Relative stabilities of wild-type and mutant glial fibrillary acidic protein in patients with Alexander disease

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Alexander disease (AxD) is an often fatal astrogliopathy caused by dominant gain-of-function missense mutations in the glial fibrillary acidic protein (GFAP) gene. The mechanism by which the mutations produce the AxD phenotype is not known. However, the observation that features of AxD are displayed by mice that express elevated levels of GFAP from a human WT GFAP transgene has contributed to the notion that the mutations produce AxD by increasing accumulation of total GFAP above some toxic threshold rather than the mutant GFAP being inherently toxic. A possible mechanism for accumulation of GFAP in AxD patients is that the mutated GFAP variants are more stable than WT GFAP and thereby causes accumulation of GFAP above a critical toxic threshold. This view of how the presence of mutant GFAP leads to these defects is that, rather than being toxic per se, it is more stable than the WT and thereby causes accumulation of GFAP above a critical toxic threshold. The origin of this view was the observation that mice expressing elevated levels of WT GFAP from a human GFAP transgene display features of AxD. In addition, the presence of this WT transgene dramatically enhances the pathology displayed by AxD model mice engineered to express an endogenous mutated GFAP. Also contributing to the toxic threshold hypothesis is the analysis of transgenic mouse lines expressing different levels of the GFAP gene. This work was supported by NINDS, National Institutes of Health Grant P01NS42803 (to M. B.). Funds for the Sciex 5600 TripleTOF mass spectrometer came from National Institutes of Health Shared Instrumentation Grant S10 RR027822 (to S. B.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains Table S1.

Results

General approach

We investigated whether mutant GFAP is more stable than WT GFAP by using MS to determine the relative amounts of mutant and WT GFAP in AxD patients harboring the R79C, R239H, or R416W mutation. A peptide unique to either the mutant or WT protein was quantified, and its molar concentration was compared to that of total GFAP obtained by quantifying astrocytes from human patients and animal models display multiple defects (reviewed in Ref. 7), including decreased glutamate and potassium transport, decreased proteasome activity, increased production of nitric oxide and proinflammatory chemokines, defective calcium signaling, and aberrant mechanosensitive signaling. A widely held view of how the presence of mutant GFAP leads to these defects is that, rather than being toxic per se, it is more stable than the WT and thereby causes accumulation of GFAP above a critical toxic threshold. The origin of this view was the observation that mice expressing elevated levels of WT GFAP from a human GFAP transgene display features of AxD. In addition, the presence of this WT transgene dramatically enhances the pathology displayed by AxD model mice engineered to express an endogenous mutated GFAP. Also contributing to the toxic threshold hypothesis is the analysis of transgenic mouse lines expressing different levels of the GFAP gene. This work was supported by NINDS, National Institutes of Health Grant P01NS42803 (to M. B.). Funds for the Sciex 5600 TripleTOF mass spectrometer came from National Institutes of Health Shared Instrumentation Grant S10 RR027822 (to S. B.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains Table S1.

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3 The abbreviations used are: AxD, Alexander disease; GFAP, glial fibrillary acidic protein; iPSC, induced pluripotent stem cell.

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Instability of mutant GFAP in Alexander disease patients

Identification of WT- and mutant-specific peptides

The mutant and WT diagnostic peptides were selected based on their efficient detection by LC-MS/MS and sequence uniqueness to GFAP in the predicted human proteome using the PROWL algorithm ProteinInfo tool to search the NCBI database (http://prowl.rockefeller.edu).4 For the R79C case, we observed a GFAP tryptic peptide specific for WT GFAP (FASYIEK, residues 80–86). For the R416W patient, we observed a tryptic peptide specific for the mutant (TVEMWIDGEVIK, residues 412–422). For the R239H patient, we were unable to detect either a WT-specific or mutant-specific peptide using trypsin digestion, but by using chymotrypsin, we detected the mutant-specific peptide KEIHTQY (residues 236–242). Spectra for these three diagnostic peptides are shown in Fig. 1.

