Cells of the Neuronal Lineage Play a Major Role in the Generation of Amyloid Precursor Fragments in Gelsolin-related Amyloidosis*

Tina Paunio§, Hannele Kangas§, Outi Heinonen‡, Marie-Hélène Buc-Caron‡, Jean-Jacques Robert§, Susanna Kaasinen†, Ilkka Julkunen**, Jacques Mallet§, and Leena Peltonen‡ ‡‡

From the §Department of Human Molecular Genetics, Institute of Biomedicine, University of Helsinki and National Public Health Institute, 00280 Helsinki, Finland, the §Laboratory of Genetic Neurotransmission and of the Neurodegenerative Processes, CNRS, 75013 Paris, France, and the **Department of Virology, National Public Health Institute, 00280 Helsinki, Finland

Gelsolin-related amyloidosis or familial amyloidosis, Finnish type (FAF) (OMIM No105120) is a hereditary amyloid disease caused by a mutation in a precursor protein for amyloid (gelsolin) and characterized by corneal dystrophy and polyneuropathy. In vitro expression of the FAF-mutant (Asp187 → Asn/Tyr) secretory gelsolin in COS cells leads to generation of an aberrant polypeptide presumably representing the precursor for tissue amyloid. Here, we provide evidence that this abnormal processing results from defective initial folding of the secreted FAF gelsolin due to the lack of the Cys188-Cys201 disulfide bond, normally formed next to the FAF mutation site. We compared cells of different tissue origin and discovered a dramatic difference between the amount of cleavage of FAF gelsolin to the amyloid precursor in neuronal and non-neuronal cells. More than half of the mutant gelsolin was cleaved in PC12 and in vitro differentiated human neuronal progenitor cells. In contrast, human fibroblasts and Schwannoma cell cultures showed only a limited capacity to cleave FAF gelsolin, although the cleavage mechanism per se seems to be similar in the various cell types. The present findings of processing and distribution of secreted FAF gelsolin in the neuronal cells emphasize the role of neurons in the tissue pathogenesis of this amyloid polypeathy.

Ameloidosis represents a group of diseases in which abnormal fibrillar protein deposits, derived from biochemically distinct proteins are found in the extracellular space of patients' tissues (1). Gelsolin-related amyloidosis or familial amyloidosis of the Finnish type (FAF), is a representative example of a hereditary amyloid polyneuropathy. The main symptoms in FAF include corneal lattice dystrophy (type II) and progressive cranial and peripheral neuropathy (2, 3). The cell type responsible for amyloid deposits found in organs such as cornea, nerves, skin and kidney (3–9) has been a topic of debate. FAF is caused by the Asp187 → Asn or Asp187 → Tyr mutations of gelsolin (7, 10, 11), an actin-modulating protein. Intracellular and secreted forms of gelsolin differ by the presence of a disulfide bond between amino acids 188 and 201, a signal sequence, and a short amino-terminal extension in the secreted form (12, 13). In addition, a novel gelsolin isoform of intracellular gelsolin-3, has been recently described in oligodendrocytes (14).

Transient expression of the wild-type and mutant gelsolin in COS-1 cells results in the abnormal cleavage of the secretory form of FAF-mutant gelsolin. Consequently, secretion of both the full-length (83 kDa) and the 68-kDa carboxyl-terminal fragment (GSN-c68) of gelsolin consisting of amino acids 173–755 is observed (15, 16). Interestingly, GSN-c68 has been found also in the cerebrospinal fluid (CSF) and plasma of FAF patients (16–18). The second putative cleavage of this fragment at amino acid position 244 is likely to generate the FAF amyloid (amino acids 173–243) (19, 20) and, consequently, a 60-kDa carboxyl-terminal fragment also observed in the serum of the patients and CSF (17, 21) (see Fig. 1). These in vitro and in vivo findings have led to the conclusion that GSN-c68 represents the immediate precursor for FAF amyloid, and thus, generation of this polypeptide seems to be the first crucial event in the molecular pathogenesis of FAF.

