ASTROCYTE-SPECIFIC EXPRESSION OF THE $\alpha_1$-ANTICHYMOTRYPSIN AND GLIAL FIBRILLARY ACIDIC PROTEIN GENES REQUIRES ACTIVATOR PROTEIN-1*.

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Running title: AP-1 and astrocyte-specific expression.

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An amyloid-associated serine proteinase inhibitor (serpin), $\alpha_1$-antichymotrypsin (ACT), is encoded by a gene located within the distal serpin subcluster on human chromosome 14q32.1. The expression of these distal serpin genes is determined by tissue-specific chromatin structures that allow their ubiquitous expression in hepatocytes; however, their expression is limited to a single ACT gene in astrocytes. In astrocytes and glioma cells, six specific DNase I hypersensitive sites (DHSs) were found located exclusively in the 5' flanking region of the ACT gene. We identified two enhancers that mapped to the two DHSs at -13 kb and -11.5 kb that contain activator protein-1 (AP-1) binding sites, both of which are critical for basal astrocyte-specific expression of ACT reporters. In vivo, these elements are occupied by c-jun homodimers in unstimulated cells and c-jun/c-fos heterodimers in IL-1-treated cells. Moreover, functional c-jun is required for the expression of ACT in glioma cells since both transient or stable inducible overexpression of dominant-negative c-jun (TAM67) specifically abrogates basal and reduces cytokine-induced expression of ACT. Expression-associated methylation of lysine 4 of histone H3 was also lost in these cells, but the DHS distribution pattern and global histone acetylation was not changed upstream of the ACT locus. Interestingly, functional AP-1 is also indispensable for the expression of glial fibrillary acidic protein (GFAP), which is an astrocyte-specific marker. We propose that AP-1 is a key transcription factor that, in part, controls astrocitespecific expression of genes including the ACT and GFAP genes.

The eleven genes encoding the serine proteinase inhibitors (serpin) are clustered on human chromosome 14q32.1 and occupy ~370 kb region (1) (2). This serpin cluster can be divided into three subclusters that contain four, three and four genes, respectively. The distal subcluster consists of the genes encoding the $\alpha_1$-antichymotrypsin (ACT), kallistatin (KAL), protein-C-inhibitor (PCI), and the recently identified KAL-like protein (2). All of these genes are highly transcribed in hepatocytes and hepatoma cells, and their promoters are accessible to DNase I digestion (3). In contrast, only the ACT gene is expressed in brain astrocytes and glioma cells (3). Selective expression of ACT in these cells correlates with DNase I accessibility at the gene's 5' flanking region while non-expressed PCI and KAL genes are localized to DNase I inaccessible chromatin (3).

In the liver, hepatocyte-specific gene expression is determined by transcription factors belonging to the hepatocyte nuclear factor (HNF) and CAAT-enhancer binding protein (C/EBP) families, with HNF-1 and HNF-4 being critical, but not sufficient, for the expression of the genes from the proximal serpin subcluster (4). The presence of binding sites for these transcription factors near the promoters of the distal serpin genes suggests that they play a critical role in their expression in hepatic cells. However, HNF-1, -3, -4 and C/EBP-$\alpha$, the predominant member of the C/EBP family found in non-stimulated hepatocytes, are not expressed in brain...
astrocytes. Therefore, yet to be identified, astrocyte-specific factors likely determine the astrocyte-specific chromatin structure of the distal serpin subcluster and the astrocyte-specific expression of the ACT gene in both astrocytes and glioma cells. Glial fibrillary acidic protein (GFAP), an intermediate filament protein, is exclusively expressed in astrocytes and the 2.2 kb long 5' flanking region of the GFAP gene is widely used to specifically express transgenes in astrocytes (5). In addition, several other genes including the gene encoding glial-specific cytokine S100β and a glial-selective promoter of the human polyomavirus JC are expressed in both astrocytes and oligodendrocytes (6) (7). However, the precise mechanisms that allow the tissue-specific expression of these genes are not understood.

Increased expression of the ACT gene is found in the brains of patients suffering from Alzheimer’s disease (AD) (8) (9). This serpin, primarily produced by astrocytes, colocalizes with the β-amyloid peptide found in plaques in the brains of AD patients. In astrocytes the expression of ACT is regulated by pro-inflammatory cytokines including IL-1, OSM and complexes of IL-6 and soluble IL-6 receptors (10) (11). We have previously identified the IL-1-responsive enhancer (at -13 kb) and the IL-6/OSM-responsive proximal elements both located upstream of the coding region of the ACT gene that mediate the responsiveness to cytokines (10) (11). However, the molecular mechanism that allows astrocyte-specific basal expression of ACT is not known.

We have previously reported the presence of six DNase I hypersensitive sites (DHS) in the 5' flanking region of the ACT gene in astrocytes and glioma cells (3). We initiated this study with the aim of identifying key trans-acting factors that bind to regulatory elements within these DHSs, and to determine their effect(s) on astrocyte-specific ACT expression. Here we identify two AP-1 binding elements that are indispensable for the basal expression of the ACT gene in astrocytes. In addition, we show that AP-1 is also indispensable for the expression of GFAP, which is an astrocyte-specific marker.

