Protein aggregates in astrocytes that contain glial fibrillary acidic protein (GFAP), small heat shock proteins, and ubiquitinated proteins are termed Rosenthal fibers (RFs). The major molecular constituents of RFs are the intermediate filament protein, glial fibrillary acidic protein (GFAP), the small heat shock proteins (shsps), αB-crystallin, and hsp 27, as well as ubiquitin (1). Overexpression of normal human GFAP in the astrocytes of transgenic mice resulted in cytoplasmic inclusions that appeared identical to the RFs in AxD (2, 3), raising the possibility that genetic defects in the GFAP gene were a primary cause of AxD. Sequencing analysis revealed that almost all AxD patients are heterozygous for non-conservative mutations in the coding region of GFAP (4), a finding subsequently confirmed by other investigations (reviewed in Refs. 5 and 6). Although mutations in GFAP are regarded as causative for AxD, little is known of the mechanisms by which GFAP accumulates in RFs or the effects of GFAP accumulation on astrocyte function.

Earlier work in our laboratory focused on protein inclusions resulting from expression of GFAP in cell lines. Transient transfection of wild type (WT) human GFAP caused filamentous, cytoplasmic inclusions in cultured primary mouse astrocytes, NIH3T3 cells, and rat and human glioma cells (7). Inclusion formation was further confirmed by Hsiao et al. (8), who expressed WT and R239C mutant (mt) human GFAP in Cos7 cells, which contain vimentin (Vim) but not GFAP, and also in SW13 Vim− cells, which contain no endogenous intermediate filament. Introduction of both WT and mt GFAP led to a variety of GFAP aggregation phenotypes, including a filamentous pattern, a diffuse pattern, irregular coils, and aggregates or large inclusions scattered around the nuclei.

Both altered proteasome function and activated stress pathways might be important consequences of GFAP accumulation. First, small hspss are major constituents of RFs, and AxD tissues contain high levels of shsp transcripts (9), suggesting that GFAP accumulation results in the up-regulation of stress pathways. Second, protein aggregation is a signature pathology of a number of neurodegenerative disorders, including Alzheimer disease, Parkinson disease, and polyglutamine disorders, and is accompanied by a decrease in proteasome activity, indicating that the proteasome system might be a critical pathway through which cellular dysfunction occurs (10).

Thus, to gain insights into the effects of GFAP accumulation, we investigated the possibility that it leads to a cellular stress response and decreased proteasome function. Since the mutation generating an amino acid change at Arg-239 is the most common in AxD and produces severe disease, we focused on the expression of R239C mt GFAP. When compared with WT, the mutation exacerbated the aggregation of GFAP. GFAP accumulation resulted in an impaired proteasome function and also in the activation of the JNK pathway. In turn, both the proteasome hypofunction and JNK activation increased GFAP accumulation and cellular stress responses.
accumulation, leading to a positive feedback loop that produced further protein accumulation, and finally, an increased susceptibility of the cell to stressful stimuli.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Cell culture medium (Dulbecco’s modified Eagle’s medium), F12, and molecular biological reagents were obtained from Invitrogen and Qiagen. Primary antibodies included anti-GFAP polyclonal antibody (DAKO), anti-GFAP monoclonal antibody, anti-αβ-crystallin polyclonal antibody, anti-ubiquitin monoclonal antibody (Chemicon), anti-FLAG (Sigma), anti-p-JNK, total JNK, p-MLK3, and Hsp70 antibody (Cell Signaling Technology), anti-GAPDH monoclonal antibody (Enco), and anti-20S polyclonal antibody (Biomol). The fluorescence-conjugated and horseradish peroxidase-conjugated secondary antibodies were from Chemicon and Amersham Biosciences. MG132, lactacystin, camptothecin, anti-FLAG M2-agarose affinity gel, and protein G-Sepharose were from Sigma.

**Brain Tissue**—Tissues stored at −80°C from cerebral hemispheric white matter of control subjects or AxD cases with different mutations were obtained postmortem. For immunohistochemistry, the tissue was fixed in formalin and then embedded in paraffin. All AxD cases were diagnosed based on histopathological examination and confirmed by the molecular genetic analysis for GFAP mutation. Controls included frozen central nervous system tissue from two children, one with no neurological disease (Control I) and one with Werdnig-Hoffman disease (non-AxD, non-leukodystrophy neurological disease without RFs) (Control II).