This study was carried out to further clarify the molecular mechanisms underlying FAF with particular emphasis on (i) the impact of the FAF mutation on the initial folding of gelsolin and (ii) on the role of the processing of FAF gelsolin by different cell types. By providing evidence for the specific role of neurons in the generation of precursor protein for FAF amyloid, our data give interesting insight into the vigorously studied issue of the cellular pathogenesis of an amyloidosis affecting the nervous system.

EXPERIMENTAL PROCEDURES

Cell Lines and Cultures—COS-1 and Hep2C cells as well as cultured primary fibroblasts from a control individual and from a 55-year-old male FAF patient with the G654A mutation of gelsolin (22) were cultured in Dulbecco's modified Eagle's medium and A549 and Madin-Darby canine kidney (MDCK) cells modified Eagle's medium supplemented with 10 or 5% fetal calf serum (FCS). Undifferentiated PC12 cells were cultured on plates coated with a 1:40 dilution of Matrigel adenovirus encoding for the FAF form of sGSN; MDCK, Madin-Darby canine kidney cells; HS, horse serum.

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‡ These authors contributed equally to this work.
†† To whom correspondence should be addressed. Tel.: 358-9-47448393; Fax: 358-9-47448480; E-mail: Leena.Peltonen@ktl.fi.
§ The abbreviations used are: FAE, familial amyloidosis of the Finnish type; sGSN, secretory gelsolin; GSN-c68, 68-kDa carboxyl-terminal fragment of FAF gelsolin; CSF, cerebrospinal fluid; FCS, fetal calf serum; NGF, nerve growth factor; HNF, human neuronal telencephalic progenitor (cells); p-sGSNwt, expression plasmid encoding for wild-type secretory gelsolin; p-sGSNAsn187, expression plasmid encoding for FAF form of sGSN with Asp → Asn mutation; p-sGSNAsn187, expression plasmid encoding for sGSN with Cys188 Ser mutation; p-sGSNAsn187/Ser188, expression plasmid encoding for sGSN with Asp → Asn and Cys → Ser mutations; Ad-sGSNwt recombinant adenovirus encoding for the wild-type sGSN; Ad-sGSNASn187, recombinant adeno-
(Becton Dickinson Labware, MA) and in RPMI 1640 medium supplemented with 10% normal horse serum (HS) and 5% FCS. For differentiation, a medium with 1% HS and 50 ng/ml nerve growth factor (NGF) (Alomone Labs, Israel) was used.

Human macrophages and monocytes were obtained as described previously (23). 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 with histopathological diagnosis of neurilemmoma atypical and neurilemmoma as published earlier (24). Staining with S-100 antibody, a marker for Schwann cells, was performed to confirm the phenotype of the cultured cells. Human peripheral blood monocyte progenitor cells (HPCs) were obtained after the legal abortion of an 8-week-old fetus and cultivated as earlier described (25) with the addition of dibutyl cyclic AMP (1 mM) (Sigma) to the culture medium. This medium allowed differentiation of the progenitors into cells of neuronal lineage, as was confirmed by the staining of most of the cells with antibodies against the microtubule-associated protein, MAP-2

**Construction of the Expression Plasmids and Recombinant Adenoviruses**—Site-directed mutagenesis changing nucleotide G658 to C (Cys^{188} → Ser) was performed with a Chameleon™ double-stranded, site-directed mutagenesis kit (Stratagene) on pcDX-EX expression vectors containing either the wild-type (Asp^{188}) or FAF-mutant (Asn^{187}) secretory gelsolin cDNA (15). The mutant clones (p-sGSNwt and p-sGSNSer^{188}) were identified using solid-phase minisequencing.

cDNAs encoding for the wild-type and the FAF-mutant gelsolin were ligated to a shuttle vector between the long terminal repeat from Rous sarcoma virus and the SV40 polyadenylation signal sequence, flanked by regions derived from the adenoviral genome. Linearized shuttle plasmid and the large ClaI fragment of Ad-5 DNA with deletion in E3 regions were co-transfected to the 293 cell line. After homologous recombination, the recombinant E1/E3 deleted adenoviruses were plaque-purified and expanded in 293 cells (26). The correct structures were verified by restriction enzyme digestion and Southern blotting as well as by a minisequencing test for gelsolin (22).

