Astrocytes have become a focal point for research in neurobiology, especially regarding their purported ability to regulate neuronal communication and survival. The present study addressed a poorly understood but important focus in this area, the mechanism(s) underlying astrocyte-induced survival of neurons. The results of the study show that soluble factors in astrocyte-conditioned media (ACM) protect murine GT1–7 neurons from serum deprivation-induced cell death and that this neuroprotection is correlated with enhanced activation/phosphorylation of the AP-1 transcription factor, c-JunSer-63. A parallel and correlated activation of the upstream kinases, c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinase kinase-4 (MKK4) was also demonstrated. Furthermore, co-administration of JNK inhibitors, but not a MEK inhibitor, significantly attenuated ACM-induced phosphorylation of c-JunSer-63 and blocked its neuroprotective action. Gel shift analysis demonstrated that ACM enhanced AP-1 binding, an effect that appears functionally important, since an AP-1 binding inhibitor significantly attenuated the neuroprotective action of ACM. Further studies implicated transforming growth factor (TGF)-β1 and TGF-β2 as critical active soluble factors released by astrocytes, since both were demonstrated in ACM, and immunoneutralization of the conditioned media with a panspecific TGF-β antibody significantly attenuated the enhanced AP-1 binding and neuroprotective action of the ACM. Furthermore, exogenous application of TGF-β1 and TGF-β2 was found to enhance c-JunSer-63 phosphorylation and to be neuroprotective, and co-administration of JNK inhibitors or an AP-1 binding inhibitor blocked TGF-β-induced neuroprotection. Taken together, these studies suggest that astrocytes can protect neurons from serum deprivation-induced cell death, at least in part, by release of TGF-β and activation of a c-Jun/AP-1 protective pathway.

Astrocytes, the major nonneuronal cells in the brain, have been implicated in the segregation, maintenance, and support of neurons. Recent work also implicates a role for astrocytes in the reduction of neuronal cell death following a variety of cellular stresses, such as excitotoxicity and oxidative stress (1–4). Along these lines, during acute ischemic stroke, neuronal cell death rapidly progresses following the loss of astrocytes, suggesting that astrocytes are critical for support of neuronal survival in vivo in injury situations (5). This suggestion is further supported by the fact that administration of a gliotoxin, which abolishes astrocytic function, increases the sensitivity of neurons to cellular stressors (6) and by the observation that targeted ablation of astrocytic function in vivo results in increased neuronal cell death as well as increased susceptibility to ischemic stroke damage (7, 8). Collectively, these findings have led to the suggestion that astrocytes possess potent neuroprotective capability; however, the mechanisms underlying these neuroprotective effects are poorly understood.

Both soluble and insoluble factors could play a mediatory role in astrocyte-induced neuroprotection. Our laboratory and others have focused on soluble factors released by astrocytes and their potential roles in central nervous system function. Along these lines, recent work has demonstrated that astrocytes can produce and release the growth factor, transforming growth factor-β (TGF-β)1 (9–11), which could play a role in mediating astrocyte-induced neuroprotection. TGF-β is a member of the TGF-β superfamily and was the first family member isolated, having been discovered over 20 years ago (10). Abundant work since then has implicated a role for TGF-β in such diverse processes as regulation of growth, differentiation, extracellular matrix formation, and immune regulation as well as induction of neuronal survival and repair following injury (12, 13). TGF-β exists as three isoforms in mammals, designated TGF-β1, TGF-β2, and TGF-β3, each of which is the result of a separate gene. Studies of the TGF-β cell signaling pathway have shown that the TGF-β type II receptor, a constitutively active kinase, binds TGF-β and initiates signal transduction following the activation of the TGF-β type I receptor (14). Although TGF-β activates numerous diverse signaling pathways, recent studies demonstrated that AP-1 activation is required for TGF-β to regulate a number of its target genes (15–18). AP-1 is a family of transcription factors that form homodimeric and/or heterodimeric complexes consisting of C-Fos and c-Jun family members. The function of AP-1 transcription factors during brain injury is unclear, but increased c-Jun expression has been noted in surviving hippocampal neurons following ischemic brain injury (19). Additionally, prolongation of AP-1 binding has been shown to protect vulnerable neurons in the CA1 region of the hippocampus following ischemia (20),