**Cell Cultures and Transfection**—cDNA clones encoding WT GFAP or R239C mt GFAP were inserted into a pcDNA3-FLAG expression vector for expressing a GFAP-FLAG fusion protein or into the EGFP-C1 expression vector, resulting in a fusion of GFP to the N-terminal of GFAP. pcDNA3- HA-AvMLK2, pcDNA3- HA-AvMLK3, pcDNA3- HA-Av ASK1, and pCMV-d/N-C-Jun were constructed as before (11). The GFP-U plasmid was a gift from Prof. Ron R. Kopito. Five cell lines were used in this study, including three GFAP-negative cells (Cos7, SW13 Vim−, and 293T) and two GFAP-positive cells, immortalized human astrocytes (IM) and U251 astrocytoma cells. Among them, Cos7 and SW13 Vim− cells were transiently transfected with the GFAP-FLAG plasmid using Lipofectamine Plus. 48 h after transfection, cells were harvested or fixed for either Western blotting or immunostaining. MG132 (10 μM), lactacystin (10 μM), or camptothecin (CPT) (10 μM) were added to cultures at 24 h after transfection and incubated for a further 24 h, with Me2SO as the vehicle control.

To generate permanent cell lines, IM and U251 cells were transfected with the GFAP-GFP plasmid using calcium phosphate. The transfected cells were then cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, antibiotics, and 500 μg/ml G418. Clonal lines of stably transfected cells were isolated and confirmed by fluorescence-activated cell sorter sorting or Western blot analysis. The 293T cells stably expressing GFP-U were obtained using the same protocol.

**Immunoprecipitation and Western Blotting**—For protein extraction and fractionation, brain tissue or cells were homogenized in total cell lysis buffer (2% w/v SDS, 6.25% mm Tris (pH 7.5), 5 mm EDTA supplemented with the protease inhibitor mixture tablet (Roche Applied Science)) for total protein or in a fractional lysis buffer to separate soluble and insoluble fractions. Briefly, cells or brain tissues were homogenized in S1 buffer (0.5% v/v Triton-X, 2 mm Tris, 2 mm EDTA, and sucrose implemented with Complete Mini protease inhibitor mixture (Roche Applied Science)). After centrifugation for 20 min at 15,000 rpm at 4°C, the supernatant was collected as the soluble fraction, and the pellet (insoluble fraction) was recovered in S2 buffer (2% w/v SDS, 2 mm Tris, 2 mm EDTA). Protein concentration was determined using a Bio-Rad protein assay kit according to the manufacturer’s instructions.

For immunoprecipitation, equal amounts of protein from whole cell extracts were diluted in immunoprecipitation lysis buffer (11) and were incubated with M2-conjugated protein A-Sepharose or with anti-GFAP antibody and protein G-Sepharose overnight at 4°C. Immunoprecipitates were then separated by SDS-PAGE and subjected to Western blotting as described previously (11).

**Analysis of Cell Survival**—The cells were plated into 96-well plates, and cell viability was measured by methylthiazol tetrazolium (MTT) assay according to standard protocol. All results were confirmed by the trypan blue test. Data were presented as the percentage of living cells. All experiments were performed in triplicate.

**Proteasome Activity Assay**—Cell debris were homogenized in ice-cold proteolysis buffer (10 mm Tris-HCl, pH 7.2, 0.035% SDS, 5 mm MgCl2, 5 mm ATP, and 0.5 mm dithiothreitol). The homogenates was incubated with proteasome substate II (S-II, Z-LEL2-AMC) or III (S-III, Suc-LLVY-AMC) to detect the post-glutamy peptidase hydroxase (PGPH) and chymotrypsin-like peptidase activities, respectively. Fluorescence was monitored at 360 nm excitation and 450 nm emission in a fluorescence plate reader (Bio-Tek FL600).

**Immunostaining and Immunohistochemistry**—Immunostaining of cultured cells was performed at 48 h after transfection, as described before (8). In some experiments, cultures were exposed to a cytokinester buffer (PHEM buffer: 100 mM Pipes, 2 mm EGTA, 1 mm MgCl2, 0.5% Triton X-100, pH 6.8) to remove soluble proteins prior to immunostaining. Fluorescent labeled cells were visualized using a Zeiss LSM510 confocal microscope.

Immunohistochemistry was performed according to general protocols. 10-μm-thick consecutive sections were prepared. Sections were blocked in Tris-buffered saline with Tween/5% bovine serum albumin for 1 h and subsequently incubated with primary antibodies overnight at 4°C. Negative control sections were held in phosphate-buffered saline during the primary incubation. Sections were then incubated with horseradish peroxidase-labeled secondary antibody 1 h at room temperature and developed with 3,3′-diaminobenzidine.

**Statistical Analysis**—Results are expressed as mean ± S.D. Statistical analysis was performed using the Student’s t test. p < 0.05 was considered statistically significant.