**Transfection and Transduction**—Transfection with the plasmid constructs as well as collection of the transfected cells and their media were performed as described earlier (15, 16). Cells were infected for 1–2 h in serum-free medium using the recombinant adenoviruses at different amounts of multiples of infectivity (1–100) in order to avoid a plausible artifact caused by the varying capability of the vectors to transduce different types of cells. After infection, medium supplemented with 2% FCS or 1% FCS and 1% HS was added to all cell types except for the HNP cells, the medium of which contained no FCS. In protease inhibition experiments, EDTA (1.2 or 10 mg/ml) was added to serum-free medium, and the cells were incubated for 4 h. Similar efficiency of transduction in COS-1 and PC12 cell cultures was confirmed by immunocytochemical staining of gelsolin in the transduced cells.

**Immunological Techniques**—Monoclonal anti-gelsolin (GS-2C4) (27), anti-MAP 1 (HM-1), anti-MAP 2 (HM-2) (Sigma), and monoclonal anti-synaptophysin (SY 38, DAKO, Denmark) antibodies were used in the immunocytochemical studies using dilutions provided by the manufacturer. The polyclonal antibodies for gelsolin (K-572, AM904, and NH951) have been described in detail elsewhere (15, 16). To obtain a polyclonal COOH961 antibody (amino acids 420–755 of gelsolin), gelatin-Sepharose, both at 4 °C for 1 h. In temperature-shift experiments, the cells were labeled for 2 h at 19.5 °C (16). The cells and media were mounted in GelMount (Biomeda Corp., Foster City, CA), Vectashield mounting medium (Vector Laboratories), or glycerol gelatin (Sigma) and viewed with a Leica confocal microscope or a Zeiss Axiophot light microscope using a 40 × 63 objective.

**RESULTS**

**The Disulfide Bond between Cys^{188} and Cys^{201} Is Crucial for the Normal Processing of Secreted Gelsolin**—We wanted to determine whether the abnormal cleavage of FAF secretory gelsolin to GSN-c68 fragment results from disruption of the Cys^{188}–Cys^{201} bond formed in the immediate vicinity of the FAF-causing mutation in secretory gelsolin (13). A Cys^{188} → Ser (G658C) mutation was introduced into expression plasmids coding for both wild-type and FAF-mutant gelsolin. The plasmids were expressed in COS-1 cells, and the cells and media were analyzed by Western blotting using antibodies recognizing different epitopes of gelsolin (Fig. 1).

Cells transfected with either of the two plasmids containing the cysteine mutation secreted not only the full-length gelsolin but also the truncated polypeptide of the expected size and immunoreactivity as that of the GSN-c68 gelsolin fragment (Fig. 2). Based on densitometric scanning, approximately 30% of the mutant gelsolin was processed to the abnormal 68-kDa form (GSN-c68). The cell fraction contained only the full-length gelsolin, and the aberrant fragmentation could be inhibited by EDTA (Fig. 2). Thus the results obtained for the secreted gelsolin with the disrupted cysteine bridge Cys^{188}–Cys^{201} were identical to those we had obtained earlier for gelsolin carrying the FAF mutation (15).

**The Processing of Mutant Secreted Gelsolin in Neuronal and Non-neuronal Cells**—To monitor the processing of secreted FAF gelsolin in various cell types, we utilized recombinant adenoviruses encoding for the wild-type or FAF form of secretory gelsolin (Ad-sGSNwt and Ad-sGSNSer^{188}, respectively) and infected cells of different tissue origin with these constructs. The processing of mutant gelsolin in COS-1 cells was found to be similar both in the adenovirus-driven and in the transient expression experiments (Fig. 3, left).

