A New Splice Variant of Glial Fibrillary Acidic Protein, GFAPε, Interacts with the Presenilin Proteins*

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We describe a new human isoform, GFAPε, of the intermediary filament protein GFAP (glial fibrillary acidic protein). GFAPε mRNA is the result of alternative splicing and a new polyadenylation signal, and thus GFAPε has a new C-terminal protein sequence. This provides GFAPε with the capacity for specific binding of presenilin proteins in yeast and in vitro. Our observations suggest a direct link between the presenilins and the cytoskeleton where GFAPε is incorporated. Mutations in GFAP and presenilins are associated with Alexander disease and Alzheimer’s disease, respectively. Accordingly, GFAPε should be taken into consideration when studying neurodegenerative diseases.

Glial fibrillary acidic protein (GFAP) is a 432-amino acid-long polypeptide of 55 kDa encoded by the human astrocytic mRNA of 2.9 kb representing the dominating astrocytic splicing and a new polyadenylation signal, and thus GFAPε has a new C-terminal protein sequence. This provides GFAPε with the capacity for specific binding of presenilin proteins in yeast and in vitro. Our observations suggest a direct link between the presenilins and the cytoskeleton where GFAPε is incorporated. Mutations in GFAP and presenilins are associated with Alexander disease and Alzheimer’s disease, respectively. Accordingly, GFAPε should be taken into consideration when studying neurodegenerative diseases.

Three additional minor isoforms, termed GFAPβ, γ, and δ, have been described in the rodent. Isoform GFAPβ transcribes a 169-nucleotide downstream of GFAPα and was described in Schwann cells of the peripheral nervous system (9, 10); GFAPγ mRNA is about 2.4 kb and lacks exon 1 but includes the last 126 nucleotides of intron 1. GFAPδ is expressed outside the brain (11); the mRNA of GFAPδ is 4.2 kb and includes all 9 GFAP exons and, in addition, the last 1255 bp of intron 7 spliced in frame to exon 7 (12). The derived hypothetical GFAPδ protein has a new tail domain where the normal C-terminal 42 amino acids encoded by exons 8 and 9 have been replaced by the 33 amino acids encoded by intron 7 sequences used as exon (12).

Missense mutations in the tail domain and the rod domain of GFAP have been implicated in the neurodegenerative process of Alexander disease where astrocytes accumulate GFAP-containing cytoplasmic aggregates (13). Here we describe a novel human GFAP isofrom designated GFAPε. GFAPε has a novel C-terminal tail domain, the integrity of which is required for binding to the Alzheimer’s disease-associated transmembrane proteins presenilin 1 and 2 in yeast and in vitro (14, 15). Thus, GFAPε represents a functionally distinct isoform in terms of presenilin interactions, and this finding suggests a direct link between presenilins and the cytoskeleton.

EXPERIMENTAL PROCEDURES

Plasmids—Details on individual plasmid constructs, which were all verified by sequencing, are available upon request. Human cDNA for GFAP was cloned by PCR from a brain cDNA library. Point mutations were generated as described for QuickChange site-directed mutagenesis (Stratagene). For yeast two-hybrid assays, DBD and AAD plasmids expressing AAD or different AAD fusion proteins were expressed from the yeast multicopy plasmids pBRM165 (16) and pGAD10 (CLONTECH), respectively. His6-tagged constructs were obtained by subcloning the indicated cDNAs into pRSETB In vitro. For GST pull-down experiments and far Westerns, cDNA was subcloned into pGEX2TK (Amersham Biosciences). For mammalian expression pSG5 (16), pcDNA3 (Invitrogen) or green fluorescent fusion vector pEGFP (CLONTECH) was used.

cDNA Library Screening and Yeast Transactivation Assays—A yeast GAL4 activation domain tagged cDNA library derived from human fetal brain (CLONTECH) was introduced by LiAc transformation into the Saccharomyces cerevisiae L40 strain expressing the fusion protein LexA-PS-1 (1–85) from the pBRM165 vector. Approximately 6 × 10⁶ yeast transformants were selected on Trp− Leu− YP plates containing 2 mM 3-aminoimidazole. After 5 days His− clones that have lacZ expression on X-gal indicator plates were isolated. Library plasmids were rescued in Escherichia coli strain JM110 (leuB6) and introduced back into L40 expressing either unfused LexA or different LexA fusion proteins. Positives for interaction specifically with LexA-PS-1 (1–85) were DNA sequence analyzed. Yeast L40 transformants were grown exponentially in selective medium. Yeast extracts were prepared and assayed for β-galactosidase activity essentially as described by Rose et al. (17).