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The abbreviations used are: TGF, transforming growth factor; ACM, astrocyte-conditioned medium; JNK, c-Jun N-terminal kinase; C6 CM, C6 glial cell-conditioned medium; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MKK4, mitogen-activated protein kinase kinase-4.
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e and c-Jun, the major AP-1 binding partner, exerts a neuroprotective function in PC12 neurons in vitro (21). Together, these findings suggest a potential role for AP-1-mediated transcription in neuroprotection. Based on these observations, we proposed that astrocyte-derived TGF-β could mediate, at least in part, the ability of astrocytes to exert neuroprotection and that this mediation could involve activation of a c-Jun/AP-1 protective pathway. To test this hypothetical mechanism for a possible mediatory role in astrocyte-induced neuroprotection, a serum deprivation/cell death model was employed using GT1-7 neurons. TGF-β receptor characteristics. Neuronal cell lines used extensively in the testing of neuroprotective compounds and in the study of mechanisms of neuronal cell death (22–25). The results of the study reveal a role for TGF-β-c-Jun/AP-1 signaling in mediation of the neuroprotective action of astrocytes.

EXPERIMENTAL PROCEDURES

Materials—All cell culture supplies were purchased from Invitrogen. Porcine TGF-β1 and the pan-specific TGF-β neutralizing antibody were purchased from R&D Systems (Minneapolis, MN). Dicumarol, a JNK inhibitor (26) and curcumin, an AP-1 binding inhibitor (27), were purchased from Sigma, whereas SP600125, a new highly specific JNK inhibitor (28), and PD98059, an inhibitor of MEK activation (29), were purchased from Biomol (Plymouth Meeting, PA). A TGF-β type II receptor dominant negative construct was created in which a mutation in the kinase domain was made, which rendered the receptor kinase inactive. The resulting construct could bind ligand but could not phospho-rylate downstream targets, thus, in effect, acting as an antagonist. To construct the TGF-β type II receptor dominant negative, the human TGF-β type II receptor gene was isolated from a human brain cDNA PCR library (Clontech) using the following primers: 5′/H11032 GGT TGA ACT AAG CTT CTG CTG-3′. This region, that encompasses the kinase domain, was used as a probe in PCR library (Clontech) using the following primers: 5′/H11032 GGT TGA ACT AAG CTT CTG CTG-3′. This region, that encompasses the kinase domain, was used as a probe in

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay—Nuclear extract preparation and electrophoretic mobility shift assay were performed as described previously (32, 33). Briefly, GT1-7 neurons were washed with ice-cold phosphate-buffered saline and resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol). Cells were allowed to swell for 10 min and homogenized in a Dounce homogenizer. The suspension was centrifuged at 5000 × g, and the nuclei were extracted by resuspending in low salt buffer (20 mM HEPES, 20% glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) and quickly mixed with high salt buffer (same as low salt buffer except 1.2 mM KCl). After a 20-min incubation on ice, lysates were centrifuged at 14,000 × g, and the supernatants were aliquoted and stored at −70 °C. DNA binding reactions were performed by incubation of 1–5 μg of nuclear extract with 2 × 106 cpm of 32P-labeled oligonucleotide probe for 15 min at room temperature in binding buffer containing 10 mM HEPES, pH 8.0, 0.1 mM EDTA, 2 mM dithiothreitol, 2 mg of poly(dI-dC), 4 mM KCl, 0.1% Nonidet P-40, 2 mM spermidine, and 8% glycerol. Complexes were separated on 5% nondenaturing polyacrylamide gels in 0.5 × TBE. Gels were dried and visualized by autoradiography. The probe used to analyze AP-1 DNA binding contains the consensus AP-1 binding site (26) and 5′-GGG ATC CAT GGG TCG GGG GCT GCT CAG G-3′ and 5′-CGT AGT GAG CTC TTT GGT AGT GTG TAG G-3′. The results were expressed as means ± S.E. A p value of <0.05 was considered significant.

