A Novel Mechanism of Tissue Inhibitor of Metalloproteinases-1 Activation by Interleukin-1 in Primary Human Astrocytes[*]

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Reactive astrogliosis is the gliotic response to brain injury with activated astrocytes and microglia being the major effector cells. These cells secrete inflammatory cytokines, proteinases, and proteinase inhibitors that influence extracellular matrix (ECM) remodeling. In astrocytes, the expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) is up-regulated by interleukin-1 (IL-1), which is a major neuroinflammatory cytokine. We report that IL-1 activates TIMP-1 expression via both the IKK/NF-κB and MEK3/6/p38/ATF-2 pathways in astrocytes. The activation of the *TIMP-1* gene can be blocked by using pharmacological inhibitors, including BAY11-7082 and SB202190, overexpression of the dominant-negative inhibitor of NF-κB (IκBαSR), or by the knock-down of p65 subunit of NF-κB. Binding of activated NF-κB (p50/p65 heterodimer) and ATF-2 (homodimer) to two novel regulatory elements located −2.7 and −2.2 kb upstream of the TIMP-1 transcription start site, respectively, is required for full IL-1-responsiveness. Mutational analysis of these regulatory elements and their weak activity when linked to the minimal *tk* promoter suggest that cooperative binding is required to activate transcription. In contrast to astrocytes, we observed that TIMP-1 is expressed at lower levels in gliomas and is not regulated by IL-1. We provide evidence that the lack of TIMP-1 activation in gliomas results from either dysfunctional IKK/NF-κB or MEK3/6/p38/ATF-2 activation by IL-1. In summary, we propose a novel mechanism of TIMP-1 regulation, which ensures an increased supply of the inhibitor after brain injury, and limits ECM degradation. This mechanism does not function in gliomas, and may in part explain the increased invasiveness of glioma cells.

The remodeling of the extracellular matrix (ECM),[2] including the degradation of the ECM by matrix metalloproteinases (MMPs) and its subsequent resynthesis, is critical during normal physiological processes, such as angiogenesis, embryonic development, organ morphogenesis, bone remodeling, and ovulation (1, 2). During these processes the proteolytic activity of MMPs is tightly controlled at the transcriptional level by growth factors, hormones, and cytokines, and at the protein level by proteolytic cleavage of inactive zymogens, and the inhibition by specific inhibitors, including tissue inhibitors of metalloproteinases (TIMPs) (2). The delicate balance between the activities of MMPs and TIMPs is critical to limit deleterious outcomes of uncontrolled degradation, which is manifested in pathological conditions such as periodontal disease, arthritis, tumor cell invasion, fibrosis, and neurodegenerative disorders (2–4). These pathological conditions often represent chronic inflammatory diseases suggesting that inflammatory mediators, including inflammatory cytokines, may disrupt the intricate balance between MMPs and TIMPs.

In the central nervous system (CNS), infection and injury induce a histopathological response known as reactive astrogliosis, which is the primary cause of regenerative failure in the mature CNS (5–7). During this response, activated astrocytes and microglia secrete MMPs, TIMPs, and a plethora of cytokines and growth factors that drastically change the proteolytic balance and affect ECM remodeling (8–10). IL-1 is one of the major neuroinflammatory cytokines that is detected in the CNS after brain injury, and affects the expression of several MMPs and TIMPs in astrocytes, microglia, and brain endothelial cells (10–12). Thus far, four members of the TIMP family (TIMP-1 through TIMP-4) have been identified in mammals (2). Among TIMPs, TIMP-1 is encoded by a highly inducible gene, and is up-regulated in several cell types by IL-1, IL-6, tumor necrosis factor, epidermal growth factor, transforming growth factor, and oncostatin M (OSM) (13). In the CNS, TIMP-1 expression is up-regulated in astrocytes following intracranial injury, with TIMP-2 expression up-regulated in microglia and neurons (8). Astrocytic TIMP-1 expression is also up-regulated in experimental autoimmune encephalitis, and in transgenic animals expressing cytokines in the brain (14). However, chronic inflammation associated with HIV-1-associated dementia is actually characterized by the decreased levels of

[2] The abbreviations used are: ECM, extracellular matrix; AP-1, activating protein-1; ATF-2, activating transcription factor-2; CAT, chloramphenicol acetyltransferase; CNS, central nervous system; EMSA, electromobility shift assay; ERK, extracellular stress-regulated kinase; IκB, inhibitor of NF-κB; IKK, IκB kinase; IL, interleukin; MEK, mitogen-activated protein kinase kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor κB; OSM, oncostatin M; TIMP, tissue inhibitor of metalloproteinase.

TIMP-1 in both cerebrospinal fluid and brain tissue, suggesting that prolonged activation of astrocytes leads to TIMP-1 down-regulation and, in turn, ECM degradation (10). *In vitro*, IL-1 significantly increases TIMP-1 expression in astrocytes, which recapitulates the *in vivo* findings from acute injury models (10, 15).

Because TIMP-1 up-regulation in astrocytes prevents excessive ECM degradation, the mechanisms of TIMP-1 regulation may lead to the identification of new therapeutic targets. We initiated this study with the aim of identifying the molecular mechanism that regulates TIMP-1 expression in astrocytes exposed to IL-1.

EXPERIMENTAL PROCEDURES

Cell Culture—Human cortical astrocyte cultures were established using dissociated human cerebral tissue established exactly as previously described (16). Cortical tissue was provided by Advanced Bioscience Resources (Alameda, CA), and the protocol for obtaining postmortem fetal neural tissue complied with the federal guidelines for fetal research and with the Uniformed Anatomical Gift Act. Human glioblastoma U373-MG cells were obtained from American Type Culture Collection, whereas human glioma A172, U251, and T98G cells were obtained from Dr. Jaharul Haque (Cleveland Clinic Foundation, Cleveland, OH). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, antibiotics, sodium pyruvate, and non-essential amino acids.

Cytokines and Cell Stimulation—Cells were stimulated with 25 ng/ml OSM (R&D, Systems, Inc., Minneapolis, MN) or 10 ng/ml IL-1 (a gift from Immunex Corp., Seattle, WA). For inhibitor studies, cells were pretreated with 1 μM SP600125, 10 μM BAY-117082, 10 μM SB202190, 5 μM parthenolide, 5 μg/ml actinomycin D (all from Sigma), 10 μM GF109203X, or 1 μM CAY10470 (EMD Biosciences, Inc. San Diego, CA), and then treated with IL-1.

RNA Preparation and Northern Blot Analysis—Total RNA was prepared by phenol extraction exactly as described previously (17). 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 TIMP-1 labeled by random priming (18). 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.

Quantitative PCR—TIMP-1 mRNA levels were measured using TaqMan technology (Applied Biosystems, Foster City, CA) according to the supplier's instructions. Briefly, 1 μg of total RNA was reverse-transcribed using the High capacity cDNA archive kit (Applied Biosystems). Subsequently, the cDNAs were diluted 100-fold (TIMP-1) or 10,000-fold (18 S rRNA). For real-time PCR, pre-mixed primer probe sets and TaqMan Universal PCR Master Mix were purchased from Applied Biosystems, and cDNAs were amplified using ABI 7900HT cycler.

