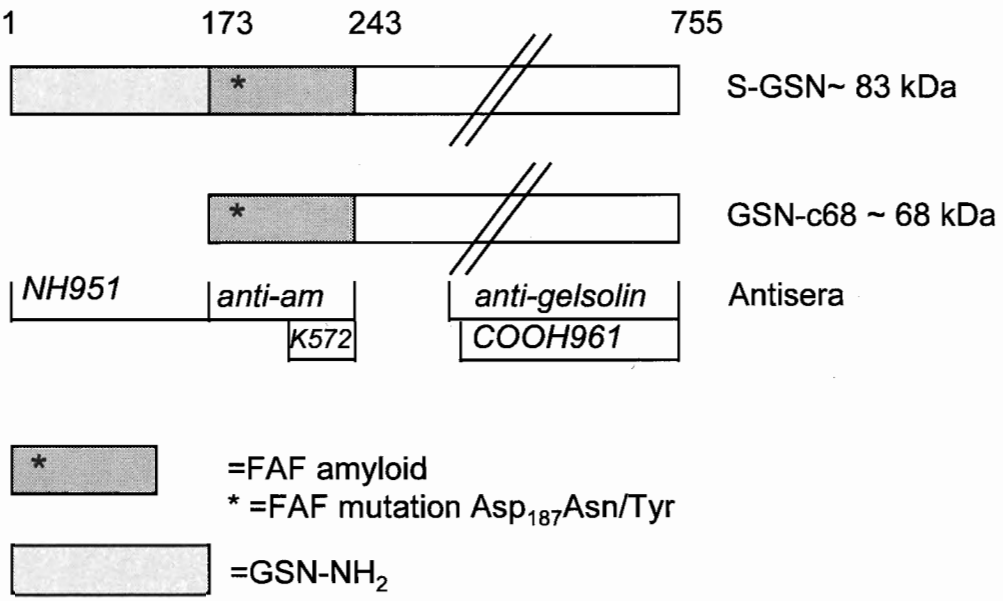

Figure 10. Schematic representation of secretory gelsolin, the FAF amyloid region, the GSN-c68 and GSN-NH_2 cleavage fragments of mutant secretory gelsolin and the regions used for raising the gelsolin-specific antibodies. S-GSN = secretory gelsolin, GSN-c68 = the 68 kDa cleavage fragment of gelsolin representing the FAF amyloid precursor, GSN-NH_2 = the 15-25 kDa aminoterminal cleavage fragment of gelsolin.

The full-length 83 kDa gelsolin polypeptide was secreted into the culture media by COS-1 cells expressing the wild-type form of gelsolin. In contrast, an aberrant 68 kDa gelsolin polypeptide (GSN-c68) in addition to the full-length 83 kDa polypeptide was secreted into the culture medium by cells expressing the FAF mutant (Asn_{187} or Tyr_{187}) gelsolin (Fig. 11). About 15-40% of the mutant gelsolin was cleaved to the GSN-c68 polypeptide (**I**). This polypeptide could be identified with K572, anti-am and anti-gelsolin antibodies, but not with the NH951 antibody, which suggested that it carried the FAF amyloid sequence at its aminoterminus and might thus represent the precursor protein for FAF amyloid (**I, II**) (Fig. 10). This was confirmed by aminoterminal sequencing, which

revealed that the first amino acid residue of this fragment is alanine 173 of gelsolin (**II**), the first amino acid of FAF tissue amyloid. Only full-length gelsolin was found in the cell extracts of cells expressing the wild-type or mutant secretory gelsolin. In addition, FAF-amyloid was not detected in the cell extracts or the medium of the cells expressing the mutant forms of secretory gelsolin (**I**).

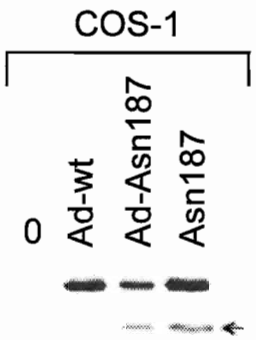

Figure 11. Processing of secretory gelsolin in COS-1 cells. COS-1 cells were transfected with pCD-X expression vector encoding for the secretory mutant gelsolin (Asn187) or infected with adenoviruses encoding for the secretory forms of wild-type (Ad-wt) or mutant (Ad-Asn187) gelsolin and the media were analyzed by Western blotting using the anti-gelsolin antibody. The 83 kDa full-length gelsolin is found in the media of cells expressing the wild-type and FAF mutant secretory gelsolin and the 68 kDa polypeptide (GSN-c68), indicated by an *arrow*, only in the media of cells expressing mutant FAF gelsolin. 0 = background of COS-1 cells (**I, III**).

The GSN-c68 fragment was secreted from the cells at the same rate as the normal full-length gelsolin, as shown by pulse chase analysis (**I, II**). GSN-c68 was not detected in the extracts of cells expressing mutant FAF gelsolin when the cell cultures were incubated at 20°C, a temperature which prevents the protein transport from the trans-Golgi network (Saraste and Kuismanen 1984). When the medium containing mutant FAF gelsolin was collected and further incubated at +37°C, the proportion of GSN-c68 did not increase (**I**).

To inhibit fragmentation of the mutant gelsolin to GSN-c68, different protease inhibitors were added to the culture medium of the wild-type or mutant gelsolin expressing cells. Interestingly, the metalloprotease inhibitors EDTA and phenanthroline diminished the fragmentation of mutant gelsolin to GSN-c68 from 35% to 5% and to 10-15%, respectively. Phosphoramidion, which inhibits many bacterial proteinases but only a few metalloendoproteinases of mammalian origin (Beynon and Salvesen 1990), did not inhibit the cleavage of mutant gelsolin to GSN-c68. Neither cysteine, nor serine nor aspartic acid protease inhibitors affected the processing of mutant gelsolin to GSN-c68 (**I**)

An 60 kDa carboxyterminal cleavage fragment of gelsolin has been observed in the serum and cerebrospinal fluid (CSF) of patients with FAF (Sunada et al. 1994; Paunio et al. 1994). In addition, a 65-70 kDa gelsolin fragment has been found in the CSF of patients

with FAF (Paunio et al. 1994). Immunoblotting analysis showed that the 60 kDa fragment was smaller than GSN-c68 (**I**) but that the higher molecular weight fragment (65-70 kDa) most likely corresponds to the GSN-c68 fragment (**II**) (Figs. 4 and 10).

