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The Composition of Rosenthal Fibers, the Protein Aggregate Hallmark of Alexander Disease

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Abstract

Alexander disease (AxD) is a neurodegenerative disorder characterized by astrocytic protein aggregates called Rosenthal fibers (RFs). We used mouse models of AxD to determine the protein composition of RFs to obtain information about disease mechanisms, including the hypothesis that sequestration of proteins in RFs contributes to disease. A method was developed for RF enrichment, and analysis of the resulting fraction using iTRAQ mass spectrometry identified 77 proteins not previously associated with RFs. Three of five proteins selected for follow-up were confirmed enriched in the RF fraction by immunoblotting of both the AxD mouse models and human patients: receptor for activated protein C kinase 1 (RACK1), G1/S-specific cyclin D2, and ATP-dependent RNA helicase DDX3X. Immunohistochemistry validated cyclin D2 as a new RF component, but results for RACK1 and DDX3X were equivocal. None of these was decreased in the non-RF fractions compared to controls. A similar result was obtained for the previously known RF component, alphaB-crystallin, which had been a candidate for sequestration. Thus no support was obtained for the sequestration hypothesis for AxD. Providing possible insight into disease

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Supporting Information

Table S-1, significantly enriched Rosenthal fiber fraction proteins detected in the mouse models; Table S-2, detection details for mouse RF-associated proteins listed in Table S-1; Table S-3, enriched Rosenthal fiber fraction proteins detected in the AxD patient samples.

Notes

The authors declare no competing financial interest.

progression, the association of several of the RF proteins with stress granules suggests a role for stress granules in the origin of RFs.

Keywords

Rosenthal fiber; Alexander disease; protein aggregates; neurodegenerative disease; astrocyte; proteomics; mass spectrometry (MS); stress granules

INTRODUCTION

Alexander disease (AxD) is a fatal neurodegenerative disorder caused by dominant gain-of-function mutations in the gene encoding glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed almost exclusively in astrocytes.¹ The clinical signs and progression of AxD are highly variable, depending primarily on age of onset.² Typical early onset patients exhibit symptoms including psychomotor retardation, seizures, abnormal myelination, and megaloccephaly; whereas later onset patients tend to display bulbar and pseudobulbar signs such as ataxia and difficulty swallowing and speaking. The defining pathological feature of AxD is the abundant presence in astrocytes of GFAP-containing protein aggregates, called Rosenthal fibers (RFs), especially in the subpial, perivascular, and subventricular regions of the central nervous system. In addition to GFAP, RFs have been reported to contain alphaB-crystallin, heat shock protein 27 (also known as heat shock protein beta-1), ubiquitin, vimentin, plectin, c-Jun, the 20 S proteasome, and synemin.³⁻⁶

The composition of disease-associated protein aggregates has received attention for the insight it might provide into the disease processes. For example, studies to characterize protein aggregate components have been reported for polyglutamine aggregates in Huntington disease,⁷ Lewy bodies in Parkinson disease⁸ and amyloid plaques in Alzheimer's disease.⁹ Of particular interest is the possibility that aggregates may sequester proteins crucial for normal cell activity, rendering them unable to perform a critical function. Reports have suggested that such sequestration occurs in amyloid-like aggregates¹⁰ and polyglutamine aggregates.^{11,12} For example, sequestration of mdm-2 in polyglutamine aggregates has been suggested to cause upregulation of p53 activity and increased cytotoxicity.¹¹

This study seeks to identify proteins associated with the RFs present in AxD using a proteomics approach, in contrast to the more restricted candidate protein approach previously taken. In addition to human tissues, mouse AxD models were used for greater control of sample quality. These models were GFAP^{TG} mice, which express a wild type human GFAP transgene; R236H(+/-) mice (designated more simply as R236H for the remainder of this paper), which are heterozygous for a knock-in mutation in the endogenous mouse GFAP gene that is homologous to the particularly severe human R239H mutation; and R236H/GFAP^{TG} mice, which carry both changes.^{13,14} All three lines form RFs, but the R236H/GFAP^{TG} line does so more abundantly. Also, whereas the GFAP^{TG} and R236H mice live a normal lifespan, the R236H/GFAP^{TG} mice die by about 35 days of age, with a median lifespan of about 25 days. The unbiased protein analysis was performed using the 8-plex isobaric tags for relative and absolute quantitation (iTRAQ)¹⁵ method. In this procedure,

peptides are produced from eight different samples, each set is labeled with a specific isotopic tag, and the eight samples combined and analyzed simultaneously by liquid chromatography followed by MS/MS.

MATERIALS AND METHODS

Animals and Human Tissue

The use of human tissue in this study was approved by the Institutional Review Boards of the University of Alabama at Birmingham (UAB) and Columbia University, and animal protocols by the Institutional Animal Care and Use Committees of UAB and Columbia University. Descriptions of the human brain tissues used for the proteomic analyses are provided in Table 1. The AxD mouse models have been described previously,^{13,14} and were either on an FVB background or C57BL/6J that had been backcrossed to FVB for 3 generations. Whole brains, including cerebellum, brain stem and olfactory bulb, were harvested by rapid decapitation, immediately frozen on dry ice and stored at -80°C until use.

Rosenthal Fiber Enrichment

Whole brains of 25 day old mice were Dounce homogenized using 20 strokes of pestle A followed by 20 strokes of pestle B in 3 mL of ice cold buffer consisting of 0.5% Triton-X 100 (w/v), 2 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1 mM PMSF, Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN), and 1% (v/v) of both phosphatase inhibitor cocktails 2 and 3 (Sigma, St. Louis, MO)[modified from Hagemann et al (2006)¹⁴ by addition of the phosphatase inhibitors]. An 800 μL aliquot of homogenate from each mouse was transferred to two 1.5 mL microfuge tubes followed by centrifugation at $17,000 \times g$ for 20 min at 4°C . The resulting Triton-X soluble fractions were frozen on dry ice and stored at -80°C . The two pellets were each resuspended in 800 μL of ice cold buffer containing 6 M urea, 20 mM Tris-HCl, pH 7.4, 1 mM PMSF, 5 mM EDTA, 1 mM EGTA (urea buffer) by drawing in and out of a 1 mL pipet tip ten times and then centrifuged at $3,000 \times g$ for 10 min at 4°C . The resulting urea soluble fractions were frozen on dry ice and stored at -80°C . One of the pellets, designated as the RF fraction in this report, was solubilized by resuspension in 800 μL 10% (w/v) SDS, boiled for 30 min and then frozen at -80°C until used for immunoblotting and iTRAQ LC-MS/MS sample preparation. The second pellet was resuspended in 800 μL of PBS to preserve RFs and used for immunostaining. These latter suspensions were centrifuged at $3,000 \times g$ for 10 min at 4°C and the resulting pellets were resuspended in 800 μL of DNase buffer at room temperature containing 20 mM Tris-HCl, pH 8.0, 10 mM MgCl_2 , 20 mM CaCl_2 , 1 mM PMSF, 0.2 mg/mL DNase I (Roche) and incubated for 15 min followed by centrifuging at $3,000 \times g$ for 10 min at 4°C . The resulting pellets were resuspended in 800 μL of PBS for immunostaining.

A slightly different procedure was used to enrich for RFs from the human samples. These analyses were done before the mouse studies, but are presented second in the Results because the high variability among human samples prompted us to use the mice for more controlled analysis. The human studies thus served for confirmation rather than for discovery. The procedure used a buffer containing sodium deoxycholate to solubilize non-

aggregated GFAP followed by treatment with DNase I and a final extraction with 6 M urea. This method works equally well as that for the mice to eliminate non-aggregated GFAP, but the sodium deoxycholate buffer in the initial step solubilizes some filament GFAP and therefore did not distinguish between soluble and polymerized GFAP. All human preparations analyzed were produced by Dounce homogenization of 200 mg of cerebral cortex in 5 mL of ice cold HEB buffer¹⁷ consisting of 20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% (v/v) NP-40, 0.5% sodium deoxycholate w/v, 1 mM PMSF, complete protease inhibitor tablet (Roche, Indianapolis, IN), 1% (v/v) of both phosphatase inhibitor cocktail 2 and 3 (Sigma, St. Louis, MO) followed by centrifuging at $3,000 \times g$ for 10 min at 4 °C. The resulting pellets were then treated with DNase I, centrifuged as described above, and the pellets resuspended in urea buffer and processed the same as the mouse samples.

Immunostaining

RF fractions were immunostained by pipetting a 20 μ l volume of each RF fraction in PBS onto Fisherbrand Superfrost Plus microscope slides (Fisher Scientific) and leaving overnight at room temperature to air dry. The following day, slides were washed 3 times in PBS and then blocked by adding blocking buffer consisting of 1% BSA (w/v), 5% normal goat serum (w/v), 0.1% Triton-X (w/v) and incubated at room temperature for 1 h. Slides were washed in PBS, tapped vertically to remove excess liquid, and costained by incubating for 1 h at room temperature with a mixture of a cocktail of monoclonal GFAP antibodies (SMI-26, Sternberger Monoclonals, Lutherville, MD) and rabbit anti-Cryab polyclonal antibody (PA1-16950, Pierce, Rockford, IL), each diluted 1:1000 in blocking buffer. Slides were washed 3 times in PBS, tapped vertically, and incubated for 1 h at room temperature in the dark with an Alexa Fluor anti-mouse 594 secondary antibody and Alexa Fluor anti-rabbit 488 secondary antibody (Invitrogen, Grand Island, NY), both at a 1:500 dilution in blocking buffer. The slides were washed 3 times in PBS, tapped vertically and allowed to air dry for 1 h at room temperature prior to imaging. Fluorescent images were obtained using a Zeiss confocal microscope with a 20x objective.