Synthetic Oligonucleotides—The following oligonucleotides were synthesized to amplify the DNA fragments from the 5'-flanking region of the *TIMP-1* gene: (−1.0 + 1.0); 5'-AGG-T<u>CCATGG</u>GGGAGGGGGCAGGG-3' and 5'-GGGG<u>CCAT</u>-<u>GG</u>TGGGTTCTGTGGGG-3', (−4.2–0.8); 5'-ACCT<u>GGT</u>-<u>ACC</u>AGGGTTGTAACTCAGG-3' and 5'-AAAG<u>GGTAC</u>-<u>CC</u>GTCCAATCAAGAGAC-3', (−5.3–4.1); 5'-CCAA<u>GG</u>-<u>TACC</u>TTACAGCTTAGAAG-3' and 5'-AGTA<u>GGTACCC</u>-GGTTCTGTGGAGTG-3', (−7.7–7.0); 5'-CGGA<u>GCATG</u>-<u>C</u>GGCAGAGGAATGGAG-3' and 5'-TCTGGGTCATAC-AGAACCAG-3', (−9.4–8.9); 5'-ACCT<u>CGGTACC</u>CCCA-GCTCAAGTAAG-3' and 5'-TGGGGCATGCTAGAGAG-AGACAAGG-3', (-2.9–2.1); 5'-TGGCT<u>GGTACC</u>TGTAA-TCCCAGCACTTTGG-3' and 5'-AGCT<u>CTCGAGA</u>TGGT-CACACACCCC-3', (−2.9–2.7); 5'-ACCT<u>GGTACC</u>TCCG-AGGGAGAAGTGAGG-3' and 5'-GCAT<u>CTCGAGG</u>CAG-CGGGCCAGGGAAAC-3', (−3.5–2.7) 5'-ACCT<u>GGTACC</u>-TCCGAGGGAGAAGTGAGG-3' and 5'-GCAT<u>CTCGAG</u>-GCAGCGGGCCAGGGAAAC-3', (-3.9–2.7); 5'-ACCT<u>GG</u>-<u>TACC</u>TCCGAGGGAGAAGTGAGG-3' and 5'-ACAA<u>CT</u>-<u>CGAG</u>ACACCCACAACTCAGTTTG-3'. The following oligonucleotides were synthesized to generate the plasmid phT(-0.7m)CAT: 5'-C<u>AGATCT</u>CTCGAGG<u>CATGC</u>GTA-3' and 5'-TAC<u>GCATGC</u>CTCGAGAGA<u>TCT</u>GGTAC-3'. The activating transcription factor-2 (ATF), activating protein-1 (AP-1), and nuclear factor κB (NF-κB) double-stranded oligonucleotides used both to generate ATF, AP-1, and NF-κB reporter constructs, and also in EMSA had the following sequence: AP-1; 5'-GATCTGTGC<u>TGACTCA</u>GGTTA-3' and 5'-GATCTAACC<u>TGAGTCA</u>GCACA-3', NF-κB; 5'-GATCT-GGCA<u>GGACTTCCCC</u>TGCCA-3' and 5'-GATCTGGCA<u>GG</u>-<u>GGAAGTCC</u>TGCCA-3', ATF; 5'-GATCTCGCT<u>TGAGCTC</u>-AGGAGA-3' and 5'-GATCTCT<u>CCTGAGCTCA</u>AGCGA-3', upperNF-κB; 5'-GATCTCCT<u>GGGCACTCCC</u>ACCCA-3' and 5'-GATCTGGGT<u>GGGAGTGCCC</u>AGGA-3', upperATF; 5'-GATCTTGACT<u>GGCGTCA</u>TTATA-3' and 5'-GATCTATA-AT<u>GACGCCA</u>GTCAA-3'. The mutations within the ATF, AP-1, and NF-κB elements were generated using the following primers: AP-1 mutant; 5'-TGAAA<u>TCCGGA</u>CACAGAGTGT-TGCCGAC-3' and 5'-TGCTG<u>TCCGGA</u>GTTTCAAAGAGT-TGGCGTCAG-3', NF-κB mutant; 5'-GGCAGC<u>TCGAG</u>TC-CTGCCTTGGCCCCTC-3' and 5'-CAGGA<u>CTCGAG</u>CTGC-CCTCACTTCTCCCTC-3', ATF mutant; 5'-CTTG<u>CATAT</u>-GGGAGTTCAAGACCAGTCTGG-3' and 5'-TCC<u>CATATG</u>-CAAGCGATCTGCCTGCCTCAGC-3', upperNF-κB mutant; 5'-CCTGGGC<u>CTGCAG</u>ACCCCAACACACATACAGTC-3' and 5'-GGGT<u>CTGCAG</u>GCCCAGGAATGTTTTCTGC-AAAC-3', upperATF mutant; 5'-TGACTG<u>GTCGAC</u>TTA-TGAGGTGGGCTGAGAGG-3' and 5'-ATAA<u>GTCGA</u>-<u>C</u>CAGTCATTTGGGAAATGAGG-3'.

Plasmid Construction—Plasmid phT(−1.0)inCAT (containing promoter, first exon, first intron, and part of the second exon of the *TIMP-1* gene) was generated by inserting the 2-kb NcoI-digested PCR product (−1.0 + 1.0) into the NcoI-digested pCAT3promoter vector (Promega, Madison, WI). Plasmid phT(−0.7)inCAT derives from the plasmid phT(−1.0)intCAT from which 300-bp KpnI fragment was deleted. Subsequently, this plasmid was digested with NcoI and PstI, ends were blunted, and religated to yield plasmid phT(−0.7)CAT. The plasmid phT(−4.2−0.8) was generated by cloning a KpnI-digested PCR product into the KpnI sites of phT(−0.7)CAT. To generate all the other reporter plasmids
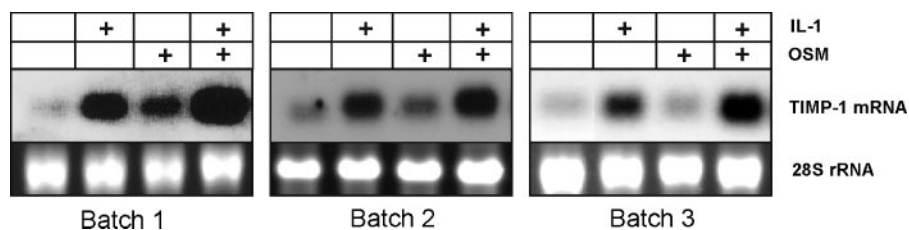
FIGURE 1. **IL-1 up-regulates the expression of TIMP-1 mRNA in human astrocytes.** Human astrocytes were treated with IL-1 or OSM for 18 h. Subsequently, RNA was isolated and subjected to Northern blot analysis using TIMP-1 cDNA as a probe. The *lower panels* show 28 S RNA stained with ethidium bromide on the membrane. Results of three independent experiments are shown using three different batches of primary human astrocytes.

containing different fragments of the 5′-flanking region of the *TIMP-1* gene, we first constructed a plasmid phT($-$0.7m)CAT by inserting a double-stranded oligonucleotide into the KpnI/ BstZ17I sites of phT($-$0.7)CAT (this introduced BglII, SphI, and XhoI sites). This plasmid was digested with KpnI, SphI, or StuI to accommodate KpnI-, SphI-, or StuI-digested PCR products, and yielded the plasmids shown in Fig. 3. Plasmid phT(ΔSac)CAT was generated by a deletion of SacI-SacI fragment from the plasmid phT($-$4.2–0.8)CAT. Plasmid phT($-$4.2–0.8) served as the template to construct plasmids with mutated AP-1, NF-κB, and ATF sites using the QuikChange XL site-directed mutagenesis kit (Strategene, La Jolla, CA) according to the manufacturer's instructions. Plasmids p5xATFCAT, p7xAP-1CAT, and p3xNFkBCAT were generated by cloning corresponding double-stranded oligonucleotides into the BamHI site of ptkCATΔEH (16). The expression plasmid pRSVIκB was a gift from Dr. K. Brand (Munich, Germany).