2 INTRACELLULAR FORM OF GELSOLIN IS NOT PROCESSED TO FAF AMYLOID PRECURSOR (II, IV)

FAF mutation is shared by both the intracellular and the secretory forms of gelsolin (Fig. 5). To study whether the intracellular mutant FAF gelsolin also contributes to the amyloid formation in FAF, COS-1 cells were transfected with expression vectors encoding for the intracellular wild-type or FAF mutant forms (Asn/Tyr_{187}) of gelsolin. The cells and media were analyzed by Western, immunoprecipitation, or immunofluorescence analysis (**II**).

Both the wild-type and the FAF mutant forms of gelsolin were similarly and homogeneously distributed througout the cytoplasm of transfected COS-1 cells, based on immunofluorescence analysis. Western analysis revealed that the gelsolin polypeptide staining patterns of the wild-type and FAF mutant gelsolin-expressing cell cultures were identical. The 80 kDa band corresponding to the full-length intracellular gelsolin was detectable with all the gelsolin-specific antibodies used. Some smaller fragments of gelsolin were also detected, but none of them were specific for either the wild-type or the mutant forms of gelsolin. In particular, no polypeptides corresponding to the size or immunospecificity of GSN-c68 or FAF amyloid was detected in these analyses (**II**). Further, the intracellular mutant FAF gelsolin was not cleaved to GSN-c68 or FAF amyloid in the gelsolin-null fibroblasts transduced with the recombinant retroviruses encoding for the wild-type or FAF mutant intracellular gelsolin (**IV**).

3 THE DISULFIDE BOND BETWEEN CYSTEINES 188 AND 201 OF GELSOLIN IS CRUCIAL FOR THE NORMAL PROCESSING OF SECRETED GELSOLIN (III)

The results of the expression analysis of FAF mutant secretory and intracellular gelsolin raised an interesting question why only the mutant secreted and not the intracellular gelsolin gets aberrantly cleaved. A disulfide bond is located in the immediate vicinity of the FAF mutation site (Asp_{187}) between amino acids 188 and 201 in the secretory, but not in the intracellular, gelsolin (Wen et al. 1996) (Fig. 9). To study whether the abnormal processing of mutant FAF gelsolin to GSN-c68 might result from disruption of this disulfide bond, we introduced a $Cys_{188}Ser$ mutation into the expression plasmids encoding for the wild-type or FAF mutant secretory gelsolin and expressed them in COS-1 cells (**II**).

A truncated 68 kDa polypeptide, which corresponded in size and immunoreactivity to that of the GSN-c68 fragment in Western analysis, was secreted, in addition to the full-length 83 kDa gelsolin polypeptide from the cells transfected with either of the two plasmids containing the $Cys_{188}Ser$ mutation. About 30% of the mutant ($Asp_{187}Ser_{188}$ and $Asn_{187}Ser_{188}$) gelsolin was cleaved to the 68 kDa GSN-c68-like polypeptide. This

fragment was not detected in the cell extracts in which only the 83 kDa gelsolin polypeptide was found (**III**). The fragmentation could be inhibited by EDTA, as had been shown earlier for mutant FAF gelsolin. Thus, the processing observed for the secretory gelsolin with the disrupted cysteine bridge 188-201 was similar to that of the mutant secretory FAF gelsolin (**I, III**).

4 NEURONAL CELLS PRODUCE LARGE AMOUNTS OF FAF AMYLOID PRECURSOR (III, IV)

To characterize the roles of the different cell types in the pathogenesis of FAF, recombinant adeno- or retroviruses encoding for the wild-type or FAF mutant secretory gelsolin (Asn_{187}) were used to infect cells of various origins. The corresponding cell lysates and media were either immunoprecipitated or analyzed by Western blotting. The intracellular distribution of gelsolin was analyzed by its immunofluorescence (**III, IV**). The processing of mutant gelsolin was similar in adenovirus-driven (Fig. 11 left), retrovirus-driven and transient expression experiments in COS-1 cells (Fig. 11, right) (**I, III, IV**).

Mutant FAF gelsolin was cleaved to the FAF amyloid precursor in canine kidney (MDCK), human lung carcinoma (A549), and hepatocarcinoma (Hep2c) cells. The 83 kDa full-length gelsolin was secreted into the culture media of MDCK, A549, and Hep2c cells expressing wild-type gelsolin. In contrast, the cells expressing mutant FAF gelsolin secreted not only the full-length gelsolin but also the GSN-c68 fragment. The amount of GSN-c68 was highest in MDCK (27% of total gelsolin) and lowest in the Hep2c (6%) cells. An aminoterminal 15-25 kDa cleavage fragment (GSN-NH_2) of gelsolin was occasionally found in the media of MDCK and A549 cells expressing mutant FAF gelsolin (see Fig. 10 for the different fragments of mutant gelsolin) (**III**).

The GSN-c68 fragment was not detected in the media from the adult human fibroblasts transduced with recombinant adeno- or retroviruses encoding for the mutant secretory FAF gelsolin or from fibroblasts derived from an FAF patient (**III, IV**). Only full-length gelsolin was secreted into the culture media of these cells. In addition, exogenously delivered mutant gelsolin polypeptides were not further processed to GSN-c68 in the nontransduced fibroblasts. Similar results were also observed with nontransduced cultured monocytes (**III**).

In contrast to the findings with adult human fibroblasts, the FAF mutant form of secretory gelsolin was cleaved to GSN-c68 when produced in embryonic fibroblasts derived from gelsolin knock-out mice (GSN$^-$EF) or AGA knock-out mice (AGA$^-$EF). In addition to the full-length gelsolin, GSN-c68 and GSN-NH_2 fragments were also found in the media of GSN$^-$EF or AGA$^-$EF cells expressing the FAF mutant gelsolin. About 40% of the mutant gelsolin was cleaved to GSN-c68 in these cells. Surprisingly, GSN-c68 was also found in the cell extracts of the GSN$^-$EF or AGA$^-$EF cells expressing mutant FAF gelsolin (**IV**) (Fig. 10).