RESULTS

Astrocyte-conditioned Medium Protects GT1-7 Neurons from Cell Death—As shown in Fig. 1, serum deprivation induced significant cell death of GT1-7 neurons. Decreased cell viability was observed in the serum-deprived group as early as 24 h after serum deprivation, with progressive, highly significant decreases in cell viability continued at 48 and 72 h after serum deprivation (Fig. 1, A and B). Of note, the addition of C6 glial cell-conditioned medium (C6 CM) or astrocyte-conditioned medium (ACM) completely prevented the serum deprivation-induced cell death of GT1-7 neurons, such that C6 CM- and ACM-treated serum-deprived groups were not significantly different from the serum control group with respect to cell viability (Fig. 1, A and B). Morphologically, serum-deprived neurons showed cellular rounding, neurite withdrawal, and cellular detachment as compared with serum control neurons (Fig. 1, compare C and D), whereas ACM-treated serum-deprived neurons maintained their cell shape, had preserved neurite connections, and reflected a healthy appearance and phenotype similar to that observed in the serum control neurons (Fig. 1, compare E and C).

Astrocyte-derived TGF-β Mediates Neuroprotection—Table I shows levels of transforming growth factors present in the ACM and C6 CM. As shown in Table I, significant levels of total TGF-β1 and TGF-β2 were observed in ACM and C6 CM, with slightly higher levels observed in C6 CM. In contrast, TGF-α was undetectable in both ACM and C6 CM. To determine whether the TGF-β present in ACM and C6 CM could mediate the neuroprotective effects of ACM and/or C6 CM, immunoneutralization of ACM and C6 CM was performed using a pan-specific TGF-β antibody (which neutralizes all three isoforms of TGF-β; i.e. TGF-β1, TGF-β2, and TGF-β3), and the effect on the protective ability of ACM and C6 CM against
serum deprivation-induced cell death was determined. As shown in Fig. 2, immunoneutralization of C6 CM or ACM with a panspecific TGF-β antibody completely reversed the neuroprotective effect of the C6 CM and ACM upon serum deprivation-induced cell death of GT1–7 neurons (Fig. 2, A and B). Further work demonstrated that significant levels of TGF-β/2 are present in serum, with TGF-β1 being predominant (TGF-β1, 2.4 ng/ml; TGF-β2, 160 pg/ml). We thus sought to determine whether the protective effects of media containing serum could be due, at least in part, to TGF-β present in serum. Toward this end, immunoneutralization of media containing serum with the panspecific TGF-β antibody was performed, and the effect on the protective ability of media containing serum on GT1–7 neurons was determined. The results suggest that TGF-β present in serum is an important contributor to the prosurvival effects of media containing serum, as immunoneutralization with the panspecific TGF-β antibody significantly decreased GT1–7 neuronal survival in a manner similar to serum deprivation (Fig. 2C).

Exogenous TGF-β Protects against Neuronal Cell Death—To further confirm that TGF-β1 and/or TGF-β2 can exert neuroprotection against serum deprivation in GT1–7 neurons, the effect of exogenous TGF-β1 and TGF-β2 treatment was examined. As shown in Fig. 3A, TGF-β1 (1 ng/ml) rescued GT1–7 neurons from serum deprivation-induced cell death in a manner similar to ACM. TGF-β2 also rescued GT1–7 neurons from serum deprivation-induced cell death, although it was slightly less potent than the effect observed with TGF-β1 treatment (Fig. 3B). Similar to the situation observed with ACM treatment, both TGF-β1 and TGF-β2 preserved normal elongated cell shape and neurite extensions of the GT1–7 neurons, which is in sharp contrast to the phenotypically unhealthy appearance of the serum-deprived vehicle-only treated cells, which displayed cellular rounding, neurite retraction, and cellular detachment (Fig. 3, C–E). Additionally, transfection of a TGF-β type II receptor dominant negative construct into GT1–7 neurons resulted in the cells displaying a phenotype similar to serum deprivation although they were in serum control medium (i.e., rounding up of cells, withdrawal of neurites, and significant cellular detachment (Fig. 3F). Transfection of an empty vector had no significant effect on the phenotype of the GT1–7 cells (Fig. 3G).