Transient Transfections—Cells were transfected in 12-well clusters using FuGENE6 transfection reagent (Roche Applied Science), according to the supplier's instructions. Plasmids (400 ng of the reporter CAT plasmid and 100 ng of expression plasmid encoding β-galactosidase) and 0.6 μl of FuGENE6 diluted into 50 μl of serum free medium were used for each well containing cells growing in 500 μl of culture medium. One day after transfection, the cells were stimulated with cytokines, cultured another 24 h, and harvested. Protein extracts were prepared by freeze-thawing (19), and the protein concentration was determined by the BCA method (Sigma). Chloramphenicol acetyltransferase (CAT) and β-galactosidase assays were performed as described (20). CAT activities were normalized to β-galactosidase activity and are means + S.E. (3–7 determinations).

Down-regulation with siRNA—The expression of p65 and ATF-2 was down-regulated using SMARTpool siRNA purchased from (Dharmacon, Inc. Lafayette, CO). siRNAs were transfected into cells using Dharmafect 1 (Dharmacon, Inc.).

Nuclear Extract Preparation and Electromobility Shift Assays (EMSA)—Nuclear extracts were prepared as described (21). All oligonucleotides used for EMSA were designed to contain four bases single-stranded 5′ overhangs at each end after annealing. Double-stranded DNA fragments were labeled by filling in 5′-protruding ends with Klenow enzyme using [α-^{32}P]dCTP (3000 Ci/mmol). EMSA was carried out according to the published procedures (22, 23). Briefly, 5 μg of nuclear extracts and ~10 fmol (10,000 cpm) of probe were used. Polyclonal anti-ATF-1, anti-ATF-2, anti-CREB, anti-c-Fos, anti-c-Jun,

anti-p65, and anti-p50 antisera were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Western Blotting—Cells were lysed in 10 mM Tris, pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 0.5% Nonidet P-40, 1% Triton X-100, 1 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Applied Science). Samples were resolved using SDS-PAGE and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Polyclonal anti-ATF-2, anti-p65, and anti-phospho-c-Jun antisera were purchased from Santa Cruz Biotechnology, Inc. while anti-phospho-p38, anti-phospho-p65(Ser^{536}), anti-phospho-ATF-2, and anti-phospho-MEK3/6 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antigen-antibody complexes were visualized by enhanced chemiluminescence according to manufacturer's instructions (Pierce).

RESULTS

Activation of TIMP-1 Expression by IL-1 Requires Ongoing Transcription and Is Mediated by Multiple Signaling Cascades—IL-1 has previously been shown to up-regulate the expression of TIMP-1 in primary human astrocytes (10, 15). To verify these findings, we analyzed TIMP-1 mRNA expression in response to IL-1 using three independent preparations of primary human astrocytes. For comparison, we also treated these cells with OSM, which has previously been shown to upregulate TIMP-1 expression in a number of cell types (24, 25). In agreement with previous observations, we found that IL-1 up-regulates expression of TIMP-1 mRNA (2.5–5 fold depending on the batch of astrocytes), whereas OSM was less effective (Fig. 1). Activation of TIMP-1 expression by IL-1 was blocked by actinomycin D (Fig. 2*A*), which indicates that ongoing transcription is needed to increase TIMP-1 mRNA levels. Interestingly, the half-life of TIMP-1 mRNA was greater than 18 h in both the control and IL-1-treated cells (Fig. 2*B*), indicating that mRNA stability was not enhanced by IL-1.

Therefore, the mechanism regulating TIMP-1-enhanced transcription in astrocytes could potentially be targeted using pharmacological approaches to ensure sustained up-regulation of the inhibitor, which is critical to restrain activity of MMPs. We examined this mechanism using pharmacological inhibitors that specifically target signaling pathways known to be activated by IL-1 in other cell types. Surprisingly, multiple inhibitors blocked the IL-1-mediated activation of the *TIMP-1* gene, suggesting that multiple pathways are involved in its regulation (Fig. 2*C*). The effective inhibitors included BAY11–7082 (an inhibitor of IkB kinase (IKK)), GF109203X (an inhibitor of protein kinase C (PKC)), and SB202190 (an inhibitor of p38 kinase), whereas SP600125 (an inhibitor of c-Jun N-terminal kinase) was ineffective.
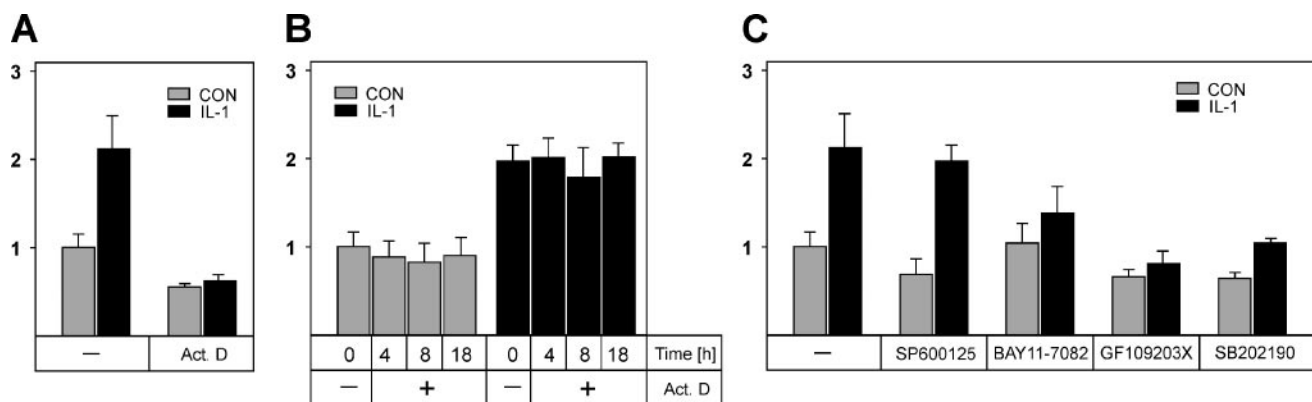
FIGURE 2. **Effect of inhibitors on IL-1-activated expression of TIMP-1 mRNA.** Human astrocytes were pretreated with 5 μg/ml actinomycin D (*A*) or 1 μM SP600125, 5 μM BAY11–7082, 10 μM SB202190, 10 μM GF109203X (*C*) for 1 h, and subsequently stimulated with IL-1 for 18 h. *B*, astrocytes were treated with IL-1 for 18 h, and then 5 μg/ml actinomycin D was added for the indicated time periods. RNA was isolated, and TIMP-1 expression was quantified by real time PCR (using TaqMan technology), normalized to 18 S rRNA, and expressed as a ratio to untreated cells. Duplicate experiments were repeated three times.

Identification of the IL-1 Regulatory Elements of the TIMP-1 Gene—Multiple regulatory elements have been described within both the 5′-flanking region and the first intron of the *TIMP-1* gene (25–30). To examine if IL-1 responsiveness is mediated by these elements, we generated reporter constructs containing either the 1 kb or 0.7 kb long 5′-flanking region of the *TIMP-1* gene fused to a CAT reporter gene. In addition, we also generated a construct containing a 0.7 kb long 5′-flanking region, the first exon (containing the 5′-untranslated region (UTR)), the entire first intron, and part of the second exon that contains the sequence coding for the first codon of the TIMP-1 protein followed by a CAT reporter gene. Transcription from this construct, followed by splicing, should result in the production of a chimeric mRNA containing the TIMP-1 5′-untranslated region, and the protein coding sequence that encodes the entire CAT protein. These reporters were not responsive to IL-1 when tested in transfection experiments of astrocytes (Fig. 3*A*); however, all constructs possessed low levels of CAT activity, including the "splicing" construct. This indicates that the chimeric mRNA was properly transcribed and spliced, while the CAT protein synthesized was expressed in an active form. Because neither the 1-kb long promoter nor the first exon and intron can mediate IL-1 responsiveness, we cloned DNA fragments from the 5′-flanking region of the *TIMP-1* gene in front of the 0.7-kb TIMP-1 promoter. This approach enabled the analysis of the cloned DNA fragments, together with the elements specific for the TIMP-1 promoter, allowing any specific interactions between the IL-1-induced transcription factor(s), and the factors binding to the TIMP-1 promoter. Analysis of these constructs in transient transfections (Fig. 3*A*) yielded one reporter containing the −4.2 to −0.8 fragment of the *TIMP-1* gene that was responsive to IL-1. Therefore, we analyzed the shorter DNA fragments derived from this fragment and found that a 0.8-kb long fragment located at −2.9 to −2.1 still conferred responsiveness to IL-1; however, further truncations led to a decrease or loss of responsiveness (Fig. 3*B*).