Since neuropathy is a characteristic feature of FAF, analyses of the processing of FAF mutant gelsolin in cells of neuronal origin was considered of special interest. Only 0-7% of mutant FAF gelsolin was cleaved in human schwannoma cell cultures derived from two patients, whereas the majority (60-90%) of the mutant FAF gelsolin was cleaved to FAF amyloid precursor by both undifferentiated and nerve growth factor-induced rat pheochromocytoma PC12 cells. In addition, GSN-c68 and GSN-NH_2 fragments were found in both the medium and the cell extracts of mutant gelsolin expressing PC12 cells. In primary cultures of human telencephalic progenitor cells, most of the FAF gelsolin was also cleaved to the FAF amyloid precursor (Fig. 10). No signs of endocytosis of GSN-c68 polypeptides were observed in pulse chase analysis of PC12 cells. Only full-length gelsolin was detected in the transduced cells expressing mutant FAF gelsolin in temperature block analysis, in which the proteins were restricted from exiting the trans-Golgi network at 20°C (Saraste and Kuismanen 1984). The fragmentation of mutant FAF gelsolin to GSN-c68 could be inhibited by EDTA, although the concentration needed for inhibition was higher in PC12 cells (**III**) than in COS-1 cells (**I**). The cellular distribution of EDTA taken from the medium into cells is not completely known. It seems likely that EDTA could inhibit extracellular metalloendoproteases but the capacity of EDTA to inhibit proteases in different cellular compartments remains unknown.

The intracellular distribution of both the wild-type and the FAF mutant secretory gelsolin were identical in the nerve growth factor-induced PC12- or human neuronal progenitor cells. Gelsolin immunostaining was seen in the soma, along the processes and at the tip of the extensions. In PC12 cells, the gelsolin immunostaining pattern most closely resembled that of immunostaining with synaptophysin, a presynaptic marker. PC12 cells and human neuronal progenitor cells expressing the secretory wild-type or FAF mutant gelsolin were morphologically similar and no signs of induced cell death were observed in the cells expressing mutant gelsolin (**III**).

5 FAF MUTATION DOES NOT DISTURB THE NORMAL ACTIN-MODULATING FUNCTION OF GELSOLIN (IV)

Although it is known that the intracellular mutant FAF gelsolin is not aberrantly processed to FAF amyloid precursor (**II**), the consequences of the FAF mutation for the functioning of intracellular gelsolin have been unknown. Since the FAF mutation is located on a functionally active region of gelsolin (Table 3), we decided to study the function of mutant intracellular gelsolin in cell cultures. GSN$^-$EF cells were infected with recombinant retroviruses encoding for the intracellular (or secretory) wild-type or FAF mutant (Asn_{187}) gelsolin, and the actin network was monitored with Oregon green 488 phalloidin staining (**IV**).

Immunostaining of gelsolin polypeptides showed that the intracellular wild-type and mutant FAF gelsolins were equally distributed in the GSN$^-$EF cells. Intracellular gelsolin was distributed mainly perinuclearly and more faintly throughout the cytoplasm, whereas

the localization of the secretory gelsolin was perinuclear. Nontransduced control GSN⁻EF cells showed only a faint background staining.

The actin stress fiber-type stainings were similar in both the control nontransduced AGA⁻ EF cells and in the AGA⁻EF cells transduced with a recombinant retrovirus encoding for the AGA cDNA. In the majority of the nontransduced GSN⁻EF cells, the actin filaments were organized as characteristic stress fibers. A minority of the nontransduced cells (15%) contained very few, if any, actin fibers. In contrast to this finding, the majority (70%) of the GSN⁻EF cells transduced with the retrovirus encoding for the wild-type or FAF mutant intracellular gelsolin revealed weaker actin filament staining and the cells contained only a few organized actin filaments. Surprisingly, in 50% of the cells transduced with the secretory wild-type or FAF mutant gelsolin cDNA weak actin fibers or no fibers at all were found. This result was most likely due to the synthesis of intracellular gelsolin polypeptides from the mRNA for secretory gelsolin because of the leaky scanning mechanism for translation initiation (Kozak 1989) (see also Fig. 5 and 6).

6 EFFECTS OF GELSOLIN PEPTIDE (GSN 150-169) ON THE CLEAVAGE OF SECRETORY FAF GELSOLIN

Gelsolin peptide (GSN150-169) from segment 2 of gelsolin binds avidly to polyphosphoinositides (PPI's) and competes with gelsolin for binding to phosphatidylinositol 4-monophosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) (Janmey et al. 1992). Cleavage of mutant FAF gelsolin to GSN-c68 might involve the interaction of PPI's with mutant gelsolin. Binding of mutant FAF gelsolin to PIP_2, on the Golgi membrane, for example, might allow the enzyme responsible for the first cleavage of mutant FAF gelsolin to bind to mutant gelsolin and cleave it. To inhibit the putative interaction of mutant FAF gelsolin with PPIs, PC12 cell cultures expressing wild-type or mutant FAF gelsolin were incubated in the presence of 20 or 50 μM gelsolin peptide (GSN150-169) for 30 min. The cells and media were analyzed by immunoprecipitation analysis, using anti-am and COOH961 antibodies (see Fig 10).

The cleavage of mutant FAF gelsolin to GSN-c68 was not inhibited when PC12 cells expressing FAF mutant secretory gelsolin were incubated in the presence of the 20 μM gelsolin peptide (GSN150-169). The 50 μM gelsolin peptide was toxic, since this peptide markedly inhibited the synthesis of both the wild-type and FAF mutant full-length gelsolin polypeptide and the GSN-c68 polypeptide (Paul Janmey and H. Kangas, unpublished results).

DISCUSSION

1 PATHOGENESIS OF FAF AT THE CELLULAR LEVEL

During this thesis work we were able to elucidate some important issues concerning the cellular pathogenesis of FAF. The most important findings of these studies can be summarized as follows:

- Proteolytic processing of secretory mutant FAF gelsolin is the most important event in the molecular pathogenesis of FAF.
- The normal formation of the disulfide bond in the immediate vicinity of the FAF mutation site of gelsolin seems to be crucial for the normal processing of the polypeptide.
- FAF tissue amyloid is solely derived from secretory gelsolin. Further, the role of different cell types varies in the tissue pathogenesis of FAF and neurons seem to have a significant role in the generation of FAF amyloid precursor.
- The actin-modulating function of intracellular mutant FAF gelsolin appears to be normal.

This novel data is discussed in more detail in the following sections.

