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Soluble Factors Secreted by Activated T-Lymphocytes Modulate the Transcription of the Immunosuppressive Cytokine TGF-β2 in Glial Cells

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Abstract Coordination of the immune response to injury or disease in the brain is postulated to involve bi-directional discourse between the immune system and the central nervous system. This cross communication involves soluble mediators, including various growth factors, cytokines, and neuropeptides. In this report, we demonstrate that the supernatant from activated T-lymphocytes is able to induce the transcription of a potent cytokine, TGF-β2 in glial cells. The activating stimulus invokes signaling mechanisms distinct from known kinase or protease pathways. Activation of TGF-β2 transcription correlates with the loss of binding activity for an 80 kDa glial labile repressor protein, GLRP, to a responsive region within the TGF-β2 promoter. Although GLRP shares some characteristics with the inducible transcription factor AP-1, it appears to be distinct from known AP-1 family members. These data along with previous observations demonstrating the potent immunosuppressive activity of TGF-β2, support a model for a feedback mechanism between the activated T-lymphocytes and astrocytes via TGF-β2 to regulate the immune response.

Key words: GLRP, T-lymphocyte, immune response, central nervous system

Neurodegeneration is seen in a broad spectrum of central nervous system (CNS) ailments, including cerebral trauma, tumors, degenerative, and infectious diseases. The immune response to neurodegeneration is largely mediated by resident microglia/macrophages, and the occasional B- and T-lymphocytes in the brain. The immune response in the CNS is delicately orchestrated, due to the anatomical complexity of the CNS, its low regenerative potential, and its exquisite vulnerability to damage by soluble mediators. Immunedysregulation has been evoked as the etiology of several CNS disorders, including multiple sclerosis.

Precise control of the immune response in the brain is thought to be shaped by the resident cells of the CNS [reviewed in Lotz and Schwartz, 1994; Owens et al., 1994]. Microglia and astrocytes can be induced to express MHC class 1 and 2 antigen, function as antigen presenting cells, and serve as primary sources in the CNS of multiple growth factors and cytokines, including interleukin (IL-1), tumor necrosis factor-alpha (TNF-α), and the transforming growth factor-beta (TGF-β). Indeed, altered levels of several inflammatory mediators are seen in several pathological conditions, including AIDS dementia, Alzheimer's disease, and CNS tumors [Finch et al., 1993; Kekow et al., 1990; Sher et al., 1992]. Recently, proinflammatory cytokines like TNF-α and IL-1β have been shown to contribute to neuronal toxicity [Gelbard et al., 1993]. On the other hand, TGF-β is postulated to play a crucial role in neuroprotection and in organizing responses to neurodegeneration, as a function of its ability to regulate the immune system at multiple levels [Finch et al., 1993; Kehrl et al., 1986; Kehrl 1991; Palladino et al., 1990; Ruscetti et al., 1993]. In vitro, TGF-β functions

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vitro, TGF-β functions as a chemotactic agent for monocytes, potently inhibits proliferative and secretory T- and B-lymphocyte responses to stimuli, blocks cytotoxicity against oligodendroglia and neurons, alters complement levels, and decreases γ-interferon mediated activation of MHC class II antigen expression in glial cells [reviewed by Lotz and Seth, 1993; Prehn and Krieglstein, 1994; Wahl 1992]. Additionally, systemic administration of TGF-β in vivo downmodulates organ inflammation in several experimental models of inflammatory diseases [reviewed by Fontana et al., 1992; Wahl, 1992]. Further, TGF-β1 transgenic knockout mice develop a massive inflammation of several organs, confirming the immunosuppressive role for this cytokine [Shull et al., 1992].

This potent pleiotropic cytokine, TGF-β actually represents a family of structurally related isoforms that serve vital functions in development, physiology, and immunity [Benzakour, 1994; Kingsley, 1994; Roberts and Sporn, 1990]. Three characterized mammalian isoforms, TGF-β1, TGF-β2, and TGF-β3 exhibit high individual conservation (>98% identity) across species, but significantly differ from each other (74–78% identity). The various isoforms are generally functionally interchangeable in most biological assays [Roberts and Sporn, 1990], but appear to be distinct in their spatial and temporal expression, with regard to both developmental-stage and tissue-specificity [Flanders et al., 1991; Pelton et al., 1990; Roberts et al., 1991]. For example, peripheral blood lymphocytes have been shown to express TGF-β1 and TGF-β3 but not TGF-β2 [Roberts and Sporn, 1990]. On the other hand, certain astrocytic cell cultures express TGF-β1, -β2, and -β3 mRNA but secrete only the latent TGF-β2 protein [Bodmer et al., 1989; Constam et al., 1992]. The secreted TGF-β2 is activated upon post-translational modification, as demonstrated in vitro by acidification, alkanization, heat, denaturation, or proteolysis [reviewed by Miyazono et al., 1990]. As evident in these cases, the expression of TGF-β appears to be regulated at multiple levels including transcription, translation, and post-translational modification.

In this study we attempted to analyze the effect of the immune response on TGF-β gene expression in glial cells. Supernatant from uninfected but activated T-lymphocytes was used to mimic the immune response in vitro. We examined the regulation of the predominant isoform in glial cells, TGF-β2, which, in fact, was initially characterized as an immunosuppressive factor secreted by glial tumor cells [de Martin et al., 1987; Wrann et al., 1987]. We observed that the supernatant from activated T-lymphocytes increased TGF-β2 expression in a glial cell line. Using deletion analysis, we mapped the responsive domain to a region encompassing nucleotides -117 to -77 of the TGF-β2 promoter. Interestingly, this promoter region co-segregates with a phorbol ester responsive region. Mobility shift assays reveal the loss of binding of a protein complex to the responsive region upon treatment of glial cells with T-cell supernatant or phorbol esters. The data suggest that a transcriptional repressor of TGF-β2 expression present in glial cells is sensitive to stimuli from activated T-cells. Based on previous studies and our observations, a negative feedback loop model is proposed for regulation of the immune response by the cells of the CNS.

MATERIALS AND METHODS

Plasmid Constructs

pB2-528, pB2-347, pB2-257, pB2-187, pB2-117, and pB2-77 are a series of deletion constructs of the 5′-promoter region of the human TGF-β2 gene cloned into pGEM4-SV0CAT vector [O'Reilly et al., 1992a] (provided by Drs. A. Roberts and M. O'Reilly, NCI), and are structurally depicted in Figure 3. The pCAT-κB, p3E2F-CAT, and pCAT-GRS constructs were previously described [Raj and Khalili, 1994]. All constructs were verified by sequencing, and prepared using commercial kits (Qiagen). The TGF-β2-CAT construct is equivalent to the pB2-528 and the pTGF-β1-CAT construct represents the phTG2 construct [Kim et al., 1992].

