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Nuclear Factor I Regulates Brain Fatty Acid-Binding Protein and Glial Fibrillary Acidic Protein Gene Expression in Malignant Glioma Cell Lines

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Glial fibrillary acidic protein (GFAP), an intermediate filament protein normally found in astrocytes, and the radial glial marker brain fatty acid-binding protein (B-FABP; also known as FABP7) are co-expressed in malignant glioma cell lines and tumors. Nuclear factor I (NFI) recognition sites have been identified in the *B-FABP* and *GFAP* promoters, and transcription of both genes is believed to be regulated by NFI. Here, we study the role of the different members of the NFI family in regulating endogenous and ectopic *B-FABP* and *GFAP* gene transcription in human malignant glioma cells. We show by gel shifts that all four members of the NFI family (NFIA, NFIB, NFIC, and NFIX) bind to *B-FABP* and *GFAP* NFI consensus sites. Over-expression of NFIs, in conjunction with mutation analysis of NFI consensus sites using a reporter gene assay, supports a role for all four NFIs in the regulation of the *GFAP* and *B-FABP* genes. Knock-down of single or combined NFIs reveals promoter-dependent and promoter-context-dependent interaction patterns and suggests cross talk between the different members of the NFI family. Our data indicate that the NFI family of transcription factors plays a key role in the regulation of both the *B-FABP* and *GFAP* genes in malignant glioma cells.

Keywords: nuclear factor I; brain fatty acid-binding protein; glial fibrillary acidic protein; malignant glioma; gene regulation

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Introduction

Malignant gliomas, comprising grade III and grade IV astrocytomas (also called anaplastic astrocytoma and glioblastoma multiforme, respectively), are the most common brain tumors in adults.[1] These highly invasive tumors are usually fatal within 2 years of diagnosis. Histopathological analysis of malignant gliomas has shown that increasing anaplasia correlates with reduced levels of the intermediate filament protein glial fibrillary acidic protein (GFAP).[2,3] Manipulation of GFAP levels in malignant glioma cells suggests an association between GFAP expression and a reduced transformed state.[4–9]

Brain fatty acid-binding protein (B-FABP; also known as FABP7 or BLBP) is a marker of radial glial cells.[10,11] B-FABP has been implicated in the establishment of the radial glial fiber system required for the migration of neurons to their correct location in the central nervous system and in glial cell differentiation.[10,11] It is generally believed that neural stem cells give rise to radial glial cells, which in turn become mature astrocytes once neuronal migration is complete.[12,13] However, radial glial cells can also give rise to neurons and have been proposed to function as neural stem cells.[14–16] B-FABP expression has recently been shown to be associated with increased cell migration in malignant glioma cells and with a worse clinical prognosis in high-grade astrocytomas.[17–19] Of note, malignant glioma cell lines that express B-FABP also express GFAP,

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Abbreviations used: GFAP, glial fibrillary acidic protein; B-FABP, brain fatty-acid-binding protein; NFI, nuclear factor I; AP-2, activating protein 2; CAT, chloramphenicol acetyltransferase; HA, hemagglutinin; br, binding region; ChIP, chromatin immunoprecipitation; RT-PCR, reverse transcription polymerase chain reaction; siRNA, small interfering RNA; PVDF, polyvinylidene fluoride.

suggesting either a functional or a regulatory link between these two proteins.[20]

Nuclear factor I (NFI) has been implicated in the regulation of both the *B-FABP* and the *GFAP* genes.[21–23] NFI is a family of transcription factors that includes four genes: *NFIA*, *NFIB*, *NFIC*, and *NFIX/NFID*.[24] Additional diversity within this family comes from alternative splicing and post-translational modification (reviewed in Ref. 25). NFI proteins bind to the consensus sequence 5′-TTGGCN$_5$GCCAA-3′ as homodimers or heterodimers with the same apparent affinity.[26,27] NFIs are widely expressed in different tissues and cell types, although the distribution pattern of each NFI varies from tissue to tissue.[28] NFI consensus binding sites are found in many brain-specific gene promoters/enhancers, and NFI transcription factors have been proposed to play a role in the determination of gene expression in glial cells.[29–34]