Identification of common peptides

The total amount of GFAP in the patient samples was measured by identifying proteotypic GFAP peptides not affected by the mutations. For the R79C and R416W patients, these were the tryptic peptides FADLTDAAAR (residues 261–270), LADVQAEELR (residues 112–121), ALAAELNQLR (residues 96–105), and LLEGEEENR (residues 369–376). For the R239H patient sample, these were the chymotryptic peptides RQEADEATL (residues 173–181) and ARQQVHVEL (residues 216–224). Because of difficulty in detecting ARQQVHVEL (Table 1), for R239H we also used IEKVRF (residues 84–89), which is also present in desmin and peripherin. Both of these nontarget proteins are at extremely low concentrations in the brain compared to GFAP (The Human Protein Atlas, https://www.proteinatlas.org (44)).4 The fragment ions used for quantitation of each common peptide are listed in Table S1.

Examination of degraded GFAP

GFAP Immunoblots of the patient brain extracts displayed the typically observed pattern of a major band at the expected position for intact human GFAP of 50 kDa and a series of bands in the 35- to 45-kDa range that are presumed to be partially degraded GFAP (Fig. 2) (24–27). Because the WT and mutant GFAP composition of the degraded protein might be a sensitive indicator of their relative stabilities, this material was analyzed in addition to that in the intact region for the R79C and R416W patients (attempts to perform this analysis for the R239H patient were unsuccessful, probably because of the reduced ionization efficiency or digestion of the chymotryptic peptides relative to the tryptic peptides used for the R79C and R416W patients).

Use of an internal standard for quantitation

The signals obtained for the various peptides were quantified by comparison to signals obtained from a known amount of full-length 15N-labeled human GFAP added to each sample. The 15N-labeled GFAP was either the mutant or WT form, depending on whether the type-specific peptide used for identification was from the mutant or the WT. Thus, WT 15N-labeled GFAP was added to the R79C patient sample, and 239H and 416W mutant 15N-labeled GFAP was added to the R239H and R416W patient samples, respectively (see “Experimental procedures” for details). These 15N-labeled GFAP standards were added to the samples prior to gel electrophoresis. However, because these were full-length protein standards lacking significant degradation products, and so would not be present in the degraded bands following electrophoresis (Fig. 2), the same amount of 15N-labeled GFAP standard was run in a separate lane in the gel and then excised and combined with the excised patient degradation bands prior to extraction for analysis (see “Experimental procedures” for details).

Calculation method

The fraction of total GFAP that was mutant (or WT) was obtained by (A) determining the amount of the unique mutant (or WT) peptide in the patient sample relative to the same peptide in the 15N-labeled GFAP internal standard, (B) determining the amount of total GFAP relative to the 15N-labeled standard using GFAP peptides common to both mutant and WT forms, and (C) dividing A by B. For uniformity, results are presented in Table 1 as the percentage of total GFAP attributable to the mutant form (percent mutant); thus, for R79C, the experimentally determined WT percentage was subtracted from 100.

Control experiments

The calculation method used should yield the same result for any amount of 15N-labeled standard added to the samples, provided that the amount was sufficient for its peptides to be detected. The data presented in Table 1 confirm this expectation. Varying the added 15N-labeled standard up to 10-fold had no apparent effect on the percent mutant quantitation. Table 1 also shows results from testing whether the duration of proteolytic digestion was appropriate. As shown in Table 1, reducing the time of trypsin digestion from our standard of 40 h to 18 h or the time of chymotrypsin digestion from our standard of 72 h to 48 h had a marginal, if any, effect on quantitation.

Relative level of mutant GFAP in AxD patients

None of the three AxD patients had a higher percentage of mutant GFAP than WT in the intact band (Table 1), counter to expectation if mutant GFAP contributed to disease by having greater stability. The percentages of total GFAP attributed to the mutant form for the R79C patient (21%) and R239H patient (29%) were actually much lower than that of the WT, suggesting that these mutant GFAPs are less stable rather than more stable. Similarly, less mutant GFAP than WT was observed in the

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Figure 1. MS/MS spectra used for identifying peptides specific for WT or mutant GFAP. A–C, peptides are diagnostic for WT GFAP in an R79C patient (A), R416W mutant GFAP (B), and R239H mutant GFAP (C). In each panel, the first spectrum is for the unlabeled peptide and the second is for the corresponding 15N-labeled peptide. The ion m/z ratios were calculated using the Protein Prospector MS Product tool (http://prospector.ucsf.edu).4
**Instability of mutant GFAP in Alexander disease patients**