Northern Blotting and RT-PCR—For Northern blotting experiments a mouse 298-bp fragment specific for exon 7a was amplified by PCR,
purified, and randomly labeled with radioactivity. The probe was hybridized onto a mouse tissue MTN blot (CLONTECH) overnight at 65 °C in 5× SSPE (0.9 mM NaCl, 0.05 mM sodium phosphate, 4 mM EDTA), 0.02 mg/ml carrier DNA, 5× Denhardt’s solution, and 0.5% (w/v) SDS. The filter was washed twice for 10 min at room temperature in 2× SSPE, 0.1% (w/v) SDS; twice for 15 min at 65 °C in 1× SSPE, 0.1% (w/v) SDS; and for high stringency twice for 10 min at 65 °C with 0.1× SSPE, 0.1% (w/v) SDS. The blot was revealed by autoradiography. For RT-PCR mouse brain RNA was prepared according to the protocol for the TRI Reagent (Sigma). RT-PCR was done according to the protocol of the Titanium One-step RT-PCR kit (CLONTECH). Briefly, 1 μg of mouse brain RNA was reverse-transcribed at 50 °C for 1 h and subsequently PCR-amplified (94 °C, 30 s; 62 °C, 30 s; and 68 °C, 1 min) for the indicated number of cycles. The following primers were used for the PCR: mGFAP exon 7 forward (PE7f), CATCACATTCTCTGGACATCTTC; mGFAP exon 8 reverse (PE8r), CCACGATGTTCCTCTTAGGGTGAGCCTG. The RT-PCR products were analyzed by 2% agarose gel electrophoresis.

**In Vitro Binding Assays**—GST and GST-PS-1(1–85) fusion proteins were expressed in *E. coli* XL1-blue, purified on glutathione-Sepharose beads, and GST pull-down assays performed essential as described (18, 19). His epitope-tagged GFAP fusion proteins were expressed in *E. coli* BL21(DE3) and purified on Ni²⁺-chelating columns (Amersham Biosciences). Labeling of GST fusion proteins and far Western experiments were done exactly as described (20).

**Immunological Methods**—GFAP antibody was raised in rabbit against a fusion protein GST-GFAP(390–431) consisting of GST fused to amino acids 390–431 of GFAPs. The polyclonal antibody was designated pAb-GFAPe. In Western blotting the antibody was used in a 1:800 dilution. Green fluorescent protein (GFP) antibody (Roche Molecular Biochemicals) was used in a 1:1000 dilution. His tag antibody (Santa Cruz Biotechnology) was used in a 1:2000 dilution. In epi-immunofluorescence experiments pAb-GFAP was used in a 1:300 dilution; FLAG antibody 1B11 (18) was used in concentration 1:750; and fluorescein isothiocyanate or TRITC-labeled goat anti-rabbit or rabbit anti-mouse secondary antibodies (Molecular Probes) were diluted 1:200. For epi-immunofluorescence analysis cells were grown in slide flasks (Nunc), and to induce cytoskeletal rearrangement medium was changed to serum-free media 24 h after transfection. After a further 24 h of incubation, cells were washed twice in PBS, and fixed in 2% paraformaldehyde, 0.1% glutaraldehyde, 0.1% Triton X-100 for 30 min on ice. The fixed cells were washed in PBS, and antibody incubations were done in PBS, 5% fetal calf serum for 1 h at 4 °C.