The wild-type and FAF-mutant gelsolin were then expressed in telencephalic derivate from canine kidney (MDCK), human lung carcinoma (A549), and hepatic carcinoma (Hep2c). In each case, wild-type gelsolin was secreted in the full-length form, whereas the media from the Ad-sGSNSer^{188} virus-infected cultures contained the additional, aberrant GSN-c68 fragment (Fig. 3). We observed, however, some variation in the different types of cells, the proportion of GSN-c68 being highest in the media of
MDCK cells (27% of total gelsolin) and lowest in the media of Hep2c cells (6%). In contrast, human fibroblasts transduced with Ad-sGSNAsn187 similarly to fibroblasts derived from a FAF patient secreted only the full-length gelsolin into the culture media (Fig. 5). To ascertain whether fibroblasts have any interest in analyzing the processing of FAF gelsolin in cells of neuroectodermal origin. Schwannoma cell cultures obtained similarly in the MDCK and A549 cells expressing FAF gelsolin (Fig. 5).

The cleavage of FAF gelsolin (83 kDa) into GSN-c68 should also generate an amino-terminal 15 kDa polypeptide (Fig. 1). We detected this polypeptide both in the cells and media of NFG-induced PC12 cells expressing FAF gelsolin. The polypeptide proved to be unstable because it was not found in the COS-1 cells or media and only occasionally in the media from the MDCK and A549 cells expressing FAF gelsolin (Fig. 5).

Kinetics of Processing of Mutant Gelsolin in PC12 Cells—Pulse-chase experiments revealed that the GSN-c68 polypeptide was secreted from the PC12 cells at the same rate as the full-size gelsolin while no signs of endocytosis of gelsolin polypeptides were observed (Fig. 6). This was further confirmed by the finding of only the 83-kDa gelsolin in the media and no endocytosed gelsolin polypeptides from non-transduced PC12 cells that had been incubated with medium derived from fibroblasts expressing FAF gelsolin (data not shown). Pulse-chase experiments on a temperature block of 19.5 °C (28) showed that secreted FAF gelsolin gets cleaved in the PC12 cells after the trans-Golgi network but prior to secretion (Fig. 6). Finally, a high concentration (10 mg/ml) of EDTA had some inhibitory effect on the fragmentation of FAF-gelsolin (Fig. 6C). Thus the results obtained here are very similar to those we obtained earlier in the COS-cells (15), suggesting that the enzymatic process cleaving FAF gelsolin is most likely similar in the PC12 and COS cells.

The Distribution of Secretory Gelsolin in the NFG-induced PC12 and Human Telencephalic Progenitor Cells—In order to examine the distribution of wild-type and mutant secretory

FIG. 3. Processing of FAF-mutant gelsolin in cell lines derived from kidney, lung, and liver as well as in fibroblasts. Media of COS-1, MDCK, A549 and Hep2c cell lines and of fibroblast infected with Ad-sGSNwt and Ad-sGSNAsn187 were subjected to Western analysis with anti-gelsolin antibody. The 83-kDa band corresponding to the full-length gelsolin was found in the medium from cells expressing the wild-type and mutant gelsolin and the GSN-c68 polypeptide (indicated by an arrow) only in the mutant culture medium of COS-1, MDCK, A549, and Hep2c cells. The proportional amount of GSN-c68 was highest (27%) in MDCK and lowest (6%) in Hep2c cells. Immunostaining of the media from control fibroblasts transduced with Ad-sGSNwt and Ad-sGSNAsn187 and the concentrated control (Co) and patient (P) fibroblast medium with anti-gelsolin antibody revealed only the 83-kDa gelsolin. 0 = background of COS-1 cells, MDCK, A549 and Hep2c, and fibroblast cells.