**Table 1**

<table>
<thead>
<tr>
<th>Amt 15N Std</th>
<th>Total GFAP</th>
<th>Peptide Ratios (Patient/15N Std)</th>
<th>Peptide Ratios (Patient/15N Std)</th>
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<tr>
<td>ng</td>
<td>Digestion</td>
<td>Intact</td>
<td>Degraded</td>
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<td>R79C patient, trypsin digestion</td>
<td>50</td>
<td>120</td>
<td>40</td>
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<td></td>
<td>100</td>
<td>170</td>
<td>48</td>
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<tr>
<td></td>
<td>100</td>
<td>170</td>
<td>40</td>
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<tr>
<td></td>
<td>Average ± SD</td>
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<td>23% ± 2%</td>
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<tr>
<td>R416W patient, trypsin digestion</td>
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<td>90.5</td>
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<td></td>
<td>36</td>
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<td>18</td>
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<td>135.5</td>
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<td>Average ± SD</td>
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<td>50</td>
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<td>Average ± SD</td>
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</table>

**Figure 2.** GFAP immunoblot of 15N-labeled GFAP proteins and patient total homogenate samples. For each 15N-labeled GFAP standard (lanes 1–3), 7.5 ng of purified standard was loaded on the gel. The amounts of total protein applied for the R239H, R416W, and R79C patient samples (lanes 5–7) were 200 ng, 400 ng, and 270 ng, respectively. These patient protein amounts reflected differences in GFAP content, which were determined by densitometric comparison of GFAP staining intensity in pilot gels with that of the 15N-labeled GFAP standards. The negative control (lane 4) had no protein loaded. The positions of relevant molecular mass markers run concurrently indicated.
explanation for the otherwise puzzling observation that total GFAP levels are actually lower in the spinal cord of R236H mice than in the WT (33).

A mechanism for reduced stability of mutant GFAP arises from an analysis of the crystal structure of the human GFAP 1B rod domain (34). The structure obtained led the authors to conclude that many of the mutations in the central rod domain producing AxD would destabilize filament formation, particularly at the initial step of dimerization (a caveat, however, is that this prediction was also made for V115I, which is not believed to be disease-causing (35)). The suggested defect in polymerization of mutant GFAP would result in a greater fraction of the mutant form than WT being present as monomers and thus more available for degradation. This mechanism could explain not only the instability of R79C and R239H GFAP but also why the stability of R416W GFAP is similar to that of the WT; R79C and R239H are in the central rod domain and thus might affect dimerization, whereas R416W is in the tail region and is believed to only affect subsequent steps in polymerization (21). Several prior observations are consistent with compromised polymerization of mutant GFAP. Using a yeast two-hybrid system, Nielsen et al. (36) observed decreased dimerization for the several common AxD GFAP mutations they tested. Tian et al. (37) found that, in U251 cells expressing either GFP-tagged WT or tagged R239C GFAP, the fraction of GFAP present in the soluble fraction (monomers and short oligomers) was significantly greater for the mutant.

The possibility that analysis of the partially degraded forms of GFAP might provide a more sensitive measure of relative stabilities was not realized. Instead of containing more of the presumptively less stable R79C mutant GFAP, the percentage of R79C mutant GFAP in the partially degraded GFAP was similar to that in the intact GFAP (23% versus 21%). In the case of the R416W patient, the calculated percentage of mutant GFAP in the degraded band (21%) was even less than that in the intact band (46%) (Table 1). A likely explanation for the lower calculated percentage of mutant GFAP in the degraded R416W GFAP is that some of the cleaved GFAPs had the R416W proteotypic peptide removed. The sequences of the degradation bands present in our AxD patient samples are not known, but the series of bands observed is quite similar to those seen in a variety of other control and diseased post-mortem samples (24–27) and has been attributed to digestion by calpain (27, 38, 39). N-terminal sequencing of several of these large degradation products obtained from normal brain white matter, multiple sclerosis plaques, leucotomy scars (24) and spinal cords of patients with ALS (38), and by complete in vitro calpain digestion of GFAP (39), yielded cleavage sites after Ala-40, Asn-59, and Thr-383. Cleavage at Ala-40 or Asn-59 and/or Thr-383 could generate the series of degradation bands in our samples.

