Self-assembly of the Cytoskeletal Glial Fibrillary Acidic Protein Is Inhibited by an Isoform-specific C Terminus*

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The predominant isoform of glial fibrillary acidic protein (GFAP), GFAPα, is the characteristic building block of the cytoskeletal intermediate filaments in astrocytes. Isoform GFAPε, produced by alternative splicing of the GFAP gene, includes a new tail domain that confers a presenilin binding capacity. We here show that the GFAPε tail prevents GFAPε homodimerization and homomeric filament formation, whereas the ability to form heterodimers and filaments with GFAPα is retained. Furthermore, GFAPε shows decreased affinity for several GFAPε-interacting proteins. A GFAPε tail mutation that results in gain of GFAPε dimerization and filament formation abolishes presenilin binding. This mutation also abolishes interaction between the tail and the coiled-coil domain of GFAPα. Together, this indicates that direct interaction between the coiled-coil and tail domains may serve as an inhibitory mechanism for homomeric dimerization and filament formation. We propose that the GFAPε isoform represents a new functionally distinct component of GFAP intermediate filaments.

Intermediate filament proteins have a characteristic structure composed of a highly conserved α-helical rod domain flanked by nonhelical head and tail domains. The α-helical rod domain mediates dimerization and higher order structures during filament formation, and the head and tail regions participate in the regulation of this process (1, 2). Whereas the central rod domain is well conserved between different intermediate filament proteins, the head and tail sequences diverge both in length and amino acid composition. However, substitution of a few amino acids in the head region can be deleterious for correct filament formation.

Intermediate filaments are, in general, polymers of more than one intermediate filament protein. Glial fibrillary acidic protein (GFAP), vimentin, and nestin are the main intermediate filament proteins in astrocytes, and they are differentially regulated (1, 2). Partnership between vimentin and nestin is characteristic of intermediate filament of the immature astrocytes, whereas vimentin is expressed together with GFAP in the mature astrocyte, and especially GFAP is up-regulated in activated astrocytes, as observed during aging and in Alzheimers disease (3–5). Studies of mice with targeted mutations have shown that vimentin and nestin require a partner to form filaments, whereas GFAP can form filaments on its own (6). Mutated human GFAP, as found in Alexander disease, forms abnormal cytoplasmic fibers, termed Rosenthal fibers, in the brain of patients with this neurodegenerative disease (7–9), and such mutations can directly interfere with normal GFAP dimerization (10).

Human GFAP is a 432-amino acid-long polypeptide of 50 kDa (11, 12) and is encoded by the GFAP gene on chromosome 17q21. The gene extends over 10 kb, and the predominant splice form, termed GFAPα, consists of nine exons (13). We have recently isolated a new splice form of the GFAP transcript encoding a protein isoform termed GFAPε (14). The alternative splicing is a result of the usage of a cryptic exon, exon 7a, embedded in intron 7 (14). The alternative spliced GFAPε mRNA represents about 5% of the total amount of GFAP mRNA. Exon 7a carries its own polyadenylation signal, and usage inhibits expression of exons 8 and 9. These two exons 8 and 9 encode the evolutionarily well conserved 42-amino acid-long tail domain of GFAPα. When exon 7a is spliced, GFAPε is generated with a new 41-amino acid-long tail domain. Exon 7a sequences have only been identified in mammalian GFAP genes, and they appear to be under a different evolutionary pressure than the sequences of the other GFAP exons (15). GFAPε was functionally isolated by the constitution of a binding surface for the presenilin proteins (14). GFAPα does not have this capacity, and mutation analyses show that the new tail domain of GFAPε, together with sequences in the coiled-coil region, is required for binding to the presenilins (14). The GFAPε tail is also characterized by the absence of the RDG motif, which has been proposed to be involved in correct filament formation (16).

Here we show that GFAPε, in contrast to GFAPα, cannot bind to itself to form homodimers and homomorphic filaments but can form heterodimers and heteromeric filaments with GFAPα. The inhibition of GFAPε dimerization is directly linked to the capacity of the GFAPε tail to interact with the coiled-coil region of the protein. Our results elucidate a possible new mechanism for selective dimerization of intermediate proteins.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Details on individual plasmid constructs, which were all verified by sequencing, are available upon request. Human cDNA for GFAPα was cloned by PCR from a brain cDNA library. Specific mutations were generated as described for QuikChange site-directed mutagenesis (Stratagene). For yeast two-hybrid assays, DNA binding domain and acidic activation domain (AAD) fusion proteins were expressed from the yeast multicycop plasmids pBTM116m (17) and pGAD10 (Clontech), respectively. His6-tagged constructs were obtained by subcloning the indicated cDNAs into pRSETB (Invitrogen). For GST pull-down experiments and far Westerns, cDNA was subcloned into pGEX2TK (Amersham Biosciences). For mammalian expression, we used pSG5 (18), pcDNA3 (Invitrogen), pNCFLAG (18), or N- and C-terminal green fluorescent fusion pEGFP vectors (Clontech). The Gen...
Bank™ accession number for the human GFAPε exon 7a sequence is AJ306447.