**RESULTS**

**Overexpression of WT or AxD Mutant GFAP Leads to Protein Accumulation and Intracellular Inclusions**—WT and mt GFAP were overexpressed in four different cell lines, each with different intermediate filament constituents: Cos7 cells (GFAP−,
the Cos7 and SW13 Vim− cells were transiently transfected with FLAG-tagged GFAP. Because of the low transfection efficiency of astrocytes, we established permanent GFAP overexpressers in the IM and U251 cells, using a GFP-GFAP construct that expresses a fusion protein. Intracellular inclusions were found in all lines (Fig. 1A). A statistical analysis of the various organizational patterns observed in Cos7 cells revealed a significant difference between WT and mt GFAPs (Fig. 1B); the WT generally formed filamentous structures, although some cells also contained inclusions, especially the large ones located next to nuclei. The mt GFAP largely formed inclusions or diffuse patterns composed of tiny dot-like aggregates and far fewer filamentous patterns. The mt GFAP formed more inclusions than did the WT in all cell backgrounds. Note that in astrocytic lines (IM and U251), WT GFAP usually formed filamentous bundles and occasionally very few aggregates (Fig. 1A), whereas mt GFAP formed punctate aggregates predominantly.

The transfection of both WT and mt GFAP constructs led to GFAP protein accumulation, as assayed by Western blotting. The increases were apparent in both the Triton X-100-soluble and the Triton X-100-insoluble fractions of the transfected cells (Fig. 2, A–D). Note that in the IM or U251 cells stably expressing WT or mt GFAP-GFP, the anti-GFAP antibody recognized both the endogenous WT GFAP (50 kDa) and the exogenous GFAP-GFP fusion protein (∼80 kDa, Fig. 2, C and D). Interestingly, in the two astrocytic lines, transfection of both WT and mt GFAPs caused an accumulation of the endogenous GFAP of the cells (Fig. 1, C and D). This observation is consistent with our model in which the overexpression of GFAP leads to further accumulation of GFAP (see below).

We noted a high molecular weight band immunoreactive to GFAP that was more prominent with the mt than the WT protein. The major band migrated with an apparent molecular weight of approximately

**FIGURE 1. Overexpression of WT or mt GFAP leads to intracellular inclusions.** A, representative GFAP inclusions in Cos7, SW13 Vim− cells, IM, and U251 astrocytoma cells; B, the percentages of cells with different organizational phenotypes and examples of each phenotype (Cos7). Results are averages ± S.D. from three independent experiments, with at least 400 transfected cells counted in each. When compared with WT and by student’s t test, *, p < 0.05, **, p < 0.01.
twice that of GFAP, suggesting a dimerization. We did not observe any more slowly migrating bands. This GFAP band appeared not only in cells transfected with FLAG-tagged (Fig. 2, A and B) or GFP-tagged mt GFAP (Fig. 2, C and D) but also in non-tagged mt GFAP-expressing cells (not shown), indicating that it was not a function of the tag.

We also asked whether the endogenous GFAP is recruited to the GFAP inclusions formed by exogenously introduced mt GFAP in the astrocytic cell lines. Because of the lack of antibody that specifically recognizes WT or R239C mt GFAP, we established permanent IM or U251 cells stably expressing GFP-tagged WT GFAP at a low level. Very few GFAP aggregates were seen in those WT GFAP overexpressers. Transient transfection of FLAG-tagged mt GFAP constructs led to formation of GFAP aggregates. By immunostaining, we found both WT GFAP-GFAP and exogenous FLAG-tagged mt GFAP in these aggregates.

**GFAP Accumulation Elicits a shsp Response**—Western blot analysis revealed an increased level of αB-crystallin in WT and mt GFAP-expressing Cos7 and SW13 Vim—cells (Fig. 3). The accumulation of αB-crystallin was also seen in U251 or IM stably expressing WT or mt GFAPs (Fig. 3). In contrast, the level of Hsp70, another heat shock protein and also an ubiquitin proteasome system (UPS) component, was not increased due to GFAP overexpression (data not shown), consistent with our previous findings for astrocytes (3) and AxD brain tissues (9).

**GFAP Accumulation Activates the SAPK/JNK Pathway**—Due to the massive amounts of αB-crystallin that accumulate in the brains of children with AxD and the increased levels in cell cultures overexpressing GFAP, we speculated that GFAP accumulation evokes a cellular stress response. Here we addressed whether the JNK pathway is activated in response to the overexpression of GFAP by monitoring the activated form of JNK (phosphorylated JNK (p-JNK)). Transfection with the vector alone did not activate JNK, but overexpression of WT or mt GFAP resulted in significant increases of p-JNK at 24 and 48 h after transfection (Fig. 4A), whereas levels of total JNK did not change during this period. Persistent elevation of p-JNK also existed in IM astrocytes and U251 cells expressing mt than cells expressing WT GFAP. It is also interesting to note that some of the p-JNK co-localized with the GFAP inclusion bodies in each of the transfected cell lines (data for Cos7 and IM cells shown in Fig. 4E). We explored the associ
A strong association between p-JNK and GFAP inclusions existed in both WT and mt expressers (Fig. 4E).

