Nuclear Factor-1-X Regulates Astrocyte-specific Expression of the α1-Antichymotrypsin and Glial Fibrillary Acidic Protein Genes

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ACT belongs to the serine protease inhibitor (serpin) family of proteins and is also expressed in the liver and secreted into the plasma (5). The gene encoding ACT is clustered with 10 additional serpin genes on human chromosome 14q32.1 and resides within the distal serpin subcluster that also contains genes encoding kallistatin, protein C inhibitor, and the kallistatin-like protein (6, 7). The expression profile of the distal serpin subcluster is dramatically different between astrocytes and hepatocytes. All four genes are expressed in hepatocytes, whereas only the ACT gene is expressed in astrocytes (8). Investigations of the regulatory mechanisms controlling the selective expression of ACT in astrocytes and glioma cells demonstrated that the ACT gene is localized to the DNase I-accessible chromatin, whereas the promoters of the non-expressed protein C inhibitor and kallistatin genes are enzyme-inaccessible (8). In contrast to astrocytes, the entire distal subcluster resides within decondensed chromatin in hepatoma cells with all promoters easily accessible to DNase I (8).

During normal physiological conditions, ACT secreted into the plasma by hepatocytes is separated from the brain-derived ACT by the blood-brain barrier. Its expression in hepatocytes is likely determined by liver-specific transcription factors belonging to the hepatocyte nuclear factor and CAAT enhancer-binding protein families (9). In contrast to hepatocytes, the constitutive astrocyte-specific expression of ACT requires the activator protein-1 (AP-1) transcription factor, which is composed of the Jun and Fos family members (10). Two AP-1 binding sites have recently been identified at −13 kb and −11.5 kb in the 5′-flanking region of the ACT gene, which are critical for astrocyte-specific expression (4, 10). However, glioma cells overexpressing dominant-negative c-jun(TAM67), which quenches functional AP-1 complexes and abolishes basal ACT expression, retained the astrocyte-specific chromatin structure of the distal serpin subcluster (10). Several models have been hypothesized to explain this phenomenon, including the one that proposes the presence of an additional transcription factor(s) that, by virtue of its cooperation with AP-1, determine both astrocyte-specific chromatin structure and ACT gene expression.

We initiated this study with the aim of identifying the hypothetical transcription factor(s) needed to determine chromatin structure and expression of the ACT gene in astrocytes and glioma cells. This study resulted in the identification of NFI-X, which is indispensable for the astrocyte-specific expression of the ACT gene. Furthermore, we provide evidence that NFI-X also regulates the expression of glial fibrillary acidic protein (GFAP), which is an astrocyte marker protein. Analysis of the 5′-flanking regions of both genes highlighted the presence of additional transcription factor(s) that, by virtue of their cooperation with AP-1, determine both astrocyte-specific chromatin structure and ACT gene expression.

**EXPERIMENTAL PROCEDURES**

### Cell Culture

Human hepatoma HepG2 and glioblastoma U373-MG cells were obtained from American Type Culture Collection (Rockville, MD).
NFI-X and Astrocyte-specific Expression

Cells were transfected with GeneJuice transfection reagent (Novagen, Darmstadt, Germany), according to the supplier’s instructions. Plasmids (350 ng of the CAT reporter plasmid and 50 ng of the β-galactosidase expression plasmid) and 5 μl of GeneJuice diluted in 50 μl of serum-free medium were used for each well containing cells growing in 1 ml of culture medium. One day after transfection, cells were stimulated for 24 h and harvested. Protein extracts were prepared by freeze thawing, and the protein concentration was determined by the BCA method (Sigma Co.). Chloramphenicol acetyltransferase and β-galactosidase assays were performed as described (11), and chloramphenicol acetyltransferase activities were normalized to the β-galactosidase activities. Experiments were repeated three to five times. NFI expression was knocked down using either general NFI siRNA (Santa Cruz Biotechnology, Inc.) or isoforms-specific SMARTpool siRNAs, which were specific for the NFI-A, NFI-B, NFI-C, and NFI-X (Dharmacon, Inc., Lafayette, CO).

