Lithostathine Quadruple-helical Filaments Form Proteinase K-resistant Deposits in Creutzfeldt-Jakob Disease*

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The pathological hallmark of many neurodegenerative diseases are fibrillar deposits of proteins and polypeptides in brain. Many of these deposits are characterized by histochemical staining properties similar to those of starch and hence were called amyloid structures. Although the causal relationship between fibrillar deposits and pathogenesis has not yet been established in all cases, there is increasing evidence that these fibrils can exert a deleterious effect. Their clinical relevance has been shown for instance in cataract formation (1) and in familial encephalopathy with neuroserpin inclusions (2); also, it is highly likely in early onset Parkinson’s disease (3). In addition, there are some recurring correlations between fibrillar pathologies and disease-associated mutations, as in some familial systemic amyloidoses, Huntington’s disease, and early onset Parkinson’s disease (4). These correlations strongly suggest that fibril formation initiates the pathological events and that it is not an unavoidable epiphenomenon. In addition, the early aggregates of disease-associated polypeptides have been shown to be cytotoxic (5, 6). Understanding the process of fibril formation and the means by which amyloid deposits can be cleared in vivo therefore represent two major scientific challenges.

Among proteins that form clinical fibril deposits, we have been working on lithostathine. We have studied its physical characteristics and observed that it readily polymerizes into fibrils after self-proteolysis of its N-terminal undecapeptide (7). On the basis of the x-ray structure of the monomer (8) and high resolution electron microscopy, we showed that fibrils formed a quadruple-helical filament (QHF-litho); see Ref. 9. Interestingly, these fibrils were found to be present in the pathological lesions of Alzheimer’s disease (senile plaques and neurofibrillary tangles), and it is overexpressed during the very early stages of the disease before clinical signs appear (10). We therefore believe that lithostathine constitutes an important protein to investigate to understand the deposition of polypeptides in vivo in relation to neurodegenerative diseases. It has remained unclear, however, whether stacking involves conformational changes comparable with those occurring during PrPsc (prion “scrapie”) formation (11), whether these fibrils are amyloid in nature, and how fibrils stack together to produce large fibers. In this paper, we have used Fourier-transform infrared spectroscopy, circular dichroism, optical absorbance, mass spectrometry, atomic force microscopy, molecular modeling studies, and immunohistochemistry to explore the structural characteristics of lithostathine fibrils, the formation of large fibers, and strategies by which this process can be disturbed. In particular, we have been interested in their potential structural and clinical relation to amyloid diseases, most notably Creutzfeldt-Jakob disease (CJD).
EXPERIMENTAL PROCEDURES

Recombinant Lithostathine—Lithostathine was prepared as described previously (7). In brief, recombinant lithostathine was produced in CHO cells. A CHO cell line overexpressing lithostathine was stably transfected with a retroviral vector containing the lithostathine cDNA, and was selected with Geneticin (G418). lithostathine positive clones were isolated and expanded. Lithostathine was produced using a serum-free medium for 48 h. Samples were then dialyzed against 10 mM Tris base, pH 7.4, dialyzed against 200 mM Tris base, pH 8.0, dialyzed against 200 mM Tris base, pH 8.0, and stored at −80 °C until required. Lithostathine samples were then frozen in liquid nitrogen and stored at −80 °C until required. For experiments concerning fibril formation, 100 μg of lithostathine (1 mg/ml) was incubated in phosphate-buffered saline at 37 °C for 2 weeks. After centrifugation for 5 min at 13000 × g the supernatant was discarded. The pellet was then washed twice and resuspended in 100 μl of water, frozen in liquid nitrogen, and stored at −80 °C until required.

Congo Red Binding—The capacity of lithostathine fibrils and of amyloid fibrils formed from glucagon to bind Congo Red (CR) was assessed by mixing 50-μl aliquots of solutions containing amyloid fibrils from glucagon with 450 μl of a 20 μM solution of the dye in 1× Tris-Cl at pH 7.4. The final polypropylene concentrations were as indicated in the plot. The stock solution containing glucagon amyloid fibrils was prepared by incubating glucagon at a concentration of 1 mg/ml in 50 mM sodium phosphate, pH 2.5, at room temperature for 2 weeks. Lithostathine fibrils were prepared from stock solution (1 mg/ml) as described above. The absorption measurements were carried out at room temperature in a Cary 3 UV-visible spectrophotometer (Varian Inc., Palo Alto, CA).