To determine whether there was a correlation between the level of overexpressed GFAP and extent of activation of JNK, four lines of U251 cells stably expressing WT or mt GFAP, at low, medium, and high levels were studied. JNK activation was evident in cells with medium levels of either WT or mt GFAP and was enhanced in cells expressing high levels of GFAP (Fig. 4C).

The JNK activation state was also assessed in brain tissue from AxD and brain tissues without RFs from non-AxD subjects. A monoclonal anti-p-JNK antibody recognized and co-localized with the RFs (Fig. 4F).

**FIGURE 5. Activation of the SAPK/JNK pathway increases GFAP accumulation.** A, phosphorylation of endogenous MLK3 in Cos7 cells overexpressing GFAP. 48 h after transfection, cells were lysed and examined for endogenous p-MLK3 by Western blotting. V, vector-transfected. B, GFAP accumulation in the insoluble fraction is regulated by the SAPK/JNK pathway. Cos7 cells were transfected with either WT or mt GFAP alone or along with active MLK2, MLK3, or ASK1 (left panel). Cos7 cells were transfected with either WT or mt GFAP alone or along with dominant negative c-Jun (right panel). 48 h after transfection, cells were lysed and 5 (left panel) or 15 (right panel) μg of protein from the insoluble fraction were loaded and probed sequentially with anti-GFAP and anti-GAPDH antibodies.
pathway promoted GFAP accumulation. We studied the effects of activated MLK2, MLK3, and ASK1, three kinases upstream of JNK, on GFAP levels. Cos7 cells were transfected with WT or mt GFAP alone or together with plasmids encoding constitutively activated (Av) forms of MLK2, MLK3, or ASK1. The introduction of activated kinases led to a dramatic increase in GFAP levels (Fig. 5B, left panel). Examination of co-transfected cells by immunofluorescence showed that MLK2, MLK3, or ASK1 increased the numbers of cells with one or more GFAP inclusions (data not shown).

We also co-transfected the cells with GFAP constructs along with a dominant negative mutant form of c-Jun, an immediate downstream component of the JNK pathway. Suppression of the JNK pathway by dominant negative c-Jun led to a decrease in both WT and mt GFAP accumulation in the insoluble fraction (Fig. 5B, right panel), indicating that blocking the JNK pathway inhibits GFAP accumulation.

GFAP Accumulation Impairs Proteasome Function—We next asked whether proteasome function is impaired by GFAP accumulation. We examined the effects of GFAP overexpression on proteasome function by transfecting WT or mt GFAP plasmids into 293T/GFP-U stable cell lines, a model system for evaluating function of the proteasome pathway. This cell line permanently expresses a reporter peptide consisting of a short degron fused to the C terminus of GFP (12). Ordinarily, the ubiquitin-GFP hybrid protein has a very rapid turnover. By monitoring the fluorescence in the GFP-U cells transiently expressing WT or mt GFAP, we found that both WT and mt GFAP overexpression increased steady-state GFP-U fluorescence levels (Fig. 6A). We also observed by Western blotting an accumulation of ubiquitinated GFAP-U protein in cells expressing WT or mt GFAP (Fig. 6B) or, as a positive control, in empty vector-transfected cells treated with MG132 (not shown).

We further examined the proteasome activity with a different, independent assay: the levels of chymotrypsin-like peptidase and PGPH activities (Fig. 7C). Both the PGPH and the chymotrypsin-like peptidase activities were reduced in cells overexpressing WT and mt GFAP when compared with control cells transfected with vector alone. The decreased proteasome activity caused by GFAP accumulation was not the result of a reduction in proteasome complexes.3

Inhibition of Proteasome and Activation of the JNK Pathway Have Synergistic Effects on GFAP Accumulation—When we treated cells with MG132, a proteasome inhibitor, or CPT, a SAPK/JNK activator, we found that both compounds led to increased GFAP accumulation (Fig. 7A), and also an increased level of phospho-JNK (Fig. 7A). We asked whether there existed a synergistic effect of these two pathways on GFAP accumulation. We first addressed the interplay between proteasome inhibition and the activation of the JNK pathway. In non-GFAP-expressing Cos7 cells, which were transfected with vector only, inhibition of the proteasome pathway did activate SAPK/JNK. As shown in Fig. 7A, cells treated with the proteasome inhibitor MG132 showed an increased level of p-JNK when compared with non-drug-treated cells. This observation is in accord with a previous report showing that proteasome inhibition may act as a potential upstream regulator of JNK activation (13). Overexpression of constitutively activated MLK2 or MLK3 in 293T cells stably expressing GFP-U led to the accumulation of the short-lived protein GFP-U, indicating an impaired proteasome function following the activation of the JNK pathway (Fig. 7B).