Identification of Regulatory Elements Binding IL-1-induced Factors—The entire −4.2 to −0.8 fragment of the *TIMP-1* gene, which conferred IL-1 responsiveness was searched for the presence of putative binding sites for transcription factors using the Mat Inspector program. We identified six putative

binding sites, including one for AP-1, two for NF-κB, and three for ATF within this fragment (Fig. 3*B* and 4*A*). Three of these elements (−2711 to −2702 NF-κB element, −2174 to −2167 ATF element, and −2767 to −2761 AP-1 element) were located within the 0.8-kb long fragment that conferred minimal IL-1-responsiveness (Fig. 3*B*). To evaluate the contribution of the six putative binding sites to the overall responsiveness to IL-1, we generated reporter constructs with mutations introduced into each of the putative regulatory sites (Fig. 4*A*). As expected, mutations introduced into the elements located outside of the minimal 0.8-kb long IL-1-responsive element had no effect on the activity of reporters. In contrast, mutations introduced into putative −2711 to −2702 NF-κB and −2174 to −2167 ATF diminished the responsiveness to IL-1, whereas mutation of the AP-1 element had no effect (Fig. 4*A*). We also fused putative ATF, NF-κB, and AP-1 elements of the *TIMP-1* gene to the minimal *tk* promoter, which is not responsive to IL-1, and examined the activity of these reporters. The NF-κB element conferred IL-1 responsiveness when linked to the *tk* promoter, while the AP-1 and ATF elements were ineffective (Fig. 4*B*). We also noted that the intrinsic activity of the AP-1 reporter was greatly enhanced (Fig. 4*B*), as observed previously for other AP-1 elements (15, 31). We conclude that both the NF-κB and ATF elements are needed for the full response of the *TIMP-1* gene to IL-1; however in contrast to the NF-κB element, the ATF element is ineffective by itself.

These findings elucidate that both elements are binding sites for their corresponding transcription factors, which likely bind cooperatively to regulate *TIMP-1* gene expression. We tested this prediction by EMSA, and observed that the ATF element was constitutively bound by the ATF-2 homodimer (Fig. 5, *A* and *B*). In contrast, treatment of astrocytes with IL-1 induced the binding of the p65/p50 heterodimers to the NF-κB element (Fig. 5, *A* and *B*). The AP-1 element was also constitutively bound (by c-Jun) (Fig. 5, *A* and *B*), but this element did not mediate the IL-1 response, as shown by mutational analysis (Fig. 4*A*).