**Yeast Transactivation Assays**—L40 yeast cells grown in yeast extract/peptone/dextrose were transformed by the lithium acetate procedure. For β-galactosidase activity measurements, yeast transformants were grown exponentially for about five generations in selective medium, and extracts were prepared and assayed essentially as described by Rose et al. (19). For qualitative β-galactosidase activity measurements, cotransformants were plated on minimal X-gal indicator plates, and color appearance was monitored.

**In Vitro Binding Assays**—GST, GST-GFAPε (391–431), GST-GFAPε (391–432), GST-encoplakin (29), and GST-periplakin (20) fusion proteins were expressed in Escherichia coli XLI-1 blue and purified on glutathione S-Sepharose beads (Amersham Biosciences) as described (18, 21). His, epitope-tagged GFAP fusion proteins were expressed in *E. coli* BL21 (DE3) and purified at denaturing conditions (all buffers included 8 M urea) on Ni²⁺-chelating columns (Amersham Biosciences) as described (14, 21). Proteins were renatured by dialyzing against buffer NEB (25 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitor mixture (Roche Applied Science)), and insoluble material was removed by centrifugation in a table centrifuge (12,000 rpm, 15 min, 4°C). Pig brain extract was prepared as described (14). GST pull-down assays were performed essentially as described (14). Briefly, purified proteins were quantified by Coomassie staining after SDS-PAGE and by Bradford protein assay. Five μg of GST or GST fusion proteins loaded on glutathione S-Sepharose beads for 2 h at 4°C in NEB buffer were incubated with 0.5 μg of His-tagged protein. Incubation was performed overnight at 4°C with gentle agitation. The beads were washed six times with 750 μl of NEB buffer, resuspended in 50 μl of Laemmli buffer, and boiled for 10 min, and proteins were analyzed by SDS-PAGE. Procedures for Western blotting and antibody revealing by chemiluminescence were as described (14, 21).

**Mammalian Cell Lines and Transfections**—Human SW13cl.1 and SW13cl.2 adrenal carcinoma cell lines with the presence and absence of vimentin expression, respectively, were described previously (22). All transfections were done with Superfect (Qiagen). Unless otherwise indicated in the figure legends, 2 μg of DNA was used for each transfection.

**Immunological Methods**—His tag antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used in a 1:2000 dilution for Western blotting. In epi-immunofluorescence experiments, rabbit anti-GFAP (Dako) was used in a 1:400 dilution, and fluorescein isothiocyanate or TRITC-labeled goat anti-rabbit secondary antibodies (Molecular Probes, Inc., Eugene, OR) were diluted 1:200. For epi-immunofluorescence analysis, cells were grown in slide flasks (Nunc). After 48 h of incubation, cells were washed twice in PBS and fixed in 2% para-formaldehyde, 0.1% glutaraldehyde, 0.1% Triton X-100 for 30 min on ice. The fixed cells were washed in phosphate-buffered saline, and antibody incubations were done in phosphate-buffered saline supplemented with 5% fetal calf serum for 1 h at 4°C. Cells were counterstained with Hoechst 33258 to visualize the nuclear compartments.

**RESULTS**

**GFAPε Lacks Homodimerization Capacity**—The amino acid sequences of the tail domains of GFAPα and GFAPε are similar in length but show no clear amino acid homology to each other, and their isoelectric points are very different (Fig. 1A). The tail confers GFAPε with a capacity for presenilin binding not found in GFAPα (14). Because GFAPα is a building block of the cytoskeleton, we were interested in examining whether the tail provided specific functions to GFAPε with respect to protein interaction at the level of dimerization. We utilized the yeast two-hybrid assay, where the proteins to be examined are expressed in fusion to either a DNA binding domain (LexA) or an AAD. The ability of forming protein interactions and thereby generating a chimeric transcription factor can be determined by co-transformation into the *Saccharomyces cerevisiae* strain L40 harboring integrated copies of *(LexA)₇-his* and *(LexA)₇-LacZ* indicator genes. cDNA encoding the 432 amino acids of GFAPε, the 431 amino acids of GFAPα, or a truncated GFAP without the alternative spliced tail sequences (residues 1–390, GFAPdt) was inserted into the LexA DNA binding domain fusion yeast expression vector pBTM116m. GFAPα, GFAPε, or GFAPdt were fused to an AAD in the yeast expression vector pGAD10. In a yeast two-hybrid assay, the fusions were tested for all possible interactions. GFAPα fused to LexA interacted readily with AAD-GFAPα and AAD-GFAPdt, but, to our surprise, no measurable interaction was observed toward AAD-GFAPε (Fig. 1B). The same pattern of interaction was observed when the LexA-GFAPdt fusion was tested for the similar interactions (Fig. 1B). LexA-GFAPα showed a low level of dimerization toward AAD-GFAPα and AAD-GFAPdt, but we never observed interaction with AAD-GFAPε (Fig. 1B). A DNA binding domain and AAD orientation-dependent variation in interaction capacity, as observed in this assay, has previously been described for intermediate filament proteins (29). These results show that, in yeast, GFAPε has some capacity to form heterodimers with GFAPα but has no detectable homodimerization potential.