Fourier Transform Infrared Spectroscopy (FTIR) Experiments—FTIR spectra were recorded on a Bruker IFS28 spectrometer equipped with a liquid nitrogen-cooled MTC detector (Bruker Optics Inc., Billerica, MA). The spectra (1000—2000 scans) were recorded at a spectral resolution of 4 cm−1 and were analyzed using the OPUS/IR2 program (Bruker). To ensure solubility and fibrillar lithostathine, spectra were recorded with dry samples. For this purpose, samples were deposited on to fluorine plates, and the solvent was allowed to evaporate.

Spectroscopic Studies—Baseline-corrected absorbance spectra in the range of 275 to 300 nm were recorded at 37 °C with a Cary 3E spectrophotometer (Varian Inc.) characterized by a high spectral reproducibility (S.D. < 0.02 nm). Data acquisition was in steps of 0.1 nm with an acquisition time of 1 s per data point. The instrument was equipped with a home-built double sample compartment, thermostated at a Haake F3-Q circulating water bath (Thermo Haake, Karlsruhe, Germany). Protein incubation was performed in a 1-mL, 1-cm light path quartz cuvette under the following conditions: 0.47 mg/ml lithostathine in 0.5 μl Tris-Cl, pH 8. The fourth derivatives of the UV spectra were evaluated with the optimized spectral shift method as described previously (12) with an automated transform program designed in the laboratory.

MALDI-TOF Mass Spectrometry—MALDI-TOF analyses were performed using a Voyager DE-STR mass spectrometer (PerSeptive Biosystems, Framingham, MA). The instrument was calibrated with bovine carbonic anhydrase B (22951) and cytochrome c (12361) at 4 × 10−10 m and 10−10 m, respectively. An α-cyano-4-hydroxy-cinnamic acid matrix solution was prepared as a saturated solution in 50% acetonitrile and 0.1% trifluoroacetic acid. 0.5-μl aliquots of a lithostathine solution (1.2 mg/ml in 20 mM phosphate buffer, pH 8) were deposited directly on the sample plate. 0.5-μl aliquots of the matrix solution were then pipetted onto each drop, which was then allowed to dry. Positive-ion mass spectra were collected in reflectron mode at an acceleration voltage of 25 kV and an extraction delay time of 350 ns. Raw data were analyzed and processed using Grams software (PerSeptive Biosystems). Electronic Microscopy Studies—A solution of lithostathine (50 μg/ml) in 100 μM Tris-Cl, pH 7.5, was mixed with a trypsin solution (2.5 mg/ml) and left for 5 min at room temperature, and [6,6]-[1H2]benzothiazol-2,2'-diamine (PGL-034; Merck KGaA, Darmstadt, Germany) was added (1 μl in 100% MeSO, final concentration 20 μM). The mixture was incubated at 37 °C for 16 h. Samples were then stained successively with 1% uranyl acetate for 1 min. The molar ratio of lithostathine/PGL-034 was 3:20, slightly more than used previously for EM analysis of PGL-034 with huntingtin (1:5:20; see Ref. 13). The final preparation was then viewed on a Philips CM100 EM (FEI Company, Hillsboro, OR).