While treating the cells pharmacologically with CPT, we also observed a decreased protease activity in Cos7 cells transfected with empty vector (Fig. 7C, p < 0.05). CPT treatment further exacerbated the effects of GFAP overexpression. Thus, after CPT treatment, WT GFAP expressers had decreased chymotrypsin-like protease activity by an additional 67%, and mt GFAP expressers had decreased this activity by an additional 58%. WT expressers decreased PGPH activity an additional 44%, and mt GFAP expressers had decreased this activity an additional 47% (Fig. 7C, p < 0.05). Thus, the activation of JNK in the context of GFAP overexpression resulted in further proteasome inhibition.

We then asked whether inhibiting proteasome activity by GFAP overexpression or by JNK activation by CPT in GFAP-overexpressing cells would lead to the accumulation of ubiquitinated GFAP. Cells were treated with MG132 or CPT, and GFAP was immunoprecipitated from whole cell lysates. Proteins were separated by PAGE and transferred to nitrocellulose, on which they were blotted first with an anti-ubiquitin antibody and then reprobed with an anti-GFAP antibody. In non-drug-treated cells (control), there was a high molecular weight smear for ubiquitinated species in WT and mt GFAP transfectants (Fig. 7D), not seen in empty vector controls or in control samples precipitated without antibody (not shown). After JNK activation by CPT, the GFAP-overexpressing cells increased the accumulation of ubiquitinated GFAP (Fig. 7D). Used as a positive control, MG132 also increased the accumulation of ubiquitinated GFAP (Fig. 7D).

Taken together, these data suggest that proteasome inhibition, which was already produced by GFAP overexpression, can activate the SAPK/JNK pathway and subsequently be further inhibited by JNK activation itself. Further proteasome inhibi-

3 Tang, G., in preparation.
GFAP Accumulation, SAPK/JNK, and Proteasome Pathway

**FIGURE 7.** Effects of proteasome inhibition or JNK activation on GFAP accumulation. A, Cos7 cells were transiently transfected with WT or mt GFAP. 24 h after transfection, cells were treated with 10 μM MG-132 or 10 μM CPT for a further 24 h. Total cell lysates were prepared and subjected to immunoblot analysis with antibodies against GFAP, p-JNK, and GAPDH. DMSO, MeSO. V, vector-transfected. B, UPS function was impaired by constitutively active MLK2 or MLK3 overexpression. 293T cells stably expressing GFP-U reporter (Bence et al., 12) were transiently transfected with pcDNA3-FLAG vector (V), vector expressing constitutively active form of MLK2 or MLK3. 24 h after transfection, cells were lysed for Western blotting to assess the steady-state level of GFP-U. C, 24 h after transfection, Cos7 cells were treated with MG-132 or CPT. The PGPH and chymotrypsin-like protease activities were analyzed by using the fluorescent substrates S-II and S-III, respectively. The results are the averages of five independent experiments. **D**, proteasome inhibition or JNK activation increased the levels of ubiquitinated (Ub) GFAP. 24 h after transfection, Cos7 cells were exposed to 10 μM MG-132 or 10 μM CPT for another 24 h. Cells were lysed, and GFAP was immunoprecipitated with an anti-FLAG M2-agarose affinity gel. Immunoprecipitation products were analyzed by Western blotting with antibodies against ubiquitin (upper) and GFAP (lower).

**DISCUSSION**

**WT GFAP Is Prone to Aggregate, and the R239C Mutation Exacerbates the GFAP Accumulation and Aggregation**—In accordance with our previous observations in transgenic mice and cell cultures (3, 7, 8), we found that overexpression of both WT GFAP and the R239C mt GFAP leads to GFAP accumulation and produces cytoplasmic inclusions. Inclusions formed in GFAP-negative cell lines (Cos7 and SW13 Vim−) and in GFAP-positive cell lines (IM and U251 cells). Based on these findings, we postulate that the formation of inclusions is at least in part due to the increased levels of GFAP protein.