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Fig. 1. Effect of C6 glial cell-conditioned medium (C6-CM) and ACM on GT1–7 neuronal cell viability and morphology following serum deprivation. Both C6 CM and ACM protected GT1–7 neurons from serum deprivation-induced death (A and B). C–E demonstrate the effect of serum (C), serum deprivation (D), and ACM plus serum deprivation (E) on morphology of GT1–7 neurons. a, significantly different from all other groups at the same time point; *, p < 0.05; **, p < 0.01.

| Table 1 | TGF-β1, TGF-β2, and TGF-α concentrations in ACM and C6CM |
|---------|----------------------------------------------------------|
| Growth factor | ACM | C6CM |
| TGF-β1  | 538 | 978 |
| TGF-β2  | 202 | 363 |
| TGF-α   | ND  | ND  |

* ND, not detectable.
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of GT1-7 neurons with ACM induced an increase in c-Jun protein levels starting at 1 h and persisting until 24 h after treatment. A correlated activation of c-Jun was also observed, as indicated by a rapid increase in phosphorylation of c-Jun<sup>Ser-63</sup>, which started at 30 min and persisted until 12 h following ACM treatment. Additional studies showed that phospho-c-Jun<sup>Ser-63</sup> levels at the 0 time point were similar to levels observed for the serum-deprived group at all time points, excepting 30 min and 1 h, where the serum-deprived group had slightly lower levels than the 0 time point, and that ACM increased phospho-c-Jun<sup>Ser-63</sup> levels as early as 5 min after treatment (data not shown). As shown in Fig. 4C, the elevation of phospho-c-Jun<sup>Ser-63</sup> was mirrored by an ACM-induced increase in AP-1 binding to DNA as compared with the vehicle-treated serum deprivation group when examined at 4.5 h after ACM treatment. Further work showed that ACM induced the phosphorylation of the upstream kinases, MKK4 and JNK1 and JNK2, beginning at 30 min and lasting for the duration of the experiment (Fig. 5, A and B). Nonphosphorylated MKK4 did not show any significant fluctuation with ACM treatment (Fig. 5A), whereas nonphosphorylated JNK displayed a slight elevation at 1, 3, and 6 h after ACM treatment (Fig. 5B). Fig. 5, C and D, shows the graphical expression of the Western blot data for MKK4 and JNK (percentage of phosphoprotein/total protein) showing the ACM-induced elevation of P-MKK4 and P-JNK at all time points following ACM treatment. As shown in Fig. 6, A and C, treatment with the JNK inhibitor, dicoumarol, blocked the ACM-induced phosphorylation of JNK in GT1-7 neurons and correspondingly abolished the neuroprotective effect of ACM against serum deprivation-induced cell death. TGF-β1-induced neuroprotection in GT1-7 neurons was also abolished by dicoumarol treatment (Fig. 6B). A second recently developed highly selective JNK inhibitor, SP600125, was also tested, as was a MEK inhibitor (PD98059), to test for specificity of the inhibitory effect. As shown in Fig. 7A, treatment with a 30 or 50 µM dose of the JNK inhibitor, SP600125, completely blocked the neuroprotective effect of ACM against serum deprivation in GT1-7 neurons. In contrast, treatment with a 30 µM dose of the MEK inhibitor, PD98059, had no significant effect on the neuroprotective effect of ACM against serum deprivation (Fig. 7B). As shown in Fig. 7C, SP600125 also blocked the neuroprotective effect of TGF-β1 against serum deprivation in GT1-7 neurons, similar to the effect observed for ACM.

We next examined whether TGF-β would regulate phosphorylation of c-Jun<sup>Ser-63</sup> and whether immunoneutralization with a TGF-β antibody would attenuate the ability of ACM to upregulate AP-1 binding in serum-deprived GT1-7 neurons. As shown in Fig. 8, A and B, TGF-β1 and TGF-β2 both induced significant phosphorylation of c-Jun, with increases in phospho-c-Jun<sup>Ser-63</sup> noted at all time points but the 30-min time point by TGF-β1 and TGF-β2. Additionally, c-Jun protein levels were also enhanced by TGF-β1 and TGF-β2, demonstrating a regulation of both the c-Jun protein and its activation by TGF-β1 and TGF-β2, a situation similar to that observed for ACM (Fig. 4A). Furthermore, immunoneutralization of ACM with a TGF-β1 and TGF-β2 pan-specific antibody markedly attenuated the ACM-induced elevation of AP-1 binding in GT1-7 neurons under serum deprivation conditions (Fig. 8B). Correspondingly, co-incubation with an AP-1 binding inhibitor, curcumin, significantly attenuated the neuroprotection by ACM and TGF-β1 in GT1-7 neurons, further suggesting that AP-1-mediated transcription is important for the neuroprotective actions of ACM and TGF-β1 (Fig. 8, C and D).