Immunohistochemistry—Brain slices (8 μm) were collected onto pre-treated glass slides (Super Frost Plus; Menzel-Glaser, Braunschweig, Germany) and baked over 1 week at 57 °C. The slides were then dewaxed and used for immunohistochemistry. Endogenous peroxidase activity was inhibited with 1% H2O2 in Tris-buffered saline for 30 min at room temperature. Slides were then further incubated for 30 min in 20% goat serum, 0.3% Triton in Tris-buffered saline to block non-specific antigenic sites. Specific polyclonal antibodies to lithostathine (Romeo; see Ref. 10) were then added at 1/100 for 6 days at 4 °C. To reveal the presence of PrPsc or resistant lithostathine deposits, some slides were submitted to drastic pre-treatment before addition of antibodies. These treatments involved a combination of chemical, physical, and enzymatic methods (14) used to get rid off all the PrPsc. In brief, they were incubated in 0.1% trypsin solution (Merek, Darmstadt, Germany) for 5 min at 37 °C, 120 min at 121 °C in water, and finally in proteinase K (25 μg/ml; Merek) for 20 min at 37 °C. The presence of PrPsc was checked by incubating the mouse monoclonal antibody 8G8, at 1/2000, overnight at 4 °C. This antibody recognizes the human 95–110 PrP sequence. The VectASTAIN ABC kit (Vector Laboratories, Burlingame, CA) was used for detection, using diaminobenzidine (Sigma-Aldrich) in simple staining procedures, and the VIP kit (Vector Laboratories) was used for double staining experiments.

Sequence Alignment and Molecular Modeling—Multiple sequence alignment was carried out using the ClustalX program (15) and manually improved. Swapped loop sequences of a set of C-type lectin proteins and prion protein were selected from the Protein Data Bank and aligned with the lithostathine sequence. Homology modeling of the lithostathine dimer was performed largely according to the principles outlined by Greer (16) and as previously described in detail (17), using the software modules InsightII, Homology, and Discover from Accelrys (San Diego, CA) and a Silicon Graphics O2 work station (SGI, Mountain View, CA). The model of the lithostathine dimer connected by a swapped loop was built from the crystal Protein Data Bank structure of flavocetin-A (1C5A; see Ref. 18) from habu snake venom, a coagulation factor X binding protein heterodimer. The lithostathine dimer was then optimized with the constant valence force field. Energy minimization was carried out with the conjugate gradient algorithm, down to a maximum derivative of 0.001 kcal/Å.

RESULTS

Lithostathine Fibrils Did Not Possess the Classical Properties of Amyloid Fibrils—To establish whether lithostathine fibrils possess the generic amyloid structure, we tested for the presence of cross-β conformation using apple green birefringence upon staining with CR and x-ray diffraction. In the light of conflicting methods used for detecting amyloid in clinical diagnosis, such as dyes of low specificity, it has been agreed by the community that in particular CR green birefringence should be an essential part of the clinical detection of amyloid structures (19). Interestingly, although the morphology of lithostathine fibrils is very similar to that of many amyloid fibrils (9), they do not possess green birefringent properties. In fact, QHF-litho failed to even bind CR, and the dye was washed off readily from samples prepared from fibril pellets after centrifugation. The same result was obtained when we examined the CR absorption spectra in the presence of QHF-litho or glucagon fibrils that have been shown previously (20) to be amyloid in nature. Whereas glucagon fibrils characteristic increased the CR absorption signal at 540 nm, no such effect could be detected for QHF-litho (Fig. 1A). The spectrum of QHF-litho mixed with CR had the same shape as the one recorded on the dye alone. However, a small offset value in the baseline of the two spectra was evident, presumably arising from light scattering induced by the fibrils. Consistent with this observation, we could not obtain evidence for the presence of a cross-β structure when we examined the fibril pellet with x-ray diffraction (data not shown), and also FTIR spectroscopy could not reveal aggregated β-sheet structure in QHF-litho. The sheets of amyloid or prion fibrils appear in the infrared spectrum with an amide I maximum close to 1620 cm−1 (11, 21, 22). In the case of QHF-litho, no such maximum could be discerned. Moreover, fibrils and the soluble protein monomers produced almost the same amide I region with a main component centered at 1650 cm−1 (Fig. 1B). These data indicate that both states of the protein contain high levels of α-helical conformation (23) and that the C-terminal part of lithostathine did not undergo substantial structural transitions similar to those known to occur during amyloid fibril formation. Taken together, we conclude that if QHF-litho fibrils were amyloid structures they would represent an entirely novel and different type of amyloid fibrils, in par-
Modification of the Environment of Tryptophan Residues Examined by UV and Fluorescence Spectroscopy—We tested, in addition, whether the formation of fibrils by lithostathine involves a more subtle structural reorganization of the protein (that maintains its secondary structure composition). Ultraviolet absorbance in the fourth derivative mode was used as an intrinsic probe for both tryptophan and tyrosine residues. This technique allows one to enhance the generally low resolution of zero-order UV absorbance spectra and to obtain information about structural changes in the local environment of tyrosine and tryptophan residues (12, 24). The fourth derivative UV absorbance spectra were characterized by two maxima, reflecting mainly the tyrosine environment at 284 nm and the tryptophan environment at 290.5 nm (Fig. 2). Under conditions where fibril formation occurs, i.e. incubation of lithostathine at 37 °C up to 4 days, no substantial change in the tyrosine derivative band was observed. However, the maximum of the tryptophan derivative band shifted from 290.7 to 291.8 nm, accompanied by an increase of the intensity of absorption. This significant red shift can be explained either by a decreased interaction of tryptophan residues with water molecules or by an increased interaction with other hydrophobic or aromatic residues. To distinguish between these two hypotheses we used fluorescence as an intrinsic probe of the solvent exposure of tryptophan residues. The initial fluorescence spectrum of lithostathine (t = 0) was characterized by an emission maximum at 340 nm, indicating that most of the tryptophan residues are exposed to water. This finding is in good agreement with our structural data (8). During fibril formation, however, the maximum emission wavelength did not change significantly, suggesting that the mean exposure of tryptophans to water is not affected. However, a 25% quenching of tryptophan fluorescence was observed during incubation, indicative of conformational rearrangements in the vicinity of tryptophan residues. Altogether, these results suggested that the shift in the absorption maximum and fluorescence quenching of tryptophan residues were the result of an increased interaction with hydrophobic or aromatic residues, which could occur during fibril formation.

