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Research article

TGF-β1 induction of the adenine nucleotide translocator 1 in astrocytes occurs through Smads and Sp1 transcription factors

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Abstract

Background: The adenine nucleotide translocator 1 (Ant1) is an inner mitochondrial membrane protein involved with energy mobilization during oxidative phosphorylation. We recently showed that rodent Ant1 is upregulated by transforming growth factor-beta (TGF-β) in reactive astrocytes following CNS injury. In the present study, we describe the molecular mechanisms by which TGF-β1 regulates Ant1 gene expression in cultured primary rodent astrocytes.

Results: Transcription reporter analysis verified that TGF-β1 regulates transcription of the mouse Ant1 gene, but not the gene encoding the closely related Ant2 isoform. A 69 basepair TGF-β1 responsive element of the Ant1 promoter was also identified. Electrophoretic mobility shift assays demonstrated that astrocyte nuclear proteins bind to this response element and TGF-β1 treatment recruits additional nuclear protein binding to this element. Antibody supershift and promoter deletion analyses demonstrated that Sp1 consensus binding sites in the RE are important for TGF-β1 regulation of Ant1 in astrocytes. Additionally, we demonstrate that Smad 2, 3 and 4 transcription factors are expressed in injured cerebral cortex and in primary astrocyte cultures. TGF-β1 activated Smad transcription factors also contribute to Ant1 regulation since transcription reporter assays in the presence of dominant negative (DN)-Smads 3 and 4 significantly reduced induction of Ant1 by TGF-β1.

Conclusion: The specific regulation of Ant1 by TGF-β1 in astrocytes involves a cooperative interaction of both Smad and Sp1 binding elements located immediately upstream of the transcriptional start site. The first report of expression of Smads 2, 3 and 4 in astrocytes provided here is consistent with a regulation of Ant1 gene expression by these transcription factors in reactive astrocytes. Given the similarity in TGF-β1 regulation of Ant1 with other genes that are thought to promote neuronal survival, this interaction may represent a general mechanism that underlies the neuroprotective effects of TGF-β1.

Background

The secreted signaling molecule TGF-β1 is rapidly and chronically elevated in response to CNS injury. Astroglial cells at the site of injury become reactive and hypertrophy leading to the formation of a glial scar, a barrier for regenerating axons. TGF-β1 stimulates production by reactive astrocytes of glial fibrillary acidic protein (GFAP), the diagnostic marker of reactive astrogliosis as well as extracellular matrix molecules that contribute to the inhibition of axonal regeneration [1-5]. Using an *in vivo* filter implant model of the glial scar, we have recently shown that the expression of Ant1, a gene involved in energy mobilization, is elevated in reactive astrocytes and that astrocytic Ant1 expression is regulated by TGF-β1 both *in vivo* and *in vitro* [6].

Ant1 is a major mitochondrial inner membrane protein that exchanges mitochondrial ATP for cytosolic ADP and is thereby an important component of oxidative phosphorylation (OXPHOS) energy production. Patients with myocarditis and cardiac myopathy exhibit lower activity of the translocator [7] as well as altered levels of Ant isoform expression [8]. A familial mutation of ANT1 is associated with autosomal dominant progressive external ophthalmoplegia, characterized by large-scale mitochondrial DNA deletions [9]. In addition, mice deficient in Ant1 demonstrate characteristics of cardiac myopathy, including severe exercise intolerance and mitochondrial proliferation in the heart [10].

The importance of Ant1 function is further underscored by the highly conserved Ant genes that have been identified in a number of mammalian species, including bovine, rat, human and mouse [10-13] as well as other eukaryotes such as yeast and plants [14-16]. Unlike humans with three ANT isoforms, rodents have two Ant genes that share with each other 78% cDNA and 85% amino acid sequence identity. Interestingly, Ant1 isoforms from different species (e.g. human Ant1 and mouse Ant1) are more closely related than different Ant isoforms within the same species. Rodent Ant1 has an expression pattern similar to human ANT1, with the highest level found in brain, heart and skeletal muscles [17-20]. Rodent Ant2, on the other hand, is readily detected in all tissues except for skeletal muscles whereas human ANT2 is only weakly expressed in most tissues examined [18,20].

Examination of transcriptional regulation of human ANT genes revealed OXBOX and REBOX response elements (REs), sensitive to oxidative phosphorylation (OXPHOS) activity and redox state, respectively. These promoter elements can regulate transcription of human ANT1 and other OXPHOS genes, including ATP synthase β [21,22]. Little is known about the regulation of Ant gene expression by factors other than metabolic and redox influences, although estrogen can regulate Ant1 expression in female rat hearts [23] and may induce Ant2 mRNA expression in the rat hypothalamus [24]. We have recently shown that TGF-β1 upregulates expression of Ant1, but not Ant2 mRNA in glial scars. Despite functional similarity and sequence homology between the mouse isoforms however, Ant2 is neither upregulated in reactive astrocytes following CNS injury, nor in TGF-β1 treated primary astrocytes [6]. In the present study, we have taken advantage of this differential regulation of Ant1 and Ant2 in mouse astrocytes to examine TGF-β1 regulation of Ant1 gene expression in primary astrocyte cultures.

The best characterized mechanism of TGFβ-mediated regulation of gene expression is via the Smad family of transcription factors. Following TGFβ receptor activation, the cytosolic receptor-regulated Smads (R-Smads) 2 and 3 are phosphorylated and associate with the common-partner Smad (co-Smad), Smad 4, to form hetero-oligomers. This complex enters the nucleus to induce gene expression (reviewed by Massagué [25]). The diverse effects of TGFβ in multiple cell types, suggests that regulation of transcription by this cytokine is likely to occur at multiple levels to provide precise activation of target genes. For instance, Smad activity can be regulated by crosstalk with the MAPK signaling pathway [26,27] as well as protein kinase C [28]. Smads can also directly bind to promoter DNA response elements or interact with other transcription factors as part of a nuclear complex. A large number of nuclear Smad binding partners have been reported [reviewed by [29]]. In particular, Smads interact with Sp1 to induce transcription of p21/WAF1/Cip1 [30,31], integrin β_5 subunit [32], α 2(I) collagen [33] and plasminogen activator inhibitor-1 (PAI) [34]. Despite the well documented effect of TGF-β1 on gene expression in astrocytes, however, expression of endogenous Smads in reactive astrocytes or other CNS cell types has not been reported.

Our studies were aimed to determine the molecular mechanisms that regulate the astrocytic expression of Ant1, a protein that mobilizes mitochondrial energy. We have identified a TGF-β1 responsive element within the mouse Ant1 promoter. This response element sequence is sufficient to confer TGF-β1 responsivity to the Ant2 gene promoter, which is not normally regulated by this cytokine in astrocytes. Furthermore, the transcription factors Sp1 and Smads 3 and 4 are expressed in the CNS and in cultured astrocytes and appear to account for specific upregulation of the Ant1 gene in reactive astrocytes following CNS injury.