Figure 3. Illustration of the quantitation method. Data shown are from the 40-h tryptic digest of the intact GFAP band from the run using 36 ng of 15N-labeled mutant 416W protein standard and 113 ng of unlabeled R416W patient GFAP (Table 1). A and B, elution chromatograms for detection of the product ions from the R416W mutant–specific peptide TVEMWDEGVIK that were used for quantitation. A, unlabeled patient GFAP. B, [15N]GFAP internal standard. C and D, data for the common peptide FADLTDAAAR. Note that, as expected, the unlabeled and 15N-labeled peptides coelute. The table insets in B and D list the product ions, the peak areas of their signals, and the ratios of the peak area of each unlabeled ion to that of the labeled ion. These ratios provide measures of the amount of mutant GFAP and total GFAP, respectively, in the patient sample relative to the 15N standard. The ratio of these two values is the fraction of total patient GFAP that is the mutant form. The actual calculations summarized in Table 1 include additional values from other common peptides. Data for each individual peak area are provided in Table S1.
Instability of mutant GFAP in Alexander disease patients

(Fig. 2), with Thr–383 cleavage removing the R416W diagnostic peptide. Because this peptide identifies the mutant protein, its loss would result in underestimation of the contribution of mutant protein to the total. In contrast, the R79C site is expected to be retained in the degradation products examined. Two possible explanations for the percentage of R79C GFAP being similar in the intact and degraded bands are that the clipped mutant GFAPs may also be less stable than their WT counterparts, or that calpain degradation may occur predominantly post-mortem (39) so that the composition of the degradation products reflects that of the intact GFAP at the time of death.

A remaining question is whether our finding of less mutant than WT GFAP applies to all astrocytes or only to those affected (or unaffected) by the disorder. Using Rosenthal fiber content as an indication of affected astrocytes, our data, in conjunction with that of Heaven et al. (6), suggest that the status of the astrocytes is not a critical parameter. Using urea extraction to distinguish between GFAP in normal filaments and that in Rosenthal fibers, that study estimated that about two-thirds of the GFAP in the R79C sample was associated with Rosenthal fibers, but little to none was in the R239H sample, whereas the observed percentage of mutant GFAP was similar in both samples (21% and 29%, respectively). However, a more rigorous examination of these variables with a larger sample size is required to establish these relationships.

Our finding that the levels of mutant GFAP are similar to or lower than WT levels suggests that the mutant GFAP does not initiate AxD by causing accumulation of normally functioning GFAP, but that the mutations per se produce a toxic response. This same conclusion was reached by Jones et al. (14) in their study of astrocytes derived from AxD patient iPSCs. They observed that, compared with their gene-corrected controls, these cells displayed marked differences in both their RNA transcripts and their ability to propagate intercellular calcium waves but no difference in their GFAP protein levels, prompting the comment that “mutations in GFAP are sufficient.” An example of an effect due to a GFAP mutation rather than GFAP quantity is the finding of Tang et al. (9) that proteasome activity is inhibited more strongly by soluble R239C GFAP than by WT GFAP. Another is the finding of Jany et al. (33) that, although GFAP levels in the spinal cord of R236H mice were decreased compared with the WT, the level of GFap mRNA was increased. Thus, similar to the findings of Jones et al. (14), mutant GFAP initiates changes in gene transcription in the absence of an increase in total GFAP levels. There are also multiple reports that, at comparable levels, mutant GFAP causes significantly more physiological changes, including aggregate formation, than WT GFAP (e.g. 10, 19, 23, 40).