Cells and Tissue Culture

U-87MG is a human astrocytic glial cell line and T98G is a cell line derived from human glioblastoma multiforme both obtained from American type culture collection (ATCC). These cells are positive for glial fibrillary acidic protein (GFAP) which is the marker for astrocytes. All cell lines were maintained in Dulbecco's MEM (DMEM), supplemented with antibiotics, penicillin/streptomycin, and 10% (vol/vol) fetal calf serum (Gibco). Treatments were performed with media supplemented with fetal calf serum, SerumPlus (JRH Biosciences) or NuSerum (Collaborative Research), with similar results seen in each case.

Transfection and CAT Assays

Transient transfection assays were carried out by the calcium phosphate method [Graham and van der Eb, 1973], as previously described [Raj and Khalili, 1994]. Cells were treated with PMA (75 ng/ml) (Sigma, St. Louis, MO), or a 1:1 dilution of supernatant from activated T-lymphocytes for specified times, 16 h post transfection. Each experiment was repeated four or more times with different plasmid preparations.

Preparation of Conditioned Media

T lymphocytes were obtained from human blood samples using a previously characterized separation procedure [Julius et al., 1973]. The *cells* were then plated in RPMI (GIBCO, Long Island, NY) at a concentration of 2×10^6 cells per ml and received either no treatment, treatment with 100 ng/ml PMA, ionomycin (350 ng/ml), or co-treatment with PMA and ionomycin for 1 h. After 1 h, cells were washed with PBS and fresh medium was added. The conditioned media was removed from the T lymphocytes 18 h later. Supernatant from T-cells post-phorbol ester treatments consistently exhibited similar effects as supernatant from T-cells post-phorbol ester and ionomycin treatments. Supernatant from untreated T-cells was consistently similar to T-cells treated only with ionomycin.

Primer Extension Analysis

The oligonucleotide 5′ CCCCT CCTCC CCTGC CTCTT TCAC 3′ used in these experiments corresponded to the region on the TGF-β2 promoter overlapping with nucleotides 2402 to 2425 on the initially characterized sequence [Noma et al., 1991], where the observed transcription initiation sites are from 2278, 2282, 2283, and 2288. RNA was prepared by the hot phenol extraction method [Queen and Baltimore, 1983] and primer extension analyses were performed by the method described previously [Ausubel et al., 1989].

Analysis of Conditioned Media

Molecular weight cutoff experiments were performed both by using Ambicon centrifuge tubes or by use of dialysis bags with pore sizes that allow for proteins smaller than 12 kDa to freely move out of the supernatant. Heat treatment was performed by exposure of the supernatant to 65°C for 25 min prior to addition to the cells. Acidification of the conditioned media was performed by addition of dilute hydrochloric acid until the pH reached appropriate level.

Inhibitors and Activators

For experiments involving various inhibitors and activators, cells were pre-treated for 1 h with the specified inhibitor/activator, then washed in PBS and stimulated with conditioned media in the presence of additional inhibitor/activator for 45 min at the following concentrations: 6 μM H7, 3 μM forskolin, 1 μM calphositin C, 2 μM DAG, 10–100 μM TPCK, 10–100 μM indomethacin, and 1 μM PAO. Activators include phorbol myristate acetate (PMA) at a concentration of 75 ng/ml, TNF-α (1 u/ml), TGF-β1 (0.01–10 ng/ml), and TGF-β2 (1 ng/ml).

Nuclear Extract Preparation

Nuclear extracts were prepared by a modification of the mini-extract protocol as previously described [Raj and Khalili, 1994].

Oligonucleotides

Oligonucleotides were prepared commercially by Oligos Etc., Guilford, Conn, and their sequences are detailed below:

TGF-β2 responsive region:
5′ GCTCG TGGTC TAAGT AACGA GAGGA CTTCT GACTG TAATC 3′
3′ CGAGC ACCAG ATTCA TTGCT CTCCT GAAGA CTGAC ATTAG 5′

GRS (from JCV promoter):
5′ AGCTT GGAGG CGGAG GCGGC CTCGG 3′
3′ TCGAA CCTCC GCCTC CGCCG GAGCC 5′

NF-κB (from JCV promoter):
5′ AACAA GGGAA TTTCC TGGCC 3′
3′ TTGTT CCCTT AAAGG ACCGG 5′

NF-1 (from MBP promoter):
5′ TGCCT TGCAG GATGC CCACC CAGCT GACCC AGGGTG 3′
3′ ACGGA ACGTC CTACG GGTGG GTCGA CTGGG TCCCAC 5′

JCV-D (from JCV-promoter):
5′ AGCTT GCGAG CCAGA GCTGT TTTGG CTTGT CACCA G 3′
3′ A CGCTC GGTCT CGACA AAACC GAACA GTGGT CTCGA 5′

JCV-B (from JCV-promoter):
5′ CAGGGA AGGGA TGGCT GCCAG CCAAG CATGA GCTCA TACCTG 3′

3′ GTCCCT TCCCT ACCGA CGGTC GGTTC
GTACT CGAGT ATGGAC 5′
AP-1 (from Promega):
5′ CGCTT GATGA GTCAG CCGGAA 3′
3′ GCGAA CTACT CAGTC GGCCTT 5′.

DNA-Protein Interactions

Band shift assays and UV-crosslinking analyses were essentially carried out as previously described [Raj and Khalili, 1994]. The dissociation experiments with deoxycholate were performed essentially as described [Bauerle and Baltimore, 1988].

Sequence Analyses

All sequence analyses were performed with GenBank's signal scan program to identify potential transcription factor binding sites on the fragment of interest.

RESULTS

Activated T-Lymphocytes Increase the Expression of TGF-β2 Promoter in Glial Cells

In an attempt to mimic the immune response in vitro, supernatant from human T-lymphocytes, either untreated or treated with phorbol esters, were examined for the ability to modulate expression from various promoters (Fig. 1). Expression from a TGF-β2 reporter in U-87MG glial cells was enhanced by the addition of supernatant from activated T-lymphocytes (as described in Materials and Methods) (Fig. 1A, compare lane 3 to lane 1), but not by the addition of supernatant from untreated T-lymphocytes (Fig. 1A, compare lane 2 to lane 1). Supernatant from activated but not untreated T-lymphocytes was similarly able to boost the promoter activity of a pCAT-κB construct containing the cytokine-responsive NF-κB site (Fig. 1A, lanes 4–6), but had no effect on a control promoter containing multiple copies of the E2F site (Fig. 1A, lanes 7–9). Further, the expression from the TGF-β1 promoter was also increased by the addition of supernatant from activated T-lymphocytes (Fig. 1A, lanes 10–12). This finding is not surprising in light of multiple AP-1 binding sites in the TGF-β1 promoter.

The activation of TGF-β2 expression by T-lymphocytes appeared to be cell-type specific since a similar pattern of responsiveness of TGF-β2 to T lymphocyte-supernatant was seen in another human glioblastoma cell line, T98G and the oligodendrocytic cell line N20.1 (data not shown). However, neither supernatant from untreated or stimulated T-lymphocytes was able to significantly affect the level of expression from the TGF-β2 promoter in Hela cells (Fig. 1B, compare lanes 2 and 3 to lane 1). The supernatant from the stimulated T-lymphocytes was able to enhance the expression from the cytokine-responsive NF-κB site (Fig. 1B, compare lane 6 to lane 4) in non-glial, Hela cells. These data suggest that the cell-type restriction of the effect of the supernatant on TGF-β2 gene expression may be a consequence of a glial-specific receptor, but further studies in large numbers of glial-derived cells are needed to further establish this possibility.