1.1 Proteolytic processing of FAF gelsolin (I-IV)

1.1.1 The cleavage of mutant FAF gelsolin to FAF amyloid precursor during intracellular secretion

Proteolytic processing of the precursor proteins for amyloid seems to be a critical factor in the disease pathogenesis in many forms of amyloidosis (Table 1). Here, we studied the proteolytic processing of mutant FAF gelsolin in various cell types.

The present results show that, in contrast to wild-type secreted gelsolin, mutant FAF gelsolin (Asn_{187} or Tyr_{187}) was cleaved to an aberrant 68 kDa polypeptide (GSN-c68) in COS cells. Studies using different antibodies and amino-terminal aminoacid sequencing of this fragment confirmed that it carries the FAF amyloid sequence at its aminoterminal end and thus most likely represents the immediate precursor protein for FAF amyloid and the 60 kDa fragment found in patients' serum (**I, II**) (Fig. 10 and 12). This first cleavage of FAF gelsolin is most probably a prefibrillogenic event and triggers the pathological cascade of events ultimately resulting in accumulation of tissue amyloid and FAF. Alternative processing of the precursor protein for amyloid has been shown, not only for gelsolin, but also APP. Cells expressing APP carrying the $Lys_{670}AsnMet_{671}Leu$ double mutation secrete more Aβ than wild-type cells (Citron et al. 1992). Other mutations in APP (Val_{717}) cause enhanced production of the longer Aβ (1-42) peptide, while cells expressing the wild-type APP mainly produce the Aβ (1-40) peptide (Suzuki et al. 1994).

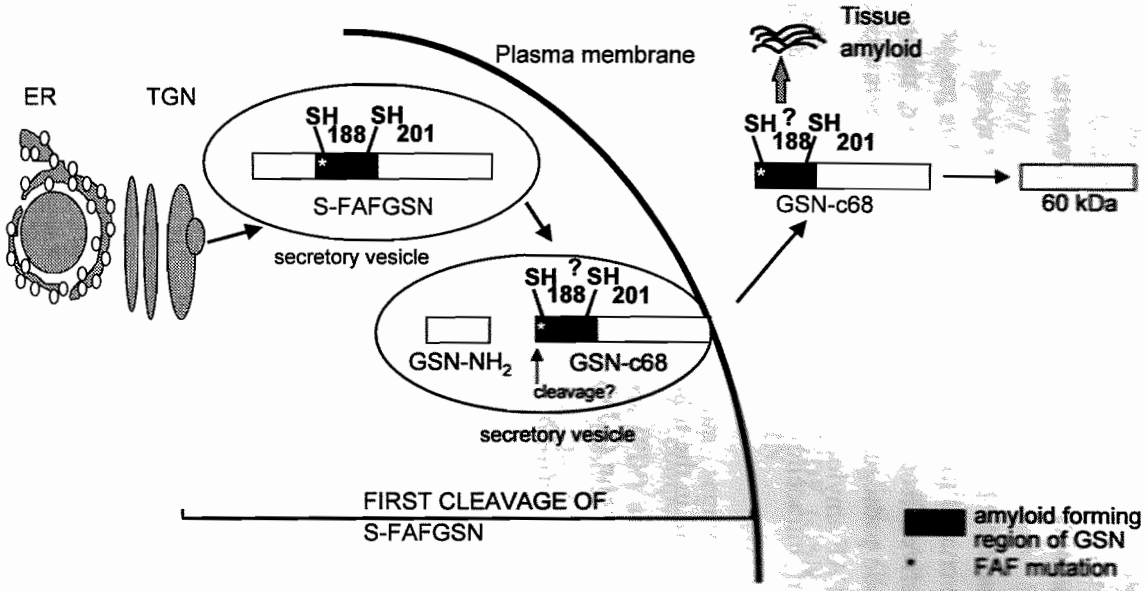

Figure 12. Hypothesis to account for the initial pathological events leading to FAF. Only FAF mutant secretory gelsolin, but not intracellular gelsolin, is proteolytically processed in cells to the FAF amyloid precursor (GSN-c68) (**I-IV**). This indicates that mutant secretory FAF gelsolin is the source of amyloid in FAF (**II**). Lack of the disulfide bond between cysteines 188 and 201 of mutant secretory FAF gelsolin most likely leads to abnormal initial folding of the protein and makes it susceptible to the proteolytic cleavage producing the GSN-c68 and GSN-NH_2 fragments (**III**). This first prefibrillogenic cleavage occurs in cells after the trans-Golgi network but before secretion (**I, III**), and does not involve the interaction of polyphosphoinositides with mutant gelsolin. The second putative cleavage results in the generation of FAF tissue amyloid (consisting of amino acids 173-244 of gelsolin (Maury 1991b)) and the 60 kDa fragment (Sunada et al. 1994; Paunio et al. 1994). The second cleavage of mutant FAF gelsolin to FAF amyloid could occur during incorporation of the mutant gelsolin into the extracellular matrix, during transport to the circulation or in the serum. The role of the cysteines in the later processing steps of GSN-c68 to FAF amyloid is unclear, but the cysteines have been suggested to help to stabilize the FAF amyloid (Burtnick et al. 1997). ER = endoplasmic reticulum, TGN = trans Golgi network, s-FAFGSN = FAF mutant secretory gelsolin, GSN-c68 = FAF amyloid precursor found in cell cultures (**I-IV**) and patients' CSF (**II**), GSN-NH_2=the 15-25 kDa aminoterminal cleavage fragment of gelsolin found in cell cultures (**III, IV**), 60 kDa = 60 kDa cleavage fragment of gelsolin found in patients' serum (Sunada et al. 1994) and CSF (Paunio et al. 1994).

In addition, it was recently shown that wild-type APP is mostly cleaved by caspase-3, whereas mutant APP ($Lys_{670}AsnMet_{671}Leu$) is cleaved by caspase-6 (Gervais et al. 1999).