Nuclear Extract Preparation and EMSA—Nuclear extracts were prepared as described (12). Double stranded fragments were labeled by filling in the 5'-protruding ends with Klenow enzyme using [α-32P]dCTP (3000 Ci/mmol) (13). Gel retardation assays were performed according to published procedures using 2 μg of nuclear extracts (14, 15). Competition experiments were performed in the presence of a 100-fold concentration of the cold oligonucleotides. Polyclonal anti-NFI antibodies that recognize all isoforms of NFI (sc-5567) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and used for supershift studies.

Chromatin Immunoprecipitation Assay—Cells were treated with cytokines for 18 h, and chromatin was cross-linked by the addition of formaldehyde to 1%, followed by a 10-min long incubation at 37 °C. Subsequently, the cells were washed with ice-cold phosphate-buffered saline containing 125 mM glycine and 1 mM phenylmethylsulfonyl fluoride. Chromatin was sonicated and immunoprecipitated using anti-NFI antibodies (sc-5567) exactly as described in the chromatin immunoprecipitation protocol from Upstate Inc. (Charlottesville, VA).

DNase I Footprint Analysis—DNase I footprinting of the −13-kb IL-1-responsive enhancer of the ACT gene was performed according to standard procedures (16). The 579-bp long probe for the footprinting was prepared from the plasmid pΔACTCAT. The plasmid was digested with HindIII, labeled with [α-32P]dCTP (3000 Ci/mmol) using T4 DNA polymerase, and subsequently digested with BglIII. The HindIII/BglII fragment was gel-purified using the gel purification kit (Qiagen, Valencia, CA). The fragment (10,000 cpm) was incubated with 15 μg of nuclear extracts from U373 cells for 10 min and subsequently digested with DNase I (1, 0.75, 0.5, and 0.3 units) for 3 min. DNA was purified by phenol extraction, followed by precipitation with ethanol, and samples were separated by electrophoresis in sequencing gels using standard procedures (13). Sequencing reactions were performed using the SequiTherm EXCEL™II DNA sequencing kit (Epitector, Madison, WI). 1000 fmol of the template DNA and 2 pmol of the primer were used for the sequencing reactions.

Identification of Transcription Factor Binding Sites—The putative transcription factor binding sites were identified using the MatInspector program. The Matrix Family Library version 5.0 was used with core/matrix similarity values set to 0.75/optimized.

RESULTS

NFI Binds at the −13-kb IL-1 Enhancer of the ACT Gene—We have recently reported that the AP-1 complex is needed for the basal astrocyte-specific expression of the ACT and GFAP genes (10). However, the precise mechanism that allows this ubiquitous transcription factor to determine astrocyte-specific expression is not known. One of the possibilities of its action could involve cooperation of AP-1 with yet to be
**NFI-X and Astrocyte-specific Expression**

Identified factors. Because AP-1 binds to two AP-1 binding elements at −13 and −11.5 kb from the transcription start site of the ACT gene (4, 10), we analyzed the fragments adjacent to the −13-kb AP-1 element for binding of novel transcription factors using DNase I footprinting. A strong footprint corresponding to the putative NFI binding site was detected using the 579-bp long DNA fragment that partially overlaps the −13-kb enhancer (Fig. 1A). This putative NFI binding site was further analyzed using nuclear extracts of both glioblastoma and hepatoma cells by EMSA. The in vitro binding of a transcription factor to the NFI putative binding site was detected in both cell types (Fig. 1B). The specificity of NFI binding was confirmed by competition, and the identity of NFI was verified using NFI antibodies (Fig. 1C). We have also analyzed the binding of NFI in the nuclei of U373 cells using chromatin immunoprecipitation assays. NFI was detected bound to its site at the −13-kb enhancer in untreated U373 cells, and IL-1 treatment resulted in some increase of this binding. We conclude that NFI binds to the newly identified element within the −13-kb enhancer of the ACT gene in glioma cells.