To generate the Triton-insoluble cell fraction enriched in cytoskeletal proteins, cells were washed twice in PBS and scraped off the culture dishes. After centrifugation at 2000 × g for 5 min, the cell pellet was homogenized in 170 mM NaCl, 600 mM KCl, 1% (w/v) Triton X-100, 6 mM sodium phosphate (pH 7.4), 1 mM EDTA, and protease inhibitor mixture (homogenate fraction, H0). After centrifugation at 8000 × g for 10 min the supernatant was restored (TSE fraction), and the pellet was washed twice in PBS and suspended in SDS-PAGE buffer (TIE fraction). Triton-insoluble extracts from brain material were similarly prepared except for the inclusion of an additional Triton extraction step.

**Accession Number**—The GenBank™ accession number for the human GFAPe exon 7a sequence is AJ306447.

**RESULTS**

**GFAPe Interacts with Presenin 1 in Yeast and in Vitro**—We used the yeast two-hybrid system to identify cDNAs encoding proteins with a capacity for interacting with PS-1. A fusion between the DBD of the LexA protein and the N-terminal 85-amino acid cytoplasmic region of PS-1, GST-PS-1(1–85), was used as a bait to screen a library of human fetal brain cDNAs fused to the yeast GAL4 activation domain (AAD). Around 6 × 10⁵ yeast transformants were screened, and those that gave a low sensitive histidine deficient yeast transformants and showed positive X-gal indicator were isolated. The isolated cDNAs were used as yeast retrotransformants, and cDNAs were sequenced from transformants that were positive for interaction with PS-1, but negative for interaction with an unfused DBD or DBD fusions to unrelated proteins. Five cDNAs isolated were shown by BLAST sequence homology searches to have high homology to the DNA sequence of the gene coding for the human GFAP. Four of the five cDNA isolates were identical and encoded an entire GFAP protein except for the three N-terminal residues. They were named GFAP46. The sequence of the fifth cDNA isolate, named GFAP21, was shorter and included in the GFAP46 sequence.

In a liquid β-galactosidase assay we tested the capacity of AAD-GFAP cDNAs and DBD-PS-1(1–85) or DBD-PS-2(1–93) in yeast strain L40 activates β-galactosidase expression. L40 was transformed with high copy number plasmids containing DBD fusions to various parts of PS-1, PS-2, HP1α, lamin, or no insert. PS-1(1–85) and PS-2(1–93) correspond to the first 85 amino acids of the cytoplasmic N-terminal region, PS-1(257–376) to the cytoplasmic loop, and PS-1(405–446) to the C-terminal region of the PS-1 molecule. Strains were retransformed with AAD alone or AAD fusions to GFAP cDNA identified in the yeast two-hybrid screen. Double transformants were assayed for β-galactosidase activity expressed from an integrated lacZ gene transcriptionally regulated by LexA-binding sites. β-Galactosidase activities are the averages from three independent transformants assayed the same day. b, PS-1 binds to GFAPe in vitro. Purified N-terminal His-tagged GFAP46, His-GFAPe, was incubated in a batch assay with GST (2nd lane) or the fusion protein GST-PS-1(1–85) (3rd lane) bound to glutathione S-Sepharose beads. Bound GFAP was monitored by Western blotting using an His tag antibody. The 1st lane shows 1/10 the amount of input His-GFAPe (indicated by an arrow).

**FIG. 1.** a, GFAP interacts with presenilins. Co-expression of AAD-GFAP cDNAs and DBD-PS-1(1–85) or DBD-PS-2(1–93) in yeast strain L40 activates β-galactosidase expression. L40 was transformed with high copy number plasmids containing DBD fusions to various parts of PS-1, PS-2, HP1α, lamin, or no insert. PS-1(1–85) and PS-2(1–93) correspond to the first 85 amino acids of the cytoplasmic N-terminal region, PS-1(257–376) to the cytoplasmic loop, and PS-1(405–446) to the C-terminal region of the PS-1 molecule. Strains were retransformed with AAD alone or AAD fusions to GFAP cDNA identified in the yeast two-hybrid screen. Double transformants were assayed for β-galactosidase activity expressed from an integrated lacZ gene transcriptionally regulated by LexA-binding sites. β-Galactosidase activities are the averages from three independent transformants assayed the same day. b, PS-1 binds to GFAPe in vitro. Purified N-terminal His-tagged GFAP46, His-GFAPe, was incubated in a batch assay with GST (2nd lane) or the fusion protein GST-PS-1(1–85) (3rd lane) bound to glutathione S-Sepharose beads. Bound GFAP was monitored by Western blotting using an His tag antibody. The 1st lane shows 1/10 the amount of input His-GFAPe (indicated by an arrow).
ings, the retained protein was analyzed by SDS-PAGE followed by Western blotting and visualized by an antibody against the His tag. The input lane (Fig. 1b) corresponds to 1/10 of the material used in each GST pull-down assay. GST-PS-1(1–85) retained a substantial amount of His-GFAP46 ensuring a direct interaction in vitro.