Results

Identification of a TGF-βI response element within the Ant1 promoter

Transcription reporter assays (TRAs) were employed to identify the region in the Ant1 promoter that responds to TGF-β1. Luciferase reporter plasmids containing overlapping fragments of the mouse Ant1 promoter were generated (Fig 1a). A reporter construct with the 7 kilobases (kb) of Ant1 promoter sequence (Ant1 Full Length) proximal to the transcription start site (TSS) exhibited a 2.3-fold induction in transcription activity following TGF-β1 treatment (Fig 1b). Given our previous finding that the level of endogenous Ant1 protein is elevated 1.7 fold *in vivo* following CNS injury, our present results suggest this construct contains the entire Ant1 promoter; insofar as TGF-β1 mediated transcription is concerned. Next, fragments of the 7 kb Ant1 promoter were subcloned into the reporter plasmid and employed to localize the majority (72%) of the TGF-β1 response to a 485 basepair (bp) promoter sequence upstream of the transcription start site (Ant1 485 bp).

Localization to this proximal upstream promoter sequence of the Ant1 is consistent with the localization of sequences involved in the TGF-β1 regulation of astrocytic GFAP [35] and PAI [36] which appear to be confined to regions 0.8 to 1.8 kb upstream of the transcription start site, respectively. The difference in transcription induction between the full length and the 485 bp reporter constructs may be due to the loss of basal transcription elements residing in the more distal 6.5 kb promoter region. Interestingly however, this upstream 6.5 kb of promoter sequence not only showed a lack of induction in response to treatment with this cytokine, but transcriptional activity from this fragment was reduced by 43% in the presence of TGF-β1 compared to untreated controls (Fig 1b). Together, these results suggest that TGF-β1 regulation of Ant1 expression is complex and that induction of gene transcription by this cytokine is principally localized to the 485 bp of promoter upstream from the transcription start site. Overlapping reporter constructs spanning the distal (Ant1Prom1), medial (Ant1Prom2) and proximal (Ant1Prom3) regions of the TGF-β1-responsive 485 bp sequence were then employed to further localize the Ant1 TGF-β1 RE (Fig 1a). TRAs with these constructs revealed that the TGF-β1 responsivity is localized entirely to the 5' half of Ant1Prom3 (Ant1Prom3-5') (Fig 1b). TGF-β1 induction of Ant1 was confirmed by immunoblot analysis demonstrating elevated expression of endogenous Ant1protein in TGF-β1 treated versus control astrocyte cultures (Fig 1c).

The Ant1 TGF-βI RE is sufficient for cytokine-mediated transcription induction in astrocytes

We have demonstrated that Ant1, but not the closely related Ant2 isoform, is upregulated following CNS injury despite the close functional and sequence homologies between these isoforms [6]. To confirm these observations in our experimental paradigm, we conducted analogous TRAs with reporter plasmids containing Ant2 promoter sequences. Reporter constructs containing approximately 6 kb of the Ant2 promoter upstream of the transcription start site and 2.2 kb of the Ant2 promoter including the start site sequence were generated (Fig 2a). Neither of these reporter constructs responded to TGF-β1 (Fig 2b and data not shown). Similarly, RT-PCR and immunoblot analyses indicate increased expression of Ant1, but not Ant2, expression in the *in vivo* glial scar and in TGF-β1 treated astrocytes [[6]; and Fig. 1c]. Together these data support the suggestion that induction of Ant1 by TGF-β1 following CNS injury is a specific response.

Activation of the Ant1 RE by TGF-β1 was further tested in the context of this non-responsive Ant2 promoter. The promoter region corresponding to the TGF-β1 responsive Ant1Prom3-5' was inserted into 2.2 kb Ant2 reporter construct for TRA analysis. Ant1Prom3-5' conferred significant TGF-β1 responsivity ($p < 0.05$) in the otherwise non-responsive Ant2 isoform promoter (Fig 2c). The decreased magnitude of induction with this construct compared with the Ant1 promoter constructs may result from the distance of the TGF-β1 RE from the point of transcription initiation. Alternatively, there may be inhibitory regulation in the context of the Ant2 promoter sequence. Nevertheless, these results indicate that the Ant1Prom3-5' promoter sequence is both necessary and sufficient for regulation of Ant1 mRNA expression by this cytokine.

TGF-βI induces binding of Sp1 to the Ant1 TGF-βI RE

The Ant1Prom3-5' promoter fragment was examined for recognition by astrocytic nuclear proteins in electrophoretic mobility shift assays (EMSAs). The sequence of this promoter fragment revealed the presence of CAAT and TATA sequences consensus transcriptional regulatory sequences located 87 and 27 bp upstream of the Ant1 transcription start site, as expected. Further analysis revealed a potential Smad binding element (SBE) as well as three consensus binding sites for the Sp1 transcription factor (Fig 3a). The first ($Sp1\text{-}1_{GC}$) and third ($Sp1\text{-}3_{GC}$) Sp1 sites conform to the classical GC-rich RE sequence for this factor. The repeated TCC sequence of the second site ($Sp1\text{-}2_{TCC}$), found in the promoters of the TGF-β1 regulated α 2(I) collagen [37] and the IL-10 cytokine [38], has also been shown to bind specifically to Sp1 and Sp3. Importantly, the localization of Sp1 and Smad binding sites in the Ant1 promoter suggests that they may