**Functional Analysis of the NFI and AP-1 Elements**—The previously identified 413-bp long ACT enhancer, located 13 kb upstream of the transcription start site, contains one functional AP-1 binding site and two NF-kB binding sites, which confer IL-1 responsiveness (4). To evaluate whether the newly identified proximal NFI element is critical for the activity of the enhancer, we introduced point mutations into the NFI element either alone or together with the mutation of the AP-1 element. Mutations within either the NFI or AP-1 sites reduced both basal and IL-1-induced expression of the ACT reporter (Fig. 2A). Moreover, mutation of both NFI and AP-1 elements within the reporter abolished its expression. These results indicate that both NFI and AP-1 are essential for the full activity of the ACT enhancer in glioma cells.

We have also generated reporter constructs containing multiple copies of either NFI or AP-1 elements linked to the minimal tk promoter driving the transcription of the chloramphenicol acetyltransferase reporter gene. These constructs were tested in transient transfections of U373 cells (Fig. 2B). Multiple copies of the AP-1 element dramatically increased the basal activity of the reporter, which was further increased after IL-1 stimulation. In contrast, the basal expression of the NFI reporter was similar to that of the reporter containing the minimal tk promoter, and this expression was not activated by IL-1. We conclude that NFI by itself is not sufficient to mediate astrocyte-specific expression; however, it likely cooperates with AP-1 to mediate the full activity of the enhancer in glioblastoma cells.

**NFI siRNA Down-regulates ACT and GFAP Gene Expression in U373 Cells**—Because the NFI element at −13 kb is occupied by NFI in glioma cells, we questioned whether depletion of NFI in these cells would affect endogenous ACT expression. In addition, we analyzed the expression of GFAP, which is an astrocyte-specific marker protein, because three
putative NFI binding sites have previously been described in its 5′-flanking region (17). The expression of NFI was knocked down to 60% using siRNA, and the expression of ACT and GFAP mRNA was analyzed by Northern blotting. Interestingly, down-regulation of NFI expression almost completely abolished the basal expression of the astrocyte-specific ACT and GFAP genes but not a ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase gene (Fig. 3). In addition, OSM-induced expression of both genes was also diminished, however, to a much lower extent, indicating that cytokine-activated trans-acting factors are sufficient to drive transcription in the absence of NFI. We have also observed a dramatic down-regulation of GFAP expression by IL-1 as previously reported (18, 19). Hence, NFI is critical for intrinsic astrocyte-specific expression of the ACT and GFAP genes.

NFI and AP-1 Colocalize with the DNase I-hypersensitive Sites at the 5′-Flanking Region of the ACT Gene—Because knock-down of NFI expression or overexpression of dominant-negative c-jun(TAM67) (10) resulted in a drastic suppression of ACT expression in glioma cells, we analyzed the 14-kb long 5′-flanking region of the ACT gene for the

FIGURE 2. AP-1 and NFI binding elements are necessary for the full activity of the −13-kb enhancer of the ACT gene. Point mutations were introduced within the putative binding sites of the −13-kb enhancer as described under "Experimental Procedures." U373 cells were transfected with pΔACTCAT, p(mutNFI)ACTCAT, p(mutAP-1)ACTCAT, and pmut(NFIandAP-1)ACTCAT (A) or p5X(NFI)CAT and p4X(AP-1)CAT (B) and β-galactosidase (β-gal) expression vector was also included in transfections as an internal control for transfection efficiency. One day after transfection, cells were stimulated with IL-1 for 24 h and harvested. Chloramphenicol acetyltransferase (CAT) activities were normalized to β-galactosidase activities (cpm/units). Duplicate experiments were repeated three times, and the mean values ± S.E. are shown. ACT and tk indicate a 247-bp long promoter of the ACT gene and a minimal promoter of the thymidine kinase gene, respectively.