We extended the study to include heterogeneous protein interactions, which have been described to involve GFAP. In yeast two-hybrid assays, dimerization of GFAPα with the intermediate filament proteins, NFL, peripherin, and internexin, was decreased as compared with the interaction observed for GFAPα (Fig. 1C). Also, GFAPε failed to interact with the GFAP filament-associated kinase PKN (data not shown). We next examined protein interactions with nonintermediate filament proteins in a GST pull-down *in vitro* assay and found that the interaction of GFAPα with periplakin and envoplakin was severely decreased as compared with the GFAPα interaction (19) (Fig. 1D). Thus, the GFAPε tail confers an inhibitory effect on multiple genetically distinct GFAP interactions.

**GFAPε Is Deficient in Homomeric Filament Formation**—We have previously shown that GFAPε overexpressed in mouse N2A cells integrates into filamentous structures as does overexpressed GFAPα (14). This may be explained by the capacity to form heteromeric complexes with the intermediate filament proteins present in this cellular environment. We therefore examined whether GFAPα can form homomeric filaments in a cell type without intermediate filaments. The SW13cl.1 and SW13cl.2 cell lines are derived from a human adrenal carcinoma tissue (22). The SW13cl.2 cells are derived from SW13cl.1 cells and lack expression of intermediate filament proteins including vimentin (22). GFAPα, GFAPε, and GFAPdt were separately transfected into SW13cl.1 cells, and their incorporation into filamentous structures was determined by immunofluorescence analysis using an antibody that recognizes all three forms of GFAP (Fig. 2). The only difference we observed between the three GFAP forms was a more numerous presence of coarse filaments in the GFAPα-transfected SW13cl.1 cells (Fig. 2 and data not shown). When the GFAP constructs were transfected into SW13cl.2 cells, however, clearly distinguishable patterns of localization were observed. GFAPα formed filamentous structures consistent with an intrinsic capacity to generate homomeric filaments (Fig. 2). GFAPdt formed unstructured filamentous bundles or discontinuous filament fragments, indicating that GFAPdt does have the capacity to generate homomeric multimers but that these multimers are incapable of correct assembling into longer filaments (Fig. 2). GFAPε was completely deficient in forming any filamentous structures and localized instead to aggregates either in the perinuclear region or to spots throughout the cytoplasm (Fig. 2 and data not shown). Thus, in mammalian cells, GFAPε is deficient in homomeric filament formation, which is consistent with the finding that GFAPε is deficient in homodimerization.

We did similar experiments with the GFAP isoforms fused to either N- or C-terminally located green fluorescent protein (GFP) tags. In SW13cl.1 cells, such GFP fusions to GFAPα, GFAPε, and GFAPdt all localized to filamentous structures (data not shown). In SW13cl.2 cells, N-terminal GFP-tagged
GFAPα formed thick filament-like structures often characterized by the presence of only one large filament, whereas N-terminal GFP-tagged GFAPs only formed aggregates (Fig. 3 and data not shown). Also, C-terminal GFP-tagged GFAPs formed only aggregate-like structures in contrast to C-terminal GFP tagged GFAPα, which formed filament-like structures (data not shown). Taken together, the GFP tag fusion results and the immunofluorescence results show that GFAPα and GFAPs are not functional equivalent with respect to filament incorporation.

We next examined the capacity of heterodimer formation in mammalian cells by monitoring the capacity of GFAPα to co-
assemble with GFP-GFAPα. As shown in Fig. 3A, GFAPα expression clearly co-assembled with GFP-GFAPα into thick filamentous bundles. Thus, albeit the GFP tags in SW13cl.2 cells influence the filament-forming capacity of GFAPα (data not shown) the localization experiment demonstrates that GFAPβ in a cellular context can associate with GFAPα in accordance with the yeast experiments that showed the capacity of forming heterodimers (Fig. 1).