**DISCUSSION**

The traditional view of astrocytes as serving primarily only a supporting role in the central nervous system has been in revision in recent years. For instance, there is a growing body of literature demonstrating that astrocytes can regulate neu-
rogenesis (34), neurite outgrowth (35, 36), and synaptic plasticity (37, 38) as well as exert neuroprotective effects (2, 39). Both soluble and insoluble factors from astrocytes have been implicated to mediate these regulatory effects, although the identity of the factors has remained elusive (2, 34–40). The present study has identified several soluble factors released by astrocytes and implicates a major role for one, TGF-β1 and TGF-β2, in mediating the neuroprotective actions of astrocyte-conditioned media. Along these lines, it was demonstrated that both TGF-β1 and TGF-β2 are present in ACM and C6 CM and that exogenous administration of TGF-β1 or TGF-β2 elicits neuroprotection similar to ACM and C6 CM. Furthermore, immunoneutralization of TGF-β in ACM with a panspecific TGF-β antibody abolished the neuroprotective ability of ACM. It also abolished the neuroprotective effect of C6 CM. The dose of TGF-β1 and TGF-β2 used in the exogenous application studies (1 ng/ml) approximates the total TGF-β levels demonstrated in ACM. Thus, a concentration of TGF-β equivalent to the total TGF-β found in ACM yielded a neuroprotection profile that was similar to ACM, and immunoneutralization of the TGF-β present in ACM abolished the ACM neuroprotective effect. Collectively, these observations suggest that TGF-β released by astrocytes represents an important soluble factor for astrocyte-induced neuroprotection.

In other studies, a lower concentration of TGF-β1 (0.25 ng/ml) was also tested and found to induce significant neuroprotection in GT1–7 neurons, but the effect was ~50–60% that of the 1 ng/ml dose. Additionally, TGF-β had no significant effect on GT1–7 neurons (A–E), and effect of a dominant negative TGF-β type II receptor upon GT1–7 morphology (F and G). Exogenous TGF-β1 or TGF-β2 (1 ng/ml) preserves morphology and cell viability of GT1–7 neurons following serum deprivation. Expression of a dominant negative TGF-β type II receptor in GT1–7 neurons leads to rounding up of neurons, retraction of neurites, and detachment and floating cells (F), whereas expression of the empty vector has no effect upon GT1–7 neuron morphology (G). Groups with different subscripts are significantly different. *, p < 0.05; **, p < 0.01.

**Fig. 3.** Effect of exogenous TGF-β upon cell viability and morphology of GT1–7 neurons (A–E), and effect of a dominant negative TGF-β type II receptor upon GT1–7 morphology (F and G). Exogenous TGF-β1 or TGF-β2 (1 ng/ml) preserves morphology and cell viability of GT1–7 neurons following serum deprivation. Expression of a dominant negative TGF-β type II receptor in GT1–7 neurons leads to rounding up of neurons, retraction of neurites, and detachment and floating cells (F), whereas expression of the empty vector has no effect upon GT1–7 neuron morphology (G). Groups with different subscripts are significantly different. *, p < 0.05; **, p < 0.01.
upon GT1–7 cell growth in serum-free conditions, confirming that the increase in cell survival was not due to a proliferative effect of TGF-β (data not shown). Of significant interest, the protective effect of TGF-β and ACM appears not to be limited to only GT1–7 neurons, since we recently found that ACM and TGF-β also protect primary rat cortical neurons in vitro from a variety of cell death-inducing agents, such as glutamate, camptothecin, and chemical hypoxia/ischemia (31). TGF-β has also been shown to protect primary rat hippocampal neurons and human hNT neurons in vitro (13, 41–44), demonstrating that its protective effect can extend to neurons from different regions of the brain and across species.