Activated T-Lymphocytes Increase the Expression of Endogenous TGF-β2 in Glial Cells

Data from transient transfections indicate that supernatant from activated T-lymphocytes could regulate the expression of TGF-β2 promoter in glial cells. In order to examine the relevance of this finding to the regulation of the endogenous transcript levels, primer extension analyses were performed (Fig. 2). The expected transcript of 137 bp was enhanced upon addition of supernatant from activated but not untreated T-lymphocytes (Fig. 2, compare lanes 2 and 3 to lane 1) in U-87MG glial cells [see Noma et al., 1991]. Additional transcripts of 94, 72, and 68 bp showed a similar pattern of regulation by the supernatant from activated T-lymphocytes (Fig. 2). These transcripts may represent shorter primer extension products due to potential secondary structure formation of the long 5′ TGF-β2 untranslated message. The cell-type specificity of the phenomenon was again evident, as the various transcripts in Hela cells did not demonstrate a significant increase in promoter level upon the addition of T-lymphocyte supernatant (Fig. 2, compare lanes 5 and 6 to lane 4). A quantitative comparison between U-87MG and Hela cell lines demonstrated the clear differences in the change in the transcript levels upon the addition of supernatant from activated T-lymphocytes (not shown).

Taken together, these data indicate that TGF-β2 is regulated at the transcriptional level by supernatant from activated T-lymphocytes.

Identification of a Supernatant-Responsive Region on the TGF-β2 Promoter

A progressive series of deletion constructs spanning the 5′ promoter region of TGF-β2

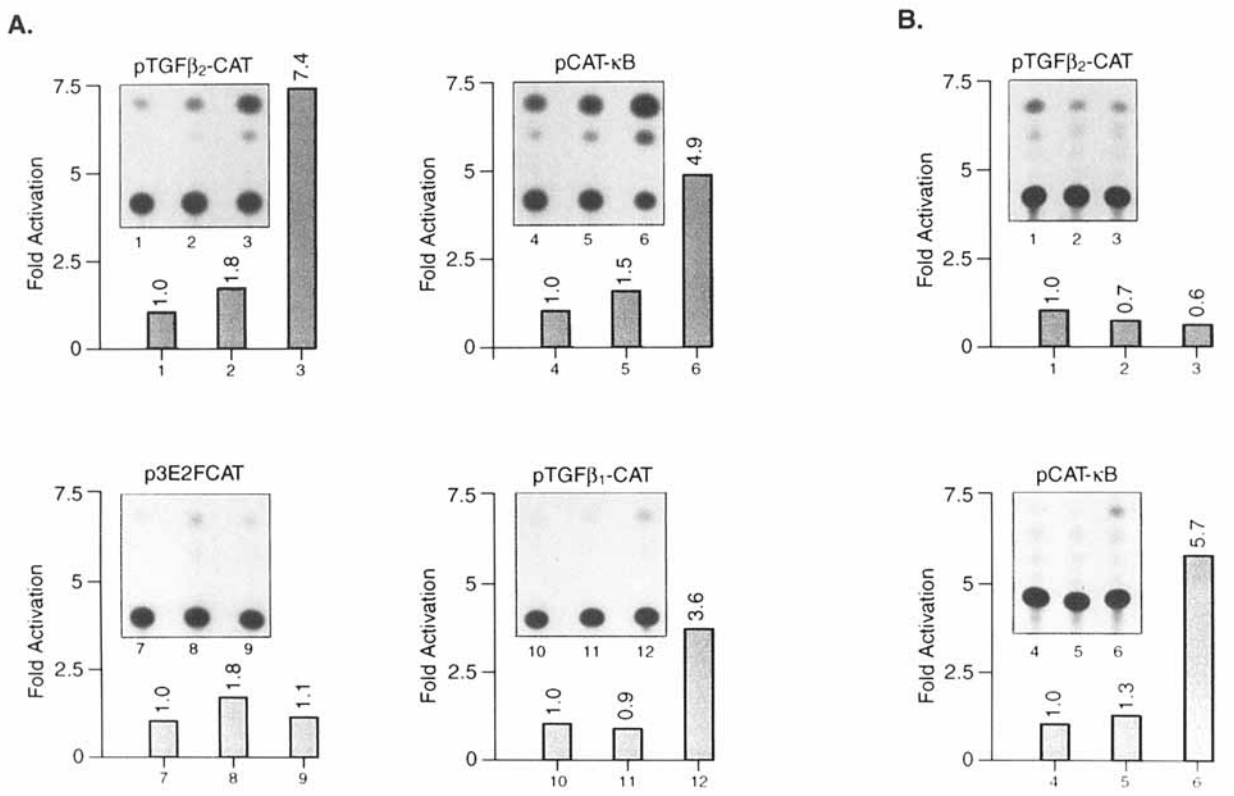

Fig. 1. Supernatant from activated T-lymphocytes increases the expression of TGF-β2 promoter in glial cells. Panels **A** and **B** illustrate both representative data (inset) and the average fold transactivation (bar graphs) of experiments examining the effect of control media (panel A, *lanes 1, 4, 7, 10* and panel B, *lanes 1 and 4*), supernatant from unstimulated T-lymphocytes (panel A, *lanes 2, 5, 8, 11* and panel B, *lanes 2 and 5*) and supernatant from activated T-lymphocytes (panel A, *lanes 3, 6, 9, 12* and panel B, *lanes 3 and 6*) on the expression of 3 μg of various promoter constructs. Experiments performed in the U-87MG glial cell line are shown in panel A, while those in the Hela cell line are depicted in panel B. The numbers above the bars represent the average fold activation. STD deviations for panel A, lane 2 is 0.3, lane 3 is 2.8, lane 5 is 0.4, lane 6 is 2.6, lane 8 is 0.2, lane 9 is 0.3, lane 11 is 0.4, lane 12 is 0.2. STD deviations for panel B, lane 2 is 0.2, lane 3 is 0.2, lane 5 is 0.2, and lane 6 is 1.2.