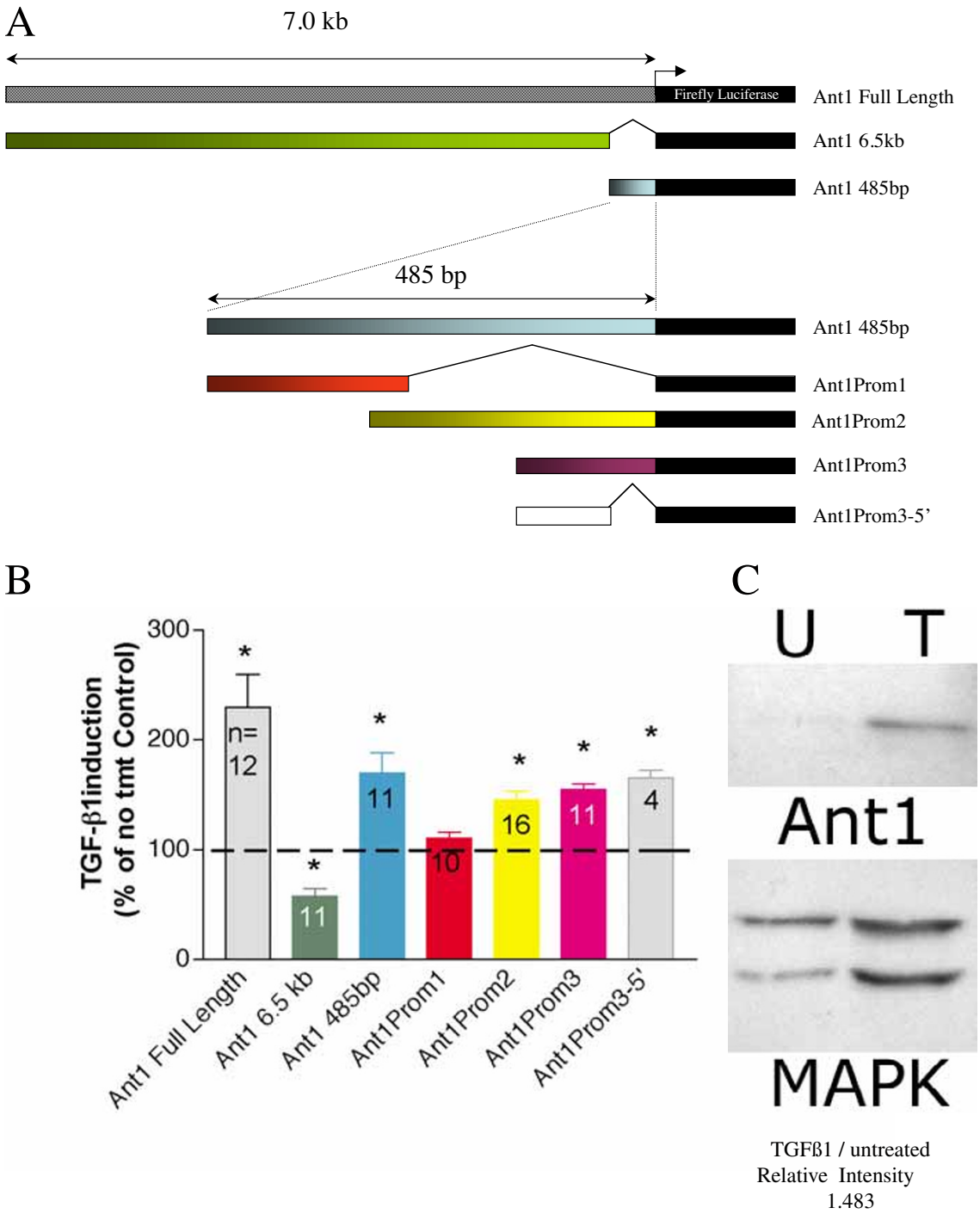

Figure 1

TGF-β1 regulation of Ant1 in primary astrocytes. A. Schematic diagram of Ant1 promoter full-length and deletion reporter constructs used in transcription reporter assays. **B**. Primary astrocytes were transiently transfected with the indicated reporter constructs. Reporter firefly luciferase activity (normalized to Renilla luciferase activity) is expressed as the mean ± SEM with the number of replicates performed for individual treatment conditions shown in each bar. The average TGF-β1 induction of each construct was compared to untreated controls transfected with the same construct by student's t-test. Asterisks denote $p \leq 0.05$. **C**. Immunoblot analysis of Ant1 protein in control untreated (U) and TGF-β1 treated (T) astrocyte cultures. Immunoblots were subsequently stripped and reprobed with an antibody to p42/44 MAPK to demonstrate the amount of protein loaded in each lane.

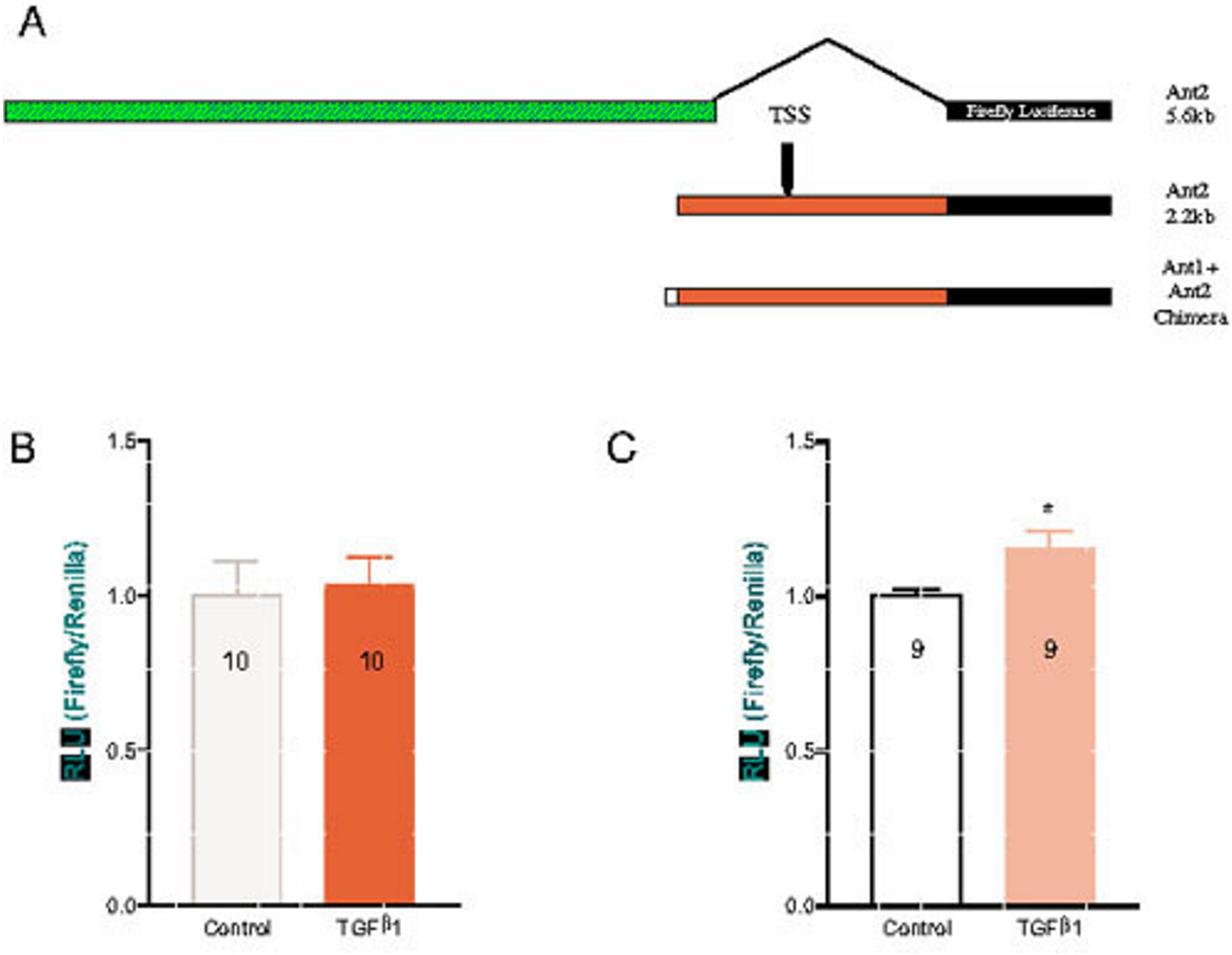

Figure 2

Isoform specific transcriptional regulation of Ant genes by TGF-β1. A. Schematic diagram of Ant2 promoter and Ant1+Ant2 chimeric constructs used in transcription reporter assays. Ant2 5.6 kb construct contains Ant2 promoter sequence (gray bar) upstream of the transcription start site (TSS; black arrow). Ant2 2.2 kb contains promoter sequence proximal to the TSS and some downstream sequence (red bar). The Ant1+Ant2 Chimera construct was generated by inserting the 88 bp Ant1 promoter sequence of Ant1Prom3-5' (white bar) upstream of Ant2 2.2 kb. **B.** TRAs with primary astrocytes transiently transfected with Ant2 2.2 kb. Reporter firefly luciferase activity (normalized to Renilla luciferase activity) is expressed as the mean ± SEM with the number of replicates performed for individual treatment conditions shown in each bar. $p \leq 0.05$ for two-tailed student's t-test. **C**. TRAs with primary astrocytes transiently transfected with Ant1+Ant2 Chimera. $p = 0.017$ for one-tailed student's t-test.

cooperate to regulate Ant1 transcription, as has been shown for TGF-β1-regulation of PAI [34] both in primary astrocytes and following CNS injury [36,39].