If mutation of GFAP per se causes disease, then how does one account for the AxD-like characteristics of mice overexpressing WT GFAP? A model proposed by Li et al. (41) provides a resolution of this seeming paradox. They hypothesized that a rate-limiting step in GFAP polymer formation is sensitive to the presence of the GFAP mutations, a suggestion now supported by the crystal structure analysis (34). Thus, either elevated production of WT GFAP or the presence of mutant GFAP would cause abnormal accumulation of precursors. Some of these precursors might then be diverted into an alternative, aberrant pathway to produce toxic complexes. Consistent with this explanation, Tang et al. (9) suggested that formation of abnormal GFAP oligomers is responsible for proteasome inhibition. In light of this kinetic bottleneck model, we suggest an alternative to the conclusion of Tanaka et al. (19) that an increase in total GFAP of between 2.7% and 30%, caused by expression of a mutant transgene, is sufficient to produce Rosenthal fiber formation. We suggest that, rather than such a modest increase in total GFAP being toxic, their observations instead define the minimum percent of total GFAP that must be the mutant form to significantly interfere with polymerization. Our data suggest that having about 20% of the total GFAP as the mutant form is sufficient to produce disease (Table 1), and a prior report of an AxD mutation that caused occasional exon skipping suggests that about 10% mutant form is sufficient (42).

The greater resistance of mutant GFAP to solubilization by various solvents was another observation that contributed to the concept that the total amount of GFAP is the critical factor for toxicity in AxD. Although our data indicate that the stability of mutant GFAP is similar to or less than that of the WT, it remains possible that GFAP complexes containing even a minority of the mutant form have increased stability. However, several observations suggest that the GFAP extraction data, which were obtained in vivo, may not reflect differences in GFAP stability in vitro. Moody et al. (43) found that GFAP in the R236H mouse actually turns over more rapidly than in the WT, and in a Drosophila AxD model, Wang et al. (23) observed no change in GFAP levels when the solvent extraction of mutant GFAP was restored to that of the WT by overexpression of αB-crystallin.

In conclusion, we found that in three AxD patients the level of mutant GFAP is lower than that of the WT, indicating that mutant GFAP is less stable rather than more stable than the WT. This observation, together with those cited above of other laboratories, suggests a reordering of events in the development of AxD. Instead of mutant GFAP having increased stability and serving simply to increase the total level of GFAP above some toxic threshold, we suggest that the initial event is toxicity of mutant GFAP per se. Although this view places toxic effects of mutant GFAP as an event occurring prior to a rise in total GFAP levels, the importance of increasing levels of total GFAP is not discounted. A number of positive feedback loops have been proposed whereby pathological changes caused by mutant GFAP, such as induction of a stress response and inhibition of proteasome activity, result in increasing GFAP production (reviewed in Ref. 2). An irreversible point in disease progression may be reached when activation of the positive feedback loops by mutant GFAP more than compensates for its relative loss because of its instability.

Experimental procedures

Tissue samples

Use of post-mortem human tissue for this study was approved by the University of Alabama at Birmingham Institutional Review Board and abided by the Declaration of Helsinki principles. The sources and characteristics of the tissues are
Instability of mutant GFAP in Alexander disease patients

Table 2
Description of Alexander disease tissues used

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sex</th>
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<th>Death</th>
<th>PMI</th>
<th>Source</th>
<th>Previous Report</th>
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<tbody>
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<td>R79C</td>
<td>M</td>
<td>3 months</td>
<td>14 years</td>
<td>7</td>
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<td>Patient 1 in Ref. 1</td>
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<tr>
<td>R239H</td>
<td>M</td>
<td>3 months</td>
<td>1 year</td>
<td>4</td>
<td>UMB BTB 1070</td>
<td>Patient 23 in Ref. 35</td>
</tr>
<tr>
<td>R416W</td>
<td>M</td>
<td>10 months</td>
<td>7 years</td>
<td></td>
<td>Goldman</td>
<td>Patient 9 in Ref. 1</td>
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</table>

The tissues analyzed were from typical cases of the infantile form of AxD that have been reported previously as indicated. The R79C and R239H tissues were obtained from the University of Maryland Brain and Tissue Bank (UMB BTB). The R79C sample was a 1-cm-thick coronal section of parietal cerebral cortex from section 12R, produced by sectioning protocol 1. The R239H sample was frontal neocortical gray matter from section 3L, produced by sectioning protocol 2. The R416W tissue was a small fragment of neocortex and underlying white matter provided by James Goldman (Columbia Medical School); its post-mortem interval (PMI) is unknown. Although the exact locations of the tissues excised for analysis are not known, information about their Rosenthal fiber content is provided in Ref.6, which used adjacent samples in a proteomics study of Rosenthal fibers. A fraction enriched for Rosenthal fibers contained 87% of the total GFAP in the R416W sample, 73% in the R79C sample, and 3% in the R239H sample, whereas 1% to 8% was observed in controls. Thus, most of the GFAP analyzed here for the R416W and R79C samples was presumably in Rosenthal fibers, but little if any of these aggregates were present in the R239H sample.