(Fig. 3A) [O'Reilly et al., 1992a] (kindly provided by Drs. A. Roberts and M. O'Reilly, NCI), cloned upstream of the CAT reporter gene were used in transient transfection analysis in the U-87MG cells to identify the supernatant-responsive region. The basal level of expression for the various deletion constructs was low, perhaps reflecting the low basal level of expression of the TGF-β2 promoter. Sequential deletions pointed to the presence of two responsive regions on the TGF-β2 promoter, a negative regulatory element (NRE) between -528 and -347 and a positive regulatory element (PRE) between -117 and -77 (Fig. 3B and Fig. 3C). Both regulatory regions contained no previously characterized binding sites for established transcription factors. Computer aided analyses showed that the NRE contained a potential CREB site, while the PRE contained several potential binding sites including a weak AP-1 binding site from -97 to -90

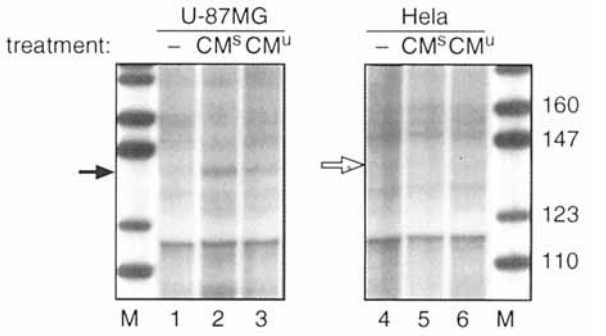

Fig. 2. Activated T-lymphocytes increase the expression of endogenous TGF-β2 in glial cells. Primer extension analyses were performed to analyze the effect of control media (*lanes 1 and 4*), supernatant from activated T-lymphocytes (CM^s *lanes 2 and 5*), and supernatant from unstimulated T-lymphocytes (CM^u *lanes 3 and 6*) on the expression of the endogenous TGF-β2 message in U-87MG cells (lanes 1–3) and Hela cells (lanes 4–6). Molecular size markers are depicted on either side (lane M).

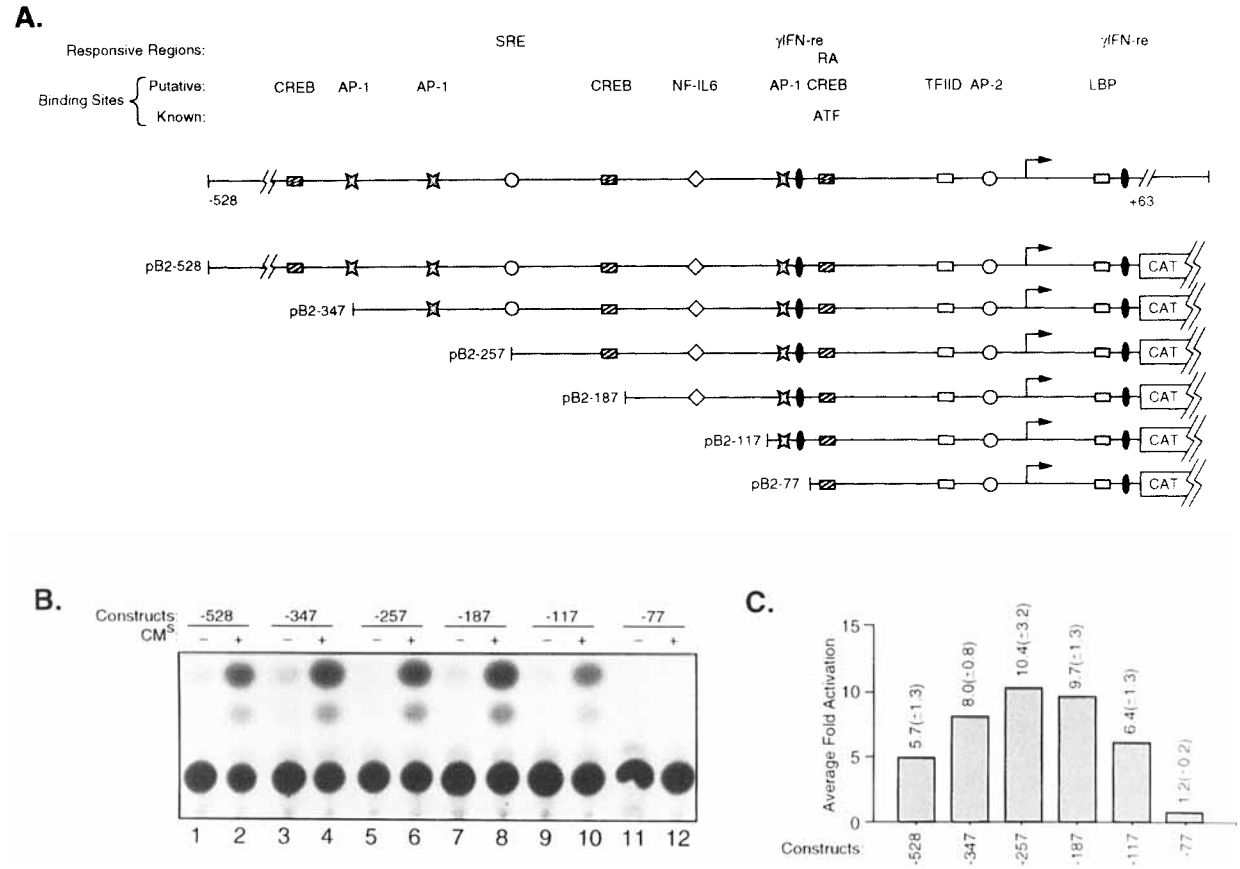

Fig. 3. Identification of a supernatant-responsive region on the TGF-β2 promoter. The schematic depicts the architecture of the TGF-β2 transcriptional control region, with both known and putative transcription factor binding sites detailed above the schematic (**panel A**) [Kim et al., 1992; O'Reilly et al., 1992a; O'Reilly et al., 1992b; Roberts et al., 1991]. The blueprint below the schematic diagrams the organizational structure of the various TGF-β2 promoter constructs, with numbers that reflect positions on the promoter relative to the transcription initiation start site. The effect of supernatant from activated T-lymphocytes on 3 μg of each reporter construct in U-87MG cells is depicted both as a representative experiment (**panel B**) and as an average fold transactivation seen over several experiments (**panel C**).

(sequence 5′ GAGAGGA 3′), and a weak γ-interferon responsive element from -83 to -77 (sequence 5′ CTGTAATCC 3′) on the TGF-β2 promoter.

Analysis of the Supernatant From Activated T-Lymphocytes

In order to characterize the nature of the activator present in the supernatant from activated T-lymphocytes, the supernatant was modified by various treatments prior to use in further transient transfection assays. Dialysis of the supernatant against an isomolar solution in a membrane with a molecular weight cutoff of 12,000 (Table I, panel A) modestly increased the expression from the TGF-β2 promoter. Exposure of the supernatant to heat (65°C for 25 min) (Table I, panel A) did not significantly affect the activation wrought by the supernatant. A reconstituted precipitate obtained by the addition of saturating levels of ammonium sulfate to the supernatant retained the ability to induce expression from the TGF-β2 promoter (data not shown). Taken together, these data suggest that the activator of TGF-β2 expression present in the supernatant appeared to be a heat-stable protein that is larger than 12 kDa. Further, the supernatant exhibited an increased activity following acidification to a pH of 2.5 (Table I, panel A). A possible explanation involves the activation of a latent factor(s) as a result of acidification, as previously documented for members of the TGF-β family. Since TGF-β isoforms have been shown to regulate expression of one another in a cell-type specific manner, activation of TGF-β might account for the increase in TGF-β2 expression seen in the acidified supernatant.