Critical Role of p65 in Regulating TIMP-1 Gene Expression—The IL-1 induced binding of the p65/p50 heterodimer to the −2711 to −2702 NF-κB element (Fig. 5*A*), and the reduced activity of the reporter lacking this element (Fig. 4*A*) suggest
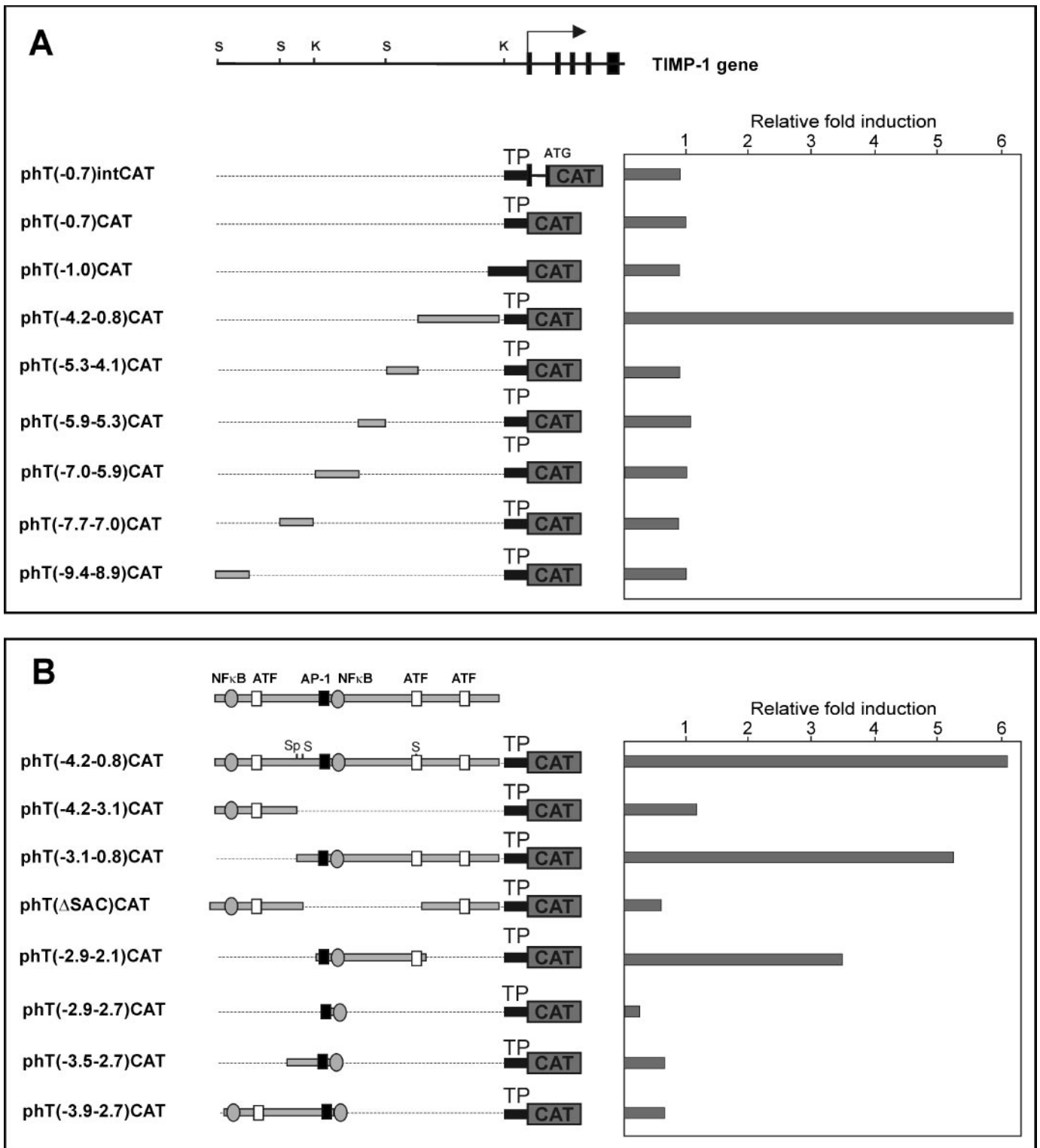
FIGURE 3. **Identification of the IL-1-responsive elements of the *TIMP-1* gene.** *A*, fragment located −4.2- to −0.8-kb upstream of the transcription start site of the *TIMP-1* gene mediates responsiveness to IL-1. Human astrocytes were transfected with reporter plasmids and a β-galactosidase expression vector. One day after transfection, cells were stimulated with IL-1, cultured for another 24 h, and harvested. CAT activities were normalized to β-galactosidase activities to account for transfection efficiency. Results are expressed as fold induction relative to the plasmid phT(-0.7)CAT that contains 700-bp long promoter of the *TIMP-1* gene indicated by TP. The model of the *TIMP-1* gene, including its 5′-flanking region, is also shown (*upper part* of the panel); *black boxes* represent exons, restriction sites for SphI (*S*) and KpnI (*K*), and transcription start site (*arrow*) are indicated. *B*, detailed analysis of the −4.2- to −0.8-kb fragment of the *TIMP-1* gene. Human astrocytes were transfected with the indicated reporters and a β-galactosidase expression vector, and processed as described above. A model of the −4.2 to −0.8-kb fragment of the *TIMP-1* gene is shown (*upper part* of the panel); putative binding sites are indicated, restriction sites for SphI (*Sp*) and SacI (*S*) are marked. A representative example of 3–6 experiments is shown.
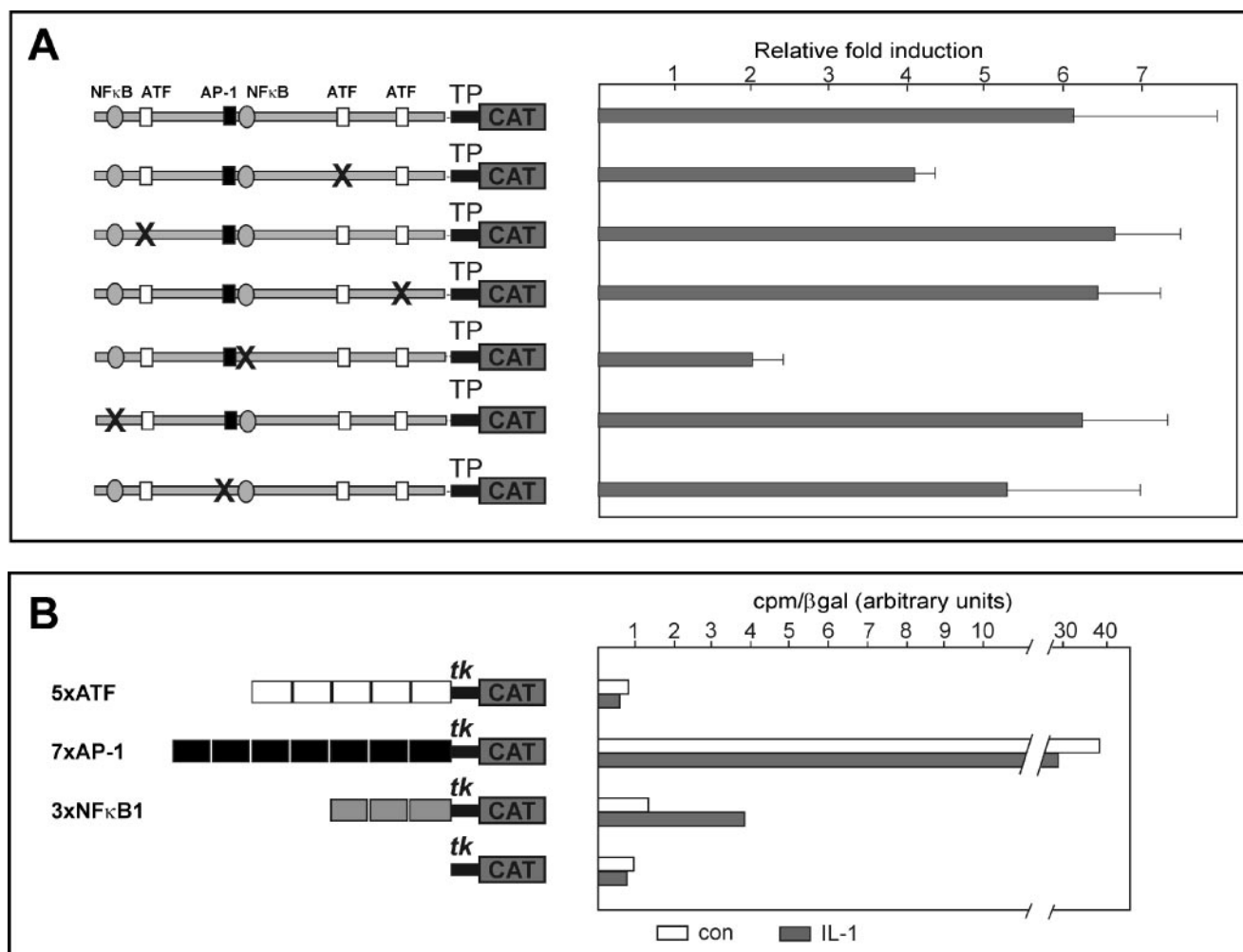
FIGURE 4. **Identification of functional AP-1, ATF-2, and NF-κB elements within the −4. 2 to −0.8 fragment of the *TIMP-1* gene.** Point mutations were introduced into the putative NF-κB, AP-1, and ATF elements of the *TIMP-1* gene (*A*) or multiple binding elements for ATF, AP-1, and NF-κB were cloned in the front of tk promoter (*B*) as described under "Experimental Procedures." Human astrocytes were transfected with the indicated reporter plasmids and a β-galactosidase expression vector, induced with IL-1 for 24 h, harvested, and processed as described in the legend to Fig. 3. Results are expressed as fold induction (*A*) or are shown in arbitrary units (untreated cells transfected with vector were set equal to 1) (*B*).
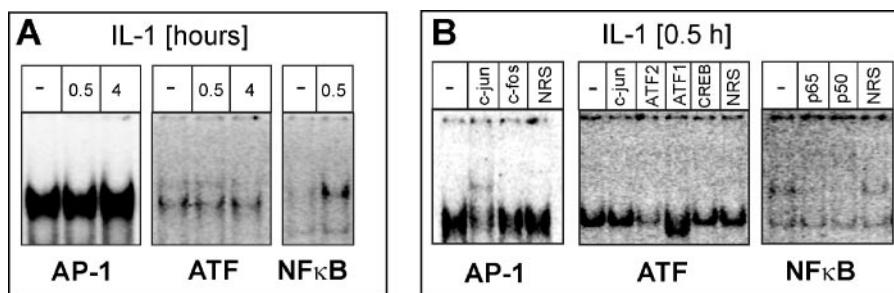

FIGURE 5. **AP-1, ATF-2, and NF-κB bind to the regulatory elements of *TIMP-1* gene in human astrocytes.** Nuclear extracts were prepared from control and IL-1 treated astrocytes as indicated. The binding was then analyzed by EMSA using the ^{32}P-labeled oligonucleotide probes derived from the 5′-flanking region of the *TIMP-1* gene (*A*). Specific antibodies or normal rabbit serum (*NRS*) were added to the binding reaction (*B*) and binding was analyzed as in *A*.