FAF amyloid precursor (GSN-c68) was not detected in the cells expressing mutant gelsolin (**I, III**) when protein transport to trans-Golgi network was stopped (Saraste and Kuismanen 1984). Nor did the proportion of this precursor increase when the medium containing mutant secretory FAF gelsolin was incubated at 37°C. These observations suggest that the first cleavage of mutant secretory gelsolin to FAF amyloid precursor most likely occurs after trans-Golgi network, during secretion into the media or on the cell membrane (**I**) (Fig. 12). The location of the FAF gelsolin-processing enzyme may thus be restricted solely to the secretory pathway. The cleavage of mutant FAF gelsolin to GSN-c68 was not inhibited when PC12 cells expressing mutant FAF gelsolin were incubated with the gelsolin peptide (GSN150-169), which competes with gelsolin for the binding to PPIs/PIP_2. This suggests that interaction of mutant gelsolin with polyphosphoinositides is not needed for the cleavage of the mutant FAF gelsolin to the FAF amyloid precursor (Paul Janmey and H. Kangas, unpublished results).

1.1.2 Enzymatic processing of mutant FAF gelsolin to FAF amyloid precursor

The enzyme(s) responsible for the first proteolytic cleavage of mutant FAF gelsolin to FAF amyloid precursor is/are unknown. To analyze the type of protease involved in the pathologic processing of mutant FAF gelsolin, various protease inhibitors were added to the culture medium of cells expressing mutant FAF gelsolin (**I**). None of the cysteine, serine, or aspartic protease inhibitors used in this study could inhibit the cleavage of mutant FAF gelsolin to GSN-c68. However, EDTA and phenanthroline, which inhibit various metalloproteases, diminished the production of GSN-c68 markedly (**I**). Structural analysis of wild-type gelsolin segment 1 complexed with actin has suggested that the FAF mutation at amino acid 187 of gelsolin could cause local rearrangements to the gelsolin structure, leaving amino acid 172 more accessible to proteolytic cleavage by trypsin-like proteases (McLaughlin et al. 1993). Our demonstration of the novel cleavage site between amino acids 172-173 of gelsolin (**II**) is in accordance with these structural studies, although the present results suggest that the cleaving enzyme might be a metalloendoprotease (**I**). Since EDTA was able to diminish the cleavage of mutant gelsolin to GSN-c68 in PC12 cells (**III**), the results suggest that metalloendoproteinases might be involved in the cleavage of mutant FAF gelsolin in the neuronal cells also.

Proprotein convertases (PCs) are a family of enzymes involved in the intracellular endoproteolytic processing of a variety of protein precursors at specific sites. Some of these PCs can cleave precursors in the constitutive secretory pathway at the level of the trans-Golgi network or cell surface (Seidah and Chrétien 1997) and are thus potential candidates for the mutant FAF gelsolin-processing enzymes. Recently, it was shown that processing of the precursor protein for familial British dementia (BRI) to amyloidogenic ABri peptides is mediated by the proprotein convertase furin (Kim et al. 1999).

1.1.3 The second cleavage of mutant FAF gelsolin to FAF amyloid

Our data on Western analysis suggest that GSN-c68 can be found not only in the cell cultures, but also in the CSF of patients with FAF (**II**). A 65 kDa gelsolin fragment detected in the plasma of homozygous FAF patients has been suggested to represent this same carboxyterminal cleavage product of gelsolin (Maury et al. 1997) (Fig. 4). At some stage after secretion, a second putative cleavage at the carboxyterminus of the amyloid-forming region of this fragment at amino acid 244 or 247 is likely to produce the FAF amyloid protein (amino acids 173-243) and the 60 kDa gelsolin polypeptide also found in patients' serum and CSF (Sunada et al. 1994; Maury 1991b; Paunio et al. 1994) (**I, II**) (Figs. 4, 10, and 12). Alternatively to serum, the second cleavage of mutant FAF gelsolin to FAF amyloid could occur during transport to the circulation or during incorporation of the mutant gelsolin into the extracellular matrix. FAF amyloid deposits can be stained not only with the FAF amyloid-specific antibody, but also with a monoclonal antibody raised against the carboxyterminal, nonamyloid-forming part of gelsolin (Haltia et al. 1990b; Kivelä et al. 1994). This might indicate that mutant gelsolin or fragments of it are codeposited with FAF amyloid or that the final processing to the FAF amyloid occurs locally (Kivelä et al. 1994). Post-fibrillogenic processing of the amyloid precursor has been demonstrated, for example, in AA-amyloidosis (Kisilevsky et al. 1994).

The two disease-associated forms of gelsolin (Asn_{187} and Tyr_{187}) were similarly processed in COS-1 cells, which indicates that the initial cellular mechanisms underlying the pathogenesis of the two molecular subtypes of FAF are similar (**I**). This is not surprising, since the clinical findings in patients' carrying either of these mutations closely resemble each other (Meretoja 1969; Boysen et al. 1979). In addition to creating the novel proteolytic cleavage site in gelsolin, FAF mutation also increases the fibrillogenic potential of gelsolin, as has been shown with mutant Asn_{187} synthetic gelsolin peptides. However, synthetic gelsolin peptides carrying the Tyr_{187} mutation required an acidic milieu to form amyloid, while Asn_{187} peptides formed amyloid-like fibrils even in water (Maury et al. 1994). It is not clear whether this difference has any biological significance. Interestingly, it has been shown that both the wild-type and the mutant (Asn_{187}) gelsolin fragments consisting of the amyloid-forming region of gelsolin (GSN 173-243) formed fibrils in acidic, but not in physiological, conditions (Ratnaswamy et al. 1999). Thus, the nonamyloidogenic nature of the wild-type gelsolin in humans seems to result from the resistance of the protein to proteolysis. The destabilization of the structure of gelsolin by the FAF mutation and subsequent proteolysis of gelsolin leads eventually to the formation of FAF amyloid fibril. This might occur in an acidic organelle such as in endosome or lysosome or via the intermediacy of the extracellular matrix (Ratnaswamy et al. 1999). However, we have never detected endosomal- or lysosomal-like staining in immunofluorescence analysis of cells expressing FAF mutant gelsolin (**I-IV**).