Immunostaining of mouse brains was performed using the R236H/GFAP^{TG} AxD model at 4 weeks of age, and the R236H mice at 4 months and 1 year of age. Mice were anesthetized with ketamine-xylazine before intracardiac perfusion with 4% paraformaldehyde in PBS. Brains were removed and kept in the fixative for 12 to 16 h at 4 °C. 40 μ m coronal sections were prepared with a vibratome (Leica VT1000S) and stored in cryoprotectant solution at -20 °C before use. Primary antibodies used were against cyclin D2 (mouse monoclonal DS3.1, Thermo Scientific, Rockford, IL, 1:1000), DDX3X (rabbit polyclonal, ab61153, Abcam, Cambridge, MA, 1:100), RACK 1 (rabbit monoclonal EPR7388, Abcam, 1:100), and GFAP (mouse monoclonal G3893, Sigma-Aldrich, St. Louis, MO, 1:1000; rabbit polyclonal Z0334, Dako, Carpinteria, CA, 1:1000). Secondary antibodies were anti-mouse Alexa Fluor 488, 594; anti-rabbit Alexa Fluor 594; anti-rat Alexa Fluor 488, 594, all from goat or donkey (Molecular Probes, Eugene, OR, 1:300). To control for the specificity of immunostaining, primary antibodies were omitted and substituted with appropriate normal serum. Slides were viewed using a Nikon A1R MP confocal microscope.

Human brain immunostaining was performed on isocortex and subcortical white matter from the autopsy of three AxD patients: patient 1 was a previously reported¹⁸ 11 month old female with an R239H mutation, patient 2 was a 3 year old female with an R239H mutation, and patient 3 was a previously reported¹⁹ 39 year old female with a splice site mutation in intron 3. Autopsy specimens of a 4-year old female with no neuropathology were used as controls. Tissues were fixed in 10% formalin, embedded in paraffin, and processed for immunohistochemistry. Primary antibodies against cyclin D2, DDX3X and RACK 1 were as described above for mouse. Stains were visualized with an ABC immunoperoxidase kit (Vector Labs, Burlingame, CA) and slides were counterstained with hematoxylin and examined with an Olympus BX40 microscope.

Electron microscopy

Vibratome sections were postfixed in 1% osmium tetroxide in 0.2 M phosphate buffer (pH = 7.4) for 1 h at 4 °C, dehydrated and flat-embedded in Epon-Araldite. Areas of interest were identified by light microscopy, excised from sections, and glued onto resin blocks. Ultrathin sections were cut using a Reichert Ultramicrotome, stained with uranyl acetate and lead citrate and examined with a JEOL 1200 electron microscope.

Immunoblotting

An equal fraction (e.g., 2%) of each sample was analyzed by immunoblotting. Samples were boiled in Laemmli sample buffer for 5 min and electrophoresed for 45 min on a 4-20% SDS-PAGE gel (Pierce, Rockford, IL) at 120 volts and then transferred to nitrocellulose membranes for 1 h at room temperature at 100 volts followed by blocking with 5% BSA in PBS for 1 h at room temperature. Blots were then incubated overnight with agitation with primary antibody, washed 3 times in 5% non-fat dry milk/0.1% (v/v) Tween-20/PBS (diluent buffer), incubated for 1 h at room temperature with secondary antibody, washed 3 times with diluent buffer, once with PBS, and then imaged on a Li-Cor Odyssey imager. Primary antibodies, all diluted 1:2,000 in diluent buffer, were SMI-26, RACK1 rabbit monoclonal (D59D5, Cell Signaling Technology, Danvers, MA), eIF4A rabbit monoclonal (C32B4, Cell Signaling Technology), Cyclin D2 rabbit monoclonal (D52F9, Cell Signaling Technology) and DDX3X rabbit monoclonal (D19B4, Cell Signaling Technology); secondary antibodies, diluted 1:15,000, were an anti-mouse 800 CW for GFAP immunoblots or an anti-rabbit 800 CW for all other blots (Li-Cor Biosciences, Lincoln, NE). All samples presented in a figure panel were run on the same gel as indicated, but for some of the mouse sample immunoblots a lane not relevant to this study was deleted.

Sample Preparation and iTRAQ labeling for LC-MS/MS

RF fractions were boiled in Laemmli sample buffer for 5 min. To facilitate comparisons, equal percentages of each RF fraction prepared starting with equal milligram amounts of human tissue or mouse whole brains were electrophoresed in a 4-20% SDS-PAGE gel (Pierce) at 120 volts for 5 min. Then the gels were rinsed three times briefly with deionized water and incubated with Coomassie Brilliant Blue R-250 protein stain [0.05% (w/v) Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid] for 1 h at room temperature on an orbital shaker. Gels were destained for 2 h in 15% (v/v) methanol, 10% (v/v) glacial acetic acid on an orbital shaker at room temperature. The destained gels were

then placed in ACROS HPLC water and each sample gel lane dissected into approximately 1 mm cubes and placed into 1.5 mL centrifuge tubes for processing at room temperature. The gel fragments were then incubated twice for 15 min in protein destaining solution containing 100 mM ammonium bicarbonate, 50% (v/v) aqueous acetonitrile followed by incubation overnight in the same buffer. The following day the protein destaining solution was carefully removed and new protein destaining solution added and the gel fragments incubated another 4 h to completely remove the Coomassie Blue stain. The protein destaining solution was then removed and acetonitrile was added for 5 min to completely dehydrate the gel pieces. The acetonitrile was then removed and the gel fragments dried in a Speed Vac for 5 min. The samples were then reduced by the addition of 10 mM dithiothreitol (DTT) for 30 min. The DTT solution was then removed and an equal volume of 100 mM iodoacetamide was added and incubated for 30 min in the dark. The iodoacetamide solution was then removed and acetonitrile was added and incubated 5 min to dehydrate the gel fragments and remove residual iodoacetamide. The gel fragments were then placed in 100 mM ammonium bicarbonate for 10 min followed by acetonitrile treatment for 5 min. The acetonitrile was then removed and the gel fragments dried in a vacuum centrifuge for 5 min. The fully dried gel fragments were digested with sequencing grade trypsin (Promega, Madison, WI), which cleaves after arginine and lysine residues except when followed by a proline. Trypsin was used at a 1:20 ratio to protein determined from a BCA assay of each fraction loaded for SDS-PAGE. After digestion overnight at 37 °C, the trypsin digest solutions were transferred to fresh tubes and the gel fragments incubated for 1 h in 50% aqueous acetonitrile, 5% formic acid. The acetonitrile/formic acid solution was then combined with the overnight trypsin digests and vacuum centrifuged for 2 h to dry the peptides. The peptides were then resuspended in 0.1% formic acid and bound to a P10 C₁₈ Zip Tip (Millipore, Billerica, MA) followed by eluting in 50% aqueous acetonitrile. The peptides were then vacuum centrifuged to dryness and resuspended in isopropanol followed by labeling with 8-plex iTRAQ reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. For analysis of the mouse samples, RF fractions obtained from four different 25 day old wild type mice were labeled with reporter ions 113, 115, 117, and 119 and four 25 day old AxD model mice were labeled with 114, 116, 118, and 121. For analysis of the human RF fractions, labels used were changed in a duplicate run to determine if the labeling process affected the results. The samples were labeled as follows [patient mutation (run 1 label, run 2 label)]: R79C (113, 121), R239H (114, 119), R239C (115, 118), S247P (116, 117), R416W (117, 116), 14 yr old control (118, 115), 50 yr old control (119, 114), and 1 yr old control (121, 113). Similar fold-enrichments for RF proteins relative to the controls were obtained with the two labeling schemes. Following iTRAQ labeling, the samples were combined, vacuum centrifuged and redissolved in 0.5% formic acid and Zip Tipped to remove excess iTRAQ reagent and eluted with 50% acetonitrile, 0.1% formic acid. The iTRAQ labeled peptides were then vacuum centrifuged to dryness, redissolved in 0.1% formic acid, and analyzed by LC-MS/MS.

LC-MS/MS Conditions and Data Analysis

The mouse samples were analyzed at North Carolina State University. Peptides were separated using an Easy nLC II (Thermo Fisher Scientific, San Jose, CA) integrated with a cHiPLC-Nanoflex system (Eksigent, Dublin, CA). Reversed phase chromatography was

employed using a trap and elute configuration with the trapping column (0.5 mm $\times$ 200 μ m i.d.) and analytical column (15 cm $\times$ 75 μ m i.d.) existing in a cHiP format and packed with ChromXP C18-CL (3 μ m, 120 Å) by the manufacturer (Eksigent). LC solvents (Burdick and Jackson, Muskegon, MI) consisted of 98% water, 2% acetonitrile, 0.2% formic acid for mobile phase A and 98% acetonitrile, 2% water, and 0.2% formic acid for mobile phase B.

Data were acquired on a Q Exactive mass spectrometer (Thermo Fisher Scientific) in data-dependent mode. AGC settings were 1E6 and 5E4 for MS and MS/MS, respectively. Maximum ion injection times were set to 30 ms and 250 ms for MS and MS/MS, respectively. Resolving power settings were set to 60,000_{FWHM} at 200 m/z and 17,500_{FWHM} at 200 m/z for MS and MS/MS acquisition, respectively. Stepped normalized collision energy was employed at 25 $\pm$ 10% NCE. Mass window was 2 m/z , the charge state exclusion was set to unknown, and the dynamic exclusion was set to 30 seconds after sequencing an m/z once.