TABLE I. Analysis of the Supernatant From Activated T-Lymphocytes and the Involvement of Signaling Pathways*

Panel A								
CM^s	−	+	+	+	+			
Treatment	−	−	D	H	A			
Fold activation	1.0	10.5	15.2	10.8	28.0			
Panel B								
Treatment	−	CM^u	CM^s	PMA	TGF β1	TGF β2	TNF α	
Fold activation	1.0	0.8	6.3	5.5	0.7	1.9	0.9	
Panel C								
CM^s	−	−	−	+	+	+		
Activator	−	F	DAG	−	F	DAG		
Fold activation	1.0	0.9	1.1	4.2	6.8	5.3		
Panel D								
CM^s	−	−	−	−	+	+	+	+
Inhibitor	−	H7	C	T	−	H7	C	T
Fold activation	1.0	0.8	0.9	0.8	3.8	4.2	4.2	3.8

*U-87MG cells transiently transfected with 3 μg of the pB-528 construct, were treated on the day following transfection with control media, unmodified supernatant from stimulated T-lymphocytes, supernatant following dialysis (D) (mw cutoff 12 kDa), supernatant following exposure to 65°C for 25 min (H) or supernatant after acidification to a pH of 2.5 (A) (panel A). The effect of other exogenous activators on the TGF-β2 expression (panel B), as well as activators (panel C), and inhibitors (panel D) of several signaling pathways is represented. The effect of diacylglycerol (DAG), or forskolin (3 μM) (F) either alone or in the presence of supernatant from activated T-lymphocytes is examined. Similarly, the effect of H7 (6 μM), calphositin C (1 μM), or TPCK (T) alone or in the presence of supernatant from activated T-lymphocytes is represented.

In order to address this question directly, other exogenous activators and individual cytokines were examined for their ability to modulate TGF-β2 expression. Phorbol ester alone was able to activate transcription from the TGF-β2 promoter (Table I, panel B) to a similar level as the supernatant from activated T-lymphocytes. Interestingly, the cytokines TGF-β1, TGF-β2, TNF-α, IL-1α or β, or IL-2 (data not shown) over a wide range of concentrations could not elevate the transcriptional level from the TGF-β2 promoter (Table I, panel B). This finding is not surprising, given earlier reports showing that the cross-regulation of TGF-β isoforms shows a distinct cell-type specificity [Bascom et al., 1989]. Further, the addition of the various isoforms of TGF-β to the supernatant from the activated T-cells did not cause a superinduction as seen with the acidified supernatant (data not shown). These data suggest that the activating factors in either the normal or acidified supernatant could be distinct from TGF-β. However, the possible effect of combination cytokine treatment and their synergistic action on TGF-β2 gene regulation cannot be ruled out by these observations. Future studies which include depletion of various cytokines in single and combinations from the conditioned media by specific antibodies, and supplementation of the depleted media with purified cytokines should provide information regarding the identity of the T-cell derived molecule(s) which stimulates transcription of TGF-β2 in glial cells.

In an attempt to delineate the signal transduction pathway mediating the TGF-β2 response to the supernatant from activated T-lymphocytes, transient transfection assays were performed in the presence of specific inhibitors or activators. Diacylgylcerol, which is structurally similar to PMA and capable of activating protein kinase C [Altman et al., 1990], was unable to directly increase or superactivate TGF-β2 expression in glial cells (Table I, panel C). The protein kinase A activator, forskolin, was also unable to affect TGF-β2 expression in glial cells either directly or in the presence of supernatant. Further, the presence of either H7, a non-specific inhibitor of protein kinases A and C, or calphositin C, a specific inhibitor of protein kinase C, did not significantly affect the level of expression of TGF-β2 directly or in the presence of the supernatant (Table I, panel D). These results supported earlier findings which indicated that TGF-β2 could not be induced by activators of protein kinase C or A [O'Reilly et al., 1992a]. Additionally, protease inhibitors like TPCK (Table I, panel D), or phosphatase inhibitors like phenylarsine oxide (data not shown) were unable to affect the level of TGF-β2 expression either alone or in the presence of supernatant.

Multiple or previously uncharacterized pathways which do not involve protein kinase A or C, TPCK-sensitive proteases, may be at work in the activation of TGF-β2 promoter by the supernatant.

Analysis of Phorbol Ester Activity in Glial Cells

Phorbol esters have previously been shown to be involved in both transcriptional as well as post-transcriptional events associated with the duration of treatment [Raj and Khalili, 1994]. Our observations demonstrating the ability of phorbol esters to activate TGF-β2 promoter prompted analyses of whether a mechanism similar to that seen with supernatant from activated T-lymphocytes is at work. Deletion analyses were performed with the various TGF-β2 promoter constructs with phorbol ester treatment for varying lengths of time. A pattern, consistent with the results described for supernatant, identified two responsive regions on the TGF-β2 promoter. The presence of several upstream consensus AP-1 sites may account for the higher level of induction by PMA seen with some of the larger promoter constructs. Additionally, the time course analysis indicated that despite minor variations, the overall response pattern was identical with induction in roughly the same ratios over 1.5, 6 and 24 h time periods (data not shown). In comparison, the supernatant from activated T-lymphocytes required a longer minimal incubation time, with significant activation seen only after 3 h of stimulation (data not shown). Additional experiments indicated that the kinase inhibitors and activators also do not affect the outcome of phorbol ester treatment (data not shown).

Based on the results described above, a forty nucleotide region spanning nucleotides -117 to -77 of the TGF-β2 promoter appeared to mediate the increase in TGF-β2 promoter activity in response to stimulation with supernatant from phorbol ester-stimulated T cells as well as with phorbol ester itself. The identification of the same responsive regions for both stimuli is not surprising, given that both stimuli are potent activators of signal transduction processes. Interestingly, although similar pathways may be utilized, the variation in the minimal time of incubation required for transactivation of the TGF-β2 promoter may reflect differences in the strength of the stimulus.

Loss of Binding to the Positive-Responsive Region is Associated With Stimulation

Analysis of DNA-protein interactions undertaken to examine the binding pattern to the positive-responsive region, detected several nucleo-protein complexes in the nuclear extract from untreated U-87MG cells (Fig. 4A, lane 1). The slower migrating complex (complex A, as designated by arrow) reproducibly showed a biphasic pattern of change in nuclear extracts prepared from U-87MG cells treated with supernatant from activated T-lymphocytes for increasing time periods (Fig. 4A, lanes 2–5). Alteration in complex A was most noticeable in the extracts prepared from cells treated with supernatant for 3 h, with a loss in binding (Fig. 4A, lane 3). Extracts from cells treated for 6 and 24 h with supernatant appeared to recover their complex A binding ability (Fig. 4A, lanes 4 and 5). The effect of the supernatant was specific for complex A as the faster migrating complexes (complex B, designated by the asterisk) did not appear to be affected by these treatments. Further, neither supernatant from resting T-lymphocytes nor control media (RPMI) appeared to have an inhibitory effect on the binding of either complex over the same time course (Fig. 4A, lanes 6 and 7). It should be noted that the increase in the level of complex A in unstimulated extract was not reproducibly observed.