Dimerization of Lithostathine Preceded or Was Concomitant to Cleavage—To understand the process of fibril formation and, hence, the mode of interaction between the protein molecules within the fibril, we have examined the early events in fibril formation using MALDI-TOF mass spectrometry. As reported previously (7), fibril formation depends on the self-cleavage of lithostathine, resulting in the release of an N-terminal undecapeptide from the C-terminal protein domain, which represents the precursor of lithostathine fibrils. The mass spectrum recorded at t = 0 (Fig. 3A), in the 1000 to 25000-Da mass range, contained a single charge state series at mass/charge (m/z) values of 16612.8, 8307.1, and 5545.0 that corresponded to the ionic states +1 to +3 of uncleaved lithostathine (S2). Note that the dispersion in the mass/charge ratio that can be observed in each charge state corresponds to the salt adducts. By contrast, the spectrum taken after 1 day (Fig. 3B) demonstrates the presence of uncleaved lithostathine, as well as substantial amounts of cleaved lithostathine (S1), which can be detected at m/z values of 15012.2, 7510.0, and 5009.98 (charge states +1 to +3). Bearing in mind that dimerization is an essential step in fibril formation (9), we also recorded spectra in the 1000 to 45000-Da mass range. After 1 day, we found a peak at 30108.3 m/z, corresponding to the mass of S1-dimers, along with a second peak at 33488.1 m/z, representing dimers of intact lithostathine (Fig. 3B, inset). Although MALDI-TOF does not allow rigorous relative quantification of the S1 and S2 dimers, it is clear that the cleaved species disappears more rapidly than full-length lithostathine (after 3 days incubation; see Fig. 3, C (10 days) and D). This observation suggests that, as soon as it is cleaved, lithostathine assembles into multimers. In addition, the intensity of the peak corresponding to the S1 dimer is always low. Taken together, these data suggest that the S1 dimer represents only a transient species on the route to the formation of higher mass multimers. This finding is different from the one obtained for dimers containing the full-length protein that were previously shown to be unable to give rise to fibrils (9). MALDI mass spectrometry thus reveals a complex
FIG. 2. UV absorbance changes during fibril formation. Lithostathine (0.47 mg/ml) was incubated in 0.5 M Tris-Cl buffer, pH 8. UV spectra were recorded at intervals of 4 days. The fourth derivative amplitude at 292 nm, representative of the tryptophan contribution to the UV absorbance, is plotted as a function of time. Inset, fourth derivative UV spectra at $t = 0$ (solid line) and $t = 96$ h (broken line). The structural changes observed were slow, with a $t_{1/2}$ of $\sim 20$ h.