The GFAPα mRNA appears to be 20 times more abundant than GFAPβ mRNA in analysis using a mouse whole brain (14). Given their differences in dimerization capacity, it is conceivable that changing the ratios between GFAPα and GFAPβ expression may have a profound effect on filament structure. Accordingly, we co-expressed GFAPα and GFAPβ in SW13cl.2 cells, and it was possible to change the GFAPα/GFAPβ ratio from 200:1 to 10:1 without any severe alterations in filament structures (Fig. 3B). Further increase in the GFAPα amount to a GFAPα/GFAPβ ratio of 3:1 resulted in collapse of the intermediate filament cytoskeleton in most cells (Fig. 3B). This ratio may represent a limit for efficient fiber formation beyond which GFAPβ does not incorporate together with GFAPα in fibers but inhibits formation of such structures. Consistent with this interpretation, we found only aggregates and no fibers in any cell when a GFAPα/GFAPβ ratio of 1:1 was used in the transfection experiment (Fig. 3B), and in none of the experiments did we observe in any cell co-existence of aggregates and elongated filaments. This might indicate that whenever the GFAPα/GFAPβ ratio is compatible with fiber formation, the two isoforms heterodimerize and incorporate into heteromeric filaments with no GFAPβ monomers left to aggregate.

To examine whether the capacity of GFAPβ to interfere with filament formation was only due to the presence in the cells of the GFAPβ tail domain or also required the additional coiled-coil region, we co-transfected SW13cl.2 cells with GFAPα and F-GFAPβα-(391–431) encoding the FLAG epitope-tagged GFAPβ tail. No effect on the filament structure was monitored in immunofluorescence analysis at a 1:1 ratio of the expression vectors (Fig. 3B, bottom). Also, in a co-transfection experiment with GFAPα and F-GFAPβα-(391–431), the GFAP filament structure was intact (Fig. 3B, bottom panel). Thus, the inhibitory effect of the GFAPβ tail requires fusion to the coiled-coil domain, which indicates that the effect is dependent of dimerization with other GFAP monomers.

GFAPβ Tail Residues Mediate a Coiled-coil Domain Interaction and Inhibit Homodimerization—Both GFAPα with a tail domain and GFAPβ without a tail domain, GFAPαt, are capable of homo- and heterodimerization (Fig. 1). It is therefore likely that the observed inability of GFAPβ to homodimerize is a direct inhibitory effect of residues present exclusively in the GFAPβ tail domain rather than the absence of residues supporting dimerization, such as the RDG motif also absent from GFAPdt. One explanation of the inhibitory effect could be the presence of molecular interactions between the GFAPβ tail domain and heterologous GFAP sequences and thereby interference with both the dimerization and filament forming capacity. To examine this hypothesis, we fused the GFAPβ tail to GST in GST-GFAPβα-(390–431) and examined its capacity to interact with full-length GFAP in a GST pull-down assay. As revealed by Western blotting, we observed a significant interaction with bacterial expressed histidine-tagged GFAPα and, to a lesser degree, with histidine-tagged GFAPβ (Fig. 4A). The lower level of interaction with GFAPβ is in accordance with competition from intramolecular GFAPβ interaction. Also, GST-GFAPα-(391–432) interacted with histidine-tagged GFAPβ and GFAPα (Fig. 4A). This interaction, however, was less strong, and especially toward GFAPβ the interaction was close to GST background interaction.

The interaction mediated by the tail domain was also detectable in a cellular extract from a pig brain, as shown by a GST pull-down assay (Fig. 4B). The antibody used in this assay did not distinguish between the two GFAP isoforms. Again the GFAPβ tail showed an interaction with GFAPβ, and the GFAPβ tail showed a much weaker GFAPβ interaction. These results clearly demonstrate that the GFAPβ tail can directly form protein interactions with heterologous GFAP sequences.

To map the interaction domain for the GFAPβ tail, we generated truncated versions of His-tagged GFAPβ. Interaction was observed with his-GFAPβc-(204–431) and his-GFAPβc-(204–390), indicating that coiled-coil 1a and 1b as well as the tail itself are not requested (Fig. 4C). Further mapping identified a region, GFAPβc-(349–390), present in all interacting fragments (Fig. 4C), which corresponds to the extreme C-terminal end of
The GFAP<sub>e</sub> Tail Inhibits Dimerization

If the inhibition of GFAP<sub>e</sub> dimerization is an effect of active participation of tail residues rather than lack of essential residues, it is envisaged that tail mutations can be identified that revert the effect. To determine residues in the GFAP<sub>e</sub> tail required for the inhibitory function, we generated a series of GFAP<sub>e</sub>-tail mutants and studied their effect on the capacity in a yeast two-hybrid assay to dimerize with GFAP<sub>α</sub>. Since especially phosphorylation has been identified as a key regulator of intermediate filament protein interactions, we also mutated all potential phosphate receptor amino acids (1, 4, 25). Of the tested mutations, AAD-GFAP<sub>e</sub>(T411E,I412E) clearly gained in binding capacity for LexA-GFAP<sub>α</sub>, as did, to a lesser degree, the AAD-GFAP<sub>e</sub>(T411A) mutant (Fig. 5A). All other mutations tested showed only weak or insignificant gain in interaction with LexA-GFAP<sub>α</sub> (Fig. 5A). When the gain of function mutations were tested for interaction with LexA-GFAP<sub>e</sub>, no effect or only a weak effect was observed (Fig. 5A).