Interestingly, TGF-β1 and TGF-β2 were also detected in serum, and immunoneutralization of serum with a pan-specific TGF-β antibody significantly attenuated GT1–7 neuronal survival. This suggests that the prosurvival effect of serum involves mediation, at least in part, by TGF-β. The protective actions of TGF-β are probably mediated by the TGF-β receptor, since previous work by our laboratory and others has demonstrated that GT1–7 neurons express both the mRNA transcript and protein for the TGF-β type I and II receptor as well as the transcript for furin, which has been implicated in the activation of latent TGF-β (9, 45–47). In further support of a role for the TGF-β receptor in mediating TGF-β-induced neuroprotection, transfection of GT1–7 neurons in serum with a dominant negative TGF-β type II receptor induced considerable cell death, as evidenced by rounding up of the GT1–7 neurons, retraction of neurites, and detachment and floating of cells. In contrast, transfection of GT1–7 neurons in serum with an empty vector had no effect upon neuronal survival. An interesting in vivo correlate to our work is the observation that expression of TGF-β is enhanced during cerebral ischemia in vivo and that this increased expression reportedly occurs in neurons and astrocytes in regions of the brain that are resistant to the ischemic insult (48). This suggests that enhanced TGF-β is protective in cerebral ischemia and that astrocytes participate in the production of this neuroprotective factor in injury situations (for review, see Refs. 49 and 50). This observation nicely parallels the findings of our present study and provides additional support for a potential protective role of astrocyte-derived TGF-β.

Our study also implicated a role for a c-Jun/AP-1 signaling pathway in the protective effects of ACM and TGF-β. Both ACM and TGF-β1 and TGF-β2 increased the levels and phosphorylation of c-Jun in GT1–7 neurons. The increased phosphorylation of c-Jun is probably due to enhanced activation/phosphorylation of the upstream kinases, MKK4 and JNK. Along these lines, ACM was shown to enhance phosphorylation of MKK4 and JNK in GT1–7 neurons undergoing serum deprivation. We also found that TGF-β1 and TGF-β2 treatment.
showed a similar enhancement of phosphorylation of MKK4 and JNK (data not shown). Furthermore, treatment with the JNK inhibitor, dicoumarol, inhibited ACM-induced phosphorylation of c-Jun, an effect correlated with a significant attenuation of the neuroprotective effect of ACM in GT1–7 neurons subjected to serum deprivation. Dicoumarol treatment also inhibited TGF-β1-induced neuroprotection in serum-deprived GT1–7 neurons. Additionally, treatment with a new highly selective JNK inhibitor, SP600125 (28), also blocked ACM- and TGF-β1-induced neuroprotection from serum deprivation, further confirming a mediatory role for JNK/c-Jun in ACM/TGF-β1 neuroprotective effects. In contrast, treatment with a MEK inhibitor, PD/98059, had no significant effect upon ACM-induced neuroprotection, suggesting that the MAPK-extracellular signal-regulated kinase signaling pathway, unlike the MKK4/JNK/c-Jun pathway, most likely does not play a significant role in ACM-induced neuroprotection.

Whereas ACM treatment showed enhancement of phospho-c-Jun and phospho-JNK levels at most time points, the effect was particularly strong at the 0.5–3-h time points. This may be important, since it prevented a clear pattern in the serum deprivation group alone for a strong down-regulation of phospho-c-Jun and phospho-JNK levels at the 1-h time point (see Fig. 4, B and D). Interestingly, the serum-deprived only group
itself displayed a recovery of phospho-c-Jun and phospho-JNK levels from 3 to 12 h, which, however, did not prevent the cell death induced by the serum deprivation insult. Thus, the ability of ACM and TGF-β/H9252 to raise phospho-c-Jun levels at the early time points may be critical for eliciting survival in the face of the serum deprivation insult, most likely by early induction of genes that promote survival. In potential support of this possibility, preliminary work in our laboratory showed that treatment with the transcription inhibitor, actinomycin D, during the first 6 h of ACM treatment, significantly attenuated the ability of ACM to induce neuroprotection in serum-deprived GT1-7 neurons.²