EXPERIMENTAL PROCEDURES

Cell culture. Human hepatoma HepG2, glioma U373-MG and A172, and cervical carcinoma HeLa cells were obtained from American Type Culture Collection (Rockville, MD). These cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, antibiotics, sodium pyruvate, and non-essential amino acids. U373 stable clones expressing c-jun (TAM67) were cultured in the presence of 10 μg/ml blasticidin, 100 μg/ml zeocin and 2 μg/ml tetracycline (all from Invitrogen, Carlsbad, CA) as needed.

Cytokines and cell stimulation. Cells were stimulated with 25 ng/ml OSM and 5 ng/ml TNFα (both from R&D, Systems, Inc., Minneapolis, MN, USA) or 10 ng/ml IL-1 (a gift from Immunex Corp., Seattle, WA, USA). One μM dexamethasone (Sigma Chemical Co., St. Louis, MO, USA) was also added to enhance cytokine action.

RNA preparation and Northern blot analysis. Total RNA was prepared by phenol extraction exactly as described previously (3). The filters were prehybridized at 65°C for 3 h in 0.5 M sodium phosphate buffer pH 7.2, 7% SDS, and 1 mM EDTA, and hybridized in the same solution with cDNA fragments of ACT, GFAP and GAPDH labeled by random priming (12). After the hybridization, nonspecifically bound radioactivity was removed by four washes in 40 mM phosphate buffer, 1% SDS, and 1 mM EDTA at 65°C for 20 min each.

Synthetic oligonucleotides. The following oligonucleotides were synthesized and subsequently used to generate PCR products containing the six DNase I hypersensitive sites. These PCR products were generated using Taq DNA polymerase, purified from gels using the gel purification kit (Qiagen, Valencia, CA), and subsequently used in gel retardation assays: DHS1; 5'-CTGGGAGGCTTCTTGCTA-3' and 5'-GACAAAACTGTGCAGTAC-3', DHS2; 5'-GATTGTATTATGAGATTTACTGG-3' and 5'-CAGGAACTGACTGG-3' and 5'-CAGGAAC
TCCGTGAGATAATC-3', DHS3; 5'-AGCA
GGATTTCTCTTCG-3' and 5'-ATTTC
GAAGATTCGAGAG-3', DHS4; 5'-CAGC
ACGAAACCAGCAGG-3' and 5'-GATCC
AGACACTCTCTCAGTG-3', DHS5; 5'-GAG
ACAGAGTCTCTCTGTG-3' and 5'-GCA
CATGCCATGCT-3', DHS6; 5'-CAAGC
ACGTATACAGAAAT-3' and 5'-GATTC
CATTCCCTGTC-3'. The following
oligonucleotides were synthesized containing
BamH1 sites to obtain PCR products which
were used to construct reporter plasmids:
DHS1; 5'-GGAGGATCCC
TGGGAGGCTT
CCTGCTA-3' and 5'-CAACGGATCC
GAAAACCTGTGTGAGTCAGC-3', DHS2; 5'-
TGTGGATCC
GATTGTATTATGAGATTT
ACTGG-3' and 5'-CCTGGATCC
CAGGACTCCGTGAGATAATC-3', DHS3; 5'-CGA
GGATCC
GAGCAGGATATTTCTGTCTC-3'
and 5'-CTGGGATCC
ATTTCAGAACTGCTGCACGC-3', DHS4; 5'-CAAAGGATCC
CA
GCACGAACCCAGCCAG-3' and 5'-ACAC
GAACG
GATCCGAGAGAACCCTGAGAA-3', DHS5; 5'-GAGGC
TCTCTCTGTGTTGCCCAGGCT-3' and 5'-ACTGGATCC
GCATGGCGCATGCCT-3', DHS6; 5'-TAg
GGATCC
CAAGCCCGTATTACCAGAAAT-3' and 5'-TAGG
GATCC
GATTCCAAAGCGTCTGTC-3'; ST1, 5-TTGAGGATCC
CT
GTGAGTAGCCCAC-3', ST2, 5'-CCCAG
ATCCCTGAGAGTCAAGAGG-3', ST4, 5'-
CAAGGGATCC
TTCTGGGATTGCCCTG-3', ST5, 5'-TCAGGGATCC
AGGATTGCTGTTG-3', posAP1; 5'-CT GGATCC
GTA
GATGACTAACACATTC-3'. The point
mutations in the AP-1 and NF-
κB sites were generated
using the following primers: 5'-GTAGAAGCTT
AACACATTTCCACAGC-3', 5'-TGTTAAGCTT
CTACTGCTGCAGAG-3', 5'-CAAGGGATCC
TTCTCGAGTTGCCCTG
GCCAACAGC-3'.