Interestingly, in extracts from Hela cells, complex A appeared to show a relative increase in binding upon treatment with supernatant (Fig. 4B, lanes 2–5). Curiously, the enhanced binding of complex A was most apparent in the extracts after treatment with supernatant for 3 h (Fig. 4B, lane 3). Again, this response appeared to be specific for the supernatant from activated T-lymphocytes, and was not seen with supernatant from resting T-lymphocytes or control media (RPMI) (Fig. 4B, lanes 6 and 7).

An altered pattern was seen with these extracts when examined for binding to the cytokine-responsive NF-κB or GRS probes [Raj et al., 1994; Taylor et al., 1994]. A relative increase in the β complexes was seen in the binding to the NF-κB site [Taylor et al., 1994] over the same time course of treatment (Fig. 4C, lanes 2–5). Again, the supernatant from resting T-lymphocytes or control media (RPMI) were unable to direct this increase (Fig. 4C, lanes 6 and 7).

Both phorbol esters and the activated supernatant treatments caused a similar alteration in

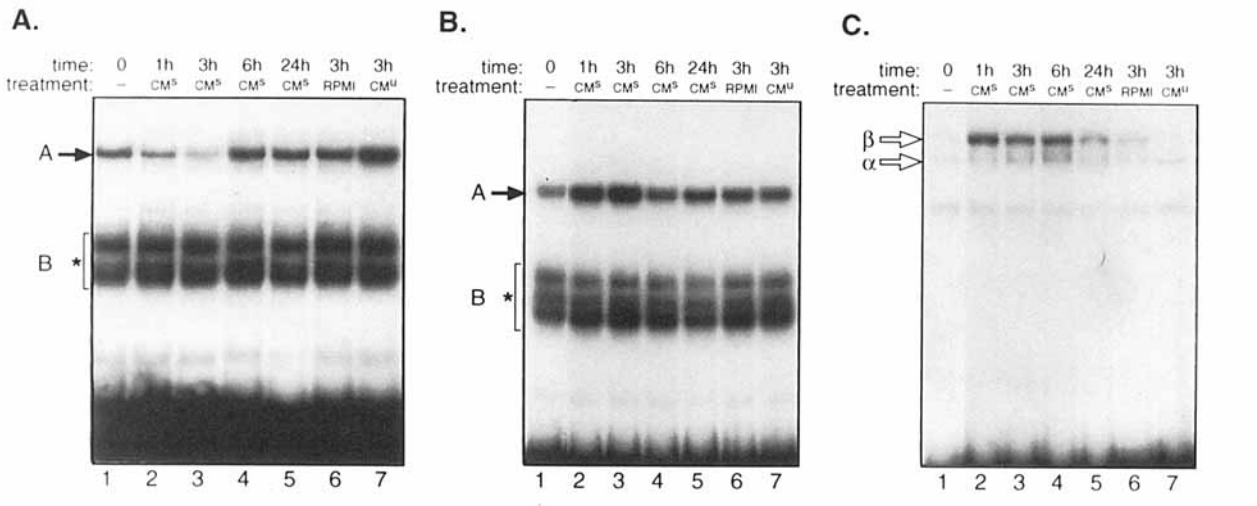

Fig. 4. Analysis of binding to the responsive region following treatment of cells with supernatant. Nuclear extracts prepared from cell lines following treatment with supernatant from activated T-lymphocytes for various periods of time, were examined for binding to the responsive region on the TGF-β2 promoter (**panels A and B,** *lanes 2–5*) or to the NF-κB site (**panel C,** *lanes 2–5*). The pattern of binding in nuclear extracts from untreated cells (panels A, B, and C, *lane 1*) or cells following treatment with control media (panels A, B, and C, *lane 6*) or supernatant from unstimulated cells (panels A, B, and C, *lane 7*) is also shown. Panels A and C represent the binding pattern seen with extracts from U-87MG cells, while panel B depicts the pattern seen with extracts from Hela cells.

the binding to the various responsive regions (Fig. 5). Both treatments induced a loss in binding of the slower migrating complex to the responsive region on the TGF-β2 promoter (Fig. 5A, lanes 1, 3, and 4), an increased binding of the GBP-i complex to the GRS probe (Fig. 5B, lanes 1, 3, and 4), an increased binding of the β-complex to the NF-κB probe (Fig. 5C, lanes 1, 3, and 4), but no difference in the binding to either single strand of the TGF-β2 responsive region or to an E2F-probe (data not shown). Interestingly, direct phorbol ester treatment exhibited a distinct kinetics of alteration in the binding to the responsive region on the TGF-β2 promoter. Complex A reproducibly disappeared within 30 min of stimulation with phorbol esters, recovered binding more quickly and did not diminish in intensity upon longer treatment durations (data not shown).

These data are consistent with a model in which the region between -117 and -77 houses functional regulators of the TGF-β2 gene expression. Complex A, whose binding activity was relieved upon stimulation, may function as a repressor, while complex B, whose binding activity did not change upon stimulation, may function as an activator. Evidence for the proposed activator function of complex B was indirectly borne by deletion analyses, which did not demonstrate a marked increase in transcription upon deletion of the -117 to -77 region [O'Reilly et al., 1992a].

Characterization of Complex A

The specificity of complex A was examined by competition mobility shift assays. Oligonucleotides representing either strand of the responsive region on the TGF-β2 promoter were unable to compete for the binding of complex A to the double stranded probe (Fig. 6A, lanes 2 and 3). The homologous double-stranded oligonucleotide was able to efficiently compete for the binding of complex A (Fig. 6A, lane 4). Additional heterologous competitors representing the GRS, JCV-D, CTF/NF-1, JCV-B, and the AP-1 elements were unable to compete for the binding of complex A (Fig. 6A, lanes 5–9). The inability of the AP-1 site to compete appears to rule out a role for the AP-1 members, fos/jun in mediating the responsiveness to both phorbol ester and supernatant from activated T-lymphocytes. These data indicated that complex A bound specifically to the double stranded oligonucleotide.