(IκBαSR), which is a mutated form of IκBα. IκBαSR is not phosphorylated, ubiquitinated, nor degraded, and thereby effectively sequesters NF-κB (32). Activation of the reporter by IL-1 was substantially diminished in the presence of IκBαSR (Fig. 6*B*), leading us to conclude that NF-κB is critical for the IL-1-mediated expression of the *TIMP-1* gene. The *in vitro* binding (Fig. 5, *A* and *B*) suggests that the p65 subunit of NF-κB may be critical for TIMP-1 regulation by IL-1. Therefore, we downregulated the expression of p65 using siRNA technology, and analyzed the response of the *TIMP-1* gene to IL-1. In fact, the basal expression was not affected but the activation by IL-1 was abrogated by the p65 siRNA (Fig. 6*C*). These results indicate that p65 is an indispensable component of NF-κB, which mediates the response to IL-1. In contrast, the knock-down of ATF-2 expres-

that NF-κB might be the prominent factor regulating TIMP-1 expression. To verify this hypothesis, we used two additional NF-κB activation inhibitors CAY10470 and parthenolide. Both of these inhibitors abrogated the activation of TIMP-1 by IL-1 (Fig. 6*A*). In addition, we blocked the activation of NF-κB by overexpressing the dominant-negative inhibitor of NF-κB
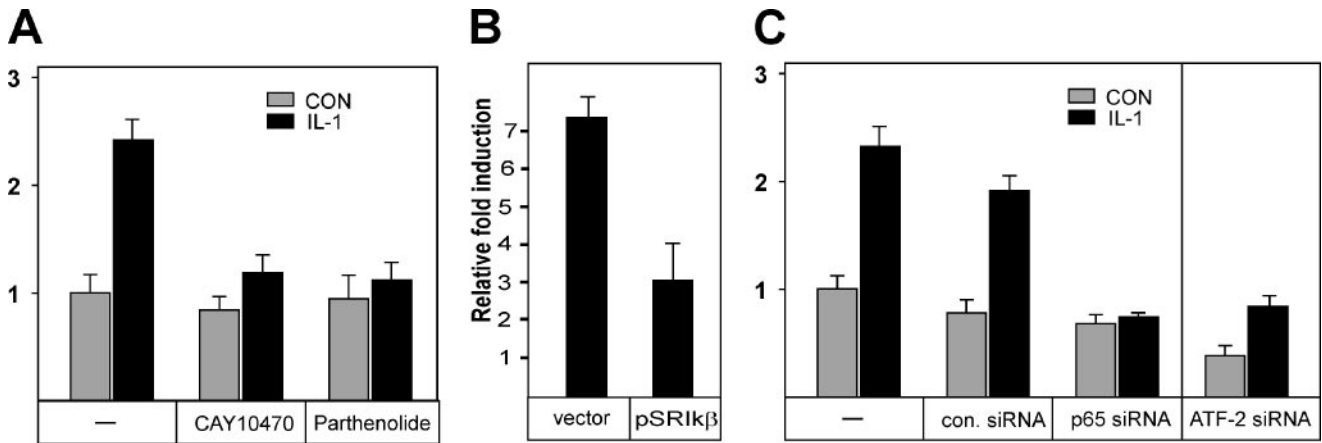
FIGURE 6. **Activation of p65 is indispensable for the IL-1 response of the *TIMP-1* gene.** Human astrocytes were pretreated with 1 μM CAY10470 or 5 μM parthenolide for 1 h, and subsequently stimulated with IL-1 for 18 h. Expression of TIMP-1 was analyzed as described in the legend to Fig. 2 (*A*). *B*, astrocytes were cotransfected with the reporter plasmid phT(−4.2−08)CAT, a β-galactosidase expression vector, and either a plasmid encoding the dominant-negative IκBα (pSRIκBα) or an empty vector. CAT activities were assessed as described earlier (3 replicates). *C*, astrocytes were transfected with control siRNA or the SMARTpool siRNA to p65 or ATF-2, and cultured for 48 h. Subsequently, cells were stimulated with IL-1 for 18 h, and TIMP-1 expression was analyzed as described in the legend to Fig. 2.
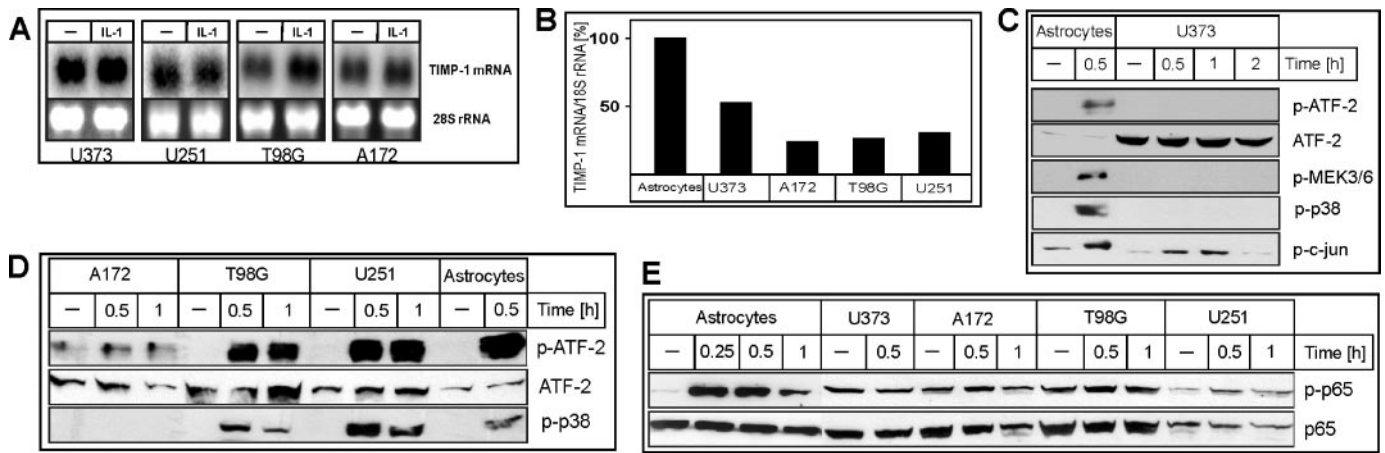

FIGURE 7. **Analysis of IL-1-activated signaling and TIMP-1 expression in human gliomas.** *A*, U373, U251, T98G, and A172 cells were treated with IL-1 for 18 h. Subsequently, RNA was isolated and subjected to Northern blot analysis using TIMP-1 cDNA as a probe. *Lower panels* show 28 S RNA stained with ethidium bromide on the membrane. *B*, RNA was isolated from confluent cultures of human astrocytes, U373, A172, T98G, and U251 cells, and expression of TIMP-1 mRNA was analyzed by real-time PCR. *C–E*, human astrocytes, U373, A172, T98G, and U251 cells were treated with IL-1 as indicated. Non-denaturing lysates were prepared, and analyzed by Western blotting with phospho-ATF-2, ATF-2, phospho-c-Jun, phospho-p38, and phospho-MEK3/6 antibodies (*C*), phospho-ATF-2, ATF-2, and phospho-p38 antibodies (*D*), and phospho-p65 and p65 antibodies (*E*), and detected by enhanced chemiluminescence.

sion had a dramatic effect on the TIMP-1 expression in control and IL-1-treated cells, but the fold activation by IL-1 was not affected (Fig. 6*C*). This implies that ATF-2 may regulate the intrinsic expression of TIMP-1.

TIMP-1 Expression Is Not Activated by IL-1 in Many Gliomas Because of the Aberrant Activation of Either NF-κB or ATF-2—Grade IV malignant gliomas (glioblastoma multiforme) are the most common primary brain tumors, and are characterized by extreme invasiveness, which makes standard treatments (including radiation and chemotherapy) ineffective. Their invasion into healthy brain tissue presumably requires extensive ECM remodeling involving the activation of MMPs. In primary astrocytes, the activity of the MMPs is tightly controlled by TIMPs, including TIMP-1, which is regulated by growth factors and cytokines, such as IL-1. The invasive phenotype of gliomas prompted us to hypothesize that TIMP-1 may be expressed at lower levels in gliomas, and IL-1 may not be able to enhance its expression. This would

result in the increased activity of glioma-derived MMPs, and in turn would likely enhance the invasiveness of gliomas. In fact, we found that IL-1 did not enhance the expression of TIMP-1 in the established glioma cell lines we examined, including U373, U251, T98G, and A172 cells (Fig. 7*A*). We also found that the intrinsic expression of TIMP-1 in glioma cell lines was significantly lower than in primary astrocytes (Fig. 7*B*).

