Role of glial filaments in cells and tumors of glial origin: a review

JAMES T. RUTKA, M.D., PH.D., MASAJI MURAKAMI, M.D., PETER B. DIRKS, M.D.,
SHERRI LYNN HUBBARD, B.S.C., LAURENCE E. BECKER, M.D., KOZO FUKUYAMA, M.D.,
SHIN JUNG, M.D., ATSUSHI TSUGU, M.D., AND KAZUHITO MATSUZAWA, M.D.

In the adult human brain, normal astrocytes constitute nearly 40% of the total central nervous system (CNS) cell population and may assume a star-shaped configuration resembling epithelial cells insofar as the astrocytes remain intimately associated, through their cytoplasmic extensions, with the basement membrane of the capillary endothelial cells and the basal lamina of the glial limitans externa. Although their exact function remains unknown, in the past, astrocytes were thought to subserve an important supportive role for neurons, providing a favorable ionic environment, modulating extracellular levels of neurotransmitters, and serving as spacers that organize neurons. 

Biological Features of Astrocytes

Astrocyte swelling represents one of the initial reactions of astrocytes to a variety of insults, the most important of which is hypoxia. Following injury to the human CNS, as a result of trauma, genetic disorders, or chemical insults, astrocytes become “reactive” and respond in a stereotypical manner termed astrogliosis. Astrogliosis is characterized by astrocyte proliferation and extensive hypertrophy of the cell body and cytoplasmic processes. The hallmark cytopathological feature of astrogliosis is a marked upregulation of cytoplasmic glial filaments (see following section). Two examples of human diseases in which the process of astrogliosis is particularly intense are multiple sclerosis and leukodystrophy.

The process of astrogliosis has been thoroughly studied.

KEY WORDS • glial filament • glial fibrillary acidic protein • cytoskeleton • intermediate filament • phosphorylation • kinase • astrocyte
Glial fibrillary acidic protein in glial cells and tumors

Intermediate Filaments: Classification and Structure

The cytoskeleton of eukaryotic cells is composed of three major protein networks: 1) the 6-nm-diameter actin microfilaments; 2) the 20-nm-diameter microtubules; and 3) the intermediate filaments (IFs) that range in diameter from 8 to 12 nm, so named because they are intermediate in size between the actin microfilaments and the microtubules. In contrast to the evolutionarily conserved actin and microtubule proteins, IF proteins are highly diverse and exhibit cell type specificity of expression. Intermediate filament gene expression is also tightly coordinated with organ development and tissue differentiation. Accordingly, IFs provide valuable tools with which to study cell determination and differentiation.

On the basis of amino acid sequence homologies and intron positions, IFs can be subdivided into at least six principal types. Keratins are the sole members of the Type I (acidic) and Type II (basic) classes. Vimentin, desmin, GFAP, and peripherin are designated as Type III IF proteins, sharing more than 70% sequence homology (Fig. 2). The neurofilament proteins are composed of light, medium, and heavy chain proteins and, together with α-internexin, are classified as Type IV IFs. Lamin is the only example of a Type V IF. A new IF protein, called nestin, which is clearly a member of the IF superfamily, based on sequence and structure analyses, and which has been localized to neuroepithelial stem cells early in neuroembryogenesis, has been described and classified as a Type VI IF.

Glial Fibrillary Acidic Protein: a Marker for Cells and Tumors of Glial Origin

Glial fibrillary acidic protein is a 50-kD intracytoplasmic filamentous protein that constitutes a portion of, and is specific for, the cytoskeleton of the astrocyte. This GFAP shares considerable structural homology with the other IFs in the central α-helical or rod domain. In sharp contrast to this highly conserved central rod domain, the NH₂- and COOH-terminal regions of all IFs have distinct amino acid sequences and widely divergent structural features (Fig. 2). Because of the variability in the NH₂-terminal segment among the several classes of IFs, this segment has been presumed to participate in the regulato-
functions of these proteins. In the case of GFAP, all four amino acid residues in the NH$_2$-terminal region that undergo phosphorylation are located here (see discussion of phosphorylation in Posttranslational Modifications in GFAP).