Plasmid construction. Plasmids ptkCATΔEH,
p'a'StCAT, pSSCAT, pACTCAT, and pStACTCAT have previously been described (11). Plasmids pDHIS1-tk-CAT, pDHIS2-tk-CAT, pDHIS3-tk-CAT, pDHIS4-tk-CAT, pDHIS5-tk-CAT, and pDHIS6-tk-CAT were generated by insertion of the corresponding BamHI-digested PCR products into the BamHI site of the plasmid ptkCATΔEH. Plasmid pmutAP-1(DHS2)-tk-CAT with the mutated AP-1 site in the DHS2 region was constructed using the QuikChange XL Site-directed Mutagenesis kit (Strategene, La Jolla, CA) according to the manufacturer’s instructions. Plasmid pS(E-S)ACT were generated by deletion of the EcoRV-BamHI fragment from plasmid pSSCAT. Subsequently, plasmids pD(A-S)ACT and pΔAatIIACT were generated by deletion of the AflII-SpeI or AflII-AflII fragments, respectively, from plasmid pS(E-S)ACT, while plasmid pS(E-S)ACT by cloning of the 1.2 kb SphI-EcoRV fragment from pSSCAT into the SphI-BamHI(blunt) digested pStACTCAT. Plasmids pST4ST2, pST1ST5, pposAP1, pmutNF, and ppmutAP were generated by insertion of the BamHI-digested PCR products into the BamHI/BglII sites of pStACTCAT. A plasmid encoding dominant-negative c-jun, pCMVc-jun(TAM67) (13), was obtained from Dr. Zendra Zehner, VCU, Richmond, VA. The expression vector was constructed using the T-REx™ system (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. The plasmid pCMVc-jun(TAM67) was digested with BamHI and the 931 base pair product containing c-jun(tam67) was cloned into the BamHI site of pcDNA4T/O.

Transient transfections. Cells were transfected using GeneJuice Transfection reagent (Novagen, Darmstadt, Germany), according to the supplier’s instructions. Plasmid DNA (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 to transfect cells growing in 1 ml of culture medium. One day after transfection, cells were stimulated, cultured another 24 h, and harvested. Protein extracts were prepared by freeze thawing (14), and protein concentration was determined by the BCA method (Sigma Chemical Co., St. Louis, MO, USA). Chloramphenicol acetyltransferase (CAT) and β-galactosidase assays were performed as described (15). CAT activities are normalized to the internal control β-galactosidase activity. Experiments were repeated 3-5 times yielding similar results and
a representative example of each experiment is shown.

**Nucleofection.** U373 cells (1x10⁶/6cm dish) were trypsinized, collected by centrifugation and resuspended in 600 μl of T nucleofactor solution™ (Amaxa, Koln, Germany). Two μg of the respective plasmids (c-jun(TAM67) or pUC19) were added to the solution, and transfection was performed using the Nucleofector device (Amaxa) with the electrical setting of T-20. One ml of warm DMEM medium was added, and cells were incubated at 37°C for 10 min and transferred to 6 cm dishes containing 5 ml of DMEM culture medium. Typical nucleofection efficiency was greater than 70% with cell viability close to 100%. One day after nucleofection, cells were stimulated with the respective cytokines for 18 hrs and processed as described earlier.

**Stable transfections** U373 cells expressing c-jun(TAM67) were generated using the T-REx™ system (Invitrogen, Carlsbad, CA, USA). U373 cells were transfected in 10 cm dishes with pcDNA6/TR using GeneJuice Transfection reagent (Novagen, Darmstadt, Germany), according to the supplier’s instructions. Four μg of pcDNA6/TR and 20 μl of GeneJuice were diluted in 650 μl of serum free medium per dish. Two days after transfections, transformants were selected in the presence of 10 μg/ml blasticidin. Stable clones were isolated after three weeks by glass cylinder cloning. Subsequently these stable cells were transfected with pcDNA4/TOc-jun(TAM67) in 10 cm dishes as described above and selected in the presence of zeocin (2 μg/ml). Positive clones were isolated and maintained in the presence of both, blasticidin and zeocin.

**Nuclear extract preparation and Electromobility shift assays (EMSA).** Nuclear extracts were prepared as described (16). Double stranded fragments were prepared by filling the 5’ protruding ends with Klenow enzyme using [α³²P]dCTP (3000 Ci/mmoll) (17). Alternatively, they were labelled by PCR in the presence [α³²P]dCTP (3000 Ci/mmoll) using Taq DNA polymerase. Gel retardation assays were performed according to published procedures using 5 μg of nuclear extracts (18) (19). Competition experiments were performed in the presence of 100-fold concentration of the cold oligonucleotides. Polyclonal anti-c-jun (sc-45), anti-junB (sc-8051), anti-junD (sc-74), anti-c-fos (sc-52), anti-fra1 (sc-605), anti-fra2 (sc-171) and anti-fosB (sc-7203) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and used for supershift studies.

**Western blotting.** U373 cells growing in 6 well plates were lysed in 200 μl of 10 mM Tris pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 0.5% NP-40, 1% Triton X-100, 1 mM sodium orthovanadate, 0.2 mM PMSF and mixture of protease inhibitors (Roche, Mannheim, Germany). These samples (20 μl) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). C-Jun and GFAP were detected using polyclonal sc-45 and sc-9065 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Antigen-antibody complexes were visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