Production of $^{15}$N GFAP standards

pET23b plasmids containing the WT and mutant human GFAP complementary DNA sequences were provided by Roy Quinlan (Durham University) (see Ref. 21 for the method of construction). The plasmids were transfected into BL21pLysS Escherichia coli and grown in medium having more than 98% $^{15}$N (Cambridge Isotope Laboratories, Andover, MA), and the recombinant GFAP was purified as described by Der Perng et al. (21).

Immunoblotting

Immunoblotting was used to estimate the GFAP concentrations in patient samples and to determine the gel position of the intact and degraded GFAP bands. Purified recombinant human GFAP, made as described above, was used as a quantitation standard. Protein samples were boiled in Laemmli sample buffer and separated by SDS-PAGE gel (Pierce) at 120 V and subsequently transferred to nitrocellulose membranes for 1 h at room temperature at 100 V, followed by Coomassie staining for 30 min and destaining in buffer consisting of 5% formic acid/50% acetonitrile. The fully dried gel slices were then digested at 37 °C for 18 or 40 h (Table 1) with sequencing-grade trypsin (Promega, Madison, WI) with a 1:20 trypsin:protein ratio, determined by comparing the stained gel region with that for 1.5 µg of BSA run on the same gel.

For chymotrypsin digestion of the R79C and R416W patient samples, the gel slices were further destained overnight in 100 mM ammonium bicarbonate/50% acetonitrile. The following day, they were washed three times in 100 mM ammonium bicarbonate, followed by dehydrocyanation in 1% glacial acetic. The fully dried gel slices were then digested at 37 °C for 18 or 40 h (Table 1) with sequencing-grade trypsin (Promega, Madison, WI) with a 1:20 trypsin:protein ratio, determined by comparing the stained gel region with that for 1.5 µg of BSA run on the same gel.

For chymotrypsin digestion of the R239H patient samples, the gel slices were further destained overnight in 50 mM ammonium bicarbonate/50% acetonitrile, washed three times in 100 mM Tris-HCl and 10 mM CaCl$_2$ (pH 8.0), followed by dehydrocyanation in 100% acetonitrile. The fully dried gel slices were then digested at 25 °C for 48 or 72 h with sequencing-grade chymotrypsin (Promega), starting with a 1:10 chymotrypsin:protein ratio. Fresh aliquots of chymotrypsin at the same 1:10 ratio were added every 24 h.

Following proteolysis, the digestion solutions were transferred to separate tubes, and the gel slices were extracted further in buffer consisting of 5% formic acid/50% acetonitrile for 1 h at room temperature. The extraction buffer was then combined with the corresponding peptide digest solution, vacuum-centrifuged to dryness, and analyzed by LC-MS/MS.
**Targeted LC-MS/MS acquisition**

Peptides were loaded onto a 15 cm × 75 μm ChromXP C18-CL 3-μm, 300 Å cHiP Nanoflex System (Eksigent, Foster City, CA) with a 250 nl/min flow rate and eluted using a 0%–50% acetonitrile/0.1% formic acid gradient over 30 min. Data were acquired using a TripleTOF 5600 system (Sciex) with an ion spray voltage of 2.3 kV, a declustering potential of 60 V, curtain gas of 20 p.s.i., nebulizer gas of 10 p.s.i., and an interface heating temperature of 120 °C. MS survey scans were acquired for 250 ms from 300–1,250 m/z, and product ion scans targeting unlabeled and 15N-labeled GFAP peptides were collected with an accumulation time of 100 ms from 100–2,000 m/z.

**Author contributions**—M. R. H. and M. B. conceptualization; M. R. H. and M. B. data curation; M. R. H. and M. B. formal analysis; M. R. H. and L. W. investigation; M. R. H. and M. B. methodology; M. R. H. and M. B. writing—original draft; M. R. H., S. B., and M. B. writing—review and editing; S. B. and M. B. supervision; M. B. resources; M. B. funding acquisition; M. B. project administration.

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**References**


Instability of mutant GFAP in Alexander disease patients