First isolated from white matter plaques of patients with longstanding multiple sclerosis, GFAP is the most widely used marker for cells of astrocytic origin under normal and pathological conditions. Highly specific polyclonal antibodies to human GFAP have been available since approximately 1975, and application of these antibodies to immunohistochemical studies in the CNS have been extremely helpful in determining brain response to injury and in refining tumor diagnosis. Notwithstanding, GFAP immunohistochemistry may lead to variable results depending on the type of antiserum used (polyclonal antiserum vs. monoclonal antibodies), shelf life of the primary antiserum used, type of fixative, interval of fixation, and embedding medium selected. McLendon, et al., have used Southern blot hybridization of somatic cell hybrids and in situ hybridization to assign the chromosomal location of the human GFAP gene to chromosome 17 (17q21). Mapping studies and sequencing have shown that monoclonal antibodies reacting to different epitopes of GFAP can be combined to enhance the specificity and sensitivity of GFAP immunostains in fixed tissue sections.

Interestingly, with increasing astrocytic malignancy, there is progressive loss of GFAP production. GFAP immunostains in fixed tissue sections. By immunohistochemical analysis, malignant astrocytomas are shown to have fewer tumor cells that stain positively and intensely for GFAP than do less malignant astrocytomas and normal brain specimens. For these reasons, GFAP has been considered a reliable marker of differentiation in normal astrocytes and tumors of astrocytic lineage. It is also well accepted, however, that GFAP may be found less commonly in CNS tumor cells that, although neuroepithelial in origin, are not strictly speaking astrocytic, including the oligodendroglioma, ependymoma, primary neuroectodermal tumor, and choroidal plexus papilloma. In these tumor types, care must be taken to distinguish between GFAP-positive neoplastic cells and entrapped or peritumoral reactive astrocytes, both of which express abundant GFAP. Interestingly, GFAP may be expressed in cells outside the CNS, such as nonmyelinating Schwann cells, the epithelium of the lens, the epithelial cells of salivary glands and their neoplasms, and neoplastic cells of Mullerian origin.

**Molecular Biology of GFAP**

The amino acid sequence of human GFAP has been deduced from the nucleotide sequence of complementary DNA (cDNA) clones encoding this protein. Mouse and human GFAP genomic genes have also been cloned and sequenced. There is high homology among rat, mouse, and human GFAP in the coding regions of the gene, but less so in the 3’ untranslated region. As such, GFAP shows 90 to 95% homology between species. Bongcam-Rudloff, et al., have used Southern blot hybridization of somatic cell hybrids and in situ hybridization to assign the chromosomal location of the human GFAP gene to chromosome 17 (17q21). Mapping studies indicate that GFAP is part of a large syntenic gene cluster including at least 22 genes located on chromosome 17.

**Transcriptional Regulation of the GFAP Gene.** Protein-encoding mammalian genes frequently contain a basal promoter region with DNA sequences that guide the transcription of the gene by assembling RNA polymerase II and other general transcription factors. In most mammalian genes, the repeated DNA nucleotide sequence of thymidine-adenosine-thymidine-adenosine (or “TATA” sequence) is found 29 to 33 bp upstream of the RNA start site. This TATA box binds general transcription factor IID, which is essential for the transcription of the gene from the TATA region and the initiation of RNA synthesis.
DNA to messenger (m)RNA. Nakatani and associates showed that GFAP has a basal promoter sequence with the "TA T" motif 29 bp from the RNA start site (Fig. 4). In addition to transcriptional regulation by basal promoter sequences such as "TATA" and "CAAT," expression of the GFAP gene is controlled, in part, by positive regulatory elements between −250 and −80 bp and between −1980 and −1500 bp, and a negative regulatory region at −650 to −360 bp. Within the positive regulatory region, the human GFAP promoter contains transcription factor binding sites for the cAMP responsive element binding protein, NF-1, AP-1, and AP-2 transcription factors. These transcription factors are among the first proteins induced by many stimuli and mediate some of the changes in gene regulation induced by hormones, growth factors, and antigens. The positive regulatory region between −1980 and −1500 bp also contains DNA sequence information that confers glial cell–specific expression of GFAP. This sequence has been termed the human GFAP consensus sequence (hgc).