1.1.4 The role of mutant intracellular FAF gelsolin in the formation of amyloid

Although amyloid deposits are defined as extracellular, it is not definitively known whether fibril formation is initiated intra or extracellularly. Intracellular amyloid has been

found, for example, in human insulinomas and in neurons infected with Semliki Forest viruses encoding for the wild-type or mutant APP (O'Brien et al. 1994; Tienari et al. 1997) (see also section 1.3.1). In some amyloid diseases, fibrillogenesis might thus initially be an intracellular event, followed by release of the amyloid fibrils into the extracellular space after cell death. Since FAF mutation is shared by both the intracellular and secretory forms of gelsolin, it was of special interest to us to analyze the role of the mutant intracellular FAF gelsolin in amyloid formation and in the pathogenesis of the disease. We found that there was no difference between the processing or cellular distribution of the wild-type and the FAF mutant forms (Asn_{187} or Tyr_{187}) of intracellular gelsolin in COS-1 cells or embryonic gelsolin knock-out fibroblasts (**II, IV**). Especially, the mutant intracellular gelsolin was not proteolytically processed to GSN-c68 or to FAF amyloid in these cells. Our results strongly indicate that intracellular mutant FAF gelsolin does not contribute to the formation of amyloid in FAF, the sole source of the amyloid being the secretory form of gelsolin (**II, IV**) (Fig. 12).

FAF amyloid deposits have been found in most tissues of the patients, but especially attached to the blood vessel walls and basement membranes of most organs (Meretoja and Teppo 1971). In FAF, the origin of the gelsolin peptides in the amyloid deposits is unknown. They may arise from the circulating plasma gelsolin, but local synthesis is also possible, especially because gelsolin is synthesized in so many tissues. Local production of mutant gelsolin may cause the characteristic skin amyloidosis in FAF, as high levels of gelsolin are expressed in the skin (Paunio et al. 1997). In addition, local production may contribute to the corneal amyloid deposits (Kivelä et al. 1994). It has also been suggested that local production of amyloidogenic gelsolin by vascular smooth muscle cells may be related to the FAF-related cerebral amyloid angiopathy (Kiuru et al. 1999). FAF amyloid deposits have not been reported from the brain parenchyma (Meretoja and Teppo 1971). Dementia is not common in FAF, but it does occur, probably as a result of some other unrelated disease (Haltia et al. 1991; Kiuru 1998). It has been shown that only intracellular gelsolin, but not plasma gelsolin, is expressed in the rat brain (Vouyiouklis and Brophy 1997). Since intracellular mutant FAF gelsolin seems not to be processed to the FAF amyloid precursor (**II, IV**), the absence of the secretory FAF amyloid precursor might explain the absence of FAF amyloid in patients' brains. *In situ* hybridization studies with human brain tissue would be useful in analyzing this interesting issue.

1.1.5 The significance of the disulfide bond between cysteines 188 and 201 of gelsolin for the processing of secretory gelsolin

The formation of the disulfide bond between cysteine residues in a polypeptide chain often stabilizes the three-dimensional structure of extracellular proteins (Alberts et al. 1994). A disulfide bond is formed next to the FAF mutation site between amino acids 188 and 201 in the secretory, but not in the intracellular, form of gelsolin (Wen et al. 1996). The restriction of proteolytic processing to the mutant secretory form of gelsolin might be a consequence of the localization of the enzyme(s) responsible for the first cleavage of the mutant FAF gelsolin solely in the secretory pathway. The FAF mutation might also

disturb the formation of the disulfide bond in the secretory mutant gelsolin. We demonstrated that secretory gelsolin, carrying the *in vitro* constructed $Cys_{188}Ser$ mutation, was processed similarly to FAF gelsolin in COS-1 cells. A 68 kDa polypeptide, which corresponded in size and immunoreactivity to that of the GSN-c68 fragment, was secreted by these cells. This would suggest that the disulfide bond between cysteines 188 and 201 is not properly formed in the secretory mutant FAF gelsolin. Therefore, we hypothesize that lack of the disulfide bond could lead to the abnormal initial folding of the mutant gelsolin polypeptide, making it susceptible to aberrant proteolytic cleavage during secretion producing the FAF amyloid precursor fragment (Fig. 12). However, it was recently shown, with a gelsolin segment two polypeptide, that the disulfide bond can form correctly in this truncated form of gelsolin despite the FAF mutation (Isaacson et al. 1999). Similar proportions, (about 30%) of the $Cys_{188}Ser$ mutant and FAF mutant gelsolin were cleaved by COS-1 cells, which was most probably due to insufficient capacity of the gelsolin cleaving enzyme(s) in these cells overexpressing the mutated proteins (**III**). However, it should be noted that it has not been shown whether the disulfide bond really exists in the full-length mutant FAF gelsolin molecule. It is also possible that the serine mutation in the wild-type gelsolin polypeptide (**III**) does not hamper the disulfide bond formation, but rather as such may cause defective folding of the polypeptide, making it susceptible to proteolytic cleavage, and producing the 68 kDa GSN-c68-like polypeptide.

Although our results strongly indicate that the disulfide bond between cysteines 188 and 201 is not present in the full-length secretory FAF gelsolin (**III**), it is possible that these cysteines have a role in the later processing steps of GSN-c68 to FAF amyloid (**III**). They might help to stabilize the FAF amyloid, as has been suggested on the basis of the three-dimensional structure of horse plasma gelsolin (Burtnick et al. 1997). For fibrillin, for example, it has been proposed, that rearrangement of the inter or intramolecular disulfide bonds could occur after secretion of the protein into the extracellular matrix (Sakai et al. 1991). Rearrangements of this kind could also occur for mutant FAF gelsolin polypeptides.

1.1.6 The production of FAF amyloid precursor in neurons

Interestingly, our results show that processing of the mutant secretory FAF gelsolin to GSN-c68 varies according to the cell types. Thus, the amount or activity of mutant FAF gelsolin-processing enzyme may be different in different cell types. In the majority of the cells, mutant secretory FAF gelsolin was cleaved to FAF amyloid precursor, but the amount of the precursor varied from 5 to 90% (**I-IV**). Only a fraction of the secretory mutant FAF gelsolin was processed to FAF amyloid precursor in the schwannoma cells representing the glial cells of the peripheral nervous system. However, most (60-90%) of the secretory mutant FAF gelsolin was cleaved to GSN-c68 in both undifferentiated and nerve growth factor-induced rat pheochromocytoma (PC12) cells, the classical model of the neuronal lineage. The GSN-c68 and GSN-NH_2 fragments of gelsolin were detected both in cell lysates and in the media. Furthermore, in primary cell cultures of human telencephalic progenitor cells, secretory mutant FAF gelsolin was extensively cleaved to

GSN-c68. Since large amounts of mutant gelsolin was processed to the FAF amyloid precursor in cells of neuronal origin, it seems likely that a constantly high level or activity of the gelsolin cleaving enzyme(s) is present in these cells (**III**).