**Isolation of nuclei and DNase I-hypersensitive site mapping.** Clones were cultured in the presence of 2 μg/ml tetracycline for 5 days. Nuclei were isolated and treated with increasing concentrations of DNase I exactly as previously described (3). DNA was isolated using a DNA isolation kit from Gentra Systems (Minneapolis, MN, USA) according to the manufacturer’s instructions. Purified genomic DNA (10 μg per gel lane) was digested with the appropriate restriction enzyme, separated in 0.8% agarose gel, and transferred to Hybond-XL membranes (Amersham Biosciences, Piscataway, NJ, USA). Membranes were hybridized to random primer-labeled SG5 and PRA7 probes (3) in 500 mM sodium phosphate pH 7.1, 7% SDS, 1 mM EDTA and 10 μg/ml herring DNA at 65°C. After the hybridization nonspecifically bound radioactivity was removed by three washes in 40 mM phosphate buffer, 1% SDS and 1 mM EDTA at 65°C for 20 min.
**Chromatin immunoprecipitation (ChIP) assay.**
Stable clones were cultured in the presence of tetracycline (2 μg/ml) for five days, and chromatin was crosslinked by the addition of formaldehyde to 1% followed by a 10 minute long incubation at 37°C. Subsequently, the cells were washed with ice-cold PBS containing 125 mM glycine and 1 mM PMSF. Chromatin was sonicated and immunoprecipitated using specific antibodies exactly as described in the chromatin immunoprecipitation protocol from Upstate Inc. (Charlottesville, VA, USA). The following antibodies were used: anti-acetyl histone H3 (ab2381), anti-acetyl-histone H4 (ab1758) (Abcam Inc, Cambridge, MA, USA), anti-dimethyl-histone H3 (Lys4) (07-030) (Charlottesville, VA, USA), anti-c-jun (sc-45), and anti-c-fos (sc-52) both from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

**RESULTS**

**Activator Protein-1 (AP-1) binds to DNase I hypersensitive sites (DHS) 1 and DHS2.**
We have previously shown that the astrocyte-specific expression of the ACT gene is associated with the tissue-specific chromatin structures at the distal serpin subcluster (3). This subcluster is easily accessible in HepG2 cells where all three genes encoding KAL, PCI and ACT are expressed. In contrast, it is accessible only near the IL-1-responsive enhancer and the ACT gene promoter in primary human astrocytes and glioma cells. We have identified six DHSs located nearby the IL-1 enhancer and the ACT promoter in astrocytes and U373 cells (3). In order to identify the astrocyte-specific factors required for the ACT expression, we analyzed the binding of proteins to the DNA fragments covering these six DHSs by EMSA. Each of these fragments was assayed for binding *in vitro* using nuclear extracts from ACT expressing glioma cells (U373 and A172), hepatoma cells (HepG2), and non-expressing carcinoma (HeLa) cells. We have obtained multiple bands and hence performed competition experiments to confirm their specificity (Fig. 1).

Simultaneously, we analyzed the fragments using the Mat Inspector program (http://www.genomatix.de) to identify transcription factor binding sites. A number of binding sites were identified including two AP-1 elements within the DHS1 and DHS2 fragments. These elements were further analyzed for the binding of AP-1, because this factor is very abundant in astrocytes (compared to hepatoma cells (20)), and it has also been suggested to regulate the expression of the GFAP gene, which is an astrocyte specific marker. We confirmed the binding of AP-1 to both fragments (DHS1 and DHS2) by competition with the AP-1 oligonucleotide in both glioma cell lines, as well as in HeLa cells, which contain abundant amounts of endogenous AP-1 (Fig. 1A, B). However, AP-1 did not bind to DHS1 and DHS2 in hepatoma HepG2 cells. The same expression pattern of ACT in both A172 and U373 glioma cells suggests that a common factor should bind to the DHSs in both cell lines. The binding pattern observed with probes DHS3 and DHS4 was different among these cells (Fig. 1C, D). Therefore, we concluded that factors binding to DHS3 and DHS4 are likely not relevant for glioma-specific expression. The DHS5 probe did not bind any protein specifically while DHS6, which corresponds to the ACT core promoter, likely binds factors from the basal transcriptional machinery (Fig. 1E, F).

In order to identify the components of the AP-1 complex that bind to DHS1 and DHS2, we performed supershift analysis using extracts from glioma and hepatoma cells. The supershifts, (Fig. 2) indicate that c-jun is the major component of the AP-1 complex, and binds to both DHS1 and DHS2 in U373 cells, while amounts of c-fos were much lower. Neither c-jun nor c-fos binding was detected in HepG2 cells as none of the DNA-protein complexes were supershifted. We conclude that AP-1 specifically binds to DHS1 and DHS2 in glioma cells; however, some other factor(s) bind(s) to these elements in hepatoma cells.

**DHS1 and DHS2 can function as enhancers in U373 cells.** In order to analyze if these six DHSs have a regulatory role, we linked each of the six DHSs to the thymidine kinase (tk)-
promoter driving the transcription of the CAT reporter gene and analyzed these reporter constructs in transient transfections of U373 cells. The fragments containing both DHS1 and DHS2 increased the basal activity of the reporter constructs by 4-5 fold while the other DHSs had no effect (Fig. 3a). In order to determine whether DHS1 and DHS2 fragments can also act as IL-1-responsive elements, we stimulated U373 cells (transiently transfected as before) with IL-1. The fragments containing DHS1 and DHS2 conferred IL-1 responsiveness (1.5-2 fold) onto the tk-promoter while the other fragments were ineffective (Fig. 3B).