GFAPΔ66 and GFAPΔ21 Represent a New Splice Product, GFAPΔs, of the GFAP Gene—The sequences of GFAPΔ21 and GFAPΔ66 cDNA inserts revealed identity to the 5′-region of the normal human GFAP transcript, GFAPΔs, but differed completely in the 3′-region (Fig. 2) (3, 4). This indicated that the GFAPΔ21 and GFAPΔ66 cDNAs represent a new mRNA splice product of the human GFAP gene which we designated GFAPΔs. BLAST homology searches identified homology between the 3′-region of the GFAPΔ21 and GFAPΔ66 cDNAs and sequences included in a newly identified rat 4.2-kb GFAP splice variant designated GFAPΔs (12). Sequence analysis showed that the divergence between GFAPΔs and GFAPΔs was the result of usage of a part of GFAPΔ gene intron 7 as an exon in GFAPΔs (Fig. 2b) (5). The new consensus splice acceptor site was identified in the genomic sequence (Fig. 2c). The new exon utilized in GFAPΔs was designated exon 7a. A perfect match with the polyadenylation signal, AATAAA, was present in the extreme 3′-end of exon 7a, denoted pAΔs (Fig. 2). The sequence of GFAPΔ66 cDNA showed polyadenylation 26 bases downstream of the polyadenylation signal in exon 7a which therefore was functional in human cells (Fig. 2b). Moreover, the sequence of the mouse GFAP gene showed that splice acceptor sites corresponding to the alternative exon 7a splicing and the polyadenylation signal were evolutionary conserved (Fig. 2c). By usage of pAΔs, exon 8 and exon 9 are skipped and the size of the human and mouse GFAPΔs mRNAs will be 1.8 and 2.5 kb, respectively. Note that in mouse the GFAPΔs and GFAPΔs mRNAs will have roughly same molecular weight. GFAPΔs mRNA was detected solely in the brain in Northern blot experiments by using human or mouse brain mRNA probed with exon 7a sequences (Fig. 2d and data not shown). The relative expression levels of the two GFAP isoforms were determined by RT-PCR. Mouse brain RNA was in a coupled RT-PCR amplified with either a GFAPΔs-specific, a GFAPΔs-specific, or a mixed primer set. The RT-PCR was run for a variable number of cycles to ensure reaction points in which the PCR amplification was exponentially increasing. The relative expression level of GFAPΔs mRNA was estimated to be about 20-fold less than that of GFAPΔs mRNA (Fig. 2e).

The alternative splicing of GFAPΔs mRNA was translated into a unique tail domain of GFAPΔs. The 43-amino acid C-terminal tail region of GFAPΔs encoded by exon 8 and exon 9 in GFAPΔs was replaced by the 42 amino acids encoded by exon 7a (Fig. 2f). This GFAPΔs-specific tail domain showed only little homology to GFAPΔs or other IF protein sequences (Fig. 2f).

Mapping of the Sequences in PS-1 Required for GFAPΔs Interaction—To detect the region in PS-1 responsible for the GFAPΔs interaction, we introduced a series of N- and C-terminal deletions and missense mutations in DBD-PS-1(1–85) and assayed for interaction with AAD-GFAPΔs-(3–431) in the two-hybrid system (3a). As exemplified by the fusion protein DBD-PS-1(66–85), deletion of the first 65 of the N-terminal residues does not influence the AAD-GFAPΔs interaction, and DBD-PS-1(1–85del66–72), in which a highly acidic stretch of amino acids (66–72) has been deleted, also retained the capacity for interaction. By contrast, removal of only three amino acids from the C-terminal end, as exemplified by DBD-PS-1(1–82), completely abolished interaction. The same effect was seen if these residues were changed by single or double missense mutations. We noted that interaction was abolished in the non-conservative amino acid substitutions V82K and V82E but was retained in DBD-PS-1(V82L) which carried a conservative amino acid substitution associated with familial Alzheimer’s disease. Also DBD-PS-1(A79V) that is associated with Alzheimer’s disease had no effect on GFAPΔs binding (Fig. 3a).