In PC12 cells, as in COS-1 cells the first cleavage of mutant gelsolin to GSN-c68 occurred after passage through trans-Golgi network and the proteolytic processing could be inhibited with EDTA (**I, III**). This suggests that the mechanisms leading to the generation of the FAF amyloid precursor in nonneuronal and neuronal cells are similar. No sign of increased cell death or morphologic differences were observed in the nerve growth factor-induced PC12 cells expressing wild-type or mutant secretory gelsolin. In immunocytochemical stainings of these cells, gelsolin colocalized perhaps best with synaptophysin, a presynaptic marker, which might suggest that gelsolin is secreted from the presynaptic region of neurons (**III**). However, analysis of the cellular site of secretion of gelsolin (dendrites, axon, or soma) would be important to confirm this hypothesis. In conclusion, our findings suggest a significant role for neurons when compared to other cell lines studied (**I-IV**) in generating the precursor protein for FAF amyloid and perhaps in the subsequent accumulation of FAF amyloid in the cornea close to the trigeminal nerve endings and peripheral nerves (**III**). In addition to FAF amyloidosis, a special role for neurons in generating the precursor protein for amyloid has been reported for APP. A difference between the processing of wild-type and mutant APP has been observed in neurons and astrocytes. Wild-type APP is processed to Aβ in neurons but not in astrocytes while mutant APP is processed to Aβ in both cell lines (Macq et al. 1998).

1.1.7 The production of FAF amyloid precursor in non-neuronal cells

Approximately 10-30% of the mutant FAF gelsolin was cleaved to GSN-c68 in cells of renal, hepatic and lung origin. The GSN-NH_2 fragment was occasionally found in the cell culture medium of canine kidney cells and human lung carcinoma cells. Adult fibroblasts and monocytes seem not to have a significant role in generating FAF amyloid, since mutant FAF gelsolin was not apparently cleaved to FAF amyloid precursor in these cells (**III, IV**). Our results indicate that, in contrast to the situation in adult human fibroblasts, the enzyme(s) responsible for the first cleavage of mutant FAF gelsolin to FAF amyloid precursor are present in embryonic mesenchymal cells. Mutant FAF gelsolin was cleaved to GSN-c68 in embryonic mouse fibroblasts in which the proportion of GSN-c68 was relatively high, 45%. In addition, GSN-c68 was found both in the culture medium and cell extracts of embryonic mouse fibroblasts. Moreover, the GSN-NH_2 fragment was also secreted from these cells. FAF gelsolin was similarly cleaved to GSN-c68 in mouse embryonic gelsolin knock-out fibroblasts. This indicates that wild-type gelsolin is not needed for the proteolytic processing of mutant FAF gelsolin to the FAF amyloid precursor (**IV**), as can also be concluded from the existence of two homozygous FAF patients (Maury et al. 1992). Our results also suggest that the underlying disease mechanism in FAF is different from the prion diseases or AA-amyloidosis, in which the amyloid formation may start from the endogenously expressed precursor protein in the

presence of triggering molecules such as prion or amyloid enhancing factor (Kisilevsky and Fraser 1997).

FAF amyloid was never detected in neuronal or non-neuronal cell cultures expressing mutant FAF gelsolin. This could indicate that the enzyme responsible for the second cleavage of the FAF amyloid precursor to FAF amyloid was not synthesized in the cell lines used. Or additional environmental factors, such as changes in the pH or addition of a nidus (for example mutant gelsolin peptides) to the cell cultures, might be needed for FAF amyloid formation to occur in cell cultures.

1.2 Function of FAF gelsolin (IV)

Although our results strongly imply that the mutant intracellular FAF gelsolin is not involved in the actual formation of amyloid in FAF (**II**), we were interested in studying the effect of the FAF mutation on one of the major functions, namely the actin-modulating function, of intracellular gelsolin. The FAF mutation is located in a functionally active area of gelsolin, on segment 2, which is involved, for example, in F-actin binding, severing and capping (Yin and Stossel 1979; Yin et al. 1980; Yin et al. 1981) (Fig. 9, Table 3). Gelsolin knock-out fibroblasts (GSN$^-$EF) provided us with an ideal background for studying the actin-modulating function of the intracellular mutant FAF gelsolin *in vivo* (**IV**).

Confocal microscopic studies showed that intracellular wild-type and FAF mutant gelsolin were similarly distributed in the GSN$^-$EF cells around the nucleus and throughout the cytoplasm. In the majority of the nontransduced GSN$^-$EF cells, actin filament staining was strong and prominent and the filaments were organized as characteristic stress fibers. In contrast, in the majority of the transduced GSN$^-$EF cells expressing either wild-type or FAF mutant gelsolin, the actin filaments were weak and thin or the cells had no filaments at all (**IV**). This result was most probably due to the actin-severing activity of recombinant gelsolin. Since the actin filament staining was similar in the wild-type and FAF mutant intracellular gelsolin-expressing GSN$^-$EF cells, it appears that the FAF mutation does not disturb the actin-modulating function of gelsolin in these cells (**IV**).

It has recently been reported that overexpression of a carboxyterminally truncated wild-type but not the FAF mutant plasma gelsolin prevented neural sprouting in neural crest-derived Paju cells (Westberg et al. 1999). This was proposed to be due to the defective actin-severing activity of mutant cytoplasmic gelsolin (Westberg et al. 1999). Curiously, conclusions were drawn from the function of intracellular gelsolin, although the constructs used in that study were derived from carboxyterminally truncated plasma gelsolin, apparently containing the signal sequence. However, it is possible, that the actin-modulating function of mutant FAF gelsolin might be different in neuronal and non-neuronal cells and that, in different cell types and tissues, the pathogenic mechanisms underlying FAF may vary.

It has been shown with plasma samples from homozygous patients with FAF that FAF mutant plasma gelsolin has defective actin-severing and nucleating activity *in vitro* (Weeds et al. 1993). This could be due to the abnormal fragmentation of the mutant plasma gelsolin in the analyzed samples. It is also possible that while the function of the intracellular FAF gelsolin remains intact (**IV**) the lack of the disulfide bond in the plasma gelsolin (**III**) could result in the abnormal folding of the mutant gelsolin molecule and thus influence its normal actin-severing and nucleating activity.