A previously identified 413bp long IL-1-responsive enhancer located 13 kb upstream the transcription start site of the ACT gene contains two NF-κB and one AP-1 binding site (11). The 160 bp long DHS1 fragment partially overlaps this IL-1-responsive enhancer including its AP-1 binding sites. It has already been shown that mutation of this AP-1 site (at -13 kb) leads to a 50% decrease in the IL-1 responsiveness. In order to determine whether the other AP-1 site located within the DHS2 fragment (at -11.5 kb) is also required for full transcriptional activity of this new enhancer, we mutated this site in the corresponding reporter plasmid. This mutation resulted in a dramatic loss of basal activity of the reporter plasmid when transfected into U373 cells (Fig 3. C).

We have antecedently reported that the reporter containing 413bp long IL-1-responsive enhancer (located at -13 kb) is less responsive to IL-1 than the reporter containing 8 kb long fragment, which suggested the presence of an additional regulatory element (11). Therefore, we generated a series of deletion reporters and tested them in transfection experiments (Fig. 4). This analysis resulted in the identification of an additional IL-1-responsive fragment that contained putative NF-κB and AP-1 binding sites. Interestingly, this fragment partially overlaps with the DHS2 fragment, and the AP-1 site we identified by this deletion analysis is identical to the site we identified by EMSA. Mutation of the AP-1 element within this reporter also abolished the responsiveness to IL-1 while mutation of NF-κB element had no effect. From these results we conclude that both DHS1 and DHS2 fragments containing two AP-1 binding elements are critical for the full basal transcriptional activity.

C-jun, c-fos, fosB and fra1 are major components of the AP-1 complex binding to the ACT enhancer elements in glioma cells. The AP-1 family of transcription factors include the Jun and Fos family members. C-jun can form stable homo- and hetero-dimers with other members of the AP-1 family while members of the Fos family do not homodimerize, but form stable heterodimers with Jun proteins (21). We analyzed the composition of AP-1 complexes that bind to the newly identified AP-1 binding site at -11.5 kb (DHS2) in untreated U373 cells and cells treated with IL-1, -OSM, and -TNF. The binding of AP-1 to this oligonucleotide was competed off by cold AP-1 probes (both -13 kb and -11.5 kb AP-1 elements), but not by cold oligonucleotides containing binding sites for NF-κB, STAT or C/EBP (Fig. 5A). The AP-1 complexes in untreated U373 cells contained c-jun and a limited amount of fra1, while IL-1 stimulation resulted in induction of some c-fos and fosB (Fig 5B, C). Hence, we conclude that c-jun homodimers are likely bound to the –11.5 kb AP-1 element in untreated cells, while IL-1 stimulation results in the heterodimerization of c-jun with either c-fos, fosB, or fra1.

The elements at -11.5 kb and -13 kb are bound by AP-1 complexes in vivo. Next, we analyzed the binding of AP-1 to the elements located at -13 kb (DHS1) and -11.5 kb (DHS2) in vivo using ChIP analysis in different cell lines (Fig. 6). C-jun binding was detected to both elements in untreated U373 cells, while the binding of c-fos was marginal. IL-1 treatment resulted in a decrease of c-jun and an increase in c-fos binding, mostly at the -11.5 kb element, indicating replacement of c-jun homodimers with c-jun/c-fos heterodimers. In contrast to glioma cells, no binding of c-jun or c-fos was detected in vivo in HeLa cells, even though they express abundant amounts of endogenous AP-1. This
discrepancy can be explained because both of these enhancers (-11.5 and -13 kb elements) are localized to the inaccessible chromatin in HeLa cells (3). We conclude that c-jun is the critical AP-1 component binding to both enhancers in vivo in untreated U373 cells.

**Generation of stable U373 cells expressing dominant negative c-jun(TAM67) in a tetracycline (tet) inducible system.** The binding of AP-1 to both the -13 and -11.5 kb elements in vivo, and the dramatic decrease of basal activity after mutating the AP-1 binding sites within the reporter constructs suggested that AP-1 may be critical for the basal ACT expression in glioma cells. In order to test this hypothesis in vivo, we generated a stable U373 cell line which inducibly overexpresses a dominant-negative c-jun(TAM67) that lacks amino acids 3 to 122 within the transactivation domain. Several clones that showed inducible c-jun(TAM67) expression in the presence of tetracycline, but differed in the levels of expression of an astrocytic marker GFAP were obtained (Fig 7A). We analyzed ACT expression in two of these clones either untreated or stimulated with IL-1 or OSM in the presence or absence of tetracycline using Northern blot analysis (Fig. 7B). The basal expression of ACT was completely abolished when expression of c-jun(TAM67) was induced using tetracycline (Fig. 7B, long exposure). However, there was a considerable drop in the ACT mRNA levels after IL-1 and OSM treatment in the presence of tetracycline (Fig. 7B, short exposure). This effect was specific since the GAPDH mRNA levels were unaffected. We infer that AP-1 is critical for both basal and cytokine induced expression of the ACT gene in glioma cells.