To determine whether the presence of the FLAG or GFP tags affected the capacities of the tagged GFAPs to form inclusions, we compared the staining profiles of Cos7, SW13 Vim− cells, and astrocytes transfected with the tagged constructs to those seen when they are transfected with the corresponding non-tagged GFAP construct (7, 8). Both tagged and non-tagged WT or mt GFAP could form aggregates in vitro, although there might be a possibility that tags increase the tendency of GFAP to form more aggregates and thus increase the pathology. This might compromise our ability to detect the differences between effects of overexpressing WT and mt GFAP. However, there are relatively many more aggregate-bearing cells after mt GFAP transfection than after WT transfection, despite the presence of tags (as seen in this report and also in Hsiao et al., 8, where no tags were used). The tendency for mt GFAP to form more aggregates was much more apparent in stable astrocytic cell lines expressing GFAP. In IM or U251 cells, WT GFAP formed filamentous bundles, whereas mt GFAP formed punctate aggregates predominantly. In response to accumulation of the exogenously introduced mt GFAP, endogenous WT GFAP was up-regulated and was even incorporated into aggregates. Our immunoblot analysis also revealed a high molecular weight

4 Quinlan, R., manuscript in preparation.
GFAP Accumulation, SAPK/JNK, and Proteasome Pathway

FIGURE 8. Effects of GFAP overexpression on cell viability. Cell numbers and viability were determined by MTT assay and trypan blue exclusion. Values are means ± S.E from three independent experiments. **, p < 0.01 when compared with respective untransfected controls. A, Cos7 cells were transiently transfected with pcDNA3, pcDNA3-wtGFAP, or pcDNA3-mtGFAP. At 48 h after transfection, cell numbers were measured by trypan blue (left) and MTT (right). C, non-transfected control; V, vector-transfected. B, U251 cells and those stably expressing WT or mt GFAP were plated at 80% confluency. 24 h later, they were treated with CPT at three different concentrations (10, 25, and 40 μM) for 48 h. Living cells were counted by trypan blue test and MTT test. Data are expressed as a mean percentage of cell counts in control wells. Each result represents a mean ± S.D. of three independent experiments carried out in triplicate. **, by student’s t test, and when compared with vector-transfected cells, p < 0.05. C, representative Western blot analysis of p-JNK in cells exposed to CPT at different concentrations (left) and the normalized level of p-JNK (right). The increase in p-JNK was observed in three separate experiments.

band that existed exclusively in the mt GFAP overexpressers, suggesting that the mt GFAP protein has an increased stability to SDS. Together with the fact that the R239C mutant GFAP is more prone to form more aggregates and more resistant to high salt extraction (8), we speculate that the mutation itself might confer increased stability to protein-protein interactions in the GFAP polymers and thus exacerbate protein aggregation. Consistent with this hypothesis, we found more activation of JNK (Fig. 4, A–C) and a more dramatic decrease in proteasome activity (Fig. 7C) in cells expressing mt when compared with WT.

GFAP Accumulation Induces a Stress Response—A number of studies indicate that protein accumulation activates cellular stress pathways (14–17). We found that the accumulation of either WT or an AxD mutant GFAP activates MLKs and JNK in cultured cells and that the accumulation of GFAP is associated with activated JNK in the AxD brain. WT GFAP accumulation in cultured cells and the overexpression of WT hGFAP in transgenic mice both produce cytoplasmic protein inclusions and increased shsps, elements of a stress response (18), suggesting that too much GFAP protein per se can elicit a stress response. Consistent with this idea, the levels of activated JNK correlate positively with levels of both WT and mt GFAPs in the astrocytoma cells stably expressing GFAP.

GFAP Accumulation Inhibits Proteasome Activity—We have provided several pieces of evidence that GFAP accumulation impairs proteasome activity. First, at least some of the overexpressed GFAP protein was degraded through the UPS in an ubiquitin-dependent manner. Second, overexpressing either WT or R239C GFAP led to impaired proteasome function, assayed either by GFP-U accumulation or by in vitro proteolytic assays. One possible mechanism for this inhibition is that the accumulated GFAP saturates the capacity of free cytosolic ubiquitin or molecular chaperones required for UPS function. Another possible mechanism might involve a direct interaction between proteasome and GFAP filaments that might retain proteasomes in GFAP inclusions.3 Thus, a competition between the excess GFAP and other cellular proteins for proteasome degradation might occur, leading to slowed protein turnover in general.

GFAP Accumulation Increases shsp Levels—In the case of AxD and GFAP accumulation, it is primarily the shsps (αB-crystallin and hsp27) that are up-regulated (2, 9, 19). These shsps interact with intermediate filaments, possibly serving a debundling or disaggregating purpose (7, 20). Transcript and protein levels of shsps are markedly elevated in brains of AxD patients. Similarly, we found that overexpression of WT or R239C GFAP increased the levels of αB-crystallin in cultured cells with different intermediate filament constituents.