The human samples were analyzed at the University of Alabama at Birmingham in duplicate, changing the iTRAQ reporter labeling between the 2 sets as described above. The human iTRAQ labeled peptides were loaded onto a 15 cm $\times$ 75 μ m ChromXP C18-CL 3 μ m 300 Å cHiP nanoflex system (Eksigent, Foster City, CA) with a 250 nl/min flow rate. Peptides were eluted using a 0-50% acetonitrile/0.1% formic acid gradient over 60 min. Data were acquired using a TripleTOF 5600 system (SCIEX, Toronto, Canada) with an ion spray voltage of 2.3 kV, declustering potential of 60 V, curtain gas of 20 PSI, nebulizer gas of 10 PSI, and an interface heating temperature of 120 °C. Charge states of +2 through +5 were used, and the exclusion list was set to 15 seconds after sequencing an m/z twice. IDA survey scans were acquired for 250 ms from 400-1250 m/z and 20 product ions scans were collected within an accumulation time of 50 or 100 ms from 100-2000 m/z . The collision energy was set by selecting adjust for iTRAQ reagents parameter and the rolling collision energy was set to 5 eV. The complete iTRAQ dataset has been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002448.

ProteinPilot Software v. 4.2 and the Paragon algorithm (SCIEX, Foster City, CA) were used to process the raw files and search against the human or mouse Swiss-Prot database released on 7/9/2012. The mouse Swiss-Prot database had 16,768 protein sequences (not including decoys) and the human Swiss-Prot database contained 20,193 protein sequences (not including decoys). For the search, N-terminal and K residue iTRAQ 8-plex labeling and carbamidomethylation of C residues were set as fixed modifications and oxidation of Met residues was set as a variable modification. A maximum of two missed cleavages was allowed. The human data were analyzed with the 5600 TripleTOF with precursor and MS/MS tolerances of 0.05 daltons, and the mouse data were analyzed with the Orbitrap with precursor and MS/MS tolerances of 3 ppm. Each detection of a peptide was treated as an independent event, and the fold-difference for the AxD samples relative to the controls determined from the relative intensities of the reporter ions. The median values were then determined for each protein after eliminating peptides with confidence scores below 95% or peptides that could not be used for quantitation according to ProteinPilot due to shared, weak, discordant, or low confidence MS/MS spectra. Protein abundance relative to wild type

(fold change) was determined for the mouse samples by dividing the average peak height of the 4 AxD mouse model samples by the average peak height for the 4 controls for each detection of a peptide by LC-MS/MS. Results for all peptide determinations assigned to a given protein were pooled for the 3 replicate runs, and the median fold change value selected. Fold changes for the human AxD patients were calculated in a similar way, except that in each run the single peak height for each patient was divided by the average of the 3 controls, and the median value selected from the pooled data of the 2 replicate runs. Statistical significance was determined using an unpaired two-tailed Student's T-test. p-values were calculated for each peptide for the mice by comparing the iTRAQ reporter intensities of all 4 of the AxD model mice to those for the 4 wild type mice for each MS/MS peptide scan, and for the human dataset by comparing the intensities of all 5 of the patients to the 3 controls for each scan. The p-values for all peptide determinations assigned to a given protein were pooled for the replicate runs and the median value selected.

The stoichiometry of proteins in the RFs was estimated from the number of spectral counts for each protein by the method of Mosley et al. (2011).²⁰ An estimate of the relative concentration of each protein was obtained by dividing the number of its spectra by the number of amino acids it contains, and an estimate of its mole fraction obtained by dividing this relative concentration by the sum of the relative concentrations for all proteins detected. The fraction by weight of each component was calculated by simply dividing the spectral counts for that protein by the sum of the spectral counts for all the proteins. However, the spectral count inputs for both these calculations were not the raw spectral counts obtained for each protein in the iTRAQ analyses, since these raw counts had contributions from both the Alexander disease model or human patient samples and the control samples (C). To obtain an estimate of the spectral counts attributable to the AxD samples, we used the fold-enrichment data obtained from the iTRAQ reporter signals that are specific to each sample. Assigning a value of 1.0 unit to each control C, the relative total units of spectral counts for protein i is $T_i = n + (\text{sum of } F_{ij})$ where n is the number of control samples and F_{ij} is the fold-increase of protein i in each disease sample j . The iTRAQ runs for the mouse samples each had 4 wild type controls and 4 AxD model mouse samples of the same kind. Since the values for the 4 samples in each group were averaged to obtain F_{ij} , the relative total spectral counts for the mouse samples are $1 + F_i$, and the fraction of total spectral counts attributable to the AxD model sample is $F_i/T = F_i/(1+F_i)$ and the total spectral counts for the AxD model sample is $T_i F_i/(1+F_i)$. However, some of the spectral counts in the AxD sample are due to non-RF proteins present in the preparation. To correct for this, the relative contamination in the AxD sample is assumed to be the same as that for the control value for that protein in the sample, which is 1.0. Thus the spectral counts assigned to contamination are $T_i/(1+F_i)$, and the net spectral counts for a protein i attributable to the disease state is $T_i F_i/(1+F_i) - T_i/(1+F_i) = T_i(F_i-1)/(F_i+1)$. These corrected spectral counts were the input values for the stoichiometry calculation described above, but the corrections proved to have relatively minor effects, changing the mole % values by an average of only 19%. For the human samples each iTRAQ run included 3 control and 5 different patient samples. No systematic differences were observed among the control samples, so these values were assumed identical for the correction calculation. Again assigning a relative value of 1.0 to each of the 3 control samples, the total relative spectral counts is given as $3+S$ where $S =$

$F_{i1}+F_{i2}+F_{i3}+F_{i4}+F_{i5}$ with F_{in} being the fold enrichment for protein i in patient sample n . The spectral counts for protein i attributable to the AxD patient samples is thus $T_iS/(3+S)$ and the contamination in each of the 5 patient samples is $T_i/(3+S)$, yielding net spectral counts for protein i attributable to the disease state in the patient samples as $T_iS/(3+S) - 5T_i/(3+S) = T_i(S-5)/(S+3)$.

RESULTS

Rosenthal Fiber Enrichment Procedure

Brain tissue fractions used for previous analyses of RF components did not separate the aggregates from normally formed GFAP intermediate filaments.⁴ Thus to increase the sensitivity and specificity of our proteomic analysis, a more stringent isolation procedure was developed, using three criteria to evaluate the quality of the Rosenthal fiber enrichment: (1) removal of GFAP in preparations from wild type mice, (2) retention of GFAP in AxD model mice, and (3) retention of aggregates with the characteristic appearance of RFs as judged by GFAP and alphaB-crystallin immunostaining. A concentration of 6 M urea provided the best compromise between removal of GFAP from the wild type mice and retention of GFAP in the AxD mouse model samples among several extraction systems tested, including sodium deoxycholate¹⁷ and high concentrations of sodium and potassium chloride.⁴ Other urea concentrations ranging from 2-8 M either solubilized too little GFAP from the wild type or too much GFAP from the AxD extracts. Figure 1 illustrates the method and shows the results obtained for the wild type and lethal R236H/GFAP^{TG} AxD model mice. In the wild type mice, 9% of the total amount of GFAP is solubilized by the Triton buffer, 75% by the 6 M urea, and 16% remains in the 6 M urea insoluble pellet, which will be referred to as the RF fraction (for the wild type it actually contains no RFs) (Figures 1B,C). In contrast, the lethal R236H/GFAP^{TG} AxD model mice have 12% of the total GFAP solubilized by the Triton buffer, 32% by the urea extraction, and 56% of the total GFAP remains in the RF fraction. Note that these percentages are for the distribution of GFAP among the three fractions; the actual total amount of GFAP is much greater in the AxD model mice than the controls due to accompanying reactive gliosis (Figure 1B). Thus 6 M urea markedly reduced retention of GFAP in the RF fraction prepared from wild type mice compared to that from AxD model mice. To determine if aggregates with the characteristics of RFs remain intact after the urea treatment, the preparation was immunostained for GFAP and alphaB-crystallin. As shown in Figure 1D, aggregates with the appearance of RFs²¹ are indeed present in the preparation made from the AxD model mice, but absent from the wild type control. The abundance of RFs in the brains of the AxD model mice has been observed to be in the order of $R236H < GFAP^{TG} < R236H/GFAP^{TG}$;²² and a similar ordering was observed from the relative abundance of aggregates observed in the resuspended urea pellets and the percent of the total GFAP they contained, 16% for the wild type, 41% for the R236H mice, 48% for the GFAP^{TG} mice, and 56% for the lethal R236H/GFAP^{TG} mice. It should be noted that although the urea extraction serves the purpose of removing a substantial fraction of normally polymerized GFAP from the RFs, this preparation still contains a large quantity of other proteins; of the total spectral counts detected, only about 20% were for proteins significantly enriched (p-value = 0.05) in the AxD model samples compared to the controls.

MS Analysis of Mouse AxD Models

It was our initial intent to determine the composition of RFs using human AxD patient tissue. However, the limited tissue samples available and the sampling variability amongst them compromised statistical analysis, so instead the discovery phase of this investigation was performed with the mouse models. The human data were then used for validation and to prioritize samples for follow-up. All three mouse models described in the Introduction were used, GFAP^{TG}, R236H and R236H/GFAP^{TG}. Inclusion of GFAP^{TG} and R236H mice permitted comparison of RFs resulting from expression of an AxD-associated mutant GFAP to those formed as a result of overproduction of wild type GFAP; whereas inclusion of R236H/GFAP^{TG} mice provided a model with more abundant RFs and also permitted a comparison between RFs in lethal and non-lethal mouse models. Mice were analyzed at 25 days of age, the median survival time for the R236H/GFAP^{TG} line in our colony, as a compromise between extent of RF formation and loss of the R236H/GFAP^{TG} animals. Each LC-MS/MS analysis was performed with the 8-plex isobaric tags for relative and absolute quantitation (iTRAQ)¹⁵ on RF fractions from four AxD mice of a given genotype and four littermate controls, and run in triplicate.