**Histone modifications at the IL-1-responsive enhancer and the ACT promoter in U373-TAM67 cells.** Hyperacetylation of histones H3 and H4, and methylation of lysine 4 of histone H3 has been previously shown to correlate with transcriptional activity of the ACT gene (3). Since we detected similar accessibility to DNase I digestion in parental and c-jun(TAM67) expressing cells, we next analyzed the acetylation status of histone H3 and H4 at fragments containing both DHS1 and DHS2, and the methylation of lysine 4 of histone H3 at the promoter of the ACT gene using ChIP assay. Indeed, we found the presence of acetylated histones H3 and H4 at both fragments containing DHS1 and DHS2 in the parental U373 and U373-TAM67 cells (Fig. 9A). However, lysine 4 methylation of histone H3 at the ACT promoter was drastically diminished in c-jun(TAM67) expressing cells (Fig. 9B). We conclude that the lack of the transactivation domain of c-jun(TAM67) results in the loss of both ACT expression and histone H3 lysine 4 methylation at the ACT promoter, but does not influence acetylation of histone H3 and H4 at the 5’ flanking region of this gene.

**AP-1 is indispensable for basal expression of the GFAP gene.** Since functional AP-1 was needed for ACT expression in glioma cells we suspected that it may be indispensable for expression of other astrocyte-, glioma-specific genes including GFAP. To test this
hypothesis, we transiently transfected U373 cells with an expression vector for c-jun(TAM67) using nucleofection technology and analyzed the expression of both GFAP and ACT. Indeed, c-jun(TAM67) drastically repressed expression of GFAP and ACT (Fig. 10A). We also analyzed GFAP expression in U373-TAM67 cells and found that GFAP expression was completely lost after c-jun(TAM67) expression was induced by tetracycline (Fig. 10B). We conclude that AP-1 is required for the GFAP and ACT gene expression in glioma cells.

**DISCUSSION**

ACT is constitutively expressed at low levels by brain astrocytes, and at moderate levels by liver hepatocytes (22) (23) (20). Expression of this serpin gene in the liver is likely determined by transcription factor(s) belonging to four conserved families: HNF-1, HNF-3, HNF-4 and C/EBP. However, the factors required for the expression of the ACT gene in astrocytes and/or glioma cells are unknown. Here we identify AP-1 as a critical transcription factor needed for the basal expression of the ACT gene in glioma cells. We conclude this from the following observations: i) there are two DHSs present in the 5' proximal region of the ACT gene that contain functional AP-1 binding sites; ii) mutation of these AP-1 sites leads to the loss of the enhancer’s activity; iii) over-expression of the dominant negative c-jun abolished the basal expression of the ACT gene; iv) AP-1 is also critical for the expression of GFAP, which is an astrocyte specific marker; and v) resting glioma cells and astrocytes express high levels of AP-1. These data suggest the necessity for AP-1 for the astrocyte-specific expression of the ACT gene. In addition, the two DHSs containing an AP-1 binding site in the 5’ flanking region of the ACT gene can act as enhancers when linked to the reporter gene. These elements bind mainly c-jun and fra1 in untreated cells. These results suggest that c-jun homodimers, and to a lesser extent c-jun/fra1 heterodimers, mediate the basal expression of the ACT gene (Fig. 6). However, an exchange of these dimers likely occurs in astrocytes stimulated with IL-1, due to the observations that c-jun, c-fos, fra1, and fosB containing complexes can bind to the AP-1 sites in IL-1 treated cells (Fig. 5).

We have previously reported that astrocyte- and glioma-specific expression of the ACT gene is determined by the astrocyte-specific chromatin structure of the distal serpin subcluster (3). In this study we propose that AP-1 is critical for the astrocyte-specific expression of the ACT gene. Our data raises the question as to whether AP-1 (specifically c-jun) is the key factor determining the chromatin structure at the distal serpin subcluster in astrocytes. Fig. 6 demonstrates the in vivo binding of c-jun to DHS1 and DHS2 in glioma cells. These c-jun homodimers likely recruit co-activator complexes containing histone acetyl transferases (HATs), CBP/p300, and/or chromatin remodeling factors as previously reported (24). This recruitment likely leads to the acetylation of histones and subsequent decondensation of chromatin at both the enhancer and the promoter, which facilitates the binding of the basal transcriptional apparatus.

This model is partially supported by the results obtained from U373 cells stably expressing the dominant-negative c-jun(TAM67), which lacks the transactivation domain. Expression of c-jun(TAM67) resulted in the complete inhibition of the basal ACT expression (Fig. 7). However, the up-regulation of ACT expression by IL-1 and OSM was retained in these cells suggesting that NF-xB and STAT3 binding to the -13 kb enhancer and promoter elements, respectively, could counteract AP-1 deficiency. Similar results were obtained in transient transfection experiments of U373 cells with c-jun(TAM67) expression vector thus ruling out the clonal differences in the expression of the ACT gene (Fig. 10). Our data (Fig. 8) raises the question as to why the c-jun(TAM67) clones retained all six DHSs (suggesting a lack of alteration in chromatin structure) even though the basal expression of ACT was completely abolished. This lack of change in the chromatin structure suggests that c-jun(TAM67) retained the ability to recruit co-activator proteins (including histone acetyl transferases and
ATP-dependent chromatin remodeling factors). In support of this, the c-jun(TAM67) truncation lacks the transactivation domain (amino acids 3-122); however, it retains approximately half of the region (amino acids 96-193) that interacts with p300 (24) suggesting that c-jun(TAM67) could still recruit p300. Another alternative is that a yet to be identified factor could cooperate with c-jun and be sufficient to recruit co-activator complexes. However, both of these possibilities could result in histone acetylation as confirmed by the presence of acetylated histones H3 and H4 at both enhancers (Fig. 9). The loss of lysine 4 methylation on histone H3, at the ACT promoter, in U373-TAM67 cells correlates with the loss of basal ACT expression. This confirms that functional AP-1 is needed for ACT expression in glioma cells. The absence of the transactivation domain in c-jun(TAM67) and the inhibition of basal ACT expression suggests that a functional enhansosome is not formed at the ACT gene.