TGF-β1 stimulation of nuclear protein binding to the Ant1 RE was demonstrated with two overlapping oligonucleotides from Ant1Prom3-5' (Oligo 1, -87 to -54; Oligo 2, -64 to -40) (Fig 3b). A shifted band with increasing intensity at one and three hours of TGF-β1 treatment was apparent with Oligo 1, which contains the tandem Sp1-1_{GC} and Sp1-2_{TCC} repeats. Indeed, an Sp1-specific antibody further retarded migration of this oligo, thus confirming the presence of Sp1 in the nuclear protein complex binding to the Ant1 TGF-β1 RE (Fig 3b). In

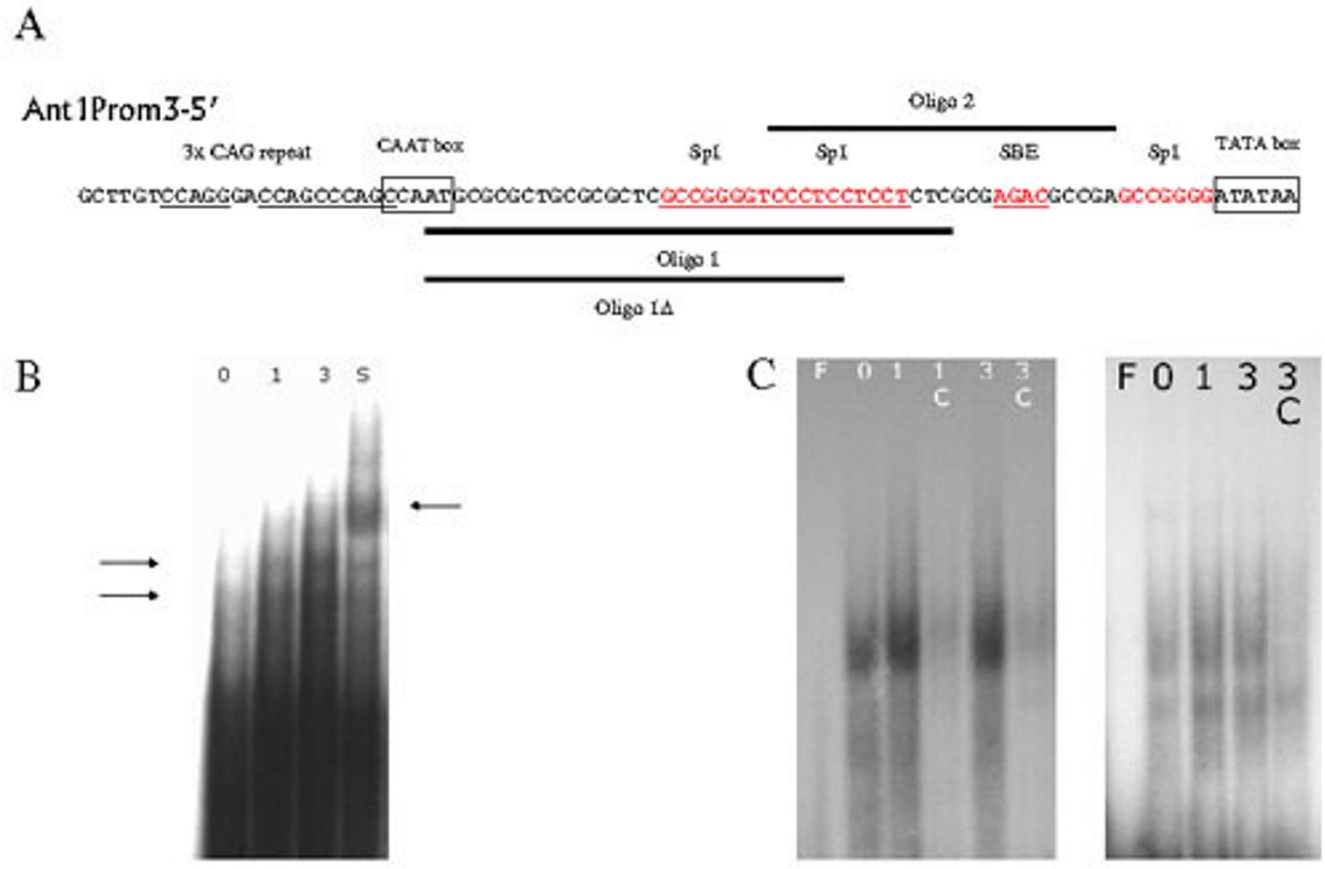

Figure 3

TGF-β1 induces nuclear protein binding to the Ant1 TGF-β1 RE. A. Sequence of the Ant1 promoter containing the TGF-β1 response element (Ant1 Prom3-5'). Boxed sequence indicates the CAAT and TATA boxes with the identified consensus Sp1 binding sites and putative SBE shown in red. Sequences that were tested in the current studies are underlined. Oligonucleotides used in EMSAs are noted in bold-face type with the corresponding promoter sequence. **B**. Nuclear extracts were prepared from untreated (0) primary astrocyte cultures and those treated with TGF-β1 for one (1) and three (3) hours. Extracts were then incubated with Oligo 1. TGF-β1 induced nuclear protein binding to this oligonucleotide (arrows on left side). Nuclear extracts from untransfected primary cultured astrocytes treated with TGF-β1 for three hours were incubated with Oligo 1 and an antibody against Sp1 (S). The arrow on the right side indicates the supershifted species. **C**. Similar EMSAs were conducted using the same nuclear extracts with Oligo 2 (left panel) and Oligo 1Δ (right panel). Although specific nuclear protein binding is detected for these oligonucletide probes, a TGF-β1 induced species was not identified in either case. Control EMSA reactions were either conducted in the presence of unlabelled oligonucleotide competitors (C) or in the absence of nuclear extracts (F).

contrast, the same nuclear extracts did not demonstrate TGF-β1 induced protein binding to Oligo 2, which includes the putative SBE and 10 out of 11 bases of Sp1-2_{TCC} (Fig 3a). These results suggest that the Sp1-1_{GC} site may be most important for nuclear protein binding to the Ant1 TGF-β1 RE and that TGF-β1 treatment likely results in recruitment of an Sp1 transcription factor species to this RE. On the other hand, the lack of inducible nuclear proteins binding to the SBE suggests that either Smads do not directly bind to this response element, or that these transcription factors bind via an as yet unidentified SBE sequence.