Because both ATF-2 and NF-κB regulate TIMP-1 expression in response to IL-1 in astrocytes, we tested whether these transcription factors are also activated in gliomas. We focused on ATF-2, for two reasons. First, NF-κB activation has been well documented in gliomas (33–37), and second, our prior inhibitor studies indicated that the inhibition of p38 (Fig. 2) significantly diminished the IL-1 induction of TIMP-1 in astrocytes. We assayed the activation of both p38 and its putative upstream kinase, mitogen-activated protein kinase kinase (MEK)-3/6, in response to IL-1 by Western blotting. ATF-2, MEK3/6, and p38

were rapidly phosphorylated following the IL-1 stimulation of astrocytes (Fig. 7*C*), while neither of these proteins was phosphorylated in U373 glioma cells. This suggests that the IL-1/MEK3/6/p38/ATF-2 pathway is not functional in these cells. Surprisingly, U373 cells contain much higher levels of total ATF-2 than astrocytes (Fig. 7*C*). The lack of ATF-2 phosphorylation seen in U373 cells in response to IL-1 was specific because c-Jun was phosphorylated in both astrocytes and U373 cells (Fig. 7*C*).

Subsequently, we analyzed three additional glioma cell lines, and found that the MEK3/6/p38/ATF-2 pathway was not functional in A172 cells; however, ATF-2 and p38 were efficiently activated in U251 and T98G cells (Fig. 7*D*). Although ATF-2 was activated in U251 by IL-1, the expression of TIMP-1 was not activated in these cells (Fig. 7, *D* and *A*), suggesting that the other arm of the cooperative IL-1 response was ineffective. Therefore, we examined NF-κB activation by evaluating the phosphorylation of p65 on serine 536, which was shown to regulate the recruitment of p300/CBP to the nuclear pool of p65 (38). In non-stimulated astrocytes, low levels of phosphorylated p65 were found; however, robust phosphorylation was rapidly induced by IL-1 (Fig. 7*E*). In contrast, glioma cells contain substantial amounts of phosphorylated p65, suggesting that NF-κB may be constitutively activated in these cells (Fig. 7*E*). This is supported because IL-1 induces only a slight increase in p65 phosphorylation, and hence p65 activity, in gliomas. In agreement with our predictions, U251 cells contained substantially lower levels of p65, much of which was already phosphorylated in control cells and barely activated in response to IL-1. We conclude that: (i) TIMP-1 is expressed at lower levels in gliomas than in astrocytes, (ii) TIMP-1 expression is not activated by IL-1 in most gliomas studied, (iii) both MEK3/6/p38/ATF-2 and IKK/NF-κB are efficiently activated in response to IL-1 in astrocytes, (iv) the gliomas studied are characterized by constitutively phosphorylated p65, and (v) in gliomas inefficient activation of either ATF-2 or NF-κB correlates with the loss of IL-1-induced TIMP-1 expression.

DISCUSSION

The precise control of the enzymatic activities of MMPs, in part ensured by the equilibrium between MMPs and TIMPs, is necessary to prevent extensive tissue damage; however, a temporary imbalance that favors MMPs over TIMPs is indispensable for the ECM remodeling that is required during many physiological processes (2, 3). In the CNS, injury induces extreme acute changes in the expression pattern of both MMPs and TIMPs, which are necessary to initiate repair processes. These expression profiles are regulated by neuroinflammatory cytokines, including IL-1. In certain pathological states such as cancer, a sustained imbalance between MMPs and TIMPs may lead to the increased migration of cells, which may possibly aid in tumor metastasis. In agreement with this, the migration of glioblastoma cells into healthy brain tissue depends upon increased proteolytic activity, as a prelude to ECM degradation, and subsequent invasion of the tumor cells. Secreted factors control this process, with IL-1 being a likely regulator since it is produced by both resident brain cells and tumor cells. Expression

of TIMP-1, which is a highly regulated soluble inhibitor, is critical in both tissue remodeling/repair and invasion (39, 40).

The expression of TIMP-1 has been shown to be transcriptionally regulated by the binding of multiple transcription factors including AP-1, ETS, EGR, STAT, and RUNX to regulatory elements present in the 5′-flanking region of the gene (25–30). The binding of these transcription factors is regulated by several stimuli, including cytokines and growth factors such as IL-6, OSM, epidermal growth factor, and transforming growth factor β. Although, IL-1 has been previously reported to activate TIMP-1 expression in both astrocytes (10, 15) and orbital fibroblasts (41), the mechanism of *TIMP-1* gene regulation remained elusive. It has been proposed that the extracellular signal-regulated kinase (ERK) pathway activates TIMP-1 expression in response to IL-1 in orbital fibroblasts, because its activation could be blocked by a MEK inhibitor PD98059 (41). However, both the overexpression of dominant-negative ERK1 and treatment with PD98059 decreased the intrinsic expression of TIMP-1, and they had a limited if any, effect on IL-1 induced TIMP-1 expression (41). To expand upon these observations, we propose that IL-1 activates TIMP-1 expression by at least two pathways that lead to the activation of NF-κB and ATF-2, which bind to newly identified regulatory elements of the *TIMP-1* gene. The activation of NF-κB by IL-1 is critical for the induction of TIMP-1 expression, since the presence of the IKK inhibitors (BAY11–7082, CAY10470, and parthenolide) (Figs. 2*C* and 6*A*) or the dominant-negative IκBαSR (superrepressor) (Fig. 6*B*) dramatically diminished TIMP-1 induction. Specifically, p65 is the component of the NF-κB that is indispensable for the induction of TIMP-1 expression, since its down-regulation abolished up-regulation by IL-1 (Fig. 6*C*). Conversely, the activation of ATF-2 by the MEK3/6-p38 pathway is needed for the intrinsic expression of TIMP-1, and the response to IL-1. These conclusions are supported by the following findings: i) inhibition of p38 by SB202190 significantly reduced both basal expression of TIMP-1, and its activation by IL-1 (Fig. 2*C*), and ii) the knock-down of ATF-2 expression drastically diminished the basal and IL-1-induced levels of TIMP-1. It is tempting to speculate that the binding of ATF-2, which possesses histone acetyltransferase activity, may induce the acetylation of nearby histones, relaxation of chromatin, and thereby enabling the efficient binding of NF-κB. Surprisingly, a general inhibitor of protein kinase C effectively blocked TIMP-1 activation by IL-1 (Fig. 2*C*). The experiments we report do not explain this observation, and we can only speculate that protein kinase C may be either needed to phosphorylate p65 (42) or to activate proteins necessary for the effective NF-κB activation such as sphingosine kinase-1 (43).