An average of 296 proteins was identified at or above 95% confidence as determined by the ProteinPilot software program for each multiplex run, and the total number of different proteins identified among all the runs combined was 533 (none of these were present only in the control samples). To evaluate the success of the iTRAQ approach for identifying RF proteins, we examined our results for the presence of the previously reported RF components. As shown in Table 2, 6 of the 8 proteins plus components of the 20S proteasome subunit previously reported associated with RFs were found to be enriched in at least one of the AxD model mouse preparations, with only c-Jun and synemin being undetected in all three of the models. Tables 2, S-1 and S-2 show the proteins whose enrichment relative to wild type reached statistical significance (p-value < 0.05) in at least one of the AxD models. In addition to previously reported RF components, 77 novel proteins were identified. Most of these were present at very low concentrations as judged by the number of times their peptides were detected by the mass spectrometer (i.e., spectral counts) as shown in Table S-1. These spectral counts can be used to approximate the stoichiometry of the RF associated proteins as described in Methods (Tables 2 and S-1). The results suggest that three proteins account for 83% of the molecules in RFs, GFAP (32.8%), ubiquitin (30.1%) and vimentin (19.9%). On a weight basis these numbers correspond to about 44% GFAP, 29% vimentin and 7% ubiquitin (Table 4). Only 4 of the 77 novel mouse Rosenthal-fiber associated proteins were present at greater than 1.0 mole percent: small nuclear ribonucleoprotein Sm D3 (2.4%), ATP-dependent RNA helicase DDX3X (2.1%), fatty acid-binding protein, brain (1.2%) and G1/S-specific cyclin-D1 (1.1%); whereas 11 were identified from a single spectral count (Table S-1). Seventy-three of the 77 novel proteins were identified in analyses of the relatively RF rich R236H/GFAP^{TG} fraction, but consistent with the low abundance of the novel proteins and the differences in RF content of the preparations, just 49 were observed in the GFAP^{TG} mouse samples and only 35 in the R236H preparations. Note also that in nearly every case the fold increase for the RF-associated proteins rises in the order R236H < GFAP^{TG} < R236H/GFAP^{TG}; probably reflecting the increasing abundance of RFs and the purity of the RF preparation.

MS Analysis of Human Patients

As noted above, tissue from the cerebral cortex of five AxD patients and three age matched controls were similarly analyzed with the intent of identifying RF components, but the small number of samples available and the large variation in RF content in the samples yielded data too variable for statistically meaningful results for even the known RF components. Both microscopic examination of the preparations and quantitation of GFAP levels in the RF preparations by western blotting indicated that the RF content for the patient samples was R239C>R79C>R416W>S247P≈R239H (data not shown), with the latter two being only marginally different from controls. For these five patient samples the percentages of the total GFAP found in the RF fraction were 76%, 73%, 87%, 15% and 3%, respectively, whereas the values for the controls ranged from 1% to 8%. Since the pathology report for the R239H patient noted abundant RFs, their low yield in our preparation likely resulted from sampling variability. The iTRAQ analysis was consistent with this ordering, with enrichments for known RF components generally following the same progression, with the S247P and R239H samples showing little difference from the controls (Tables 3 and S-3). Accordingly, we relied primarily on the results obtained from the R239C, R79C and R416W human data to verify and prioritize the proteins identified using the mouse AxD models.

Immunoblotting of Selected Proteins

Most of the components found at lower levels in the mouse were not found in the human samples, and vice-versa. Of the 77 significantly enriched mouse proteins, only 7 were detected in any of the human samples, but each of these was found elevated in at least 3 of the 5 patients (Table S-3). These proteins are cyclin D2, ATP-dependent RNA helicase DDX3X (DDX3X), guanine nucleotide-binding protein subunit beta-2-like 1 (also known as Receptor for activated C-kinase 1)(RACK1), eukaryotic initiation factor 4A (eIF4A), Serine/threonine-protein phosphatase PP1-beta catalytic subunit, NAD(P)H dehydrogenase [quinone] 1 and V-type proton ATPase subunit d 1. The first four of these were selected for further study. Cyclin D2 was chosen because its increased activity can cause both megalencephaly and hydrocephalus,²⁴ both of which are symptoms of early onset AxD. It is also of interest for its requirement for glioblastoma growth.²⁵ DDX3X, RACK1 and eIF4A were selected for being components of stress granules, which as described in the Discussion, may have a critical role in RF formation. In addition to these four proteins enriched in both the mouse and human RF fractions, we also further studied NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial (Ndufv1), which was enriched 7.9-fold in the iTRAQ analysis of the lethal R236H/GFAP^{TG} mice compared to wild type animals, but was not detected in the human samples. This protein was selected because its functional loss causes Leigh syndrome, which is sometimes misdiagnosed as AxD due to symptom similarities.^{1,26}

Immunoblotting of the RF fraction was conducted to determine if Ndufv1 and the four other selected proteins are indeed enriched in this preparation (Figure 2). These immunoblots indicated that two of the five, eIF4A and Ndufv1, were not enriched and thus likely false positives, but that the other three, cyclin D2, DDX3X, and RACK1, are highly enriched in the RF fractions of the lethal R236H/GFAP^{TG} AxD mouse model compared to wild type mice. Enrichment was also found at reduced levels in the GFAP^{TG}, and R236H models,

except that RACK1 was not observed in the R236H mice. We then immunoblotted cyclin D2, DDX3X, and RACK1 in RF fractions from human controls and AxD patients. As shown in Figure 3, all three proteins are also highly enriched in the RF fractions from the patients that had the higher levels of RFs present, R239C and R79C.

Immunostaining for cyclin D2, DDX3X and RACK1

The candidacy of cyclin D2, DDX3X and RACK1 as novel RF components was further examined by immunostaining the hippocampus of AxD mouse models and the isocortex and subcortical white matter of human patient autopsy material. R236H/GFAP^{TG} mice were examined at 4 weeks of age, and R236H mice at 4 months and 1 year of age. The human patients were an 11 month old female with an R239H mutation (patient 1), a 3 year old female with an R239H mutation (patient 2), and a 39 year old female with a splice site mutation in intron 3 (patient 3). Autopsy specimens of a 4 year old female with no neuropathology were used as controls.

Immunostaining for cyclin D2 was positive in astrocytes in the adult R236H mice, primarily appearing as small, punctate signals in the cell bodies, processes, and endfeet (Figure 4A,B). All of these puncta colocalized with GFAP (Figure 4A',B'), and their size and distribution were similar to that of RFs in these mice, as visualized by electron microscopy (Figure 5A,B). Although the proteomic analysis identified cyclin D2 as a possible RF component in 25 day old R236H/GFAP^{TG} mice, no immunofluorescent staining for this protein was observed in the hippocampus of these mice (data not shown), although RFs are clearly present (Figure 5C,D). In all three human AxD patients the antibody to cyclin D2 stained many of the RFs present in white matter and around blood vessels (Figure 4C, D), and in subpial regions (data not shown). No immunostaining was detected in astrocyte cell bodies in the human AxD sections and no staining was observed in control sections in either isocortex or white matter (data not shown).

As was the case for cyclin D2, immunostaining for DDX3X was observed only in the adult R236H mice. Staining was quite modest, although we did observe small, rare DDX3X+/GFAP+ profiles in astrocytes (Figure 6). Immunostaining of AxD human tissues revealed a granular pattern in the enlarged white matter astrocytes (Figure 6C), which was not seen in the control (data not shown). Some immunostaining was associated with RFs (Figure 6D), but the proportion of RFs that were immunostained was less than 1%. In the isocortex of both the controls and the AxD patients there was granular cell body staining of neurons and glia (data not shown).

Most of the RACK1 immunostaining of the adult R236H mice labeled the neuropil and the cell bodies of neurons, such as pyramidal cells of the hippocampus (Figure 7A,A'). Similar neuropil labeling was seen in control mice (data not shown). In a few instances, punctate objects in astrocyte cell bodies were co-labeled for RACK1 and GFAP (Figure 7A',B,B'). These were found only in the adult R236H mice, and were similar in size and form to the objects stained by cyclin D2, suggesting possible association with RFs. To explore this association further, sections were double labeled with RACK1 and cyclin D2 antibodies. All of the RACK1+ puncta in astrocytes were also cyclin D2+ (Figure 7C,D). Immunostaining for RACK1 in human tissues labeled the isocortical neuropil in a fine, granular pattern in

both the AxD patients and the control, but did not stain cell bodies of neurons or glia (not shown). In contrast, cell bodies of the enlarged astrocytes in the AxD white matter did stain, either with a diffuse pattern, a granular pattern, or both; but astrocyte processes and endfeet did not stain (Figure 7E). White matter astrocytes in the control did not immunostain. Rare RFs in white matter (fewer than 1%) were immunolabelled (Figure 7F).

Testing of the Sequestration Hypothesis

The aggregate sequestration hypothesis for disease causation holds that the function of a critical protein is compromised by it being bound by the disease-associated aggregates. To determine if the enrichment of cyclin D2, DDX3X, or RACK1 in the RF fraction is accompanied by a decrease in its concentration in the soluble fraction compared to that in wild type mice, we immunoblotted each of the fractions created during the RF enrichment procedure in wild type and lethal R236H/GFAP^{TG} AxD mice (Figure 8). A major portion of each protein was indeed found to be associated with the RF fraction from the R236H/GFAP^{TG} mice but not from the wild type mice, and significant amounts were also present in the urea soluble fraction, which includes proteins associated with normally formed GFAP filaments. However, despite these associations, the amounts in the Triton-X soluble fraction were at least equal to that from the wild type, apparently due to elevated levels of each of the three proteins in the AxD model mice. These results are thus contrary to the prediction of the sequestration hypothesis.