The consensus sequence in Oligo 2 containing all but the last base of the Sp1-2_{TCC} motif did not demonstrate a TGF-β1 induced protein binding in the EMSA study, suggesting that this Sp1 site does not directly contribute to nuclear protein complex binding to the Ant1 RE in response to TGF-β1. To further explore the relationship between the first two Sp1 sites, EMSA was performed with a truncated

version of the Oligo 1 probe (-87 to -60), lacking half of Sp1-2_{TCC} (Oligo 1Δ – Fig 3a). Surprisingly, TGF-β1-induced binding of nuclear protein to this Oligo was not detected (Fig 3c). This result suggests both Sp1-1_{GC} and Sp1-2_{TCC} must be present in order for TGF-β1 induced Sp1 binding to the RE to occur.

Expression of Smads in the in vivo *glial scar and primary astrocytes*

The TGF-β1-activated Smad transcription factors can participate in transcriptional regulation through direct physical association with DNA or they may interact with other transcription factors in a nuclear protein complex, rather than binding directly to a DNA response element (RE). For example, the TGF-β1 activated Smads 2, 3 and 4 can bind to Sp1 and effect gene transcription [31,32,34,40-42]. Given the diversity of genes regulated by TGF-β1 [reviewed by [25,29,43]], interaction with other transcription factors may provide opportunity for greater target specificity in changing cellular conditions. To address the possibility that the Sp1 transcription factors that bind to the Ant1 TGF-β1 RE (Fig. 3b) also interact with TGF-β1 activated Smads, we investigated Smad expression in astrocytes. Although Smad 2, 3 and 4 mRNAs have been detected in cultured astrocyte progenitor cells [44], and despite the prevalence of TGF-β1 regulation of gene expression following CNS injury, expression of these transcription factors in the CNS glial scar or primary astrocyte cultures has not been described. Hence we examined Smad mRNA and protein expression in the *in vivo* glial scar and astrocyte cultures. Consistent with the possibility that Smads are involved in TGF-β mediated signalling in astrocytes, RT-PCR revealed expression of mRNAs encoding Smads 2, 3 and 4 in the *in vivo* glial scar and in primary astrocytes (Fig. 4a). Immunoblot analysis using an antibody that recognizes Smads 2 and 3 further revealed a predominant band with an apparent molecular weight of 128,000 Dalton (Da) and an additional minor band of 84,000 Da in protein extracts from the filter implant, uninjured cortex, and total brain (Fig. 4b). These are also the major bands detected in both TGF-β1 treated and untreated astrocyte cultures. The monomeric forms of R-Smads 2 and 3 (predicted size between 55,000–60,000 Da [45]) were not detected; the larger species we detect likely reflect the highly stable Smad-containing complexes that have been recently described [46].

Immunostaining of R-Smads provides additional support for expression of these transcription factors in the *in vivo* glial scar and astrocyte cultures (Fig. 4c). Immunohistochemical analysis indicates the presence of cells expressing Smads 2 and/or 3 within the *in vivo* glial scar, which also exhibits strong GFAP immunoreactivity indicative of reactive astrogliosis. Expression of these TGF-β1 activated R-Smads was also demonstrated in primary astrocyte cultures. These immunocytochemical analyses further reflect the similarities between cultured astrocytes and reactive astrocytes of the *in vivo* glial scar and validates the use of primary astrocyte cultures to examine TGF-β1 regulation of Ant1. Thus the presence of TGF-β1 activated Smads in astrocytes is supported by RT-PCR, immunoblot and immunohistochemical studies. To our knowledge, these results are the first direct demonstration that these Smads are expressed by reactive astrocytes in response to CNS injury and by cultured primary astrocytes.

Functional role of Smads 3 and 4 in the TGF-βI induction of Ant1

A potential functional role for Smad transcription factors in TGF-β1 induction of Ant1 mRNA expression was also evaluated. Primary astrocytes were co-transfected with the TGF-β1 responsive Ant1 485 bp reporter construct and dominant-negative Smad (DN-Smad) 2, 3 or 4 expression plasmids (Fig 5). Dominant negative interruption of the co-Smad (Smad 4) abrogated the TGF-β1 response, indicating that TGF-β1 induction of Ant1 is mediated by Smad transcription factors. Co-transfection of DN-Smad3 also disrupted the TGF-β1 response, whereas DN-Smad2 had no effect on Ant1 induction in TGF-β1-treated astrocytes (Fig. 5). To demonstrate the specificity of the DN-Smad co-transfection approach, a reporter construct containing multiple SBEs (SBE4Lux) that is known to respond to Smad2 was transfected into primary astrocyte cultures, and was strongly induced by TGF-β1. When DN-Smad2 was co-transfected with SBE4Lux, however, regulation of the reporter gene by TGF-β1 was significantly reduced (data not shown). These results confirm the functional activity of the DN-Smad2 plasmid in disrupting TGF-β1 induction of gene expression in astrocytes and further demonstrated the differential roles of Smads 2 and 3 in gene regulation. Furthermore, the inability of DN-Smad2 to disrupt the induction of Ant1 expression by TGF-β1 rules out the possibility that the attenuation of Ant1 upregulation was due simply to overexpression of DN-Smads. Together, these results support the suggestion that Smad transcription factors are involved in the TGF-β1 induction of Ant1and indicate that Smad3 is likely to be the specific R-Smad that mediates this response.

Discussion

TGF-β is a pluripotent cytokine with well-described actions in numerous tissues during development. TGF-β exists as three isoforms with TGF-β2 and TGF-β3 predominating during development when TGF-β1 levels are low. Following CNS injury, however, TGF-β1, but not -β2 or -β3, is upregulated where it is thought to serve as a modulator of the inflammatory response and to promote neuronal survival [reviewed by [47,48]]. This cytokine also directs astrocytic expression of a number of genes including p15 (INK4B) [49], amyloid precursor protein [50],