FIG. 3. MALDI-TOF mass spectrometric analysis of lithostathine. Aliquots of lithostathine (1.2 mg/ml) in 20 mM phosphate, pH 8, incubated 0 (A), 1 (B), 3 (C), and 10 (D) days at 37 °C were withdrawn and submitted to MALDI-TOF analysis. Only uncleaved lithostathine (black squares) was present at day 0 and persisted over 10 days. Cleaved lithostathine (open circles) appeared after 1 day and then disappeared progressively. B, inset, cleaved (double open circle) and uncleaved dimers (double square).
equilibrium taking place after 1 day between intact and cleaved lithostathine, in both monomeric and dimeric forms. Interestingly, traces of S2 dimers are already present at $t = 0$, which leads us to conclude that lithostathine dimerization either precedes or is concomitant with its cleavage. By contrast, the presence of an uncleaved N-terminal undecapeptide blocks the assembly of the dimers into fibrils of high order.

**Lithostathine Is a Member of a Protein Family That Is Predisposed to Undergo Domain Swapping**—The results described above showed that the formation of lithostathine fibrils does not significantly perturb the secondary structure of the protein but could involve rearrangements of the local environment of the tryptophan residues. A possible explanation of these effects has emerged from sequence analysis and homology modeling. Previously, a segment encompassing part of the tryptophan cluster was termed a hinge region as used previously in C-type lectins from snake venoms (18). Cysteines are in dark gray boxes. By multiple sequence alignment of lithostathine based on the crystal structure of flavocetin-A (Fig. 5C). All the tryptophan residues of this model form a cluster around the swapped domain; such an arrangement would be consistent with the results of the absorbance studies. Of course, it remains to be established whether such domain swapped species exist in the case of lithostathine. Based on the structure reconstruction of QHF-litho obtained previously by electron diffraction techniques (9), however, the mobile loop would have to be on the surface of these fibrils and does not appear to be involved in stabilizing their internal structure (Fig. 5D). In this position, the loop could act as a docking surface between the swapped domain; such an arrangement would be consistent with the results of the absorbance studies.

**Lithostathine Fibrils Can Be Dissociated by [6,6’-Bibenzo- thiazoly1-2,2’-diamine—Studies were also carried out to investigate whether QHF-litho could be dissociated by compounds targeted against amyloid structures such as polyglutamine (13). Tests were carried out with one such compound, the benzothiazole, PGL-034, that has been shown previously to perturb very effectively the aggregation events associated with Huntington’s disease. We found that PGL-034 resulted in substantial degree of disruption of the stacking interactions associated with lithostathine fibrils (Fig. 6A). Control experiments without PGL-034 did not show any such effect (Fig. 6, B and C). Interestingly, PGL-034 did not dissociate the stacked tetramers of the QHF-litho quadruple-helix fibril. Consistent with our model of the interactions between juxtaposed fibrils described above, computer modeling studies suggest that PGL-034 is capable to bind to the swapping domain (not shown). Hence, it is possible that PGL-034 acts to dissociate the intermolecular interactions involved in entangling molecules of lithostathine by means of disrupting a protein surface loop corresponding to residues 89 to 105. These results suggest that PGL-034 represents a potential therapeutic agent to intervene in pathogenic processes associated with QHF-litho deposition.