Shsps are important protectors against cell death (21). In particular, αB-crystallin protects cells from apoptosis induced by a large number of stresses through interacting with the precursors of caspase-3 (22–24) or with Bax and Bcl-Xs to prevent translocation of these proapoptotic regulators into mitochondria, thus inhibiting downstream apoptotic events (25). In our cell culture system, we did not find evidence of cell death after GFAP transfection, although the activation of MLKs leads to activation of JNK and death in a number of cell types (11). However, persistent and high activation by CPT treatment compromised the ability of mt GFAP-expressing cells to withstand stressful stimuli. Further study will be directed to the
GFAP Accumulation, SAPK/JNK, and Proteasome Pathway

**FIGURE 9. A proposed model of intracellular GFAP accumulation.** GFAP accumulation also impairs the function of the UPS and activates the JNK pathway. As a positive feedback response, JNK activation and proteasome inhibition will synergistically accelerate GFAP aggregation and thus aggravate the adverse effects of GFAP accumulation. Persistent and high activation by other cellular stresses (represented here as CPT treatment) will further compromise the ability of cells to deal with stressful stimuli. Induced αB-crystallin contributes to the disaggregation of GFAP aggregates and protects cells from apoptotic events. The **circled P** represents phosphorylation.

possible protective roles of αB-crystallin in the context of GFAP accumulation.

Synergistic Interactions among GFAP Accumulation, JNK Activation, and Proteasome Inhibition—The formation of GFAP inclusions was further stimulated by activating the JNK stress-signaling cascade. This appears to be similar to the observation that inclusion bodies formed by polyQ-containing peptides or other abnormal proteins (cystic fibrosis transmembrane conductance regulator (CFTR) mutants) are increased by MEKK1 activation, a kinase that activates several downstream stress kinases (15). In our study, the expression of activated forms of ASK1 and MLK2 or MLK3, MAP kinases that activate the JNK and p38 MAP kinase signaling (11, 26), stimulated GFAP accumulation. In contrast, inhibiting pathways downstream of JNK with a dominant negative c-jun suppressed GFAP accumulation.

The reasons for the progressive accumulation of GFAP may be related to a synergistic interplay between the activated SAPK/JNK pathway and the impaired proteasome system. We observed that the inhibition of proteasome activity, with either MG132 or GFAP overexpression, increased p-JNK, suggesting a stimulatory effect of proteasome inhibition on JNK activation, as reported by Merin et al. (13). Similarly, proteasome function was impaired by JNK activation, either by CPT treatment or by the introduction of activated MLKs.

We suggest a model in which two linked pathways, proteasome-based protein degradation and JNK activation, interact to regulate GFAP accumulation in AxD (Fig. 9). GFAP accumulation, produced by the expression of the mutant and WT proteins in AxD, overexpression of WT or mt GFAP in transgenic mice, or overexpression of GFAPs in transfected cells, is the inciting pathological stimulus. This accumulation has several consequences. First, it results in the activation of the MLKs-JNK pathway, which in turn exacerbates the GFAP accumulation. The accumulation also inhibits proteasome activity. This has further consequences: more protein accumulation and further activation of the JNK pathway. As a feedback response, JNK activation further inhibits proteasome activity, thus aggravating the adverse effects of GFAP accumulation. In this feedback loop, MLKs may act as the cross-talk signal between two pathways since a direct interaction exists between MLKs and proteasome regulatory complexes PA28. Thus, GFAP accumulation elicits the activation of MLKs, and the introduction of activated MLKs impairs proteasome activity in this report.

Second, as a positive consequence, the accumulation of GFAP results in αB-crystallin accumulation. This might act to reverse the abnormal GFAP aggregation (7) and also act to protect the cell from apoptosis (24, 25). The balance between the positive and negative consequences of GFAP accumulation might define the survival or death of a cell. In astrocytic cell lines, increased vulnerability to stress occurs when JNK is highly activated using CPT as a stress inducer. It is noteworthy that the AxD brain is full of astrocytes that contain RFs. Indeed, the predominant cell type in the demyelinated or non-myelinated white matter is the astrocyte. Although studies of astrocyte death have not been performed on AxD tissues, it is possible that positive pathways, through hsps, for example, may predominate over death pathways to ensure astrocyte survival.

Acknowledgments—We are grateful to Drs. Paul Fisher and Ron Liem for providing immortalized human astrocytes and SW13 Vim− cells, to Dr. Ron Kopito for supplying the GFP-u construct, and to R. Tian for the GFAP constructs. We also thank Drs. Michael Brenner, Albee Messing, and Roy Quinlan for many helpful comments and discussion.