In addition to transcriptional regulation by basal promoter sequences such as TATA, the pattern of expression for a gene is also controlled in part by other DNA sequences that activate or inhibit transcription. Sequences that activate gene transcription are called "enhancers," whereas those that inhibit gene transcription are called "silencers." These sequences are typically found within 1 to 2 kb upstream of the RNA start site, but may be located further upstream or downstream depending on the tertiary or quaternary structure of the gene. In general, gene enhancers or silencers achieve their effects by binding proteins that interact directly with the basal transcriptional machinery or indirectly through intermediary proteins. The results from a number of laboratories now show that gene enhancer or positive regulatory regions of the GFAP gene exist between −250 and −80 bp and between −1980 and −1500 bp, and a gene silencer or negative regulatory region is found at −650 to −360 bp (Fig. 4). Within its positive regulatory region, the human GFAP promoter contains transcription factor–binding sites for the cyclic adenosine monophosphate (cAMP)–responsive element–binding protein, Sp-1, NF-1, AP-1, and AP-2 transcription factors. These transcription factors are among the first proteins induced by many stimuli and mediate some of the changes in gene regulation induced by hormones, growth factors, and antigens. As one example, the ILs, which are released during brain inflammatory responses, lead to increased levels of c-jun and c-fos. These early response genes have been shown to bind to the AP-1 binding site on the GFAP promoter, indicating that one of the effects of cytokine expression may be increased GFAP transcription.

The promoter region of the GFAP gene also contains DNA sequence information that confers glial cell–associated expression to cells of the CNS, namely the astrocytes. As for the sequence of the GFAP promoter that controls glial cell–associated expression, there has been some debate among different laboratories. However, a consensus sequence for glial cell–specific expression has been located in the positive regulatory region of the human GFAP promoter and has been termed the human GFAP consensus sequence, or “hgc.” At the present time, a yet-to-be-determined transactivating protein(s) binds this sequence and enhances GFAP transcription. Once identified, this protein, which specifically binds to the GFAP promoter and enhances glial cell–specific expression, may be used with the GFAP promoter that is linked to other genes of interest in neurobiology or neurooncology to express them specifically in astrocytes in culture or in transgenic animals (see following subsection). The information obtained from such studies could conceivably lead to interventions in brain disorders during neural development, brain injury, and glial tumorigenesis.

**Glial Fibrillary Protein–Transgenic Mouse Studies.** Attempts to elucidate the function of given proteins have
been enhanced significantly since the development of transgenic mouse technology, in which a gene encoding the protein of interest can be microinjected into a developing mouse embryo (Fig. 5). Once born, the mouse displays the phenotype of the protein, which may be expressed either ectopically or in excess. The first reported transgenic study using the GFAP promoter was reported by Mucke, et al.76 In that report and in several others that followed,11 the promoter region of the GFAP gene was found to direct the activity of a reporter gene in cells of the CNS with little, if any, expression in other organs. As one example, Campbell, et al.,14 used a GFAP-transgene cassette to express IL-6 in mouse brain astrocytes. The transgenic mouse offspring were found to have stunted growth, ataxia, and seizures. Neuropathological examination of these animals revealed loss of, and damage to, neurons and dendrites, marked astrogliosis, and some neovascularization.14 The induction of acute-phase proteins by IL-6 was thought to be responsible for the immunological responses observed in the CNS. In another study, Toggas and colleagues134 created transgenic mice from a construct in which the human immunodeficiency virus–1 coat was attached to the GFAP promoter. In these mice, considerable neuronal damage was found on neuropathological examination.114 Finally, a study by Galou, et al.,35 showed that a 2-kb fragment of the 5′ flanking region of the murine GFAP gene linked to a β-galactosidase reporter gene was activated following a stab wound to the brain in transgenic mice. These transgenic lines may thus provide a useful tool to study certain aspects of reactive astrogliosis. In all the studies just described, the ability of GFAP DNA regulatory sequences to target expression of transgenes to cells of glial origin opens exciting opportunities to influence the behavior of astrocytes or to target tumors of astrocytic origin.