The Alternative Exon 7a in GFAPΔs Is a Determinant for PS-1 Interaction in Vitro and in Yeast—To map the amino acids in GFAPΔs which take part in the PS-1 interaction, we utilized the far Western assay to monitor protein-protein interactions. Various deletions of GFAPΔs were expressed in E. coli as fusion proteins to a His tag, purified, and blotted onto a nitrocellulose filter. The filter was probed with 32P-labeled GST-PS-1(1–85), and after an extensive wash, the retained radioactivity was monitored by autoradiography. As expected, GST-PS-1(1–85) interacts with His-GFAPΔs-(3–431) and His-GFAPΔs-(204–431) which correspond to the sequences of GFAPΔ66 and GFAPΔ21, respectively (Fig. 3b, left panel). Labeled GST did not interact with these proteins (data not shown). His-GFAPΔs-(309–431) and His-GFAPΔs-(349–431) were negative for GST-PS-1(1–85) interaction showing that sequences in the C-terminal end of the coiled-coil region, which is conserved among the different GFAP splice variants, are required for PS-1 interaction (Fig. 3b, left panel). Because His-GFAPΔs-(204–390) was also negative for GST-PS-1(1–85) interaction, the GFAPΔs-specific C-terminal tail sequences were also required for PS-1 interaction (Fig. 3b). Purified full-length GFAPΔs was found not to interact with GST-PS-1(1–85) as expected because it did not include the PS-1 interaction region (Fig. 3b, right panel). By Western blotting using an antibody against the histidine tag, it was verified that an equal amount of proteins was used for the far Western analysis (Fig. 3c).

We also used the yeast two-hybrid system to define the GFAPΔs residues required for PS-1 interaction at more physiological conditions. Consistent with the far Western results AAD-GFAPΔs-(204–431) interacted with DBD-PS-1(1–85), whereas deletion of GFAPΔs sequences in the C-terminal end of the coiled-coil region in both AAD-GFAPΔs-(309–431), AAD-GFAPΔs-(349–431), and AAD-GFAPΔs-(390–431) completely abolished the interaction (Fig. 3d). The region between amino acids 204 and 309 includes the linker 1–2, coiled-coil 2A, and the beginning of coiled-coil 2B. The deletion of exon 7a encoded amino acids in AAD-GFAPΔs-(204–390) abolished PS-1 interaction (Fig. 3d). In conclusion, the PS-1 interaction domain in GFAPΔs is large or bipartite and requires both the GFAPΔs-specific tail sequence as well as sequences overlapping with the coiled-coil 2 and linker 1–2 shared by GFAPΔs and GFAPΔs. Full-length GFAPΔs was also tested for interaction with PS-1 and in agreement with the above mapping data and the far Western results (Fig. 3b) no interaction was observed between DBD-PS-1(1–85) and AAD-GFAPΔs.

GFAPΔs Is an Expressed Protein in Vivo and Incorporates into Filaments—293 cells were transfected with expression plasmids encoding GFP alone or fused to the N-terminal of GFAPΔs (pGFP-GFAPΔs) or GFAPΔs (pGFPGFAPΔs). Cellular extracts were analyzed by Western blotting using either an antibody against GFP or a pAb-GFAPΔs that was raised in rabbit against the 42-amino acid C-terminal tail region specific for the GFAPΔs isoform (Fig. 4a). Although the GFP antibody detected both GFP fusion proteins, the GFAPΔs antibody recognizes only GFAPΔs (lane 3) thus ensuring the antibody specificity. The Western blot in Fig. 4b shows GFAPΔs expression in 293 cells transfected with the expression vector pSG5 without insert (lane 1), with the GFAPΔs insert (lane 2), and a total cellular extract from the astrocyte-derived cell line SVG(P12) (lane 3). The antibody pAb-GFAPΔs detected in lane 3 an endogenous band of the expected GFAPΔs size (55 kDa) co-migrating with transfected untagged GFAPΔs (lane 2). When the antibody was
pre-adsorbed with the GFAPe antigen the endogenous band of the SVG(P12) extract disappeared, whereas pre-adsorption with GST did not affect reactivity of the antibody (Fig. 4c). By fractionation of SVG(P12) cells to obtain the Triton X-100-insoluble extract (TIE) enriched in cytoskeletal proteins, GFAPe localization was determined to be in the TIE fraction in