The results prompted us to undertake experiments where we fused the mutants GFAP<sub>e</sub>(T411E,I412E) and GFAP<sub>e</sub>(T411A) to LexA and tested their capacity for interaction with the equivalent mutations in the context of the AAD vector. Only the T411E,I412E mutation showed a gain in homodimerization capacity, reaching the level of GFAP<sub>e</sub> homodimerization (Fig. 5B) (data not shown).

To examine whether the T411E,I412E tail mutation also resulted in gain in GFAP<sub>e</sub> dimerization capacity in mammalian cells, we transfected SW13cl.2 cells with a GFAP<sub>e</sub>-expression vector. This mutant was localized, using a GFAP antibody, to rough filamentous structures (Fig. 5C). This localization was clearly distinct from the localization of nonmutated GFAP<sub>e</sub> and resembled the GFAP<sub>d</sub> tail localization (see Fig. 2A). By contrast, an array of other GFAP<sub>e</sub> mutants did not differ in their localizations as compared with nonmutated GFAP<sub>e</sub>, in accord with the yeast two-hybrid assay, which showed no gain of homodimerization capacity (data not shown).

By introducing the T411,I412E mutation, we were thus able to abolish the inhibitory effect of the GFAP<sub>e</sub> tail on homodimerization and in that respect change the GFAP<sub>e</sub> isoform to resemble GFAP<sub>d</sub> without any tail domain and thereby be more GFAP<sub>d</sub>-like.

Since the GFAP<sub>e</sub> tail to coiled-coil interaction observed in vitro (Fig. 4) could be involved in the process of inhibition of dimerization and filament formation, we examined the consequence of the T411,I412E mutation for this interaction. The T411,I412E mutation was introduced in the background of GST-GFAP<sub>e</sub>(391–431), and the produced recombinant protein was subsequently used in a GST pull-down assay with recombinant histidine-tagged GFAP<sub>e</sub>(349–431). A clear decrease in interaction to his-GFAP<sub>e</sub> (349–431) was observed for the mutant GFAP<sub>e</sub> tail as compared with the normal GFAP<sub>e</sub> tail (Fig. 5D). Thus, gain of dimerization and filament formation is monitored by epi-immunofluorescence microscopy visualizing GFAP. As control, SW13cl.2 cells transfected with either GFP-GFAP<sub>α</sub> alone or together with untagged GFAP<sub>α</sub> were included. B, alterations in the GFAP<sub>e</sub>/GFAP<sub>α</sub> ratio affect the GFAP filament forming capacity. To monitor how alterations in the GFAP<sub>e</sub>/GFAP<sub>α</sub> ratio affect filament formation, the indicated ratios of GFAP<sub>e</sub> and GFAP<sub>α</sub> expression vectors were transfected into SW13cl.2 cells. The amount of transfected GFAP<sub>e</sub> was constantly 1 μg, and the ratio of transfected GFAP<sub>e</sub> is indicated in each panel. In the bottom panel, the GFAP<sub>e</sub> tail (left panel) or GFAP<sub>α</sub> tail (right panel) was expressed as a FLAG-epitope fusion from the pSG5 vector together with GFAP<sub>α</sub>. Expressed GFAP was visualized as described above.
linked with decreased interaction between the GFAPε tail and the coiled-coil region.

Presenilin Binding Capacity and Inhibition of GFAPε Dimerization Uses Overlapping Tail Residues—Previously, we showed that the specific tail domain of GFAPε, together with determinants in the coiled-coil-2 region, was required for interaction with the presenilins, a capacity not shared by GFAPα (14). We speculated that the mutations that made GFAPε more GFAPα-like with respect to dimerization might interfere with presenilin binding so that GFAPε behaves GFAPα-like also in this respect. To map precisely the GFAPε-specific residues involved in presenilin interaction, we utilized the above described panel of AAD-GFAPε mutants in yeast two-hybrid assays. As shown in Fig. 5A, the T411E,I412E mutation had a dramatic effect, since it completely abolished presenilin-1 interaction. This same mutation, as shown above, completely abolished the mechanism that inhibits GFAPε to dimerize. Also, AAD-GFAPε(T411A) and to some extent AAD-GFAPε(L407E,K408E) had decreased presenilin-1 binding capacity (Fig. 5A). These results demonstrate that the sequences required for presenilin-1 binding overlap with sequences within the GFAPε tail that are required for inhibition of dimerization.