Posttranslational Modifications in GFAP. The authentic message (mRNA) of the GFAP gene is 3.5 kb and is known as the standard form or GFAP-a. Alternate forms of GFAP mRNA have been described and have been termed GFAP-β and GFAP-γ.10 The GFAP-β mRNA was first described in the ethynitrosourea-induced Schwann cell line, RT4-D6.53 In the peripheral nervous system, GFAP-β mRNA may be the predominant form of GFAP, whereas GFAP-α mRNA is the predominant form in the CNS.10 The GFAP-γ mRNA has been found in both CNS and non-CNS tissues including mouse bone marrow and spleen.10 At this time, relatively little is known about the function and role of the alternate forms of GFAP mRNA in terms of protein formation. After the mRNA is transl-
ed into protein, however, GFAP undergoes posttransla-
tional modifications, including blocking of the methionine
at the NH, terminus and phosphorylations. Of these, the
phosphorylation of GFAP plays a pivotal role in GFAP
structure and function.

Phosphorylation of a protein on serine, threonine, or
tyrosine residues is a common posttranslational modifica-
tion that allows for rapid and reversible alterations in pro-
tein conformation and function. Just as with other IFs and
cytoskeletal proteins, GFAP does not exist in one static
form. Rather, there is a continuous and dynamic shift from
an assembled (filamentous) state to a disassembled (solu-
ble) state.48,49,79 In particular, phosphorylation of a num-
ber of IFs has been observed to occur during mitosis.
Phosphorylation at this time point in the cell cycle may
assist with the reorganization of GFAP that occurs during
mitosis.17,18,48,68 It is currently believed that phosphoryla-
tion in the NH-terminal region of GFAP is important for
disassembly from a filamentous form into a soluble form
(Fig. 6).1,47,78,80 This disassembly of the filamentous protein
likely facilitates equal separation of cytoplasmic compo-
nents into two daughter cells during cytokinesis.

Proof that the NH-terminal head region is indispens-
able for GFAP filament formation comes from a variety of
different studies.48,89 In one study, cleavage of the NH, ter-
minus of GFAP by thrombin digestion led to failure of
GFAP assembly.90 In other studies, serine and threonine
residues in the NH-terminal region of GFAP act as in
vitro substrates for phosphorylation by a number of pro-
tein kinases including the cyclin-dependent kinase cdc2
(cdk1), CAMP-dependent kinase, Ca++-calmodulin–de-
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Glial Fibrillary Acidic Protein–Cytoskeleton Interactions.

GFAP is thought to stabilize the astrocyte’s cytoskeleton and to maintain astrocyte cell
shape through interactions between GFAP filaments, the nuclear membrane, and the plasma membrane.93
This statement is further supported by immunoelectron microscopy data that show abundant GFAP filaments at sites
of contact between astrocytic processes and neurons, endothelial or leptomeningeal basal laminae.94 Thus, GFAP
forms a structural link between the nucleus and the plasma
membrane.