**Fig. 2. Sequence characteristics and splice pattern of the new human GFAP variant, GFAPe.** a, graphical representation (not scaled) of the 3′-GFAP gene structure. Exons are indicated by rectangles, and the length of the exons and introns is shown in the lower part of the figure. The GFAPe transcription product includes exons 7a, whereas GFAPα, -β, and -γ transcripts utilize exons 8 and 9. As a result of the new polyadenylation signal in exon 7a, the transcript is truncated by 3.35 kb compared with a readthrough to the polyadenylation signal, pAα, in exon 9. b, DNA sequence of the region, designated exon 7a, in the GFAP46 and GFAP21 cDNAs that is not present in GFAPα. An arrow indicates the 3′-end of the GFAP21 cDNA. The consensus polyadenylation signal, pAα, in exon 7a is underlined, and a part of the poly(A) stretch of GFAP46 is included in the sequence. The in-frame translated peptide of GFAPe that replaces the normal GFAPα C terminus encoded by exons 8 and 9 is shown above the DNA sequence. c, DNA homology of the GFAPe exon 7a splice acceptor site (upper panel) and polyadenylation signal (lower panel) between human and mouse. The abbreviations used are as follows: y, pyrimidine; r, purine; u, uracil; and n for either pyrimidine or purine. Exon sequences are indicated by capital letters, and conserved residues are indicated by a vertical line. The splice consensus sequence is shown above the sequence, and the branch point is indicated by a dot. d, GFAPe is identified in the brain by Northern blotting experiments. By PCR a 288-bp probe specific for the mouse exon 7a was amplified, radiolabeled, and used as a probe on a multiple tissue Northern blot with mRNA from different mouse tissues. e, GFAPe represents a minor GFAP mRNA species. The relative mRNA amounts of GFAPα and GFAPe were determined by RT-PCR using mouse brain RNA. Primers specific for the two GFAP isoforms (PE7f and PE8r for GFAPα and PE7f and PE7ar for GFAPe) were either individually used or mixed in the indicated number of PCR cycles. The PCR products were analyzed by 2% agarose gel electrophoresis. Sizes of DNA marker in lane M are indicated to the left. f, sequence homology between human and mouse protein sequences encoded by exon 7a of GFAPe (upper part) and the exons 8 and 9 of GFAPα (lower panel). Identical amino acids are indicated by a vertical line and conservative amino acid changes indicated by dots.
agreement with GFAPε being an intermediate filament protein (Fig. 4d). Also in a porcine brain extract a protein with the expected size of GFAPε was observed in the TIE fraction enriched in cytoskeletal proteins together with a 35-kDa immunoreactivity of unknown origin (Fig. 4e). Thus the alternative GFAP splicing seems to be reflected in the expression of the corresponding GFAPε protein in vivo.

To examine the subcellular localization of GFAPε, we trans-
fected the mammalian N2A neuroblastoma cell line with an expression vector encoding full-length GFAP. The GFAPε protein was detected by pAb-GFAPε and a fluorescence-labeled anti-rabbit antibody. In the transfected N2A cells GFAPε was incorporated into a structural network further supporting that GFAPε is an intermediate filamentous protein (Fig. 4f).
GFAPε and GFAPα tails strongly diverge as seen in Fig. 2. The GFAP tail has been shown previously to be involved in correct filament incorporation (27). To compare GFAPε and GFAPα incorporation into filaments, we fused the cDNAs to N- and C-terminal GFP tags. N2A cells were transfected with the GFP constructs, and the subcellular localization was examined by fluorescence microscopy. Unfused GFP was dispersed throughout the entire cell (Fig. 5). GFAPε by fluorescence microscopy. Unfused GFP was dispersed throughout the entire cell (Fig. 5). GFAPε tagged in the N-terminal by GFP; b, GFAPε tagged in the C-terminal by GFP; c, GFAPα tagged in the N-terminal by GFP; d, GFAPα tagged in the C-terminal by GFP; e, neurofilament protein NFL tagged in the N-terminal by GFP; and f, unfused GFP.