**DISCUSSION**

Alternative splicing of transcripts from mammalian GFAP genes creates an isoform, GFAPε, with a tail domain that differs significantly in amino acid composition from the tail domain of the predominant isoform GFAPα (14). The relative in vivo expression levels of the GFAPα and GFAPε variants, examining mRNA levels in a total brain sample, are on the order of 20:1 (14). Here we assign a distinct regulatory function to the tail domain of GFAPε that effectively inhibits homodimerization and to some degree heterodimerization with GFAPα. This effect is rather intriguing, since it is the highly conserved coiled-coil region that is directly responsible for dimerization, whereas the tail and head domains of GFAP and of other IF proteins play important roles for correct filament assembly. The RDG motif present in the tail of GFAPα and in many other IF proteins could be involved in the assembly process (16, 24, 26), but, interestingly, the tail of GFAPε lacks the RDG motif. It is unlikely that the dimerization inhibitory effect is due only to the absence of essential amino acid residues, because the truncated GFAPδε, lacking the corresponding tail residues, has retained dimerization potential and because the introduction of specific point mutations in the GFAPε tail can restore the dimerization potential (see Fig. 5). Instead, the inhibitory mechanism may represent a new function gained by the GFAPε isoform. The mechanism is not restricted to GFAP dimerization but also has an effect on interactions with heterologous proteins, suggesting that GFAPε can exist in a protein conformation that inhibits protein-protein contacts mediated through the coiled-coil region. The observation that the GFAPε tail interacts directly with the coiled-coil region (Fig. 4) indicates that such conformation involves intramolecular interactions, and it is envisaged that such interactions compete with intermolecular interactions. One prediction from this model would be that the competition is more in favor of intramolecular interaction when only GFAPε is present, whereas the presence
Fig. 5. **GFAP tail inhibition can be reversed by specific tail mutations.** A, in a yeast two-hybrid assay, a specific mutation in the GFAP tail results in GFAP heterodimerization capacity. LexA fusions of GFAP and GFAP were examined for their capacity to interact with AAD-GFAP with mutations introduced into the tail region. The position of the mutations is shown graphically. β-Galactosidase activity from co-transformed yeast strain L40 was measured as described in the legend to Fig. 1B, and the value obtained for GFAP homodimerization was normalized to 100. Values below 10 were under the limits of measurements in this assay and are indicated as minus signs. Also, a LexA fusion to the presenilin-1 N-terminal region (amino acids 1–85), LexA-PS1, was tested for interaction with the various GFAP tail mutations. B, in yeast, the T411E,I412E GFAP tail mutation results in strong gain of homodimerization. GFAP(T411E,I412E) was recloned in the LexA vector, and was tested for homodimerization capacity in yeast strain L40. Experimental settings were as described above. C, the GFAP tail mutation T411E,I412E can result in partial gain of filament formation in mammalian cells. GFAP(T411E,I412E) was cloned in the mammalian pSG5 expression vector and transfected into SW13cl.2 cells. 48 h after transfection, cells were fixed, and GFAP was visualized by a GFAP antibody and a fluorescent labeled secondary antibody. The GFAP localization was monitored by epi-immunofluorescence microscopy, and GFAP-transfected SW13cl.2 cells were used as control. D, the GFAP tail mutation T411E,I412E results in decreased coiled-coil interaction capacity. GST-GFAP-(391–431) and GST-GFAP-(349–431) was visualized by a histidine tag antibody as described above (left panel). The input lane corresponds to one-tenth of the material used for the pull-down. The same GST fusion proteins as used for the GST pull-down were analyzed by silver staining of a SDS gel (right panel).
of the dimerization-prone GFAPα may facilitate intermolecular interactions through the coiled-coil. It remains to be determined whether GFAPe and GFAPα mRNA are translated at the same subcellular locations so that heterodimerization may proceed immediately or whether the GFAPe monomer is present for longer periods of time within cells. Clearly, overexpression of GFAPe, in the absence of GFAPα, results in aggregate formation (Fig. 2). To this end it might be significant that we have identified, in vitro and in yeast, a GFAPe-specific interaction with the presenilin proteins (14). The interaction requires both residues of the GFAPe-specific tail and residues within coiled-coil region 2 (Fig. 5) (14) and suggests a direct link between the inability of GFAPe to form homodimers and the exposure of the presenilin interaction surface. This linkage is supported by the observation that specific mutations introduced in the GFAPe tail both rescue some dimerization capacity and abolish the presenilin binding capacity (i.e. GFAPe has gained characteristics of GFAPα). It should be noted that the GFAPe coiled-coil residues required for presenilin binding (residues 204–308 (14)) and the residues required for the GFAPe tail interaction (residues 349–390 (Fig. 4C)) do not overlap, indicating that these two interactions are not mutually exclusive. The presenilin interaction might serve a function for the GFAPe monomer as chaperone and be involved in transport of GFAPe to specific cellular compartments, a function of presenilins also designated toward other proteins (27–29).