The aforementioned physical interactions are likely me-
diated through a panoply of putative IF-associated pro-
teins (IFAPs),30,39,117,118 but the details of these interactions
have yet to be fully characterized. Yang and associates121
have recently described an essential cytoskeletal linker
protein connecting neurofilaments to actin microfila-
maments. Mice deficient in this linker protein, termed
BPAG1n, will undergo sensory neurodegeneration and
will demonstrate a dystonia musculorum phenotype.121
These authors have suggested that BPAG1n is vitally im-
portant for the neuron and the host and that severing the
connections between the neurofilaments and actin microfila-
maments may adversely affect axonal transport mecha-
nisms.121 A different example of an IFAP that binds to
GFAP to aid in cytoskeletal support is plectin. Plectin is a
300-kD protein first identified in the rat C6 astrocytoma
cell line.80 Part of plectin’s primary sequence is related to
desmoplakin and bullous pemphigoid antigen. Plectin has
been shown to bind to several IFs and has been found in
virtually all cell types. Plectin colocalizes with IFs at focal
contact and actin stress fiber sites (Fig. 7).105 Using immu-
noelectron microscopy, Bohn, et al.,8 recently showed that
vimentin IFs were structurally linked to actin microfila-
maments through thin (3-nm) connecting filaments similar in
size to plectin. In cosedimentation experiments, however,
plectin has not been shown to bind to F-actin directly.105
Accordingly, it has been suggested that plectin may con-
nect with actin filaments through actin-binding proteins.105
Examples of well-characterized actin-binding proteins
include actinin, talin, and vinculin. Interestingly, these
actin-binding proteins are known to bind to the short cyto-
plasmic domains of the main mediators of cell–extracellu-
lar matrix (ECM) adhesion—the integrins (see following
subsection).

Glial Fibrillary Acidic Protein: a Link to Glial Cell Ad-
hesion? We previously observed that treatment of a GFAP-
positive cell line, U-251MG, with antisense GFAP led to
a marked reduction in cell adhesion.100 The observations
that IFs link to actin filaments by means of actin-binding
proteins and that actin is linked to the cytoplasmic do-
 mains of integrins by virtue of these same actin-binding
proteins suggest that actin and, to a lesser extent, GFAP
are physically linked to the plasma membrane and will
respond together to changes in cell morphology or the cellular microenvironment (Fig. 8). Integrins have been colocalized immunocytochemically with actin-containing microfilaments, and biochemical evidence has been obtained to indicate a direct binding of integrin cytoplasmic domains to certain cytoskeletal proteins, including talin and \( \alpha \)-actinin, at sites of contact between the cells and their substrata. The integrins are a family of Type I transmembrane proteins composed of a large extracellular domain, a hydrophobic membrane spanning segment, and a short cytoplasmic domain.\(^{46,94,105}\) Each integrin is composed of an \( \alpha \) and a \( \beta \) subunit. At present, there are at least 15 different \( \alpha \) and nine \( \beta \) subunits that can variously combine to form 21 receptors with distinct ligand specificities.\(^{56}\) Integrins recognize specific peptide regions within ECM macromolecules, the best characterized of which is the arginine-glycine-aspartic sequence of fibronectin.\(^{22–24,94}\)

A relationship between IFs and integrins has recently been elucidated via the molecular characterization of the hemidesmosome that contains keratin IFs linked to the \( \alpha 6\beta 4 \) integrin through the bullous pemphigoid antigen.\(^{16,36,110}\) It has been proposed that a reduction in integrin expression, as seen in many instances in transformed cells, leads to the dissociation of the ECM and the cytoskeleton, resulting in reduced adhesion and cytoskeletal disorganization.\(^{2,26,57}\)