FIG. 5. GFAPε is assembled into filaments. N2A cells were transfected with GFP-tagged GFAP proteins, neurofilament protein NFL, or unfused GFP. The transfected cells were visualized by epifluorescence microscopy. a, GFAPε tagged in the N-terminal by GFP; b, GFAPε tagged in the C-terminal by GFP; c, GFAPα tagged in the N-terminal by GFP; d, GFAPα tagged in the C-terminal by GFP; e, neurofilament protein NFL tagged in the N-terminal by GFP; and f, unfused GFP.

To examine subcellular co-localization of GFAPε and GFAPα, a double labeling approach was utilized where N-terminal GFP-tagged GFAPα and untagged GFAPε were transfected into N2A cells. GFAPε was specifically labeled by the pAb-GFAPε antibody and stained with a red fluorescent-labeled secondary antibody (Fig. 6). By merging the GFP and red fluorescent-stained cells, a perfect overlap in localization was observed indicating that the two GFAP isoforms were polymerized into the same filamentous structures.

Furthermore, we examined the co-localization between GFAPε and PS-1. For this N-terminal GFP-tagged GFAPε and N-terminal FLAG epitope-tagged PS-1 were transfected into N2A cells. The PS-1 localization was determined by an anti-FLAG antibody and a red fluorescent-labeled secondary antibody. As already extensively documented, PS-1 localized to the perinuclear region and cytoplasmic granules. Consequently, we observed that a subpopulation of the GFAPε pool co-localized with PS-1 (Fig. 6).

DISCUSSION
Glial fibrillary acidic protein, GFAP (isoform GFAPα), is one of the main intermediary filament proteins of the astrocytic cytoskeleton. Here we describe a novel isoform of human GFAP, designated GFAPε. The corresponding GFAPε mRNA is the result of alternative splicing where exon 8 and exon 9 of the GFAPα transcript are substituted by a new exon, designated exon 7a, located in intron 7 of the GFAP gene. Exon 7a is flanked by splice consensus sequences in the 5'-end and a polyadenylation signal in the 3'-end. In addition we identified the corresponding mouse GFAPε transcript that is expressed predominantly in the brain (Fig. 2). GFAPε mRNA was determined to be about 20-fold less expressed than GFAPα mRNA (Fig. 2), a ratio in accordance with the relative abundance of EST clones of each isoform. The GFAPε mRNA-derived human protein, GFAPε, is 431 amino acids long, one amino acid shorter than GFAPα, and has a deduced molecular mass of 55 kDa. The GFAPε protein was identified in central nervous system derived cell lines and in a porcine brain extract and had Triton X-100 extraction characteristics as expected of a cytoskeletal protein (Fig. 4). The head and rod regions of GFAPε are identical to the head and rod regions of GFAPα. But the tail region of GFAPε encoded by exon 7a is completely different from the tail region of GFAPα encoded by exons 8 and 9. This difference in the tail regions suggested different functions of GFAPε and GFAPα, and it might be significant that the tail region of GFAPα is almost 100% conserved, whereas the tail in GFAPε allows for 25% divergence between the human and mouse sequences (Fig. 2f).