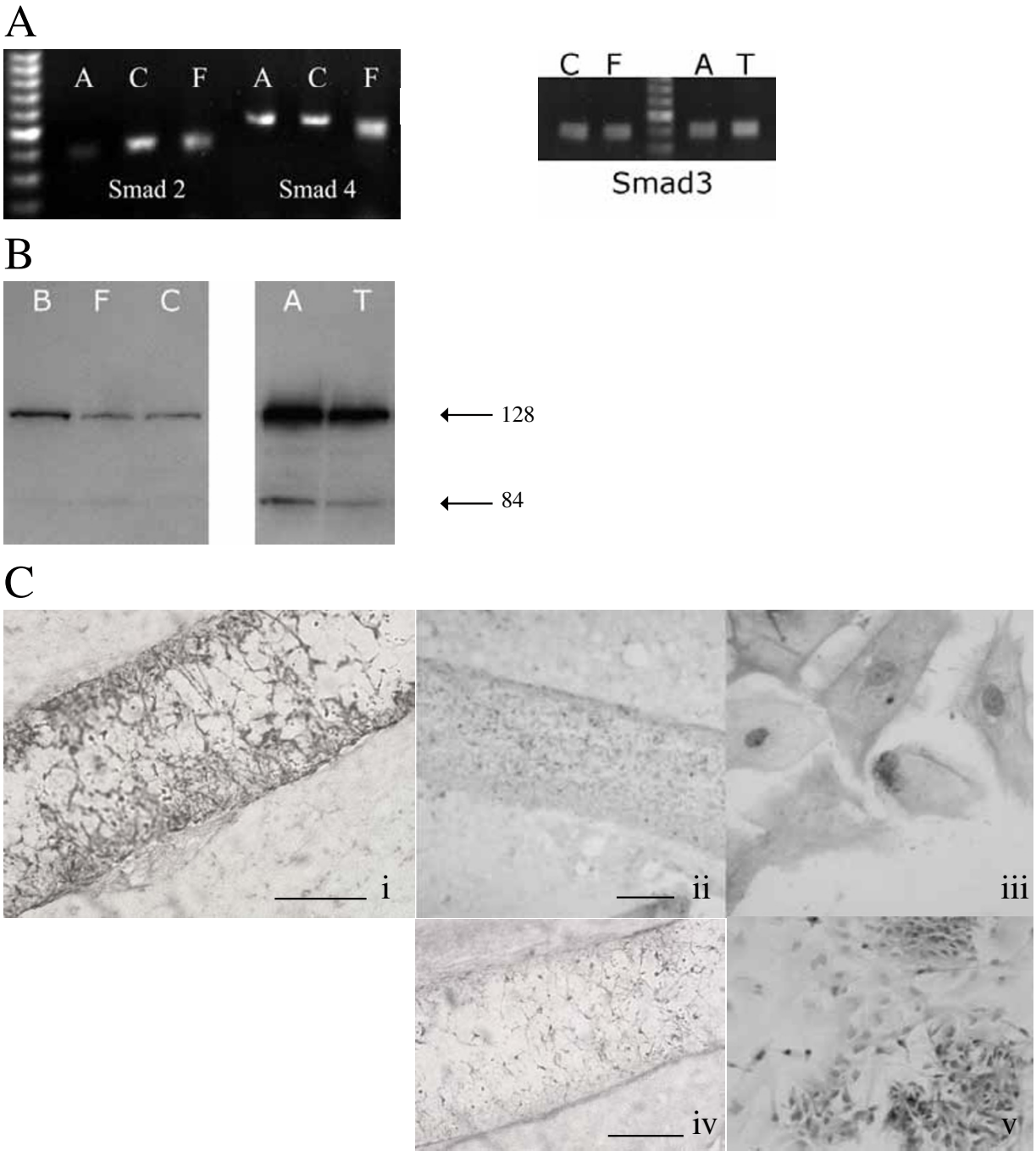

Figure 4

Smad expression in the in vivo glial scar and in primary astrocytes. A. RNA was isolated from cortex (C), *in vivo* filter implants (F), untreated (A) and TGF-β1 treated (T) cultured astrocytes. Smad mRNAs were detected by RT-PCR with primers specific for Smads 2, 3 and 4. **B**. Protein lysates from total brain (B), filter implants (F), cortex (C), untreated (A) and TGF-β1 treated (T) cultured astrocytes were examined for the presence of TGF-β1 activated R-Smads using a Smad 2/3specific antibody. **C**. Expression of Smads 2 and 3 in filter implants (ii) and cultured astrocytes (iii) was also detected by immunostaining with the same antibody. Similar immunostaining results were obtained with a specific antibody recognizing Smad2 in filter implants (iv) and in primary astrocyte cultures (v). GFAP immunohistochemistry (i) was performed to demonstrate reactive astrogliosis elicited by filter implantation. Scale bars in (i), (ii) and (iv) are 75 μm. Magnification for (iii) is 500× and for (v) is 200×.

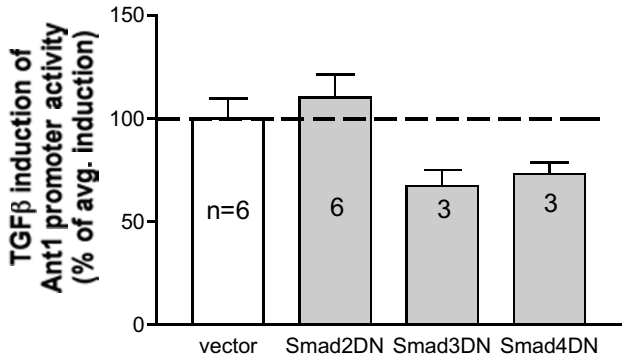

Figure 5
Effect of Smad inhibition on the TGF-β1 activation of Ant1. Primary astrocytes were transiently co-transfected with the TGF-β1 responsive Ant1 485 bp reporter construct and with expression constructs for the indicated dominant negative Smads or empty vector control. Levels of Ant1 promoter activity induction by TGF-β1 in the presence of individual dominant negative Smads and empty vector control were assessed by one-way ANOVA ($p < 0.05$). Individual dominant negative Smad treatments were compared relative to empty vector control by Dunnett's post hoc multiple comparison test.

GFAP [35,51,52] and PAI-1 [39]. The molecular mechanisms whereby TGF-β1 induces transcription in the CNS, however, are not well understood. We recently found that Ant1 is upregulated by TGF-β1 in astrocytes following CNS injury. The biological significance of this TGF-β1 mediated response is evident from in vitro studies demonstrating that glutamate uptake by Ant1 null mutant astrocytes is significantly impaired compared to uptake by wild type astrocytes [6]. Because glutamate levels can rise to toxic levels following CNS injury, regulating the expression of Ant1 may be one way TGF-β1 enhances neuronal survival. In this study, we directly examined the effects of TGF-β1 on Ant1 gene expression in primary astrocytes since this cell culture system has been extensively used as a model of the reactive astrocyte response to CNS injury [35,39,51,53,54]. As was the case in the *in vivo* glial scar, primary astrocyte cultures respond to TGF-β1 with increased expression of Ant1, but not of the closely related Ant2 isoform (Fig 1; [6]).

Transcription of a reporter plasmid construct including the transcription start stie and approximately 500 bp of the Ant1 promoter was induced 1.7-fold by TGF-β1; smaller constructs including the Ant1 TGF-β1 RE were similarly induced. The level of induction we observe for Ant1 is remarkably similar to the extent of TGF-β1 stimulation of alpha 2(I) collagen via the synergistic actions of Sp1 and Smad3 in a primary human mesangial cell culture system [40]. Surprisingly, an expression construct with 6.5 kb of Ant1 promoter upstream of the TGF-β1 responsive 500 bp fragment exhibited reduced expression in the presence of TGF-β1. These results may reflect a transcriptional inhibition of Ant1 expression that is overcome by the positive response of the TGF-β1 RE following CNS injury, thus suggesting a tightly controlled and complex regulation of Ant1 gene expression in reactive astrocytes.