On binding to extracellular ligands, integrins cluster on the plane of the plasma membrane and promote the assembly of molecular complexes containing both cytoskeletal and signaling elements.\(^{7,96,106}\) The effects that the ECM has on cellular growth and differentiation are likely mediated through various integrin signaling pathways (so-called "out/in" signaling). Although the integrin signaling pathways have not been completely characterized, many involve tyrosine kinase phosphorylation of neighboring protein species and cyclin-dependent kinases.\(^{6,42,102,111}\) As one example, the study of ECM-induced aggregation of integrin receptors at focal adhesion sites led to the identification of a phosphorylated 125-kD protein known as focal adhesion kinase (FAK).\(^{44,102}\) This FAK has been shown to associate physically with two nonreceptor protein tyrosine kinases, pp60src and pp59lyn, via their Src homology 2 domains.\(^{102}\) Interestingly, a number of recent studies have shown that induced changes in the cytoskeleton are also likely to perturb integrin-mediated signaling (so-called "in/out" signaling) (Fig. 8).\(^{57,91}\)

**Glial Fibrillary Acidic Protein and Signaling Pathways.** In general, the progression of a cell through the cell cycle is ultimately controlled by a series of protein kinases whose activities are regulated by a group of proteins called cyclins. The cyclin-dependent protein kinases (cdks) phosphorylate key substrates that are required to facilitate the passage of the cell through each phase of the cell cycle. Two important regulatory points exist in the cell cycle: one at the G\(_1\)–S interphase and the other at the G\(_2\)–M interphase. The protein kinase cdc2 interacts with cyclin B and cyclin A to regulate mitosis, and cdk2 interacts with a variety of cyclins (D1, D2, D3, E, and A) to regulate progression through the G\(_2\) phase and DNA synthesis.\(^{107}\)

Intrinsic alterations of cell cycle genes and their expression have now been identified in a wide variety of human cancers.\(^{7,73}\) As examples, overexpression and amplification of cyclin D1 has been demonstrated in human parathyroid adenoma, leukemia, lymphoma, esophageal carcinoma, and breast carcinoma.\(^{3,4,51,52,84}\) Keyomarsi and Pardee and colleagues\(^{54,55}\) demonstrated a deranged order of appearance of cyclins in synchronized breast carcinoma
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cell lines, with mitotic cyclins appearing prior to G1 cyclins. In addition, overexpression of cyclin A has been shown to be associated with anchorage-independent cell growth, another hallmark of cancer cells in vitro.42 There has been one previous report on the interaction of cyclin-dependent kinases and IFs.108 In that paper, the activation of p34cdc2 coordinated the mitotic reorganization of the vimentin IF network both by severing IF–IF connections mediated by IFAPs and by disassembling individual IFs into protofilaments. Because there are now reports that link IFs and integrins to cell cycle gene expression,42,111 future experiments in this field will undoubtedly clarify the role of GFAP in complex cellular responses such as process formation, cell adhesion, and cell motility.