An estimation of the size of the nucleo-protein complexes in complex A was gained by UV-crosslinking experiments (Fig. 6B). One dimensional crosslinking experiments indicated that several nucleoprotein complexes, including an 81 kDa, and several 41–46 kDa complexes (shown by an arrow and a bracket, respectively) were present in untreated nuclear extracts. The 81 kDa nucleoprotein complex was more consistently decreased in nuclear extracts from cells

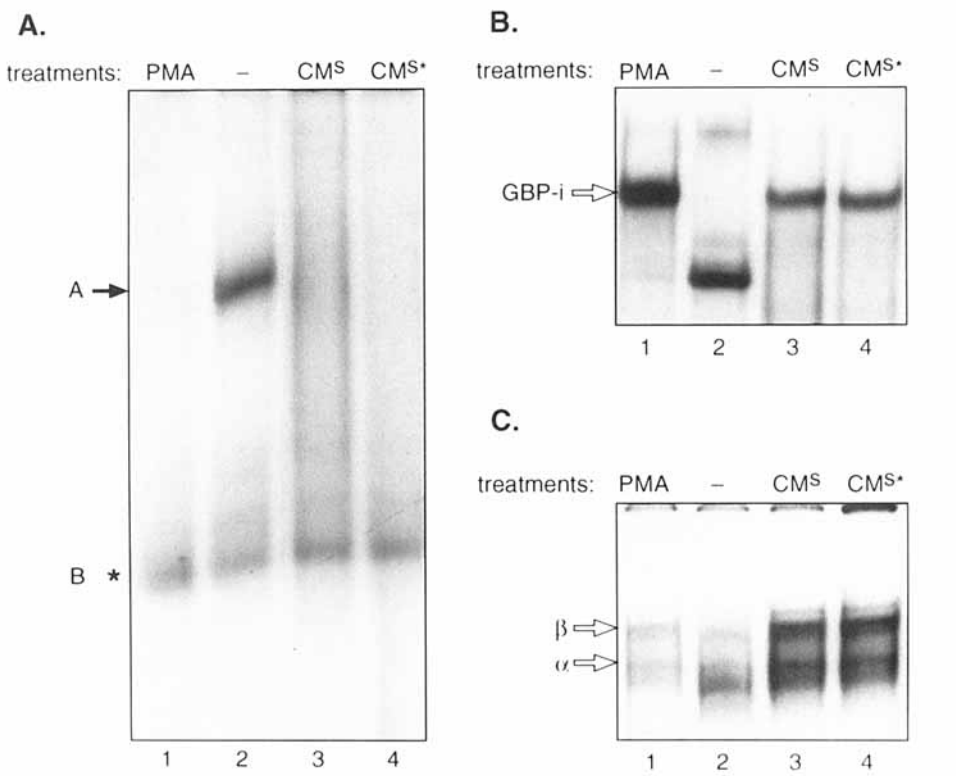

Fig. 5. Effect of various stimulants on the binding to the responsive region. Nuclear extracts prepared from U-87MG cells, after treatment with control media (**panels A, B, and C,** *lane 2*), treatment for 30 min with phorbol esters (panels A, B, and C, *lane 1*), or treatment for 3 h with supernatant fromT-lymphocytes either stimulated with phorbol esters alonepanels A, B, and C, *lane 3*), or phorbol esters in the presence of ionomycin (panels A, B, and C, *lane 4*) were examined for binding to either the responsive region on the TGF-β2 promoter (panel A), the GRS site on the JC virus promoter (panel B), or the NF-κB site (panel C).

stimulated with supernatant from treated but not untreated T-lymphocytes (Fig. 6B, lanes 2–3). Also, the levels of 41–46 kDa complexes were irreproducibly affected in the treated cells. Two dimensional UV-crosslinking analyses confirmed that complex A contains the 81 kDa species (Fig. 6C, lane 2), while complex B contains the 41–46 kDa species (Fig. 6C, lane 3). Figure 6D illustrates two dimensional UV crosslinking analysis using Hela nuclear extract. As shown in this figure, treatment of control Hela cells with the supernatant of the stimulated cells exhibits no effect on the formation of the 81 kd complex.

Dissociation experiments were performed with increasing concentrations of non-ionic detergent, deoxycholate (DOC) added to nuclear extracts from either untreated or supernatant stimulated cells. Addition of DOC to the extract did not significantly affect the binding of complex A (data not shown) suggesting a high affinity of DNA-protein interaction, like that seen for fos-jun.

Further, various inhibitors blocking selectively protein kinases A and C (H7), protein kinase C (calphositin C), serine proteases (TPCK), (PAO), or activators of the protein kinase A (forskolin) or protein kinase C (DAG) were unable to influence the loss of binding to the TGF-β2 responsive region. These data further support the earlier observations suggesting that these signaling pathways were unable to significantly modulate the activity from the TGF-β2 promoter.

Taken together, these binding analyses support a model for a functional role for the selective loss in binding of the 81 kDa protein to the responsive region on the TGF-β2 promoter in mediating the increased transcriptional level seen from the TGF-β2 promoter.

DISCUSSION

Isolated behind the physiological wall that is the blood brain barrier, the CNS has long been regarded as an immunologically privileged site [reviewed in Streilein, 1993]. In healthy mam-

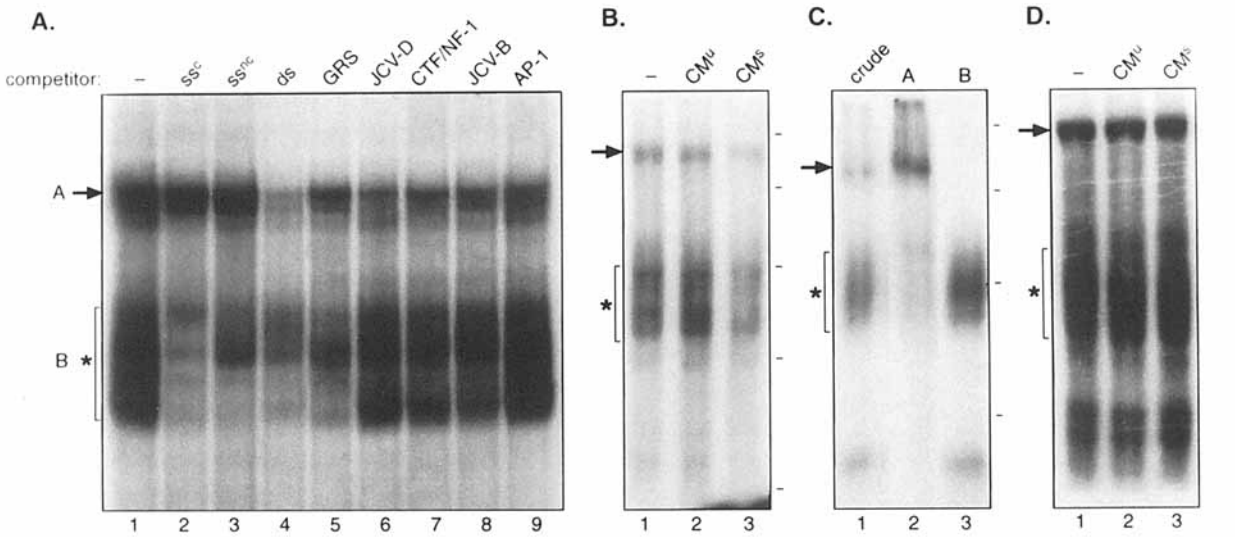

Fig. 6. Characterization of the labile complex. Competition mobility shift assays were performed with 100 ng homologous oligonucleotides representing the coding, non-coding, and double stranded versions of the responsive region on the TGF-β2 promoter to examine the specificity of nucleo-protein interaction (**panel A,** *lanes 2–4*). Further competition with 100 ng of heterologous oligonucleotides representing consensus binding sites for various transcription factors is also shown (panel A, *lanes 5–9*). The binding pattern in the absence of competitor is also shown (panel A, *lane 1*). UV-crosslinking experiments were performed to identify the size of the different DNA-protein complexes, as previously described [Raj and Khalili, 1994]. Nuclear extract from U-87MG cells treated with control media (**panel B,** *lane 1*), supernatant from unstimulated T-lymphocytes (panel B, *lane 2*), or supernatant from stimulated T-lymphocytes (panel B, *lane 3*) were crosslinked and resolved by SDS-PAGE. Individual complexes were examined by 2-D UV-crosslinking experiments (**panel C**). The various complexes were excised from the native gel, eluted, and then individually resolved by SDS-PAGE (panel C, *lanes 2 and 3*). The location of molecular weight markers are shown on the side, which corresponds to 97 kd, 68 kd, 46 kd, and 23 kd. **Panel D** represents the results of 2-D UV-crosslinking experiments performed using Hela nuclear extract as a control.