Following CNS injury, TGF-β1 treatment has been reported to promote neuronal survival [47,48] but the mechanisms of this neuroprotective effect have not been elucidated. Recently, TGF-β1 directed expression of PAI-1 in astrocytes following injury has been shown to protect neurons from glutamate excitotoxicity [55]. Our recent demonstration of the role of Ant1 in astrocytic glutamate uptake [6] further supports a role for TGF-β1 in promoting neuronal cell survival in the injured CNS. Given that both Smads and Sp1 are involved in the induction of Ant1 and PAI-1 [34] in astrocytes, this partnership of transcription factors may represent a general mechanism for TGF-β1 enhancement of cell survival following CNS injury. In addition to Ant1 and PAI-1, TGF-β1 mediated induction of the extracellular matrix molecule neurocan by astrocytes has also been documented [5,56]. The neurocan promoter also contains consensus binding sites for Sp1 [57] and for Smads (-103 to -100 and -325 to -322 relative to transcription initiation), again consistent with the possibility that these transcription factors cooperate in TGF-β1 induced neurocan gene expression following CNS injury. TGF-β1 also stimulates the astrocytic expression of GFAP, the hallmark of reactive astrogliosis following CNS injury [58,59]. Recent studies examining the TGF-β1 regulation of GFAP expression in astrocytes [35] only identified an NF-1-like response element. Hence even in the reactive astrocyte response to CNS injury, TGF-β1 may direct target gene expression via multiple pathways.

The molecular mechanisms of TGF-β1 directed transcription have been studied extensively in many non-CNS systems with transformed cell lines and many Smad interacting partners identified [reviewed by [29]]. For example, in cooperation with Sp1, Smads mediate the expression of alpha 2(I) collagen [33], PAI [34], β_5 integrin [32], and p21/WAF1/Cip1 [31]. Our results demonstrate that Smads also interact with Sp1 to induce Ant1 gene expression. Interestingly, Smad-Sp1 interactions with all of these genes occur predominantly through Smad3 rather than Smad2; although each of these R-Smads can be activated by TGF-β1. Specific interaction between Smad3 and Sp1 may represent a general mechanism for Smad-Sp1 interaction. The apparent interaction of both Smads 2 and 3 in TGF-β1 induction of p15 (INK4B) however, indicates that this is not an exclusive mechanism [42].

Although human ANT2 mRNA is weakly expressed in most tissues, this gene is induced in highly proliferative cell types [60]. This observation coincides with the upregulation of mouse Ant1 in reactive astrocytes that may proliferate following CNS injury during the formation of the glial scar. The similarity between these two genes is also evident in the structure of the gene promoters. The human ANT2 gene promoter contains three Sp1 sites flanking the TATA box, which are important in the regulation of human ANT2 mRNA expression [61]. To our surprise, the mouse Ant1 TGF-β1 RE reported here also contains three Sp1 sites near the TATA box. Given the similarity in promoter sequence structure of human ANT2 and mouse Ant1, we hypothesize that the inducibility of both genes may be partly due to the presence of tandem Sp1 repeats. This hypothesis is consistent with reports demonstrating that the TGF-β1 regulated α 2(I) collagen [40] and PAI-1 [34] gene promoters also contain multiple Sp1 sites. Further, our studies indicate that TGF-β1 specifically regulates the expression of rodent Ant1 but not Ant2. This may be partly explained by a paucity of Sp1 sites in the mouse Ant2 promoter, which contains only one Sp1 element on the non-coding strand at -32. This suggestion is supported by our finding that a chimeric construct generated by addition of the Ant1 TGF-β1 RE containing the three Sp1 sites to the Ant2 promoter transcription reporter construct confers TGF-β1 responsivity. Taken together, these observations suggest that human ANT2 may respond to TGF-β1 following CNS injury, analogous to mouse Ant1.

Conclusions

Increased production of TGF-β1 after CNS injury is thought to regulate the expression of a number of genes involved in tissue repair and neuronal protection. While TGF-β mediated gene expression typically involves the Smad family of transcription factors, the specific molecular mechanisms utilized during the induction of specific genes needs to be characterized. The results of the present study demonstrate that TGF-β1 mediated induction of Ant1 by astrocytes requires the cooperative interaction of both Smad 3 and multiple Sp1 binding sites and that these binding elements are located within 500 bps immediately upstream of the transcriptional start site. This induction is specific since the closely related Ant2 isoform, which does not contain similar binding elements, is not induced by TGF-β1. The similarity in TGF-β1 regulation of Ant1 and other genes including PAI, which has also been implicated in promoting neuronal survival by astrocytes, may represent a general mechanism that underlies the neuroprotective effects of TGF-β1. Thus, the results of the present study provide novel insights into the astrocytic regulation of Ant1 following CNS injury.

Methods

Filter implantation

Nitrocellulose filters were implanted into the cerebral cortex of adult TMG129 mice (≥30-day-old) as previously described [5,62,63]. The filters were left in place for 14 days prior to removal. Filter implants from 10–20 animals were pooled for RNA or protein extractions. Animal care was in accordance with guidelines established by the Institutional Animal Care and Use Committee at Emory University.

Cell culture

Purified populations of neonatal cortical astrocytes were prepared according to previously described methods [5]. The cells were cultured in DMEM-F-12 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 100 IU/ml penicillin-streptomycin, and were maintained at 37°C in 5% CO_2. All experiments were performed on primary astrocytes cultured for one to three months.

Immunoblotting

Total proteins were purified from nitrocellulose filter implants, uninjured cortex and primary astrocyte cultures as described [5]. Transferred nitrocellulose membranes were blocked at 4°C for one hour at room temperature or overnight. Ant1 and Smad 2/3 immunoblots were blocked in KPL milk blocking reagent in PBS. Ant1 proteins were detected by an affinity-purified polyclonal antisera [6,18]. The Smad2/3 antibody (Santa Cruz, cat. # 6032) and the Ant1 antisera were used at 1:200 dilution in KPL milk blocking buffer and incubated overnight at 4°C. MAPK proteins were detected by a p42/44 MAPK antibody (New England Biolabs). Blots were washed three times with PBS, incubated with the appropriate secondary antibodies diluted in blocking buffer and detected by ECL chemiluminescence (Amersham).

Immunostaining

Adult mice were subjected to cortical nitrocellulose filter implantation to generate a glial scar. Fourteen days after implantation, animals were perfused with 4% paraformaldehyde in PBS (pH 7.0) and 10 μm frozen sections of injured cortex containing the filter implant obtained. Sections were washed twice with PBS and blocked with 0.3% H_2O_2, 3% normal horse serum in PBS at room temperature for 10 minutes, washed again and incubated with blocking buffer containing 3% normal horse serum and 0.04% Triton X-100 in PBS for 1 hour at room temperature. Sections were again subjected to PBS washes and incubated with Smad2/3 or Smad2 goat antibodies (1:100; Santa Cruz) overnight at 4°C. After similar washes, sections were incubated with a biotinlyated anti-goat secondary antibody (1:1000; Jackson ImmunoResearch Laboratories) and developed with 3,3'-diaminobenzidine (Vector). Primary astrocytes were grown on