The cooperative activation of the *TIMP-1* gene by IL-1 in astrocytes via activation of both the IKK/NF-κB and MEK3/6/p38/ATF-2 pathways partially resembles the NF-κB- and p38-dependent mechanism that regulates MMP-1 and MMP-3 expression in synovial fibroblasts (44), and the NF-κB-, C/EBP-, and ATF-2-dependent mechanism of nitric-oxide synthase activation in rat glial cells (45). The mechanism we propose (Fig. 8) for the regulation of TIMP-1 in astrocytes likely controls other genes in these cells, and may also function in other cell types.
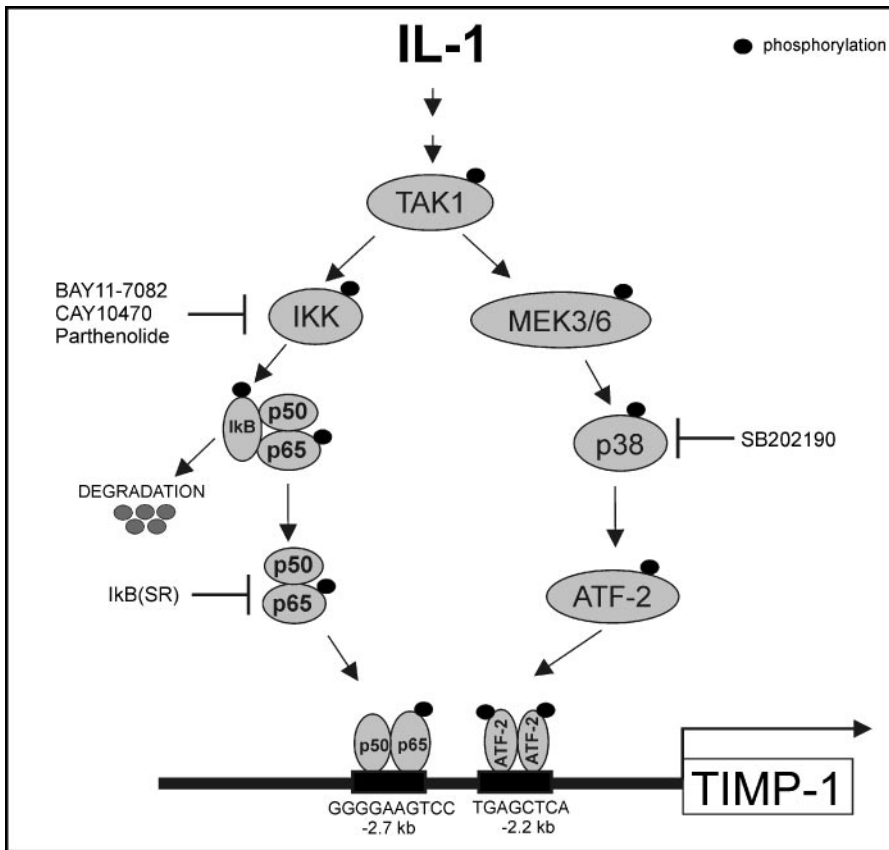
FIGURE 8. **Model of the activation of the *TIMP-1* gene in human astrocytes.** In astrocytes, IL-1 activates the signaling pathways that lead to the phosphorylation of IKK (which can be blocked by BAY11–7082, CAY10470, and parthenolide) and MEK3/6 (both likely phosphorylated by TAK1). Activated IKK phosphorylates IkBα, which leads to its subsequent ubiquitination and degradation (this can be blocked by expressing dominant-negative IkBα). Liberated p65/p50 complexes translocate to the nucleus, and bind to the TIMP-1 regulatory element at −2.7 kb (knock-down of p65 expression abrogates this signaling). Concurrently, activated MEK3/6 phosphorylates p38 kinase (this can be blocked by SB202190), which in turn phosphorylates ATF-2 bound to its TIMP-1 regulatory region at −2.2 kb. Both ATF-2 and p65/p50 complexes are needed for the full activation of the *TIMP-1* gene expression in astrocytes.

The newly identified ATF and NF-κB binding elements of the *TIMP-1* gene likely bind cooperatively ATF-2 homodimers and p65/p50 heterodimers, which may be needed to form a functional enhanceosome and activate transcription. This is supported by the findings that both of these elements are required for the full IL-1-induced transcriptional activity of the TIMP-1 reporter constructs harboring mutated binding elements (Fig. 4*A*). Furthermore, an array of ATF binding elements was not able to confer IL-1 responsiveness onto the minimal *tk* promoter, while three copies of the NF-κB element were effective (Fig. 4*B*). All of these results suggest that single ATF and NF-κB elements represent binding sites which likely cooperate to activate transcription of the *TIMP-1* gene. In contrast, mutation of an AP-1 element at −2.7 kb had no effect on IL-1 responsiveness (Fig. 4*A*), but an array of the AP-1 elements dramatically increased the intrinsic activity of the AP-1-*tk* reporter (Fig. 4*B*) while having a negligible effect on the IL-1-induced activity. Both of these results suggest that this element may therefore be important for the intrinsic expression of the endogenous gene by recruiting coactivators necessary for chromatin modification and remodeling, which cannot be properly recapitulated using reporter constructs.

Although IL-1 increases the phosphorylation of c-Jun in astrocytes (Fig. 7), the ATF binding element does not bind ATF-2/c-Jun heterodimers *in vitro* (Fig. 5*B*), but selectively binds homodimers of ATF-2. In contrast, c-Jun effectively binds the AP-1 element of the *TIMP-1* gene (Fig. 5). As suggested for other genes (46), the selective binding of the ATF-2 homodimers may represent another level of specificity in the *TIMP-1* gene regulation.

IL-1 is continuously produced and regulates normal physiological processes in the brain. Also, the expression of IL-1 is greatly increased after brain injury and during the progression of neurodegenerative diseases, such as Alzheimer and Parkinson disease (47, 48). Furthermore, IL-1 is also expressed by glioma cells and in cells surrounding necrotic tumors, such as glioblastoma multiforme (49). Because IL-1 enhances the expression of several MMPs it is tempting to propose that the net proteolytic activity secreted by gliomas is increased, and may be a cause of the extreme invasiveness of these cells. The IL-1-induced expression of TIMP-1 in astrocytes likely provides a delayed mechanism that limits the proteolytic activities of MMPs, which are needed in the initial phase following brain injury. In contrast to astrocytes, expression of TIMP-1 is not enhanced by IL-1 in glioma cells (Fig. 7*A*), suggesting that this "safety mechanism" to limit the proteolytic activities of MMPs, is not functional in gliomas. Moreover, the intrinsic expression of TIMP-1 in gliomas is significantly lower than in astrocytes (Fig. 7*B*). Our data suggest that either the IKK/NF-κB or the MEK3/6/p38/ATF-2 pathway is dysfunctional or deregulated in gliomas (Fig. 7, *C–E*). We also noted that the intrinsic levels of ATF-2 were higher in gliomas than in astrocytes (Fig. 7, *C* and *D*), but the importance of this observation is unclear since levels of ATF-2 did not correlate with its activation.

It is important to stress that the intrinsic levels of p65 phosphorylated on serine 536 were higher in gliomas than in astrocytes; however, the IL-induced increase in this phosphorylation was much lower in gliomas. These results suggest that NF-κB may be continuously activated in gliomas, which is of fundamental importance. Recently, the link between carcinogenesis and inflammation has become evident (reviewed in Ref. 50), and NF-κB is proposed to be critical for tumor promotion and progression. Activated NF-κB may therefore provide signals that allow gliomas to evade apoptosis, and increase; angiogen-

esis, self-sufficiency in growth, insensitivity to growth inhibition, and to gain limitless replicative and invasive potential (50, 51). It remains to be established if the decreased IL-1-induced phosphorylation of p65 on serine 536 correlates with these tumor promoting processes. Furthermore, the precise role of this modification needs to be addressed, because it was recently shown that it defines an IkBα-independent pathway of NF-κB activation (52), and results in accelerated NF-κB turnover (53).

In summary, we propose a novel mechanism of TIMP-1 activation by IL-1 in human astrocytes, which involves the activation of two signaling pathways; IKK/NF-κB and MEK3/6/p38/ATF-2. Activated NF-κB and ATF-2 bind to novel regulatory elements at −2.7 kb and −2.2 kb, and activate the transcription of TIMP-1. In contrast to astrocytes, this mechanism does not properly function in glioma cell lines due to the defective activation of at least one of these pathways. Lack of enhanced TIMP-1 expression in gliomas may, in part, explain their increased invasiveness.

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