To determine if DDX3X, cyclin D2 and RACK1 are increased in the R236H and GFAP^{TG} AxD model mice as well as in the lethal R236H/GFAP^{TG} model, we immunoblotted total homogenates from these lines and wild type samples (Figure 9A). Consistent with the results for analysis of the individual tissue fractions, each protein was elevated in the R236H/GFAP^{TG} mice. Each was also elevated to a similar extent in the GFAP^{TG} mice, whereas the R236H line showed an increase for cyclin D2 but not for DDX3X or RACK1. Immunoblotting of total homogenates from the human patients and controls showed a marked increase in cyclin D2 and RACK1 for the patient samples with the higher RF content (R239C and R79C), but no increase for DDX3X (Figure 9B). For both the mouse and human samples, the increase in the RACK1 immunoblot signal was nearly exclusively for a form migrating in the 50 kDa range rather than as the 35 kDa monomeric size (Figures 2,3,9). This is presumably a RACK1 dimer, which has been reported to remain intact in denaturing SDS PAGE gels and to migrate at this position.²⁷

Primary support for the sequestration hypothesis in AxD has been provided by observations of alphaB-crystallin. This protein is associated with the aggregates, and many of the phenotypes of AxD model mice are restored toward normal when its level in astrocytes is increased by expression of a transgene.²² However, as alphaB-crystallin is strongly upregulated in AxD, our results with cyclin D2, DDX3X, and RACK1 led us to ask whether its association with RFs indeed depleted it from other fractions. Immunoblots of the fractions prepared from the lethal R236H/GFAP^{TG} AxD mouse model and wild type mice did not detect alphaB-crystallin in the Triton-X soluble fraction from either group (Figure 10). However, about 80% of the protein detected is present in the urea soluble fraction,

presumably due to association with normal GFAP filaments, with only 20% present in the RF enriched fraction.

DISCUSSION

As a first step to analyzing the composition of RFs we developed a tissue fractionation protocol that reduced the amount of normally polymerized GFAP but retained GFAP aggregates. Previous methods to enrich for RFs in brain tissue⁴ did not accomplish this (J.E.G., unpublished observations), and a procedure using deoxycholate that has worked well for cultured cells¹⁷ did not remove non-aggregated GFAP from brain tissue (data not shown). Prompted by its use to solubilize protein inclusion bodies, we tried several high concentrations of urea, finding that 6 M urea provided the best balance between removing GFAP from fractions prepared from wild type mice while retaining aggregated GFAP from AxD model mice (Figure 1 and data not shown). In addition to providing a RF enriched fraction for our proteomic analysis, this method could serve as a method to quantify RF burden in AxD tissue. However, a caveat is that proteins could also become preferentially enriched in the RF fraction if their urea solubility were reduced in the AxD model mice by means other than association with Rosenthal fibers, for example by a change in binding partners or post-translational modifications.

iTRAQ mass spectrometry analysis of the RF fractions from AxD mouse models identified 77 novel proteins significantly enriched in at least one of them. Four of these were selected for further study based on results from the human patients (cyclin D2, DDX3X, RACK1 and eIF4A), and a fifth (Ndufv1) was selected due to its involvement in Leigh disease, which has similarities to AxD. Three of these, cyclin D2, which is involved in cell cycle progression and upregulated in various cancers and AxD;^{23,25} DDX3X, which functions in transcription, mRNA splicing, and RNA export;²⁹ and RACK1, which enhances Jun N-terminal kinase activation;³⁰ were confirmed by immunoblotting to be enriched in the RF fractions from each of the AxD model mice examined, with the exception that RACK1 was not found elevated in the R236H mice (Figure 2). The negative findings for Ndufv1 indicate that loss of this protein does not occur in AxD, and is thus not an explanation for the similar clinical findings in AxD and Leigh syndrome.

A likely explanation for eIF4A and Ndufv1 apparently being false positives in the proteomic analysis is the presence of contaminating peptides that are co-detected with the iTRAQ reporter,³¹ although it has also been suggested that post-translational modifications may cause disagreement between iTRAQ and immunoblotting.³² Such contamination is almost certainly the cause of the intensity of presumed reporter ion signals from AxD model mice relative to the controls differing by over 20-fold even among different detection events of the same peptide (e.g., see data for the GFAP peptides ALAAELNQLR and ITIPVQTFSNLQIR in the deposited database). This may also be the explanation for the poor agreement we found between iTRAQ and immunoblotting for determination of the fold-enrichment of several proteins in the RF preparations. As an extreme example, immunoblotting yielded a 44-fold increase in GFAP in the R416W patient (data not shown), whereas iTRAQ yielded just a 1.7-fold increase. Confirmation by immunoblotting of only about half of tested proteins (3 of 5) indicated by iTRAQ to be altered in abundance has been found by others,³³

but the generality of this success rate is uncertain because most studies report only confirmations, and do not state whether other candidate changes were tested but not verified. Similarly, most studies do not compare fold-changes determined by iTRAQ to those by immunoblotting, but among those that have, agreement has been found to vary up to 16-fold.³⁴

The uncertainty of the fold-enrichment results should not apply to the mole% values obtained, as these depend primarily on the frequency with which peptides are detected (spectral counts), with the signal intensities of the iTRAQ reporter ions having only a minor role in correcting for contamination (see Methods). Spectral counting has been found a robust method for relative quantitation [e.g., see Hoehenwarter and Wienkoop (2010)³⁵]. It is therefore notable that the apparent composition of RFs for the mice differs from that for the human samples (Table 4). These differences could be caused by true species differences, but also could be due to the maturation stage of the aggregates—the human tissues were obtained at autopsy at one year of age or later, whereas the mouse samples were obtained at 25 days of age. If maturation stage is indeed the cause, the difference in composition suggests that both activation of the stress response (increased levels of the small stress proteins alphaB-crystallin and HSP27) and ubiquitination become more severe as the disease progresses.

The three proteins confirmed enriched in the RF fraction from the AxD mouse models and in human patients, cyclin D2, DDX3X and RACK1, were further investigated by immunostaining of mouse and human tissues. Results for cyclin D2 strongly support this protein being a newly discovered RF component, whereas findings for DDX3X and RACK1 are equivocal. For both of these, the majority of staining was non-astrocytic. RACK1 staining was primarily of puncta in the neuropil, suggestive of synapses. DDX3X antibody also stained puncta, but within astrocyte cell bodies, raising the possibility that they were stress granules (see below) or nascent RFs. For both proteins there were rare instances of an association with RFs. The sparse staining of RFs could reflect a highly variable association or reflect a more general association that is difficult to reveal due to problems of antibody penetrance into the dense RF matrix, such as has previously been described for GFAP staining of RFs.³⁶ Greater clarity might be achieved by isolating RFs from acutely purified astrocytes, for example using the recently described method of Zhang Y, et al. (2016).³⁷ Whatever their association, the immunoblotting data show that each of these proteins becomes significantly associated with a Triton X-100 and urea insoluble fraction in both the AxD model mice and human patients.

One of the rationales for this study was that RFs may contribute to pathogenesis by sequestering and thereby limiting the activity of critical proteins. To test this hypothesis for the three proteins found enriched in the RF fraction, they were immunoblotted in each fraction created during the RF enrichment procedure from the lethal R236H/GFAP^{TG} AxD model mice. Although each protein was indeed found enriched in the RF fraction, their increase in the AxD model mice resulted in their levels in the Triton X-100 soluble fractions being equal or greater than that for the wild type. This suggests that loss of activity due to sequestration is not a factor in the disease for at least these proteins, although increased

binding to the GFAP monomers and small oligomers present in the soluble fraction remains a possibility.

These observations led us to perform a similar analysis for alphaB-crystallin, which has been considered a prime candidate for the sequestration mechanism in AxD because it is present in RFs and its absence exacerbates, and its transgenic over-expression rescues, many aspects of the AxD phenotype in mice.²² Immunoblotting of the fractions created during the RF enrichment procedure showed that the majority of alphaB-crystallin in the lethal R236H/GFAP^{TG} AxD mice is present in the urea soluble GFAP filament fraction rather than in the RF enriched fraction (Figure 10). Given the marked upregulation of alphaB-crystallin in the AxD mice, the amount in the urea soluble fraction was actually considerably larger in the AxD mice than in the wild type. These data indicate that the aggregate sequestration hypothesis also does not apply to alphaB-crystallin, although it is possible that the alphaB-crystallin is indeed associated with RFs, but removed by the urea treatment. In addition, our inability to detect alphaB-crystallin in the Triton soluble fraction leaves open the possibility that this protein is bound up by the excess normal GFAP filaments present in AxD. A caveat to these protein distribution findings for cyclin D2, DDX3X, RACK1, and alphaB-crystallin is that unfractionated tissue samples were used, and thus contained other cell types as well as possibly unaffected astrocytes. Indeed, immunostaining revealed extensive non-astrocytic presence of DDX3X and RACK1. This could contribute to the levels of these proteins, thereby compromising our ability to detect a reduction in the affected astrocytes. However, this caveat requires that the elevation of each of the proteins in the AxD model mice occurs in cells that do not contain RFs.