Table 1: Sense and anti-sense oligonucleotides for Smads 2, 3 and 4

Gene	Sequence (5'-3')	Position	Annealing temp (°C)
Smad2	TCAGTGCGATGCTCAAGCATGTCC	1515-1538	66
	AGACCAAGCAGCAGCACACACCTC	1926-1903	
Smad3	ACGAGCTGAACCAGCGAGTTGG	1006-1027	45 (5 cycles)
	TGGTGCACATGCGAGTCAACTGG	1407-1385	57 (35 cycles)
Smad4	ATGCCCATTGCGGACCCACAG	1755-1775	66
	GGAAGACTGGACTGCCACTTGCG	2253-2231	

laminin coated glass coverslips, fixed with 4% paraformaldehyde in PBS at room temperature for 10 minutes and processed as above except the anti-Smad2/3 primary antibody was used at 1:200 dilution. Negative staining controls using inappropriate antibodies conducted with either a monoclonal GFAP antibody (1:500; Sigma) followed by the biotinlyated anti-goat secondary antibody, or the Smad goat antibodies together with a biotinlyated anti-mouse secondary antibody did not show any staining (data not shown).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described in McKeon et. al. [5]. Total RNA was prepared from cortical tissue, primary astrocyte cultures and glial scar nitrocellulose filter implants [64]. RNA was digested with DNAseI to eliminate genomic DNA contamination. cDNA synthesis was performed from 1 μg of RNA using random hexamers and SuperScript II reverse transcriptase (RT) (Invitrogen) for one hour at 42°C. Additional cDNA synthesis reactions were conducted in the absence of RT as control for amplification from genomic DNA. The sequence of each oligonucleotide pair and conditions used to amplify the respective Smads are indicated in Table 1. PCRs for Smads 2 and 4 were performed using cDNA pools derived from Sprague-Dawley rats while Smad3 PCR was done with mouse-derived tissues.

Plasmid construction

Clones containing the promoter regions of Ant1 and Ant2 spanning 7 kb and 5.8 kb respectively were generated previously [18]. Subclones were inserted into the pGL3 firefly luciferase reporter plasmid using standard molecular biology techniques. Dominant-negative Smad expression plasmids were kindly provided by Dr. Rik Derynk (UCSF).

EMSA and supershift assays

Nuclear extracts from passages 3–7 primary astrocyte cultures were prepared according to [35]. For probe labeling, 28 picomoles of oligonucleotides were end-labeled with γ-^{32}P ATP (ICN Biochemicals) and 10 units of T4 polynucleotide kinase (Promega) at 37°C for 1 hour, followed by heat inactivation at 70°C for 15 minutes. The sequences of the oligonucleotides are shown in Table 1. Unincorporated free nucleotides were removed by micro bio-spin 30 gel exclusion chromatography (Bio-Rad) according to manufacturer's protocol. The sense and anti-sense reactions were combined and annealed at 72°C for 5 minutes and subsequently at 50°C for 40 minutes. The double-stranded probes were cooled to ≥ 37°C at room temperature and purified with a 6% acrylamide (19:1 bisacrylamide) gel in TAE buffer. Gel fragments were eluted in 200 μl TE for 1 hour, transferred to a 0.45 μm ultrafree-MC filter (Millipore) and centrifuged for 15 minutes at 11,000 × g to remove residual acrylamide. EMSAs were performed in 1× binding buffer [15 mM Hepes, pH 7.9, 50 mM KCl, 10% glycerol, 0.12 mM EDTA, 5 mM $MgCl_2$, 0.25 μg/μl BSA and 0.05% NP-40], 1 × 10^5 CPM of labeled oligonucleotides, 10 mM dithiothreitol, 200 ng of poly dI/dC and 6 μg of nuclear extracts. Reactions were incubated for 30 minutes at 4°C and separated by electrophoresis in 5% acrylamide (69:1 bisacrylamide) in Taurine buffer [10.7 mM EDTA, 1.78 M Tris-base and 0.575 M Taurine]. For supershift assays, 2 μl of a Sp1-specific antibody (Promega) was included in the reaction. EMSA experiments using an oligonucleotide corresponding to the TGF-β1 response element within the PAI promoter demonstrated inducible nuclear protein binding by this cytokine (data not shown). The specificity of nuclear protein binding was tested with unlabeled competitor oligonucleotides in 10 to 100 fold excess.

Transfections and luciferase assays

Primary astrocyte cultures were kept in 12-well dishes with 0.5 ml culture medium containing DMEM with 10% fetal calf serum and L-glutamine. Prior to transfections, 0.25 ml of conditioned culture medium was replaced with 0.25 ml of fresh culture medium. DNA cocktails consisting of 1.0 μg of firefly luciferase reporter and 0.5 μg of renilla luciferase plasmid under the control of the thromboxane synthase promoter (pTS-RL; [65]) were mixed with 3 μl Fugene 6 (Roche) previously diluted in 97 μl of OPTI-MEM (Life Technologies). The DNA-Fugene mix was incubated at room temperature for 15 minutes and added to each well. Cells were incubated at 37°C for

Table 2: EMSA oligonucleotide sequences

Oligonucleotide	Sequence (5'-3')
Oligo 1 Sense	ATGCGCGCTGCGCGCTCGCCGGGGTCCCTCCTCCTC
Oligo 1 Anti-sense	GAGGAGGAGGGACCCCGGCGAGCGCGCAGCGCGCAT
Oligo 2 Sense	CCCTCCTCCTCTCGCGAGACGCCGA
Oligo 2 Anti-sense	TCGGCGTCTCGCGAGAGGAGGAGGG
Oligo 1Δ Sense	ATGCGCGCTGCGCGCTCGCCGGGGTCCCTC
Oligo 1Δ Anti-sense	GAGGGACCCCGGCGAGCGCGCAGCGCGCAT
PAI Sense	TCGAGAGCCAGACAAGGAGCCAGACAAGGAGCCAGACAC
PAI Anti-sense	GTGTCTGGCTCCTTGTCTGGCTCCTTGTCTGGCTCTCGA

24 hours, rinsed once with 0.5 ml of serum free media and replaced with an equal volume of N2-supplemented serum free media. Transfected cells were incubated for an additional 48 hours in TGF-β1 (10 ng/ml, R&D Technologies). Cells were washed twice with 350 μl of ice-cold PBS, lysed in 100 μl of 1× passive lysis buffer (Promega), and centrifuged at 15,000 g for 20 seconds to pellet debris. Supernatants were transferred to fresh microcentrifuge tubes and stored at -70°C until assayed. Luciferase assays were performed using the Dual Luciferase Reporter kit (Promega) with 10 to 20 μl of lysates according to manufacturer's protocol. Firefly luciferase activities were normalized to renilla luciferase activity and expressed as relative light units (RLUs). Mean RLUs for TGF-β1 treated samples were compared to untreated controls by student's t-test. For dominant negative Smad co-transfection experiments, 0.5 μg of expression plasmid encoding the DN-Smad or empty vector control was added to the transcription reporter construct transfections.

Authors' contributions

AL formulated the experimental design, carried out the experiments and drafted the manuscript. RM performed the filter implants and harvesting and provided all astrocyte cultures. DG assisted with the RT-PCR and western blot analysis. SL and DW generated and provided the Ant1 and Ant2 genomic clones. CB and RM coordinated the experimental design, assisted with data analysis and co-wrote the manuscript with AL. All authors read and approved the manuscript prior to submission.

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