The GFAPe tail interaction with the coiled-coil was mapped to a region in GFAP coil 2b that shows a high degree of conservation between different intermediate filament proteins (24, 26). This coiled-coil region has been described as essential for filament formation, since even subtle amino acid alterations are deleterious (24). A mapping study of the intermediate filament protein vimentin has revealed an interaction between this coiled-coil region and the RDG segment of the tail (24), and we observed a similar interaction between the GFAPα tail and the coiled-coil region. However, this interaction was several-fold weaker than the GFAPe tail interaction with the coiled-coil, and this indicates that the presence of the GFAPe tail results in a gain of coiled-coil binding activity. Furthermore the interaction could be abolished by a tail mutation that also resulted in gain of dimerization and filament formation (Fig. 5). Our experiments demonstrate a link between the capacity of the GFAPe tail to bind the coiled-coil and to inhibition dimerization, and a simple model may account for the presence of GFAPe in a monomeric form. Given that the GFAPe tail interacting coiled-coil region is essential for the initiation of dimerization, mediated by two parallel coiled-coils, dimerization will proceed from the C terminus toward the N terminus. This process may be strongly disfavored by the presence of two GFAPe tails competing for these regions, and an equilibrium is expected to be maintained between a conformation where the GFAPe tail is associated with the coiled-coil and a conformation where coiled-coil 2b is exposed and prone to dimerize. The presence of also a GFAPα monomere, where the coiled-coil 2b is permanently exposed, may thus result in heterodimer formation. The observation that GFAPe/GFAPα heterodimers do form, but at a reduced level compared with GFAPα homodimerization, is in accordance with such a model (Fig. 1). Also, the stability of already formed heterodimers might be decreased by GFAPe tail competition for the coiled-coil region.

The GFAP filament is normally stabilized by numerous distinct interactions between the dimers/tetramers to form the 10-nm filament. If we altered the relative level of the two GFAP variants in strong favor of GFAPe, the GFAP filament disintegrated, and this might be due to increased amounts of tetramers containing a GFAPe tail at each of the ends (Fig. 3B). By contrast, we observe that a low level of GFAPe does not cause filament collapse. One explanation may be that fewer GFAPe/GFAPα heterodimers are formed decreasing the probability that such two heterodimers form tetramers. GFAPe containing tetramers are expected to be poor building blocks for further multimerization, since they could preferentially expose the GFAPe tail. The consequence for such an exposure could be a steric blocking of coiled-coil regions within the tetramers required for the multimerization process. We have no evidence yet that cell types exist with a GFAPe/GFAPα ratio close to the one experimentally found required to affect filament structure. However, even if the ratio is artificial, the presented experiments show that filament-integrated GFAPe behaves functionally nonequivalent to both GFAPα and GFAP without tail sequence.

We can only speculate as to the exact function of the GFAPe homodimerization-inhibitory capacity and whether it serves as a regulatory mechanism prior to, during, or after the filament assembly process. The inhibitory mechanism could assure that two GFAPe monomers are not positioned next to each other within the intermediate filament. Since the tails of intermediate filament proteins, sticking laterally out from the filament, are thought to constitute a candidate motif for heterologous protein-protein interactions (1, 2, 4), it is appealing to suggest that the GFAPe tail, besides the inhibitory function, also constitutes a surface for mediating new protein contacts between the GFAP filament and other cellular proteins. Such a network of protein interactions could rely on a nonclustered distribution of GFAPe tails at the filament surface for correct function.

To understand GFAP intermediate filaments and the importance and the function within cells of such filaments, it will be important to determine whether molecular pathways exist that regulate GFAPe tail-mediated inhibition of dimerization and filament formation as well as the mechanism that regulates the relative amounts of the two GFAP isoforms.

Acknowledgments—We thank Mariann Johansen for technical assistance, Dr. Robert Evans (University of Colorado Health Sciences Center, Denver, CO) for the gift of SW13c1.1 and SW13c2.2 cells, Dr. Sirpa Aho (Thomas Jefferson University, Philadelphia, PA), for the GST-ennopakin and periplakin expression vectors, and Ronald Liem (Columbia University, New York) for peripherin and internexin cDNA.