mals, T-lymphocytes are virtually undetectable in the CNS by conventional immunohistochemical methods [Hickey and Kimura, 1987]. However, much recent data indicate that activated T-lymphocytes can enter the CNS, apparently in a random manner, perhaps in some immunosurveillance role [Hickey et al., 1991; Lotan and Schwartz, 1994; Owens et al., 1994].

The immune response in the CNS is modulated by the resident microglia and astrocytes, via antigen presentation and controlled release of both positive (IL-1, IFN-γ, and TNF-α) and negative (IL-10, IL-4, and TGF-β) immunomodulators. In fact, antibodies directed against TGF-β have been shown to exacerbate the clinical course of experimental autoimmune encephalitis (EAE), while in vivo administration of TGF-β (β1 or β2) ameliorates the disease [Johns et al., 1992; Racke et al., 1993]. Expression of TGF-β by astrocytes and microglia has been postulated to play a vital role in protecting both oligodendroglia and neurons in the CNS from an overzealous immune system [Ishihara et al., 1994; Palladino et al., 1990; Ruscetti et al., 1993; Wahl, 1992].

The present study demonstrates an increase in the expression from the TGF-β2 promoter following stimulation with supernatant from activated T-lymphocytes. Analysis of the supernatant indicates that the activating factor is a heat-stable protein larger than 12 kDa (Table I), and thus distinct from the much smaller phorbol esters. The increased activity seen upon acidification of the supernatant implicates a member of a superfamily of proteins including nerve growth factor (NGF), platelet derived growth factor (PDGF), or TGF-β, which contain a similar conformational structure (the cysteine knot) that renders them resistant to denaturation or extremes of pH [reviewed in Kingsley, 1994]. The inability of individual isoforms of TGF-β to increase transcription from the TGF-β2 promoter may indicate a functional role for other members of the superfamily, or for distinct factors. Previous reports of the ability of individual cytokines, including IL-1, to induce TGF-β1 in glial cells offer several intriguing suspects [da Cunha et al., 1993]. Further studies are required to unmask the identity of the active molecules in the supernatant.

The cell type specificity of the response to supernatant is not surprising in light of previous reports that expression from the TGF-β2 promoter itself is regulated in a cell-type specific manner [O'Reilly et al., 1992b]. The striking similarity between phorbol esters and activated T-lymphocytes' supernatant in their regulation of the TGF-β2 promoter, in terms of both the level of activation and responsive regions, strongly suggests the involvement of common signal transduction pathways. Analysis of the effect of protein kinase activators or inhibitors suggests that the classical pathways of protein kinase A or C are not likely to be involved in the activation of the TGF-β2 promoter in glial cells (Table I). The activation of the TGF-β2 promoter may involve de novo protein synthesis, a post-translational modification process not involving protein kinases A or C, or both.

The responsive region on the TGF-β2 promoter lacks a previously identified PMA-responsive element. Computer aided analyses indicate a potential AP-1 site as well as a γ-interferon responsive element in this region. Indeed several nucleo-protein complexes are seen in the extracts from unstimulated glial cells. The slower migrating complex shows a decreased binding, upon treatment of the glial cells with supernatant from activated T-lymphocytes or phorbol esters. The effect of the supernatant appears to be specific for the slower migrating complex and for cells of glial origin (T98G, U-87MG) examined in this report. Coupled with the repressor function proposed for this protein, we decided to name this factor, glial labile repressor protein (GLRP). What is the identity of GLRP? The presence of a putative AP-1 binding site within the responsive region and the documented ability of both phorbol esters and TGF-β to alter expression of cellular genes through an AP-1 site strongly pointed to the involvement of an AP-1-like factor in the activation of the TGF-β2 promoter. Dissociation experiments, which reveal that the protein-DNA complex is DOC-resistant, support the association of GLRP with AP-1. Although, AP-1 has been classically shown to be an activator, many transcription factors have been shown to function as either activators or repressors depending upon the context of the responsive site [reviewed in Radler-Pohl et al., 1993]. However, other features of GLRP are not as easily reconciled with the prototypical AP-1 factor. The apparent lack of involvement of protein kinases in the activation of GLRP contradicts the classic paradigm of a kinase-dependent AP-1 [reviewed in Lin et al., 1993; Woodgett et al., 1993]. Further, the loss of binding upon stimulation does not fit the classic paradigm of increased AP-1 binding upon stimulation [Pennypacker et al., 1994]. The molecular weight characterized for the complex, 80 kDa, is distinct from known AP-1 complexes. Finally, the inability of a consensus AP-1 site to compete for the binding of GLRP to the responsive region argues against GLRP being an AP-1 factor. The mechanism of activation, the precise binding site and the very identity of GLRP await further characterization.

The nature of GLRPs as repressors of the basal level of TGF-β2 expression may account for the low level of TGF-β2 mRNA expression normally seen in certain cells. The transient nature of loss of GLRP binding to the promoter suggests a high degree of regulation of GLRP activity. The short window of loss of GLRP binding and the noticeable degree of activation seen hints at a strong repressive ability for the GLRP.

In summary, the data presented suggest that the immune response may itself contribute to the increased expression of a potent pleiotropic cytokine, TGF-β. Supernatant from T-lymphocytes activated in response to an immune trigger may act to modulate both further secretions from the T-lymphocytes as well as secretions of immunomodulators, like TGF-β, from the astrocytes, the predominant cell in the CNS. The astrocyte-secreted TGF-β can then act in an autocrine manner as a chemotactic agent, inhibitor of astrocyte proliferation, and modulator of astrocyte secretion [Benveniste et al., 1994; Chantry et al., 1989; Hunter et al., 1993; Jennings et al., 1991; Kossmann et al., 1992]. TGF-β can also act in a paracrine fashion to modulate the immune response [reviewed in Torre-Amione et al., 1990; Wahl, 1992]. This negative feedback of the immune response may represent a common scenario, that contributes to the relative immunological privilege in the CNS. Regulation of the expression of immunomodulators like TGF-β may represent potential therapeutic avenues in several afflictions of the CNS.

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