The increased levels of each of the proteins we analyzed prompted us to ask whether their encoding genes or those for any of the other proteins found in our proteomic analysis were found to be upregulated in a prior microarray study of GFAP^{TG} mice.²³ Message levels of 15 of the 77 proteins we identified in the mouse models were found changed in the mRNA study; of these, 12 were increased and 3 decreased. These are marked by the symbols ↑ and ↓, respectively, in Table S-1. Similarly, of the 18 proteins found enriched in the human RF fractions whose gene expression was found changed in the mRNA study, 11 were also increased and 7 were decreased in the mouse mRNA analysis (Table S-3). These results indicate that the levels of some of the proteins enriched in the RF fraction may be regulated by homeostatic mechanisms that increase their overall abundance to compensate for activity lost due to sequestration.

RACK1 is a scaffolding protein that facilitates interactions of mediators of a wide range of signaling pathways, including ones that regulate transcription, translation, development, stress responses, apoptosis, cell growth and NMDA channels.³⁸ The increased levels of RACK1 we observed in both the AxD model mice and the human patients were almost entirely of a presumptive dimer form (Figures 2,3,8,9), which to our knowledge is a novel observation for increased levels of this protein. Dimerization may be mediated by interactions of bound proteins, or by phosphorylation.^{39,40} A structural analysis of the dimer suggested surface changes exposing new interacting sites,⁴¹ but at present distinct roles discovered for the dimer are limited to negatively regulating N-methyl-D-aspartate channel activity by Fyn,⁴² protecting against malignant transformation,³⁹ and stimulating the

oxygen-independent proteasomal degradation of hypoxia-inducible factor 1 α .⁴⁰ This latter interaction of the RACK1 dimer with the proteasomal pathway might contribute to its association with RFs. RACK1 also supports dimerization of the MAPKKK MTK1, possibly doing so in its dimeric form. A downstream target of MTK1 is Jun N-terminal kinase (JNK), which in turn activates c-Jun.⁴² AP-1 sites present in the GFAP promoter may contribute to its expression,⁴³⁻⁴⁵ raising the possibility that RACK1 thus participates in a positive feedback loop for elevating GFAP levels in AxD. The increase in cyclin D2 could increase cell division,^{24,25} whereas increased DDX3X may either inhibit or increase apoptosis, depending on conditions.⁴⁶ Interestingly, all three of these proteins are connected to NF- κ B, which is upregulated in GFAP^{TG} mice;²³ expression of both RACK1 and cyclin D2 is enhanced by NF- κ B,^{47,48} and DDX3X is an NF- κ B coactivator.⁴⁹

Mignot et al. (2007)⁵⁰ have suggested that the RFs formed from overexpression of wild type GFAP differ from those formed from mutant GFAP by not staining for ubiquitin, and Bachetti et al. (2010)⁵¹ drew the same conclusion based on an absence of staining for HSP27 and alphaB-crystallin. Our proteomic data, however, do not support these differences. HSP27 was below the level of detection in our proteomic analysis of the RF fraction from both the wild type GFAP containing GFAP^{TG} line and mutant GFAP containing R236H line (it was detected in the R236H/GFAP^{TG} mice), but a greater rather than lesser enrichment for both alphaB-crystallin and ubiquitin was present in the GFAP^{TG} mice than in the R236H mice (Table 2). These results thus confirm previous immunohistochemical observations for RFs present in mice overexpressing wild type human GFAP.¹³ Furthermore, five of the previously described RF proteins were detected in both the GFAP^{TG} and R236H lines, alphaB-crystallin, GFAP, plectin, ubiquitin and vimentin; whereas in addition to HSP27, c-Jun and synemin were detected in neither. More generally, of the 77 novel proteins listed in Table S-1, 39 were identified in the R236H mice, and all of but 2 of these were also found in GFAP^{TG} mice. In contrast, 13 of the 77 proteins were found present in the GFAP^{TG} mice and not in the R236H mice. Although this could reflect a difference in composition between the RFs present in the two lines, it is more likely due to a difference in detection sensitivity arising from the lower RF content of the R236H fractions and the low abundance of these proteins, since all of the differences but 2 are due to absence of detection of a protein in the R236H mice (the 2 proteins that were detected in R236H and not GFAP^{TG} mice each had a single spectral count). In the data-dependent acquisition mode used for protein discovery in this study, the probability of identifying a protein in a sample is directly proportional to its abundance, and rare proteins are detected in essentially a random manner. Of the 13 novel proteins identified for GFAP^{TG} mice and not R236H mice, none had more than 10 spectral counts in the GFAP^{TG} samples (G1/S-specific cyclin-D1), and 10 of the 13 proteins had 3 or fewer. Thus these findings strongly suggest that the composition of the RFs from the two sources is identical regardless of whether the initiating cause is presence of mutant GFAP or elevated levels of wild type GFAP. This favors a common mechanism for RF formation, as previously proposed by Li et al. (2002).⁵²

The R236H and GFAP^{TG} mice were included in this study to enable comparison of their RF components as just described. The R236H/GFAP^{TG} line was included because it has the heaviest burden of RFs and was thus expected to yield a more enriched preparation, and also to permit a comparison of its RF protein composition to that of the GFAP^{TG} mice, with the

possibility that any unique components might point to proteins contributing to its lethal phenotype. As shown in Table S-1, 25 proteins were indeed found enriched in the RF fraction from the R236H/GFAP^{TG} mice, but not in the GFAP^{TG} mice. However, as in the case of the R236H comparison above, a likely explanation for this difference is detection sensitivity rather than compositional difference. All 25 of the novel proteins identified for R236H/GFAP^{TG} and not for GFAP^{TG} mice had low spectral counts. Highest was G1/S-specific cyclin-D2 with 10, and 16 of the 25 had 3 or fewer.

Two of the novel proteins we confirmed to be enriched in the RF preparation, RACK1 and DDX3X, are associated with stress granules,⁵³⁻⁵⁵ which are heterogeneous aggregates of proteins and non-polysomal mRNAs that reversibly form under various cell stress conditions and redirect translation to cope with the stressed state.⁵⁶⁻⁵⁸ DDX3X has been identified as a critical protein for stress granule formation, whereas binding of RACK1 contributes to the anti-apoptotic role of stress granules. Since RNA binding proteins are the primary components of stress granules, we note that 11 of the other proteins enriched in the RF fraction are in this class: 40S ribosomal protein S3, 40S ribosomal protein S26, 60S ribosomal protein L3, 60S ribosomal protein L23, 60S ribosomal protein L27a, cold-inducible RNA-binding protein, putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15, RNA-binding protein 14, rRNA/tRNA 2'-O-methyltransferase fibrillarin-like protein 1, tRNA-splicing endonuclease subunit Sen34 and RNA-binding protein FUS (also known as Fused in sarcoma). In addition to suggesting a relationship between RFs and stress granules, the presence of these RNA binding proteins raises the possibility that RFs bind mRNAs.

It is possible that these proteins were present in the RF fraction due to copurification of stress granules, which like RFs are insoluble in Triton X-100; however, none of the core stress granule proteins commonly used as markers for these aggregates was among the 77 novel proteins we found enriched in the RF fraction, including poly-A binding protein 1 (PABP1), T-cell-restricted intracellular antigen 1 (TIA-1), TIA-1-related protein (TIAR), RasGAP SH3-binding protein (G3BP) and tristetraprolin (TTP). Stress granules form ubiquitously in eukaryotic cells, including yeast; but although extensively studied in neurons, we find no report of their characterization in astrocytes. Aberrant stress granule formation is a primary mechanistic candidate for amyotrophic lateral sclerosis since mutations associated with this disease occur in several proteins associated with these aggregates, such as TAR DNA binding protein-43, RNA-binding protein FUS, ataxin-2, optineurin and angiogenin. Aberrant stress granules have also been associated with FTDP-17 and frontotemporal lobar degeneration (FTLD), and stress granules have been found to bind huntingtin protein and to associate with neurofibrillary tangles in Alzheimer's disease. There are several parallels between stress granules and Alexander disease in addition to the increased levels of RACK1 and DDX3X that we report here. Like RFs, stress granules contain ubiquitinated proteins,⁵⁹ and their formation is stimulated by proteasome inhibition, which occurs in AxD.⁶⁰ Stress granules also bind the inactive, non-phosphorylated form of mTORC1⁶¹ and this form is increased in a cell model of AxD.⁶² Strikingly, neuronal intermediate filament inclusion disease, which is one of the forms of FTLD, features aggregation of ubiquitinated intermediate filaments,^{63,64} analogous to the presence of ubiquitinated GFAP in RFs.⁶⁰ Relationships between stress granules and the protein

aggregates present in several neurodegenerative diseases have been suggested in which stress granules either seed the formation of the larger aggregate or coalesce to form insoluble aggregates,^{65,66} or in which both stress aggregates and disease-related aggregates initially form independently but then synergistically cross-seed each other's propagation through the binding of common components.⁵⁸ Such mechanisms could explain the recent observation that TAR DNA binding protein 43, a known stress granule component,⁵⁷ is sometimes associated with RFs in AxD.⁶⁷ Whether stress granules actually exist in Alexander disease astrocytes and have a role in this disorder will be an interesting topic to pursue, especially given the effort to develop therapeutic drugs that target their formation.^{68,69}

CONCLUSIONS

We have developed a method for the enrichment of RFs, and analyzed this preparation to provide a database of RF proteins in patients and mice as a resource for future investigations. The predictions of the sequestration mechanism for disease causality were not met for any of the four RF components that we investigated, alphaB-crystallin, cyclin D2, DDX3X and RACK1, but remain to be tested for the many other components discovered. Stress granules may play a role in the formation of RFs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Schematic of the Rosenthal fiber enrichment protocol used for mice. See Materials and Methods for details of the protocols used. (B) GFAP immunoblot of equal percentages (0.75% = 6 µl) of the Triton soluble, urea soluble, and Rosenthal fiber fractions. This corresponded to 20 µg protein for both the wild type and AxD mice Triton soluble extract, 2.4 µg for each of the urea soluble fraction and 0.3 µg for the wild type RF fraction and 0.5 µg for the AxD mice RF fraction. (C) Quantitation of the immunoblot data as a percent of the total GFAP in each fraction. Data are the average values from 2 experiments; individual values differed by no more than 4% total GFAP from the average. (D) Immunostaining of Rosenthal fiber fractions prepared from wild type and R236H/GFAP^{TG} mice for GFAP and alphaB-crystallin. Double staining of the two large aggregates is seen as yellow puncta. Scale bar is 10 µm.