Studies with gelsolin knock-out mice have shown excessive actin stress fibers in adult dermal fibroblasts, defects in platelet shape changes causing prolonged bleeding time, defects in the inflammatory response, and migration of leucocytes and dermal fibroblasts (Witke et al. 1995), while the major clinical findings in patients with FAF are ophthalmologic, neurologic, and skin changes (Meretoja 1969; Kiuru 1992). It is possible that putative malfunction of intracellular mutant FAF gelsolin might be compensated by another member of the gelsolin family with functional similarity to gelsolin. However, even if some other protein were able to compensate for the function of gelsolin in cells, the patients' symptoms would still be expected to be more severe if the function of mutant intracellular gelsolin were impaired. Thus, on the basis of our results it appears that the symptoms of the patients with FAF are caused by the accumulated tissue amyloid, and not by the disturbed function of mutant intracellular gelsolin (**IV**).

CONCLUDING REMARKS AND FUTURE PROSPECTS

This study was carried out to clarify the disease mechanisms underlying FAF at the cellular level. We have shown that the secretory mutant FAF gelsolin is abnormally processed by proteolysis to FAF amyloid precursor in various cells. This is the initial cellular step which initiates the pathological cascade of events that ultimately results in FAF. The first cleavage most likely occurs at some phase during the secretion pathway after the trans-Golgi network but before secretion from the cell and seems not to involve interaction of polyphosphoinositides with mutant FAF gelsolin. Neither is wild-type gelsolin needed for the cleavage of FAF gelsolin. The initial disease mechanism underlying the "Finnish" and "Danish" types of gelsolin-related amyloidosis (with Asn_{187} and Tyr_{187} –mutation respectively) seems to be similar. A second putative cleavage of FAF amyloid precursor at amino acid residue 244 is likely to create the FAF tissue amyloid (amino acid residues 173-244 of gelsolin) and the 60 kDa cleavage fragment detected in the serum of patients. The second cleavage of mutant FAF gelsolin to FAF amyloid has not been defined so far and may occur during incorporation of the mutant gelsolin into the extracellular matrix, in the serum, or during transport of the mutant protein into the circulation.

The enzyme(s) responsible for the first proteolytic cleavage of FAF gelsolin remain unknown. Our results suggest that metalloendoproteases might be involved in the aberrant processing, but other enzymes such as trypsin-like proteases or proprotein convertases, are putative candidates as well. Intracellular FAF gelsolin is not cleaved to FAF amyloid precursor, which suggests that the FAF tissue amyloid is derived from the secretory FAF gelsolin. This might also indicate that the enzyme responsible for the first cleavage of FAF gelsolin is located in the secretory pathway. The activity or amount of the proteolytic enzyme varies with the different cell types, although the cleavage mechanism is likely to be similar in all types of cells. Future challenges of FAF research include the characterization of the enzymes responsible for the two cleavages of secretory mutant FAF gelsolin.

We found that the cleavage of mutant FAF gelsolin to FAF amyloid precursor was most effective in neurons when compared to other cell lines studied. This result might at least partially explain the accumulation of amyloid around peripheral nerves. In addition, the composition of the extracellular matrix might also contribute to the FAF amyloid deposition in neurons. Despite the findings of the present study revealing some particular features of neurons in the processing of mutant FAF gelsolin to FAF amyloid precursor, characterization of the true role of these cells in the pathogenesis of FAF would need further investigation in the future. Analysis of the cellular site of secretion of gelsolin in neurons and more detailed analysis of different cell types (e.g. blood cells, epithelial cells, muscle cells) and tissues (e.g. biopsy samples of patients with FAF) in the disease pathogenesis would be interesting in solving this issue. Moreover, transgenic animals expressing mutant FAF gelsolin under the control of a neuron-specific or ubiquitous

promoter could be utilized to further analyze the significance of neuronal and non-neuronal cells in the tissue pathogenesis of FAF.

The formation of the disulfide bond between cysteines 188 and 201 of secretory gelsolin is apparently essential for its normal initial folding. We suggest that the lack of the disulfide bond in mutant secretory gelsolin predisposes the polypeptide to the aberrant cleavage producing the FAF amyloid precursor. Stabilization of the gelsolin structure near the mutation and disulfide sites might prevent the pair of cleavage steps that result in the formation of the FAF amyloid (Janmey et al. 1998). However, it should be noted that it is not known whether the disulfide bond really exists in the full-length FAF gelsolin molecule. This could be analysed by resolving the crystallized structure of the secreted full-length mutant FAF gelsolin molecule. Less laborous techniques for analyzing the disulfide bond structure of the mutant FAF gelsolin molecule might involve the peptide mapping and mass spectrometric analysis which were originally developed for disulfide structure analysis of wild-type gelsolin (Wen et al. 1996).

The gelsolin peptides in the FAF amyloid deposits may arise from the circulating plasma gelsolin but local synthesis in tissues like cornea, peripheral nerves, and skin is also possible. An interesting subject of future FAF research would be to analyze the pattern of expression of intracellular and secreted gelsolin in the brain. The putative lack of secretory FAF amyloid precursor in the brain might explain the lack of FAF amyloid in the brain parenchyma of patients with FAF. The FAF mutation seems not to interfere with the normal actin-modulating function of mutant intracellular gelsolin. This suggests that what causes the symptoms in patients with FAF is accumulation of tissue amyloid derived from the secreted gelsolin. However, the pathogenic mechanisms underlying FAF may vary in different cell types and tissues.

The gelsolin-expressing cell lines could be useful for initiation of the development of antisense therapy for FAF with antisense oligonucleotides or ribozymes. Our preliminary results, however, suggest that inhibition of the expression of the gelsolin gene with antisense oligonucleotides would require extensive screening of a wide variety of antisense oligonucleotides to find a potent inhibitor of gelsolin expression. The cell lines described here might be useful in testing different molecules which could inhibit the function of the yet unknown enzyme(s) responsible for the first cleavage of the mutant FAF gelsolin. Development of an animal model for FAF would be an important goal in the future as it would be essential for testing possible therapies. Since a gelsolin null-mouse already exists (Witke et al. 1995) it might be possible to use these mice for production of transgenic mice which would express mutant FAF gelsolin. The novel information from such studies could provide a basis for the development of patient therapy.

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