REFERENCES

1. Fuchs, E. (1996) Annu. Rev. Genet. 30, 197–231
2. Herrmann, H., and Aebi, U. (2000) Curr. Opin. Cell Biol. 12, 79–90
3. Porchet, R., Probst, A., Bouras, C., Draberova, E., Draber, P., and Riederer, B. M. (2005) Proteomics 5, 1476–1485
4. Eng, L. F., Ghirnikar, R. S., and Lee, Y. L. (2000) Neurochem. Res. 25, 1439–1451
5. Hol, E. M., Roche, R. F., Moraal, E., Sonnemann, M. A., Sluijs, J. A., Proper, E. A., de Graauw, P. N., Fischer, D. F., and van Leeuwen, F. W. (2003) Mol. Psychiatry 8, 786–796
6. Eliassson, C., Sahlgren, C., Berthold, C. H., Stakeberg, J., Celis, J. E., Betscholt, C., Eriksen, J. E., and Pezely, M. (1999) J. Biol. Chem. 274, 23996–24006
7. Brenner, M., Johnson, A. B., Boespflug-Tanguy, O., Rodriguez, D., Goldman, J. E., and Messing, A. (2001) Nat. Genet. 27, 117–120
8. Messing, A., Goldman, J. E., Johnson, A. B., and Brenner, M. (2001) J. Neuropathol. Exp. Neurol. 60, 563–573
9. Johnson, A. B. (2002) Int. J. Dev. Neurosci. 19, 1–4
10. Nielsen, A. L., Jørgensen, P., and Jørgensen, A. L. (2002) J. Neurogenet. 16, 175–179
11. Reeves, S. A., Helman, L. J., Allison, A., and Israel, M. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5178–5182
12. Bongcam-Rudloff, E., Nister, M., Betscholt, C., Wang, J. L., Stenman, G., Huebner, K., Croce, C. M., and Westermark, B. (1991) Cancer Res. 51, 1553–1560
13. Issac, A., Baker, M., Wavrant-De Vrieze, F., and Hutton, M. (1998) Genomics 51, 152–154
14. Nielsen, A. L., Holm, I. E., Johansen, M., Buenen, B., Jørgensen, P., and Jørgensen, A. L. (2002) J. Biol. Chem. 277, 29983–29991
15. Singh, R., Nielsen, A. L., Johansen M. G., and Jørgensen, A. L. (2003) Genomics 82, 185–193
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16. Chen, W. J., and Liem, R. K. (1994) J. Cell Sci. 107, 2299–2311
17. Le Douarin, B., Nielsen, A. L., Garnier, J. M., Ichinose, H., Jeanmougin, F., Losson, R., and Chambon, P. (1996) EMBO J. 15, 6701–6715
18. Nielsen, A. L., Ortiz, J. A., You, J., Oulad-Abdelghani, M., Khechumian, R., Gansmuller, A., Chambon, P., and Losson, R. (1999) EMBO J. 18, 6385–6395
19. Rose, M. D., Winston, F., and Hieter, P. (1990) Methods in Yeast Genetics: A Laboratory Course Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Kazerounian, S., Uitto, J., and Aho, S. (2002) Exp. Dermatol. 11, 428–438
21. Nielsen, A. L., Oulad-Abdelghani, M., Ortiz, J. A., Remboutsika, E., Chambon, P., and Losson, R. (2001) Mol. Cell 7, 729–739
22. Sarria, A. J., Lieber, J. G., Nordeen, S. K., and Evans, R. M. (1994) J. Cell Sci. 107, 1593–1607
23. Leung, C. L., and Liem, R. K. H. (1996) J. Biol. Chem. 271, 14041–14044
24. McCormick, M. B., Kouklis, P., Syder, A., and Fuchs, E. (1993) J. Cell Biol. 122, 385–407
25. Takemura, M., Gomi, H., Colucci-Guyon, R., and Itohara, S. (2002) J. Neurosci. 22, 6972–6979
26. Strelov, S. V., Herrmann, H., and Aebersold, R. (2003) BioEssays 25, 243–251
27. Thinakaran, G. (1999) J. Clin. Invest. 104, 1321–1327
28. Tandon, A., Rogaeva, E., Mullen, M., and George-Hyslop, P. H. (2000) Curr. Opin. Neurol. 13, 377–384
29. Leem, J. Y., Saura, C. A., Pietrzik, C., Christiansen, J., Wanamaker, C., King, L. T., Veselits, M. L., Tomita, T., Gasparini, L., Iwatsubo, T., Xu, H., Green, W. N., Koo, E. H., and Thinakaran, G. (2002) Neurobiol. Dis. 11, 64–82
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J. Biol. Chem. 2004, 279:41537-41545.
doi: 10.1074/jbc.M406601200 originally published online July 27, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406601200

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