Lessons Learned From Modulating GFAP Expression.
Clues as to the function of glial filaments in normal human astrocytes and glial tumors have come from a number of studies in which GFAP expression has been modulated under experimental conditions. It is known that early in neuroembryogenesis, astrocytes may synthesize vimentin instead of GFAP as their primary IF.7,109 The switch to GFAP predominance occurs prenatally in human astrocytes and is caused by factors that have yet to be elucidated. In vitro, cultured astrocytes modulate GFAP synthesis in response to cell density,38 composition of growth media,44 and hormones that stimulate CAMP production.106 Because GFAP is a cytoskeletal element that appears to play an important role in astrocytic differentiation and because it is the most specific marker for cells of astrocytic lineage, several groups have studied how the modulation of GFAP expression might affect astrocytoma growth and differentiation. In an earlier study, all-trans and 13-cis retinoic acid (10–6 M) were effective in decreasing the proliferation of GFAP-positive U-343MG-A astrocytoma cells and in increasing the amount of GFAP produced, as measured by an enzyme-linked immunoabsorbent assay.74 In another study, it was shown that ECM proteins derived from normal human leptomeningeal cells effectively stopped the proliferation of U-343MG-A astrocytoma cells and induced differentiation of these cells as calculated by a quantitative increase in cellular GFAP.96,99 The results from these studies suggest that the upregulation of GFAP in GFAP-positive astrocytoma cells is associated with alterations in astrocytoma growth and morphology. However, as the vast majority of human astrocytoma cell lines are GFAP negative and, consequently, similar to highly anaplastic astrocytomas in vivo,3,15,59,110 a study was performed in which GFAP expression was restored to GFAP-negative anaplastic astrocytoma cells. A GFAP-negative human astrocytoma cell line, SF-126, was stably transfected with an expression vector containing a cDNA for the entire coding sequence of the human GFAP gene.100 Interestingly, GFAP-immunoreactive SF-126 astrocytoma clones were characterized by elongated cytoplasmic processes containing high concentrations of GFAP and demonstrated marked alterations in cellular morphology when compared to controls. In addition, the amount of GFAP mRNA expression and immunoreactivity among stably transfected astrocytoma clones correlated inversely with astrocytoma proliferation.101 Recently, studies have been performed in which the GFAP gene has been eliminated in transgenic “knockout” mice.51,64,86 In two of these studies, GFAP-deficient mice appeared to develop and function normally, and in the areas of the brain subjected to detailed neuropathological study, no cytoarchitectural disturbances of the CNS of these mice have been found.41,108 Although these authors did not examine the long-term effects of GFAP deficiency on the mouse brain, they postulated that GFAP-deficient mice may be more prone to advanced CNS aging than normal mice. This postulate has now been proved correct by Liedtke, et al.,64 who showed that transgenic mice deficient in GFAP develop abnormal myelination in the spinal cord, optic nerve, and brain after 6 months. Ultrastructurally, the authors showed that astrocytic processes in GFAP-deficient mice were short and clublike, leading to reduced contacts between astrocytes and oligodendrocytes, and between astrocytes and myelin sheaths.64 The altered myelination of the CNS in GFAP-deficient mice suggests an important link between astrocyte function and the maintenance of myelin. In addition, GFAP-deficient mice had poor white matter vascularization, which may have resulted from the loss of inductive influences of astrocytes on endothelial cells.64 The implications from the study by Liedtke, et al., are profound because they suggest that for the CNS to retain structural and functional integrity with respect to CNS myelination, normal GFAP expression appears to be mandatory.

Conclusions
In summary, evidence is accumulating to suggest that GFAP may be involved as a collaborator in the complex cellular processes controlling astrocytoma cell morphology, adhesion, and proliferation. Glial fibrillary acidic protein may not represent a mere mechanical integrator of cellular space as has been previously thought. With several reports demonstrating that IFs may serve as “docking sites” for important kinases that recognize key cellular substrates,17,18,108,115,122 it is clear that GFAP helps to form a dynamic continuum with microfilaments, integrin receptors, and the ECM. Studies aimed at delineating how this continuum is affected by GFAP expression would be of great value in increasing our understanding of the role GFAP plays in modulating cell morphology and signaling pathways.

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J. Neurosurg. / Volume 87 / September, 1997

Manuscript received September 3, 1996.
Accepted in final form May 5, 1997.
This work was supported in part by grants from the National Cancer Institute of Canada, the Medical Research Council of Canada, the Lunenfeld Foundation, The Research Institute, The Hospital for Sick Children, and Brainchild. Dr. Dirks was supported by a Research Fellowship from The National Cancer Institute of Canada, the Medical Research Council of Canada, The Lunenfeld Foundation, The Research Institute, The Cancer Institute of Canada, the Medical Research Council of Canada, The Child and Brainchild. Dr. Dirks was supported by a Research Fellowship from The National Cancer Institute, Canadian Cancer Society, and the Medical Research Council of Canada.
Address reprint requests to: J. T. Rutka, M.D., Ph.D., The Division of Neurosurgery, Suite 1502, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8. e-mail: rutka@sickkids.on.ca.