FIGURE 3. Expression of the ACT and GFAP genes requires NFI in U373 cells. U373 cells were transfected with either control or NFI siRNA for 48 h as indicated, and cells were stimulated with IL-1 or OSM. A, RNA was isolated after 18 h and subjected to Northern blot analysis using ACT, GFAP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as probes. B, cell lysates were prepared and analyzed by Western blotting using anti-NFI or anti-β tubulin antibodies.
presence of additional NFI and AP-1 binding sites using the MatInspector program. We identified 5 putative NFI, and 13 putative AP-1 elements in this region (Fig. 4) with 4 of the putative NFI elements localized near putative AP-1 elements. Furthermore, all four of these NFI/AP-1 clusters matched the DNase I-hypersensitive sites we had previously identified in astrocytes and glioma cells (8). This analysis suggested that NFI together with AP-1 may be responsible for the generation of these DNase I-hypersensitive sites in both astrocytes and glioma cells, which leads to astrocyte-specific expression of the ACT gene.

NFI Binds to the Elements Located in the 5' Flanking Region of the GFAP Gene

The 2.2-kb long 5'-flanking region of the GFAP gene is commonly used to drive the astrocyte-specific expression of transgenes (20). Although, the molecular basis of the astrocyte-specific regulation of the GFAP promoter is not understood in detail, this promoter possesses three putative binding sites for NFI (17) (Fig. 5A). Because NFI siRNA efficiently blocked basal GFAP expression, we assayed the binding to putative NFI elements in vitro using nuclear extracts of U373 cells by EMSA. NFI was efficiently bound to the distal binding site (site 1), and it was also supershifted by the NFI antibodies (Fig. 5B). In addition, site 2 oligonucleotides neither competed with binding to site 1 nor site 2, whereas binding to site 3 was partially competed by site 1 oligonucleotides.

FIGURE 4. Analysis of the 5'-flanking region of the ACT gene. The 14-kb long 5'-flanking region was analyzed for the presence of putative binding sites for NFI and AP-1. A, the model of the ACT gene is drawn to scale with the transcription start site defined as +1. Arrows indicate the positions of the DNase I-hypersensitive sites, while gray boxes indicate the putative NFI elements, gray circles represent the AP-1 elements, and black boxes represent exons. The location of the −13 bp and −11.5 enhancers is also indicated. B, comparison of the consensus NFI binding sequence with the sequences of the putative NFI elements of the ACT gene. Nucleotides conserved between the consensus and the putative sites are underlined.

FIGURE 5. Binding of NFI to the putative binding elements located at the 5'-flanking region of the GFAP gene. A, model of the GFAP 5'-flanking region. Rectangles indicate the three putative NFI binding elements while the circle indicates the AP-1 binding element. B, nuclear extracts from U373 cells were analyzed by EMSA using labeled oligonucleotides corresponding to the three NFI sites. A 100-fold excess of oligonucleotide competitors, anti-NFI antibodies, or normal rabbit serum (NRS) was added to the binding reactions as indicated.
Sequence analysis of the GFAP 5′-flanking region revealed that an AP-1 binding site is located in the proximity of the two distal NFI sites. To evaluate the contributions of putative NFI and AP-1 binding elements, we generated reporter constructs with point mutations in each of these elements and analyzed their activities in U373 cells. We also stimulated cells with OSM, which activates GFAP expression via the STAT3 binding element located from −1518 to −1510. The reporter containing the wild type 5′-flanking region of the GFAP gene was efficiently expressed in U373 cells, and its activity was further increased by OSM (Fig. 6). However, a significant decrease in both the basal and OSM-activated expression was detected upon mutation of any of the NFI or AP-1 sites. Therefore, we conclude that the basal astrocyte-specific expression of the GFAP gene, similar to the basal astrocyte-specific expression of the ACT gene, depends on both AP-1 and NFI.