**Lithostathine Deposits Are Involved in Creutzfeldt-Jakob Disease**—Although QHF-litho are not structurally related to amyloid fibrils, there has been previous evidence for a clinical relationship between lithostathine and amyloid deposits in that lithostathine was found within the plaques formed in Alzheimer’s disease (10). Based on these observations, we in-
vestigated whether QHF-litho might also occur in other amyloidoses, most notably in Creutzfeldt-Jakob disease. Using immunological methods and tissue samples derived from normal human cerebral cortex (Fig. 7, a and b) and cerebellum (Fig. 7, c and d), we failed to detect any binding of specific Romeo antibodies generated against lithostathine (10). However, when we examined brain samples from a patient with Creutzfeldt-Jakob disease lithostathine deposits could be detected. In addition, these deposits differed in appearance depending on the brain area. In cerebral cortex, for example, focal plaques and punctiform deposits were seen (Fig. 7, e and f) whereas in the cerebellum, staining was more intense and revealed focal and punctiform deposits (Fig. 7, g and h). Experiments were also carried out to examine whether lithostathine deposits were resistant to the drastic treatments used to reveal PrP\textsuperscript{res} in immunohistochemistry experiments (Fig. 7, i–l). After such treatment (see “Experimental Procedures”), no signal was observed in the normal cerebellum (Fig. 7i) or in control experiments using CJD brain but no primary antibody to lithostathine during detection (Fig. 7j). Conversely, when Creutzfeldt-Jakob brain tissue was subjected to treatments used to reveal PrP\textsuperscript{res} and probed with a primary antibody to lithostathine we detected proteinase K-resistant deposits in the blood vessels within the cerebellum (Fig. 7k). In addition, we also observed extremely compact focal and multicentric deposits in the cerebellum (Fig. 7l). These results prompted us to explore the possibility of co-localization of lithostathine and PrP\textsuperscript{res} plaques using double staining techniques (Fig. 7, m–p). Thus experiments showed the existence of both independent and co-localized plaques. The same results were obtained in studies of the brain of another patient with Creutzfeldt-Jakob disease and of the brains of two patients with Gerstmann-Sträussler-Scheinker disease, another prion disease (not shown). Taken together, these data showed that deposits of lithostathine occur in the brain of patients suffering from spongiform amyloidotic encephalopathies.

**DISCUSSION**

Two major observations are reported in the present study. First, QHF-litho fibrils were shown not to have the characteristic properties of amyloid structures. Instead, it appears that they retain a largely native-like protein conformation during fibril formation. To form highly ordered fibril structures, we suggest that juxtaposed protein units exchange a mobile surface loop (domain swapping). Second, although these lithostathine fibrils may not be amyloids themselves there is a clinical relationship between these fibrils and prion deposits present in the transmissible spongiform encephalopathies. We can now discuss these two points in more detail.

The first point concerns the structure of lithostathine fibrils. We suggest that polypeptide fibrils can be placed into three structural groups: 1) protein fibrils, which consist of assemblies of globular protein units, such as actin or tubulin; 2) helical fibrils, for example collagen and intermediate filaments; and 3) fibrillar aggregates, such as amyloid fibrils, in which at least part of the native protein structure is lost. These amyloid fibrils are characterized by the presence of a specific type of intermolecular \( \beta \)-sheet conformation (21, 22) and differ fundamentally from globular protein structures (28). The structures of globular proteins are determined by the interactions and packing of the side chains of the constituting amino acids and, hence, by the polypeptide sequence. By contrast, aggregates and amyloid fibrils are thought to represent a generic structural form of the polypeptide chain that is dominated by the invariant properties of the main chain (backbone) and that can therefore be formed by many, if not all, polypeptide sequences (29). Consistent with these ideas, amyloid structures are found to form in vitro under solution conditions where the native protein structure is at
least partially denatured, i.e. under conditions in which the sequence-specific, globular state is disrupted (29). Based on the present data, it is evident that not all clinical polypeptide fibrils are amyloid in nature. Despite their pathological relevance, QHF-litho were not found to bind Congo Red and largely retained the secondary structural composition of the native protein. In particular, no increase in the \( \alpha \)-helical conformation. In contrast to QHF-litho, however, Ure2p fibrils can bind Congo Red and show a discernible CR green birefringence (31). Interestingly, \( \alpha \)-antitrypsin deficiencies also are associated with fibrils that give rise to CR green birefringence but retain the polypeptide chains in an almost native and globular protein structure (32).

In this context it is important to note that the fibrils associated with \( \alpha \)-antitrypsin deficiency are thought to be stimulated by serpins undergoing a domain swapping mechanism (32). Domain swapping is a mode of protein association first described with the diphtheria toxin (33). One protein domain is exchanged with an identical domain of another subunit to produce an oligomer with identical inter-molecular contacts (34). The importance of domain swapping has been already suggested in abnormal protein assembly (for reviews, see Refs. 35–37). Interestingly, there are proline residues located in the hinge regions of lithostathine, and these residues are thought to facilitate a domain swap by increasing the rigidity of the hinge region associated with the exchange of domains, as shown by Rousseau et al. (38) for p13suc1. It is therefore possible that domain swapping can favor the entangling of polypeptide chains in some cases leading to structures with properties that differ from these of the native state, such as proteinase-resistance and toxicity.