It has been suggested recently (21–23) that PS-1, in particular the 30-kDa N-terminal fragment of the protease processed PS-1, interacts with cytoskeletal proteins. High expression of both PS-1 and GFAP is observed in astrocytes associated with cerebral infarction and astrocytoma (24) and in reactive astrocytes surrounding the senile plaques of Alzheimer’s disease (25). The GFAPε transcript was identified by screening a human fetal brain cDNA library for translation products capable of binding PS-1 (Fig. 1). The tail of GFAPε is indispensable for the PS-1 binding in yeast and in vitro and cannot be replaced by the exon 8- and exon 9-encoded tail of GFAPα (Fig. 3). Moreover, sequences overlapping coiled-coil region 2 common to GFAPε and GFAPα are required for PS-1 binding.
IF proteins form polymers where the rod domain promotes formation of a coiled-coil dimer between two parallel IF proteins. The dimers associate in an antiparallel manner to form a nonpolarized tetrameric substructure where coiled-coil region 1 from one dimer is associated with coiled-coil 2 from the other dimer. The tetrameric structure appears to be the fundamental subunit from which the IF is assembled. The head domain seems to govern both end-to-end and lateral associations, whereas the tail domain may project from the surface of the filament and mediate interaction with other cellular components (1, 2). The variable tail domain may thus confer cell-specific property of the IF proteins (26). Accordingly, PS-1 and PS-2 interact with the GFAP/H9280 tail and coiled-coil region 2. Such an interaction would leave coiled-coil region 1 free to interact with coiled-coil region 2 of another anti-parallel dimer during fiber formation and link a presenilin-containing membrane to the cytoskeleton. Note that a fraction of PS-1 is associated with the /H9280-cadherin-catenin complex, which serves as a cytoskeletal attachment site on the plasma membrane and has a function in cell-cell communication (28, 29). Similarly, the

**Fig. 6. GFAPe and GFAPe co-localize and GFAPe partly co-localizes with PS-1.** N2A cells were transfected with the indicated expression vectors, and transfected cells were visualized by epi-fluorescence microscopy. a and b, cells were transfected by GFAPe tagged in the N terminus by GFP and stained with pAb-GFAPe and a red fluorescent secondary antibody. c and d, cells were transfected by untagged GFAPe and stained with pAb-GFAPe and a red fluorescent secondary antibody. e–g, GFAPe and GFAPe co-localize. N2A cells were co-transfected with GFAPe GFP tagged in the N terminus and untagged GFAPe. Cells were stained with pAb-GFAPe and a red fluorescent secondary antibody. h and i, cells were transfected by GFAPe tagged in the N terminus by GFP and stained with a FLAG antibody and a red fluorescent secondary antibody. j and k, cells were transfected by FLAG-PS-1 and stained with FLAG antibody and a red fluorescent secondary antibody. l–n, GFAPe and PS-1 partly co-localize. N2A cells were co-transfected with GFAPe GFP-tagged in the N terminus and FLAG epitope-tagged PS-1. Cells were stained with FLAG antibody and the red fluorescent secondary antibody. a, c, e, h, j, and l, images obtained in the green wavelength; b, d, f, i, k, and m, images obtained in the red wavelength; and g and n, merged images from e and f and l and m, respectively.
observed co-localization between a subpopulation of GFAPε and PS-1 might be in agreement with a function of the GFAPε and PS-1 interaction as a linkage between different structures within the cell.

The RDG motif present in the tail of GFAPα has been implicated in correct filament formation based on C-terminal deletion studies and is evolutionarily conserved in type III IF proteins (27). The RDG motif is absent from the tail region of GFAPΔ. However, in transfected cells GFAP can assemble into filaments (Figs. 4 and 5). This filament formation was indistinguishable from the filament formation with GFP-tagged GFAPΔ. Accordingly, co-localization was observed between GFAPα and GFPΔ in transfected N2A cells (Fig. 6). The observed filamentous incorporation could be due to heteromeric assembly with endogenous intermediate filament proteins. It should be noted that we have been unable to monitor direct interactions between the neurofilament proteins and GFAPΔ at least in the yeast two-hybrid system, whereas GFAPα and GFAPε interact with each other (data not shown).

Amino acids 1–65 of the PS-1-(1–85) fragment can be removed without any effect on the binding capacity of GFAPΔ, whereas amino acids 85–88 are essential for the binding capacity. Also valine at position 82 seems critical because 85 are essential for the binding capacity. Also valine at position 82 seems critical because 85 are essential for the binding capacity.

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