**REFERENCES**

1. Johnson, A. B. (2002) *Int. J. Dev. Neurosci.* 20, 391–394
2. Eng, L. F., Lee, Y. L., Kwan, H., Brenner, M., and Messing, A. (1998) *J. Neurosci. Res.* 53, 353–360
3. Messing, A., Head, M. W., Galles, K., Galbreath, E. J., Goldman, J. E., and Brenner, M. (1998) *Ann. J. Pathol.* 152, 391–398
4. Brenner, M., Johnson, A. B., Boespflug-Tanguy, O., Rodriguez, D., Goldman, J. E., and Messing, A. (2001) *Nat. Genet.* 31, 117–120
5. Li, R., Messing, A., Goldman, J. E., and Brenner, M. (2002) *Int. J. Dev. Neurosci.* 20, 259–268
6. Li, R., Johnson, A. B., Salomons, G., Goldman, J. E., Naidu, S., Quinlan, R., Cree, B., Ruyle, S. Z., Banwell, B., D’Hooghe, M., Siebert, J. R., Rolf, C. M., Cox, H., Reddy, A., Gutierrez-Solana, L. G., Collins, A., Weller, R. O., Messing, A., van der Knaap, M. S., and Brenner, M. (2005) *Ann. Neurol.* 57, 310–326
7. Koyama, Y., and Goldman, J. E. (1999) *Am. J. Pathol.* 154, 1563–1572
8. Hsiao, V. C., Tian, R., Long, H., Der Perng, M., Brenner, M., Quinlan, R. A., and Goldman, J. E. (2005) *J. Cell Sci.* 118, 2057–2065

Xu, Z., unpublished.
9. Head, M. W., Corbin, E., and Goldman, J. E. (1993) *Am. J. Pathol.* **143**, 1743–1753
10. Kopito, R. R. (2000) *Trends Cell Biol.* **10**, 524–530
11. Xu, Z., Maroney, A. C., Dobrzanski, P., Kukekov, N. V., and Greene, L. A. (2001) *Mol. Cell Biol.* **21**, 4713–4724
12. Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001) *Science* **292**, 1552–1555
13. Meriin, A. B., Gabai, V. L., Yaglom, J., Shifrin, V. I., and Sherman, M. Y. (1998) *J. Biol. Chem.* **273**, 6373–6379
14. Merienne, K., Helmlinger, D., Perkin, G. R., Devys, D., and Trottier, Y. (2003) *J. Biol. Chem.* **278**, 16957–16967
15. Meriin, A. B., Mabuchi, K., Gabai, V. L., Yaglom, J. A., Kazantsev, A., and Sherman, M. Y. (2001) *J. Cell Biol.* **153**, 851–864
16. Savage, M. J., Lin, Y. G., Ciallella, J. R., Flood, D. G., and Scott, R. W. (2002) *J. Neurosci.* **22**, 3376–3385
17. Richter-Landsberg, C., and Bauer, N. G. (2004) *Int. J. Dev. Neurosci.* **22**, 443–451
18. Hagemann, T. L., Gaeta, S. A., Smith, M. A., Johnson, D. A., Johnson, J. A., and Messing, A. (2005) *Hum. Mol. Genet.* **14**, 2443–2458
19. Iwaki, T., Kume-Iwaki, A., Liem, R. K., and Goldman, J. E. (1989) *Cell* **57**, 71–78
20. Head, M. W., Hurwitz, L., Kegel, K., and Goldman, J. E. (2000) *Neuroreport* **11**, 361–365
21. Arrigo, A. (1998) *Biol. Chem.* **379**, 19–26
22. Kamradt, M. C., Chen, F., and Cryns, V. L. (2001) *J. Biol. Chem.* **276**, 16059–16063
23. Kamradt, M. C., Lu, M., Werner, M. E., Kwan, T., Chen, F., Strohecker, A., Oshita, S., Wilkinson, J. C., Yu, C., Oliver, P. G., Duckett, C. S., Buchbaumer, D. J., Lobuglio, A. F., Jordan, V. C., and Cryns, V. L. (2005) *J. Biol. Chem.* **280**, 11059–11066
24. Mao, Y. W., Xiang, H., Wang, J., Korsmeyer, S. J., Reddan, J., and Li, D. W. (2001) *J. Biol. Chem.* **276**, 43435–43445
25. Mao, Y. W., Liu, J. P., Xiang, H., and Li, D. W. (2004) *Cell Death Differ.* **11**, 512–526
26. Takeda, K., Matsuzawa, A., Nishitoh, H., and Ichijo, H. (2003) *Cell Struct. Funct.* **28**, 23–29