NFI-X Is Critical for the Intrinsic Expressions of ACT and GFAP in U373 Cells—NFI is a family of transcription factors encoded by four genes (NFia, NFib, NFic, and NFix), which are expressed in numerous tissues (21, 22). To determine which of the NFI isoforms are critical for the intrinsic astrocyte-specific expression of both ACT and GFAP, we knocked down the expression of NFI-A, NFI-B, NFI-C, and NFI-X mRNAs using specific SMARTpool siRNAs. The knockdown of NFI-X expression (by 75%) reduced basal expression of ACT and GFAP by 50 and 70%, respectively (Fig. 7). However, the down-regulation of other NFI isoforms did not effect expression of either ACT or GFAP, with the exception of the knockdown of NFI-C, which down-regulated GFAP expression by 40%. This result suggests that NFI-C, in addition to NFI-X, may specifically regulate the intrinsic expression of GFAP. Thus, we conclude that NFI-X is critical for the intrinsic astrocyte-specific expression of the ACT and GFAP genes.

**DISCUSSION**

The expression pattern of the serpin genes located in the distal subcluster on chromosome 14q32.1 is determined by the tissue-specific chromatin structures of this subcluster (8). In astrocytes and glioblastoma cells, the expressed ACT gene is localized to the enzyme-accessible chromatin, whereas the neighboring genes are neither enzyme-accessible nor expressed (8). Recently, we have shown that AP-1 is indispensable for ACT expression in glioma cells; however, it is not sufficient to determine astrocyte-specific structure of the distal subcluster (10). In this study, we analyzed the binding of nuclear proteins near the −13-kb enhancer, which contains an AP-1 binding element that is critical for ACT expression in astrocytes and glioma cells. Here we use DNase I footprinting, EMSA, and chromatin immunoprecipitation assays to show that NFI binds to a binding site located in the proximity of the AP-1 binding element (Fig. 1). This newly identified NFI element significantly contributes toward the basal ACT expression in glioma cells. This is based on the following observations: (i) this element is constitutively occupied by NFI in glioblastoma cells (Fig. 1D), (ii) knockdown of NFI (or specifically NFI-X) down-regulated the expression of ACT in glioblastoma cells (Figs. 3 and 7), and (iii) mutation of the NFI element reduced the basal and IL-1-induced expression of the ACT reporter (Fig. 2). However, the NFI element does not ensure efficient expression in glioma cells by itself, because the reporter containing several copies of the NFI element linked to the minimal tk promoter was not efficiently expressed in these cells (Fig. 2B). These results suggested that the astrocyte-specific expression of the ACT gene may be determined by NFI-X and AP-1, which cooperate in a tissue-specific manner in astrocytes and glioblastoma cells. However, the precise molecular mechanism of this cooperation remains unknown.