Recently a locus control region (LCR) has been described in the proximal serpin subcluster (25). Deletion of this LCR resulted in the decrease of histone acetylation throughout the proximal subcluster (26). It is tempting to speculate that the region containing both enhancers within the distal serpin subcluster could also constitute an LCR. In the future, the deletion of this region may determine the relevance of these enhancers and the role of c-jun in determining the astrocyte-specific chromatin structure and astrocyte-specific gene expression.

Expression of the ACT gene in hepatocytes is likely controlled by HNF1, HNF3, HNF4, and C/EBP. Since the levels of AP-1 found in the hepatocytes are low (compared to astrocytes), and the levels of HNFs and C/EBPs are high one can invoke a model where HNFs and C/EBPs out compete AP-1 for closely spaced binding sites and determine the liver specific-expression pattern of these serpin genes. Since the expression levels of these transcription factors are reversed in astrocytes, we propose that AP-1 regulates the basal expression of ACT in these cells. Moreover, we show for the first time that AP-1 is also required for GFAP expression, an astrocyte-specific marker protein. Three binding sites for nuclear factor-1 (NF-1) have been identified in the 5' flanking region of this gene that are important for its astrocyte-specific expression (27). Interestingly, an AP-1 binding site is located near the two crucial NF-1 binding sites, which may suggest its functional importance for the GFAP expression.

The precise mechanism that allows ubiquitously expressed AP-1 to determine astrocyte-specific expression is not known at the moment but it may include: i) astrocyte specific post-translational modification of c-jun; ii) formation of different heterodimers in astrocytes versus hepatocytes; iii) the recruitment of astrocyte-specific co-activator complexes; and iv) cooperation with, the yet to be identified, c-jun-dependent astrocyte-specific transcription factor.

REFERENCES


FOOTNOTES

The abbreviations used are: ACT, α1-antichymotrypsin; AP-1, activator protein-1; CAT, chloramphenicol acetyltransferase; C/EBP, CAAT enhancer binding protein; ChIP, chromatin immunoprecipitation; DHS, DNase I-hypersensitive sites; EMSA, electromobility shift assay; GAPDH; Glyceraldehyde-3-Phosphate Dehydrogenase; GFAP, glial fibrillary acidic protein; HNF, hepatocyte nuclear factor; IL, interleukin; KAL, kallistatin; NF-κB, nuclear factor kB; OSM, oncostatin M; PCI, protein C inhibitor; serpin, serine proteinase inhibitor; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor α.

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FIGURE LEGENDS

Fig. 1. AP-1 binds to DHS1 and DHS2 in glioma cells. DHS map of the 5’ flanking region of the ACT gene. The map is drawn to scale, with position +1 defined as the transcription start site of the ACT gene. Exons are indicated as black boxes while gray boxes represent IL-1-responsive enhancers. Arrows indicate the position of DHS(s). (A-F). Nuclear extracts were prepared from human glioma U373 and A172, hepatoma HepG2, and carcinoma HeLa cells. The binding was
analyzed by EMSA using the $^{32}$P-labeled DHS probes as indicated. A 100-fold excess of unlabelled oligonucleotide competitors was added to the binding reactions as indicated. Arrows indicate positions of bands competed off by an AP-1 oligonucleotide.

**Fig. 2. C-jun binds to DHS1 and DHS2 in astrocytoma cells.** Nuclear extracts were prepared from U373 and HepG2 cells. Five μg of nuclear extracts were incubated with anti-c-jun, anti-c-fos antibodies, or normal rabbit serum (NRS). The binding was analyzed by EMSA using DHS1 and DHS2 probes.

**Fig. 3. DHS1 and DHS2 contain functional AP-1 binding sites.** U373 cells were transfected with indicated plasmids pDHS1-tk-CAT, pDHS2-tk-CAT pDHS3-tk-CAT, pDHS4-tk-CAT pDHS5-tk-CAT and pDHS6-tk-CAT (A), pDHS2-mutAP-1-tk-CAT (C), and β-galactosidase expression vector as an internal control for transfection efficiency. One day after transfection cells were stimulated with IL-1 (B and C) or left untreated. They were cultured for another 24 h, and harvested. CAT activities were normalized to β-galactosidase activities (cpm/units). A representative result of three separate experiments that produced similar results is shown.

**Fig. 4. Detailed analysis of a new putative IL-1-response element of the ACT gene.** (A). U373 cells were transfected with plasmids p’a’SACT, pΔACTCAT, pSSCAT, p(ES)ACT, p(B-E-S)ACT, pΔ(A-S)ACT, pΔAttIIACT, pST4ST2, pST1ST5, pposAP1, pmutNF or pmutAP, and β-galactosidase expression vector as internal control for transfection efficiency. One day after transfection cells were stimulated with IL-1, cultured for another 24 hours, and harvested. CAT activities were normalized to β-galactosidase activities (cpm/units). OP indicates 244 bp long ACT promoter, black boxes represent both enhancers, while grey boxes represent putative binding sites for NF-κB and AP-1. (B). Nucleotide sequence of the second enhancer at -11.5 kb. Putative binding elements are boxed.