FIG. 2. Role of the Cys188-Cys201 bond in processing of secreted gelsolin. COS-1 cells were transfected with wild-type (wt), p-sGSNAsn187 (Asn187), p-sGSNNser188 (set, -Cys), and p-sGSNAsn187Ser188 (Asn187, -Cys) expression plasmids, and the media were analyzed by Western blot procedure with anti-gelsolin antibody. The major band represents full-length gelsolin (83 kDa), and the lower band (indicated by an arrow) represents the GSN-c68 fragment, secreted from cells expressing the FAF-mutant or cysteine-defective forms of gelsolin. Fragmentation was inhibited by EDTA (1 mg/ml) that was added at 40 h after transfection for 8 h. +Cys = gelsolin with Cys188; −Cys = gelsolin with Ser188; 0 = background of COS-1 cells.

FIG. 4. Processing of mutant gelsolin in cells of neuroectodermal origin. Schwannoma cell cultures, NFG-treated PC12 cells, and human neural progenitor cells (HNP) were infected with Ad-sGSNwt and Ad-sGSNAsn187 and subjected to Western blot analysis and immunostaining with anti-gelsolin antibody. Left, in Schwann cells, the GSN-c68 fragment (indicated by arrow) was found in the medium of the trigeminal Schwannoma cells but not in the medium of the vestibular Schwannoma cells expressing mutant gelsolin. Middle, the GSN-c68 fragment (arrow) was found both in the medium and from the cellular extracts derived from the PC12 cells transduced with Ad-sGSNAsn187. Left, the GSN-c68 fragment (arrow) was found in the medium of HNP cells. Based on densitometric scanning, most (60–90%) of the mutant gelsolin was cleaved in PC12 and HNP cells. 0 = background of Schwann, PC12, and HNP cells.

from two patients showed a limited capacity to cleave FAF gelsolin because only 0–7% of secreted FAF gelsolin was cleaved (Fig. 4, left). However, unlike all the other cell types tested so far, a major fraction (approximately 60–90%) of the mutant gelsolin was cleaved in both undifferentiated and NFG-induced PC12 cells (Fig. 4, middle). Furthermore, the abnormal GSN-c68 polypeptide was detected not only in the medium but also in the PC12 cell extracts (Fig. 4, middle). In order to confirm the results obtained with PC12 cells, HNP cells that had been differentiated in vitro into cells of neuronal lineage were applied for transduction analyses. Also in these cells, more than half of the secreted FAF gelsolin was cleaved to GSN-c68 (Fig. 4, right).
gelsolin in neuronal cells, differentiated PC12 and HNP cells transduced either with Ad-sGSNwt or with Ad-sGSNAsn were subjected to immunocytochemical stainings using anti-gelsolin antibody. The infected and uninfected cells were found to be morphologically similar, and no signs of induced death of cells expressing mutant gelsolin was observed. The wild-type and mutant gelsolin were identically distributed so that, in either case, immunostaining was seen both in the soma and along the processes where the staining was most prominent at the tip of the extensions (Fig. 7A, B, and D–F). Double staining of the transduced PC12 cells using anti-gelsolin antibody with antibodies against either synaptophysin, MAP-1, or MAP-2 showed similar distributions to these marker proteins. The immunostaining pattern of anti-gelsolin antibody was perhaps closest to that obtained with the synaptophysin antibody (Fig. 7C).

DISCUSSION

FAP is a dominantly inherited amyloidosis caused by Asp
\[\rightarrow\] Asn/Tyr mutations in gelsolin (7, 10, 11). We have shown earlier in COS cells that the abnormal cleavage of the amyloidosis-associated forms of secretory gelsolin results in the secretion of an aberrant 68-kDa carboxyl-terminal fragment of gelsolin (GSN-c68) (15), presumably representing an immediate precursor protein for FAF amyloid. Intracellular FAP gelsolin also contains the FAP mutation but is not subject to alternative cleavage in COS cells, suggesting that the secreted form of FAP gelsolin is the only source of FAP amyloid (16).