The *B-FABP* promoter has at least two NFI recognition elements located within 500 bp of the *B-FABP* transcription start site. Using a combination of gel-shift assays and potato acid phosphatase treatment, Bisgrove *et al.* showed NFI to be hyperphosphorylated in GFAP/B-FABP-negative malignant glioma cell lines compared with GFAP/B-FABP-positive lines.[21] Phosphorylation of NFI did not seem to affect DNA binding activity *in vitro*, in agreement with a previous report.[35] Similarly, transfection and DNase I footprinting analysis revealed three footprints in the promoter region of the *GFAP* gene in the B-FABP/GFAP-positive malignant glioma cell line U251.[36,37] Putative NFI binding sites were identified in all three regulatory regions. Direct evidence demonstrating occupancy of the *GFAP* promoter by NFIs was obtained by chromatin immunoprecipitation (ChIP) using primary cortical neuroepithelial cells.[22]

All four *NFI* genes have been disrupted in mice.[34,38–41] Whereas *Nfic*-deficient animals have defects in tooth root formation, disruption of either *Nfia* or *Nfib* results in forebrain defects and loss of specific midline glial populations. In addition to having more severe forebrain defects than *Nfia*, *Nfib* knock-outs have abnormalities in lung maturation and pons development.[41] *Nfix*−/− mice show enlargement of the lateral and third brain ventricles, expansion of the entire brain along the dorsal ventral axis, aberrant formation of the hippocampus, deformation of the spine, and impaired ossification of vertebra and femur.[39,42] *GFAP* mRNA levels are decreased 10- and 5-fold in *Nfia*−/− and *Nfib*−/− mice, respectively, suggesting involvement of these two NFIs in *GFAP* regulation.[41] Activation of Notch signaling in mid-gestational neural precursor cells has recently been shown to induce NFIA, resulting in demethylation and activation of astrocytic gene promoters including *GFAP*.[43] Thus, NFIA seems to play a fundamental role in potentiating the differentiation of neural precursor cells along the astrocytic lineage.

Here, we investigate the role of NFI in the regulation of the *GFAP* and *B-FABP* genes in malignant glioma cells. We use ChIP to demonstrate the occupancy of NFIs at both the endogenous *GFAP* and the *B-FABP* promoters. We study the expression patterns of all four *NFI* genes in B-FABP/GFAP-positive and B-FABP/GFAP-negative malignant glioma cell lines and use the gel-shift assay to examine the binding of each NFI to three NFI recognition sites located at the 5′ ends of each of the *B-FABP* and *GFAP* genes. We use a combination of RNA interference, ectopic NFI expression, reporter gene assay, and analysis of endogenous *GFAP* and *B-FABP* RNA to investigate the biological activity of NFIs *in vivo*. Our results suggest complex antagonistic and compensatory interactions between the four members of the NFI family, which all seem to be involved in the regulation of *B-FABP* and *GFAP* transcription.

Results

Expression of NFI mRNA in malignant glioma cell lines

The four *NFI* genes (*NFIA*, *NFIB*, *NFIC*, and *NFIX*) are differentially expressed in various tissues and cell types. To identify which NFIs are expressed in malignant glioma cells, Northern blot analysis was carried out using poly(A)$^+$ RNA isolated from five B-FABP/GFAP-negative malignant glioma lines (A172, CLA, M021, T98, and U87) and five B-FABP/GFAP-positive malignant glioma lines (M016, M049, M103, U251, and U373) (Fig. 1). Highest levels of *NFIA* transcripts were detected in M049 and M103. *NFIB* mRNA was most abundant in B-FABP/GFAP-positive M049, M103, U251, and U373 lines. *NFIC* transcripts were found in all 10 lines. Highest levels of *NFIX* mRNA were observed in M103 and M021, with an easily detectable signal in every cell line except U87. Actin mRNA served as the loading control and was relatively uniform in the 10 malignant glioma lines. Overall, B-FABP/GFAP-positive malignant glioma lines seem to express higher levels of NFI mRNAs compared with B-FABP/GFAP-negative lines, with the most dramatic differences observed with *NFIA* and *NFIB*.

In vitro binding of proteins to GFAP NFI recognition sites

Sequence analysis of the *GFAP* promoter region revealed three putative NFI binding sites in the upstream region of the *GFAP* gene, located at −120 to −106 bp, −1585 to −1571 bp, and −1633 to −1619 bp. Each of these three sites is bound by protein based on DNase I footprinting analysis[36,37] and gel-shift assays.[23] We used gel shifts to determine whether NFIs from T98 (B-FABP/GFAP negative) and U251 (B-FABP/GFAP positive) malignant glioma lines could bind to the three putative NFI binding sites located at the 5′ end of the *GFAP* gene. Double-stranded oligonucleotides representing each of the three *GFAP* NFI binding regions [G-br1 (−126 to