**Fig. 5. Composition of AP-1 complexes in human glioma cells.** U373 cells were stimulated with IL-1, OSM and TNF for 2 hrs (A) or 1 h (C). Nuclear extracts were prepared, and binding was analyzed using the AP-1 (DHS2) oligonucleotide by EMSA. Unlabelled oligonucleotide competitors AP-1 element from (DHS1) and (DHS2), NF-κB, SIE and C/EBP were added as described earlier (A). Nuclear extracts from control (B) or IL-1-treated (C) cells were incubated with anti-c-jun, anti-junB, anti-junD, anti-c-fos, anti-fra1, anti-fra2, anti-fosB antibodies or normal rabbit serum (NRS), and binding was analyzed by EMSA.

**Fig. 6. In vivo binding of c-jun to DHS1 and DHS2.** U-373 and HeLa cells were stimulated with IL-1 for 1h, chromatin was prepared, and equal amounts of chromatin were immunoprecipitated with specific anti-c-jun or anti-c-fos antibodies. Subsequently, DNA was purified and the DHS1 and DHS2 regions were amplified by PCR in the presence of [α$^{32}$P]dCTP. PCR products were separated in 12% native polyacrylamide gels, and gels were exposed to phosphorimager screens. Input represents 2% of chromatin used for immunoprecipitation. A representative of two independent experiments is shown.

**Fig. 7. Dominant-negative c-jun(TAM67) down-regulates basal and cytokine-induced ACT mRNA expression.** (A). U373 stable clones expressing c-jun(TAM67) were cultured in the presence of tetracycline for 48 h (as indicated), lysates were prepared, and analyzed by Western blotting using anti-c-jun and anti-GFAP antibodies. (B). U373-TAM67 cells (clone 4.4 and 4.10) were cultured in the presence of tetracycline for 48 h, and then stimulated with IL-1 or OSM. RNA was isolated after 18 h and subjected to Northern blot analysis using ACT and GAPDH cDNA as probes. Both short and long exposures of the blot are shown.
Fig. 8. DHS analysis at the 5' flanking region of the ACT gene. U373-TAM67 cells (4.4 and 4.10) were cultured in the presence of 2 μg/ml tetracycline for 72h. Nuclei were isolated and digested with increasing concentration of DNase I, DNA was purified, and digested with BglII (promoter) or HindIII (enhancer). DNA samples were analyzed by Southern blotting using SG-5 “promoter” and PRA 7 “enhancer” probes.

Fig. 9. Histone acetylation and methylation at the ACT gene in c-jun(TAM67) expressing cells. U373-TAM67 cells (clone 4.10) were cultured in the presence of tetracycline (2 μg/ml) for 120 hrs as indicated. Chromatin was prepared from parental U373 cells and U373-TAM67 clones, and equal amounts were used for immunoprecipitation with anti-acetyl-H3, anti-acetyl-H4 (a), anti-H3-K4 (b) and non-specific (NS) antibodies. Subsequently, DNA was purified and the DHS1 and DHS2 fragments (A) and the ACT promoter (B) were amplified by PCR in the presence of [α³²P]dCTP. PCR products were separated in 12% native polyacrylamide gels, and exposed to phosphoimager screens. Input represents 2% of chromatin used for immunoprecipitation.

Fig. 10. Dominant-negative c-jun(TAM67) downregulates GFAP expression in glioma cells. (A). U373 cells were transiently transfected with plasmid pCMV-cjun(TAM67) or control plasmid using nucleofection technology, cultured overnight, and subsequently stimulated with IL-1 or OSM. RNA was isolated after 18 h, and subjected to Northern blot analysis using ACT and GFAP cDNA as probes. The bottom panel shows 28S RNA stained with ethidium bromide on the membrane. (B). U373-TAM67 cells (clone 4.10) were cultured in the presence of tetracycline for 48 h, and then stimulated with IL-1 or OSM. RNA was isolated after 18 h and subjected to Northern blot analysis using GFAP and GAPDH cDNA as probes.
Figure 1.
Figure 2.
Figure 3.

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- DHS1
- DHS2
- DHS3
- DHS4
- DHS5
- DHS6

B

- PDHS1
- pDHS2

C

- pDHS2
- PDHS2
- mutAP-1
Figure 5.
Figure 6.

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** clones
** tet
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** GFAP

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** tet
** IL-1
** OSM
** ACT mRNA (Long)
** ACT mRNA (Short)
** GAPDH mRNA
Figure 8.
**Figure 9.**

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- DHS1
- DHS2

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- U373
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- ACT pro
Figure 10.

A

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GFAP mRNA
ACT mRNA
28S RNA

B

Clone 4.10

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GFAP mRNA
GAPDH mRNA
Astrocyte-specific expression of the α1-antichymotrypsin and glial fibrillary acidic protein genes requires activator protein-1
Sunita M. Gopalan, Katarzyna M. Wilczynska, Barbara S. Konik, Lauren Bryan and Tomasz Kordula

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