The second conclusion of the work concerns the presence of lithostathine aggregates in amyloidoses. Although QHF-litho are not amyloid fibrils, there is increasing evidence that they are associated with diseases and particularly with clinical cases of amyloid diseases. Previous studies have shown that N-terminally truncated lithostathine forms fibrillar protein plugs in pancreatic ducts and in the center of calcified pancreatic calculi in patients with chronic calcifying pancreatitis (39). However, although lithostathine/reg cDNA has been cloned initially in pancreatic cells where it is largely expressed (40), its mRNA has also been detected in the human brain. In situ hybridization experiments show that lithostathine is synthesized and expressed in the pyramidal neurons of the cerebral cortex, i.e. in those neurons that contains the neurofibrillary tangles in patients with Alzheimer’s disease and Down syndrome (41, 42). More recently, we have obtained specific evidence for lithostathine deposits in the brain of patients with Alzheimer’s disease (10), and we have observed numerous inclusion bodies in two other patients.\(^2\)

In this paper, we report the presence of lithostathine deposits in patients suffering from CJD and Gerstmann-Sträussler-Scheinker syndrome. This finding indicates that several different forms of protein deposits can coexist in the same neurodegenerative disease. In this regard, it is already well known that some patients with Creutzfeldt-Jacob disease have amyloid deposits of the A\( \beta \) peptide, neurofibrillary tangles containing the polypeptide tau (43) and Lewy bodies that are composed of \( \alpha \)-synuclein (44). Conversely, prion deposits have also been observed in patients with Alzheimer’s disease (45). These results suggest that there need not be, in biochemical terms, a clear-cut distinction between different types of neurodegenerative diseases. Although the number of cases examined is still small, lithostathine has been found to be involved in all amyloidoses that have been examined so far. These observations suggest, therefore, that lithostathine fibrils might represent a common component of amyloid deposits and, hence, a new component of the material that coats clinical amyloid fibrils. The amyloid coat is known to be an ensemble of various

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\(^2\) E. Laurine and J.-M. Verdier, manuscript in preparation.
substances containing globular proteins, such as serum amyloid P component, and glycosaminoglycans (46). Together these substances can decorate the fibrillar polypeptide core. Many of these coat substances have been shown to modify or to enhance the pathogenic potential of the underlying polypeptide aggregates. Hence, they represent targets for therapeutic strategies (47). Taken together, these data raised questions about the role of lithostathine within these deposits. Oligomers of lithostathine could, for example, be cytotoxic themselves, or they could protect the pathogenic amyloid deposits against digestion by proteases or macrophages. It is possible, therefore, that QHF-litho contribute significantly to the pathology of at least some of the amyloidoses. The development of drugs to disassemble these particular fibrils or bundles, or to prevent their

FIG. 7. Lithostathine deposits in human CJD. No deposits were observed in the normal cerebral cortex (a, entorhinal cortex; b, Ammon’s Horn) and in the cerebellum (c, junction between granular layer and white matter; d, white matter). Focal plaques and punctiform deposits were observed in CJD entorhinal cortex (e), Ammon’s Horn (f) and in the granular layer (g), and in the junction between granular layer and white matter (h) of the cerebellum. After treatment to reveal PrPSc no signal was detected in normal cerebellum (i) or in CJD in the absence of primary antibodies (j). By contrast, using diseased brain samples and an antibody to lithostathine, proteinase K-resistant deposits were observed in the blood vessels (k) and in focal and multicentric plaques (l) of the cerebellum. Double labeling of lithostathine (blue) and PrPSc (brown) showed both independent deposits (m and n) and colocalized deposits (o and p).
formation, therefore represents a potential strategy for treating these disorders. One particularly promising approach to reach this goal is the development of novel and benzothiazole-based agents that perturb the association of individual fibrils into higher order aggregates.

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