The identification of NFI as a possible regulator of astrocyte-specific expression prompted us to analyze the expression of GFAP, which is an astrocyte-specific intermediate filament that is abundantly and almost exclusively expressed in astrocytes and glioma cells. It has already been shown that the 2.2-kb 5′-flanking region of the GFAP gene directs the astrocyte-specific transcription in cultured cells and transgenic animals (20). This 2.2-kb region contains three putative NFI binding sites (two at −1.5 kb, and a third located 70 bp upstream of the transcription start site) and one AP-1 binding element (17). The proximal NFI site has been shown to bind an unidentified factor present in the nuclear extracts of glioma cells, whereas the AP-1 element binds c-jun and c-fos family members (23). In
agreement to the latter observation, we have recently demonstrated that dominant-negative c-jun(TAM67) abrogates expression of GFAP in glioma cells (10). Furthermore, mutation of the AP-1 site within the GFAP reporter drastically reduced its basal and OSM-induced expression (Fig. 6). Here we show that NFI binds only to the 5’-distal binding site in vitro, whereas the identity of the protein(s) that binds to the proximal site remains unknown (Fig. 5B). Interestingly, our mutational analysis of the three NFI binding elements indicated that all three elements are needed for full basal expression of the GFAP reporter (Fig. 6). The loss of GFAP expression upon knocking down the expression of NFI (Fig. 3) or its isoforms (NFI-X and partially NFI-C, Fig. 7) and the decrease in reporter activity upon mutation of the NFI elements (Fig. 6) argue that NFI-X is indispensable, whereas NFI-C contributes to the transcription of the GFAP gene. Therefore, we suspect that NFI-X and likely NFI-C binds to sites 2 and 3 in vivo in a cooperative manner, which cannot be recapitulated by EMSA using short oligonucleotides. In addition, we observed a substantial drop in the GFAP mRNA levels upon induction with OSM in the presence of NFI siRNA (Fig. 3). OSM has been shown to induce GFAP expression via the STAT3 binding element located in the 5’-flanking region of the gene. Our results suggest that depletion of NFI cannot be fully overcome by OSM-induced STAT3. Together these results indicate that NFI-X is critical for the astrocyte-specific expression of the ACT and GFAP genes and likely other genes that are specifically expressed in astrocytes and glioma cells.

These results lead to the question as to how NFI mediates the expression of astrocyte-specific genes. NFI is a family of transcription factors encoded by four genes (NFia, NFib, NFic, and NFix) that are highly conserved from chickens to humans (21, 22). In vertebrates, products of these genes share a conserved N-terminal sequence, encoding DNA binding and dimerization domains, which allows binding to the canonical binding site [TTGGC(N)5GCCAA] and formation of homo- and heterodimers (24–26). Although all four NFI genes are expressed with overlapping spectra in most of the tissues, their transcripts are alternatively spliced yielding as many as nine different proteins from a single gene (27, 28). NFI isoforms either activate or suppress transcription depending on the cell-type and promoter differences (22, 29). Therefore, NFI regulates numerous cell-specific and developmentally regulated genes in many cell types, including neurons (30–34). Recently, NFia, NFib, and NFic genes have been disrupted in mice. NFia knock-out mice are characterized by dramatic neuroanatomical defects, whereas NFib is needed for both brain development and lung maturation (35, 36). In contrast, disruption of NFic causes postnatal loss of molar roots (37). The extensive defects in the brain development in NFia and NFib knock-out animals suggest that some NFI isoforms are critical for proper development of the central nervous system. In addition, overexpression of the NFI-X protein (also known as NFI class D) supports replication of the human JC polyomavirus, which specifically targets glial cells and causes demyelinating disease (38). Our results, proposing a central role for NFI-X in astrocyte-specific expression of the ACT and GFAP genes, extend previous findings indicating that NFI isoforms are critical for brain development.

Although the mechanism of NFI-X action is unclear, our data suggest it likely depends on cooperation with AP-1, which may lead to the cooperative recruitment of specific coactivator complexes. This is supported by the fact that NFI isoforms are known to cooperate with other transcription factors, including the glucocorticoid receptor and Oct1 to specifically regulate gene expression (39–41). Furthermore, adjacent or overlapping NFI and AP-1 (or AP-2) sites have been observed in the regulatory regions of several genes specifically expressed in glial cells.
and neurons, including myelin basic protein, brain fatty acid-binding protein, S100B, mouse neurofilament L, γ-aminobutyric acid type A receptor, promoter of JC virus, and proteolipid protein (42–44).

In summary, we show that NFI-X is indispensable for astrocyte-specific intrinsic expression of the ACT and GFAP genes. We propose that NFI-X cooperates with AP-1 by an unknown mechanism in astrocytes, which results in the expression of a subset of astrocyte-specific genes.

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