Figure 2.

Immunoblots of wild type and AxD mouse model Rosenthal fiber preparations for the five candidate proteins selected by iTRAQ analysis. Equal percentages (1.25%) of each preparation were loaded in each lane, corresponding to the following protein amounts: wild type, 0.50 μ g; R236H, 0.50 μ g; GFAP^{TG}, 0.65 μ g; R236H/GFAP^{TG}, 0.83 μ g. Arrows indicate the positions of relevant molecular weight standards. Note that RACK1 had a mobility comparable to a 50 kDa molecular weight standard instead of the predicted monomeric size of 35 kDa. Representative results are shown from duplicate experiments.

Figure 3.

Immunoblots of the three confirmed novel Rosenthal fiber components in Rosenthal fiber preparations from human AxD patients and controls. Equal percentages (1.25%) of each preparation were loaded in each lane, corresponding to the following protein amounts: 1 yr old control, 0.1 μ g; 14 yr old control, 0.3 μ g; 50 yr old control, 0.4 μ g; 1 yr old R239H, 0.2 μ g; 7 yr old R239C, 0.9 μ g; 7 yr old R416W, 0.5 μ g; 14 yr old R79C, 0.7 μ g; 50 yr old S247P, 0.4 μ g. Arrows indicate the positions of relevant molecular weight standards.

Representative results are shown from duplicate experiments.

Figure 4.

Immunoreactivity for cyclin D2 in 1 year old R236H mouse and human AxD brains. The mouse immunostaining included counterstaining with DAPI to display nuclei. (A,A') Mouse hippocampus immunostained for both cyclin D2 (green) and GFAP (red). The green channel (A) reveals immunopositive puncta for cyclin D2 (arrows, not all indicated) that colocalize with GFAP in astrocytes in the combined image (A') (cc, corpus callosum; DG, dentate gyrus; pyr, layer of pyramidal neurons). (B,B') The box in (A'), which is in the stratum lacunosum-moleculare, is shown enlarged with staining for cyclin D2 (B) and double stained for cyclin D2 and GFAP (B'). (C) RFs in subcortical white matter of patient 3 are immunopositive for cyclin D2 (arrows, not all indicated). (D) Immunoreactivity for cyclin D2 in subcortical white matter of patient 2 predominates in perivascular astrocyte endfeet (V, vessel), containing RFs. Scale bars, (A') 120 μ m, (C) 45 μ m, (D) 30 μ m.

Figure 5.

Electron microscopy of AxD model mice. (A,C) An astrocyte filled with RFs (arrows, not all indicated) in the hippocampus stratum radiatum of a 1 yr old R236H mouse (A) and a 28 d old R236H/GFAP^{TG} mouse (C). (B,D) The enlarged boxed areas in (A) and (C), respectively. Note the densely packed GFAP intermediate filaments around the electron dense material of the RFs. Scale bars: (A,C), 20 μ m.

Figure 6.

Immunoreactivity for DDX3X in 1 year old R236H mouse and human AxD brains. Counterstaining of the mouse samples with DAPI and of the human samples with hematoxylin was included to display nuclei. (A,A') Mouse hippocampus immunostained for both DDX3X (green) and GFAP (red). In the green channel, small, DDX3X-immunoreactive puncta are seen scattered throughout the hippocampus of the R236H mouse (A)(arrows, not all indicated), and these are double stained for GFAP (A'). (B,B') The box in (A'), which is in the stratum lacunosum-moleculare, is shown enlarged with staining for DDX3X (B) and double stained for DDX3X and GFAP (B'). (C) DDX3X-immunopositive astrocyte cell bodies (arrows) in subcortical white matter of patient 2. The insert top left is a magnification of the cell body indicated by the red arrow to show the granular nature of the staining. (D) RFs in subcortical white matter of patient 2 are revealed by weak and uniform blue-gray staining with hematoxylin. A RF immunopositive for DDX3X is shown (arrow), but most do not immunostain (asterisks). In contrast to RFs, staining of nuclei by hematoxylin is more intense and patchy (several indicated by a "+" at their right edge). Scale bars: (A') 85 μm , (C) 95 μm , (D) 60 μm .

Figure 7.

Immunoreactivity for RACK1 in 1 year old R236H mouse and human AxD brains. Counterstaining of the mouse samples with DAPI and of the human samples with hematoxylin was included to display nuclei. (A,A') Mouse hippocampus immunostained for both RACK1 (green) and GFAP (red). There is a high level of RACK1 immunoreactivity in pyramidal neurons of the CA1 subfield of the hippocampus (pyr). However, in the green channel (A) some small profiles in astrocytes are also seen (arrows), which are immunopositive for GFAP (A'). (B,B') The box in (A') is shown enlarged stained for RACK1 (B, arrows) and double stained for RACK1 and GFAP (B'). (C) Low power image of double immunostaining for RACK1 (green) and cyclin D2 (red) in the hippocampal stratum lacunosum. (D) Enlarged view of the box in (C), showing colocalization of RACK1 and cyclin D2 in some puncta (arrows). (E) Punctate pattern of immunostaining for RACK1 in an astrocyte in subcortical white matter of AxD patient 2 (arrow). This cell does not contain an RF, but there are RFs in the vicinity that are RACK1 immunonegative (asterisks). (F) Weakly RACK1 immunopositive RF (arrow) is surrounded by RACK1 immunonegative RFs in white matter of patient 2 (asterisks). The RFs are present in astrocyte processes, and thus not clearly traceable to cell bodies. Scale bars: (A') 75 μ m, (C) 80 μ m, (E) 15 μ m, (F) 50 μ m.

Figure 8.

Distribution of the three confirmed novel Rosenthal fiber components in fractions from the R236H/GFAP^{TG} mice and controls. The same equal percentages of each preparation were loaded in each lane as described for Figure 1B. Longer exposure revealed signals of equal intensity for the R236H/GFAP^{TG} and control samples for RACK1 in the Triton soluble fractions (data not shown). Arrows indicate the positions of relevant molecular weight standards. Representative results are shown from duplicate experiments.

Figure 9.

Comparative levels of the three confirmed novel Rosenthal fiber components in total homogenates of AxD model mice and AxD patients. (A) AxD model mice and controls, 3 µg protein per sample. (B) AxD patients and controls, 6 µg protein per sample. Arrows indicate the positions of relevant molecular weight standards. Similar results were observed in duplicate experiments.

Figure 10.

Relative levels of alphaB-crystallin in fractions from the R236H/GFAP^{TG} mice and controls. The same equal percentages of each preparation were loaded in each lane as described for Figure 1B. No signal was observed in the Triton soluble fraction on prolonged exposure or loading 3x more protein (the capacity of the gel). AlphaB-crystallin has previously been reported to be at very low levels in unperturbed mouse brain,²² especially in young mice (J.E.G., unpublished observations); and when up-regulated to preferentially associate with a detergent insoluble fraction.²⁸ The arrow indicates the position of a molecular weight standard. Similar results were observed in a duplicate experiment.

Table 1

Characteristics of human tissues used for proteomic analyses.

UMB #	Mutation	Age (y)	Sex	PMI (h)	Tissue Location	Cause of Death
M1931M	Control	0.9	M	10	2/5	accidental drowning
105	Control	14.8	M	16	1/8R	accident/injuries
1789	Control	50.5	F	22	2/1L	arteriosclerotic cardiovascular disease
1070	R239H	1.0	M	4	2/3L	AxD
338	R239C	6.2	M	12	1/9L	AxD
Goldman	R416W	7	M			AxD
613	R79C	14.0	M	7	1/12R	AxD
4858	S247P	50.4	F	17	2/1L	AxD

All tissues were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD (MD BTB) except the tissue with the R416W mutation, which was a gift of James Goldman, Columbia University Medical School. The UMB number is the identifier of the MD BTB. The Tissue Location is given as the Protocol Method/Slice number; e.g., 1/8R is slice 8R obtained using protocol method 1. The R416W tissue was obtained from patient 9 of Brenner et al. (2001).¹⁶ It is a small fragment of neocortex and underlying white matter; its exact location and PMI are not known.

Table 2

Selected proteins enriched in the Rosenthal fiber fractions of the mouse models.