Characteristic only to the secretory gelsolin is a disulfide bond that gets formed in close vicinity to the FAP mutation, between amino acids 188 and 201 (13, 29). Consequently, the aberrant processing restricted to the secretory FAP gelsolin could be related to disturbed disulfide bridge formation and abnormal primary folding of the molecule. We demonstrated here that secretory gelsolin carrying the Cys\[\rightarrow\] Ser mutation was processed in a fashion similar to that of the FAP mutant protein in COS-1 cells, resulting in the secretion of a truncated carboxyl-terminal polypeptide that corresponded in size and immunoreactivity to the GSN-c68 fragment. Since the introduction of this serine mutation to FAP gelsolin did not cause any other obvious changes in the protein processing or secretion, the present data would indicate that the 188–201 disulfide bond is not properly formed in the mutant secretory gelsolin. This would result in abnormal initial folding of the protein and aberrant proteolytic cleavage producing the FAP amyloid precursor fragment. A similar portion, about 30% of both the FAP and the Cys\[\rightarrow\] Ser mutated secretory gelsolin was found to become abnormally cleaved in the expression system of COS-1 cells, most probably due to the insufficient capacity of gelsolin-cleaving enzyme(s).

Our in vitro expression data are slightly in contrast with the assumption made by Burtnick et al. (29) who suggested, based on the three-dimensional structure of wild-type horse plasma gelsolin, that the Cys\[\rightarrow\] Ser mutated secretory gelsolin was found to become abnormally cleaved in the expression system of COS-1 cells, most probably due to the insufficient capacity of gelsolin-cleaving enzyme(s).
amyloid formation might still be influenced by additional factors in vivo (31).

The amyloid deposits in FAF are found in particular in the cornea, nerves, skin, and kidney in addition to the walls of vessels in various organs (3–9). In addition to the high level of production of gelsolin, for example in the skin, there seem to be additional factors affecting the characteristic tissue distribution of FAF amyloid (32). One such factor could be differential processing of the mutant secretory gelsolin in different types of cells.

Adenovirus-mediated expression provided us with a convenient means to analyze the processing of FAF-mutant gelsolin in cultured cells. In cells of renal, hepatic, and lung origins, approximately 10–30% of the mutant protein was cleaved. In contrast, fibroblasts did not process FAF gelsolin abnormally, nor did monocytes seem to degrade it, suggesting that these cells of the connective tissue and immune system do not play a major role in the generation of FAF amyloid. Interestingly, in the glial cells of the peripheral nervous system, the Schwann cells, only 0–7% of secreted FAF gelsolin was found to be cleaved, whereas a distinct majority of the mutant protein was cleaved in PC12 cells, the classical model of neuronal lineage. Furthermore, the GSN-c68 fragment was found both in the cell culture medium of the PC12 cells and inside the cells, and the amino-terminal counterpart derived from the proteolysis of full-length FAF gelsolin was reproducibly detected only in these cells and their medium. To confirm the results obtained with PC12 cells, primary cultures of human telencephalic progenitor (HNP) cells that had been differentiated and undifferentiated PC12 cells infected with Ad-sGSNwt and double-stained with anti-gelsolin and anti-synaptophysin antibodies; bar = 40 μm. D, anti-gelsolin-staining of differentiated PC12 cells infected with Ad-sGSNAsn187; bar (see in panel F) = 54 μm. E and F, anti-gelsolin staining of HNP cells infected with Ad-sGSNwt; bar = 28 μm. Both the wild-type and mutant secretory gelsolin immunostaining were localized in the soma and at the end of the neuritic processes. In panels A–C, red = gelsolin staining; green = synaptophysin staining; yellow = colocalization of gelsolin and synaptophysin staining.

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