Protein Names	R236H		GFAP ^{TG}		R236H/GFAP ^{TG}		Total Spectral Counts	Mole %
	Fold Increase	P-Value	Fold Increase	P-Value	Fold Increase	P-Value		
Glial fibrillary acidic protein[†]	2.7	0.037	5.3	0.018	17.6	0.033	2179	32.766
Ubiquitin[†]	1.7	0.120	2.5	0.077	5.1	0.049	525	30.088
Vimentin	2.8	0.051	2.8	0.066	7.5	0.073	1699	19.885
Small nuclear ribonucleoprotein Sm D3	2.3	0.158	5.7	0.075	15.7	0.022	45	2.386
ATP-dependent RNA helicase DDX3X	1.8	0.134	3.3	0.050	11.8	0.039	234	2.140
Fatty acid-binding protein, brain [†]	ND	ND	4.1	0.036	11.2	0.028	24	1.227
Alpha-crystallin B chain (Cryab)[†]	5.9	0.107	11.5	0.080	22.6	0.032	27	1.112
G1/S-specific cyclin-D1 [†]	ND	ND	9.1	0.028	37.8	0.041	44	1.098
Glutamine synthetase	1.0	0.925	2.1	0.130	5.1	0.048	63	0.780
Serine/threonine-protein phosphatase PP1-beta catalytic subunit ²	1.2	0.487	1.9	0.127	3.3	0.022	63	0.697
ADP-ribosylation factor 2; ADP-ribosylation factor 4; ADP-ribosylation factor 5 ³	1.3	0.560	1.7	0.264	3.8	0.048	23	0.484
60S ribosomal protein L23	1.2	0.663	1.6	0.404	3.5	0.034	23	0.411
Actin-related protein 2/3 complex subunit 4	ND	ND	2.3	0.109	5.5	0.041	10	0.322
Proteasome subunit beta type-4	ND	ND	1.6	0.104	2.7	0.036	18	0.295
Synaptosomal-associated protein 47	1.2	0.551	4.0	0.022	6.6	0.016	23	0.277
V-type proton ATPase subunit d 1	7.0	0.041	1.9	0.686	DIV/0	0.072	13	0.273
G1/S-specific cyclin-D2 [†]	ND	ND	ND	ND	60.1	0.029	9	0.264
Coronin-1C	1.2	0.596	1.5	0.224	2.4	0.043	51	0.238
Abhydrolase domain-containing protein 4	ND	ND	1.9	0.078	5.7	0.023	16	0.217
Prohibitin-2	1.3	0.447	2.9	0.352	6.4	0.018	17	0.209
Eukaryotic initiation factor 4A-1	1.1	0.559	3.1	0.038	6.6	0.019	17	0.197
Plectin	1.2	0.455	1.6	0.281	3.0	0.086	376	0.185
Guanine nucleotide-binding protein subunit beta-2-like 1	ND	ND	3.7	0.030	4.3	0.083	7	0.126
Proteasome subunit alpha type-4	ND	ND	4.5	0.204	DIV/0	0.037	4	0.113
Heat shock protein beta-1 (HSP27)	ND	ND	ND	ND	9.3	0.050	1	0.035
Proteasome subunit beta type-5	ND	ND	ND	ND	2.2	0.032	2	0.025
NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial	ND	ND	ND	ND	7.9	0.013	1	0.015

Proteins listed are the 20 most abundant, or are previously known Rosenthal fiber components (shown in **bold** and green shading) or were selected for further study (blue shading). Listings are in order of mole %, which was calculated as described in Methods. Fold increases were determined by dividing the average AxD model abundance by the average wild type mouse abundance for each observation and then taking the median of all observations for that AxD model. Similarly, p-values were calculated using a two-tailed Student's T-test for each observation and then taking the median of all observations for that AxD model. Proteins with a p-value < 0.05 for at least one AxD model are included in the table, with the exceptions of the known RF components vimentin (p = 0.051) and plectin (p = 0.086). Spectral counts are totals uncorrected for the contribution of

wild type samples or contamination (see Methods). Proteins that could not be distinguished by the peptides identified are grouped as a single entry. “ND” = not detected. “DIV/0” = detected only in the AxD model mice (no iTRAQ reporter ion signals observed for the wild type mouse samples). “t” indicates that the mRNA was increased in GFAP^{TG} mice.²³ Table S-1 provides a complete listing of all 77 novel RF-associated proteins, and Table S-2 the UniProtKB accession#, % sequence coverage and number of unique peptides identified and quantified for each of these proteins.

¹ Only monomeric ubiquitin peptides were detected for ubiquitin—no peptides were observed specific for any of its precursor proteins, Ubiquitin-60S ribosomal protein L40, Ubiquitin-40S ribosomal protein S27a, Polyubiquitin-B or Polyubiquitin-C. Thus the amino acid length of monomeric ubiquitin was used to calculate its mole %.

² Spectral counts include those for peptides also common to the alpha and gamma isoforms. The fraction of common peptides assigned to the beta isoform was taken as the fraction of spectral counts specific to the beta form divided by the sum of spectral specific for each of the 3 forms, which was 20/28. There were 2 beta-specific spectral counts from the R236H mice and 9 each from the GFAP^{TG} and the R236H/GFAP^{TG} mice. The p-values and fold changes are for the peptides specific for the beta isoform.

³ Spectral counts are a combination of 27 for a peptide common to ADP-ribosylation factors 2, 4 and 5, and 3 specific for factor 5. The 3 specific for factor 5 yielded p = 0.268. The 8 spectral counts of the R236H/GFAP^{TG} mice, which yielded p = 0.048, are all for the common peptide.

Table 3

Selected proteins enriched in the Rosenthal fiber fractions of human patients.

Protein/Patient Mutation	Fold Increase Relative to Controls					Spectral Counts	Mole %
	R239C	R79C	R416W	R239H	S247P		
Ubiquitin Glial fibrillary acidic protein[↑]	38.2 21.3	7.2 5.5	2.0 1.7	1.3 1.3	0.6 1.3	83 231	49.743 22.008
Alpha-crystallin B chain Heat shock protein beta-1 (HSP27)	24.8 18.8	3.7 4.3	1.9 1.3	1.6 1.0	1.3 1.5	29 13	6.947 2.498
Histone H4	2.1	1.1	1.1	1.0	1.0	30	2.189
Creatine kinase B-type	2.2	1.0	2.6	3.0	3.4	31	2.073
Serine/threonine-protein phosphatase PP1-beta catalytic subunit	9.4	1.6	1.4	1.5	1.0	12	1.091
NAD(P)H dehydrogenase [quinone] 1 [↑]	61.1	4.8	2.6	2.7	1.2	6	1.053
Aldo-keto reductase family 1 member C1	18.0	0.7	2.0	2.0	1.1	6	0.701
Serine/threonine-protein kinase Nek6 [↑]	5.9	1.1	1.5	1.3	1.3	7	0.520
Eukaryotic initiation factor 4A-I G1/S-specific cyclin-D2 [↑]	10.1 13.1	2.0 2.3	2.1 2.0	2.3 1.1	1.9 1.1	6 4	0.498 0.481
Vimentin	4.0	3.6	1.4	0.6	1.0	9	0.428
ADP-ribosylation factor 4	11.7	3.0	1.9	2.6	3.3	2	0.410
Putative elongation factor 1-alpha-like 3; Elongation factor 1-alpha 1	4.4	1.2	1.4	1.5	1.3	9	0.393
Glutathione S-transferase Mu 3 [↑]	12.2	2.4	2.3	1.4	1.8	2	0.312
Calcium signal-modulating cyclophilin ligand	44.5	3.0	1.1	0.5	2.2	2	0.310
Carbonyl reductase [NADPH] 1	5.3	2.1	1.5	2.7	1.4	3	0.291
Histone H2B type 1-L	2.9	1.8	0.8	0.8	0.9	3	0.276
Actin, cytoplasmic 2; Actin, cytoplasmic 1	1.6	0.8	1.2	2.3	1.0	10	0.275
ATP-dependent RNA helicase DDX3X	8.3	2.2	1.3	1.7	2.2	5	0.232
Guanine nucleotide-binding protein subunit beta-2-like 1	21.0	3.1	5.4	1.4	2.0	1	0.132
Synemin Plectin	15.9 4.3	2.4 0.9	2.3 2.0	2.0 1.8	1.4 1.4	5 4	0.121 0.019

Listing criteria and notes are the same as for Table 2, except that instead of requiring a p-value < 0.05, the 20 proteins with the highest mole % are included that had an increase relative to the controls of at least two-fold in any of the patients. Table S-3 provides a complete listing of all human RF-associated proteins that met this criterion.

Table 4

Mole % and weight % for previously reported Rosenthal fiber components and for novel components that were 1.0 mole % for either human or mouse.

Protein	Mole %		Weight %	
	Human	Mouse	Human	Mouse
Ubiquitin ¹ Glial fibrillary acidic protein	49.7 22.0	30.1 32.8	16.9 42.6	7.1 43.8
Alpha-crystallin B chain (Cryab)	6.9	1.1	5.4	0.6
Heat shock protein beta-1 (HSP27)	2.5	<0.1	2.3	<0.1
Histone H4	2.2	ND	1.0	ND
Creatine kinase B-type	2.1	ND	3.5	ND
Serine/threonine-protein phosphatase PP1-beta catalytic subunit	1.1	0.7	1.6	0.7
NAD(P)H dehydrogenase [quinone] 1	1.1	<0.1	1.3	<0.1
vimentin	0.4	19.9	0.9	28.8
ATP-dependent RNA helicase DDX3X	0.2	2.1	0.7	4.4
Synemin Plectin	0.1 <0.1	ND 0.2	0.8 0.4	ND 2.7
Small nuclear ribonucleoprotein Sm D3	ND	2.4	ND	0.9
Fatty acid-binding protein, brain	ND	1.2	ND	0.5
G1/S-specific cyclin-D1	ND	1.1	ND	1.0
Proteasome subunit alpha type-4 ²	ND	0.1	ND	0.1
Proteasome subunit beta type-2 ²	ND	ND	ND	ND
Proteasome subunit beta type-3 ²	ND	ND	ND	ND
c-Jun	ND	ND	ND	ND

Entries are in decreasing mole % in the human, and those for previously known components are in **bold** and green shading. The listed proteins account for 88.3% and 91.6% of the human and mouse mole % and for 77.4% and 90.6% of the human and mouse weight %, respectively.

“ND” = not detected.

¹ includes contributions from 60S ribosomal protein L40, ubiquitin-40S ribosomal protein S27a, polyubiquitin-B and polyubiquitin-C, which are indistinguishable by MS.

² components of the 20S proteasome