It is well known that phosphorylated c-Jun protein can dimerize with other proteins to form the AP-1 transcription complex, which binds with high affinity to DNA regulator elements on genes to regulate gene expression (51). We thus examined AP-1 binding in GT1-7 neurons undergoing serum deprivation. The results showed that serum deprivation attenuated AP-1 binding in GT1-7 neurons at 1 and 4.5 h and that ACM preserved AP-1 binding in the face of serum deprivation. The ability of ACM to preserve AP-1 binding appeared to be due, at least in part, to the presence of TGF-β in the ACM. This is evidenced by the fact that immunoneutralization with a TGF-β antibody significantly attenuated the ACM-induced preservation of AP-1 binding. Furthermore, treatment with an AP-1 binding inhibitor significantly attenuated the neuroprotective actions of ACM and TGF-β1, a finding that suggests a functionally important role for the ACM/TGF-β regulatory effects on the c-Jun-AP-1 complex.

² K. M. Dhandapani, M. Hadman, L. De Sevilla, M. F. Wade, V. B. Mahesh, and D. W. Brann, unpublished observation.
c-Jun/AP-1 pathway in other cell types as well, such as thyrocytes, fibroblasts, and lung carcinoma cells, and these effects have also been correlated with enhanced survival in stress and/or injury situations (52–54). TGF-β regulation of c-Jun in lung carcinoma cells, although correlated with survival, was not, however, associated with increased JNK activation, and in fact JNK activation was suppressed by TGF-β (54). This suggests that cell type-specific differences probably exist in TGF-β.
regulation of the c-Jun/JNK/AP-1 pathway. Additionally, it is clear that c-Jun is not always associated with survival effects, since there is an abundant literature for a death-promoting role for c-Jun (for review, see Refs. 51, 55, and 56). Along these lines, it is only recently that appreciation of the potential survival and regenerative actions of c-Jun have emerged to the forefront and begun to be fully appreciated (19, 20, 57–68). It has been suggested that type, strength, and duration of injury stimulus may determine whether a protective or death-promoting action of c-Jun is observed in a cell. Additionally, cell type, developmental stage, and levels of other AP-1 factors and interacting factors probably also play an important role in defining the action of c-Jun and the fate of the cell in injury or stress situations. Our observation of a protective role for c-Jun in GT1-7 neurons in serum deprivation conditions is consistent with a similar protective role suggested for c-Jun during cerebral ischemia based on the finding of c-Jun induction in resistant dentate gyrus cells and CA3 pyramidal cells, which survive the hypoxic-ischemic insult (57, 58). A similar c-Jun induction and correlation with survival has also been recently observed in axotomized medial septal neurons that survive fornix fimbria transection (59). The present study thus adds to a growing literature of a potential beneficial role of c-Jun in stress/injury situations.

Whereas the present study and others implicate regulation of the c-Jun/JNK/AP-1 pathway as a mechanism for TGF-β enhancement of cell survival, further work is needed to identify the AP-1-regulated genes that mediate ACM/TGF-β neuroprotection. The AP-1 complex has been shown to regulate several functional classes of genes, including genes for neuropeptides, neurotrophins, receptors, antiapoptotic proteins, transcription factors, and enzymes, a functional composite that could easily yield a neuroprotective effect (55, 56). Further studies utilizing gene chip arrays may prove especially helpful in identifying TGF-β1-regulated genes that participate in mediating neuroprotection.

In summary, Fig. 9 provides a diagram of the proposed signaling pathway underlying TGF-β1/ACM neuroprotective effects. Astrocytes release TGF-β, which in turn binds to TGF-β type II receptors on neurons, leading to activation of kinase activity of the type II receptor and subsequent phosphorylation of the TGF-β type I receptor. Through as yet unidentified intermediary steps, phosphorylation of MKK4 is induced, leading to its activation and subsequent phosphorylation of JNK. Activation of JNK leads to enhanced phosphorylation of c-JunSer-63, causing c-Jun activation and dimerization and formation of AP-1 complex. The AP-1 complex then binds to AP-1 regulatory elements on genes, leading to enhanced transcription of factors that aid in the survival of the cell. In conclusion, whereas astrocyte-induced neuroprotection is most likely complex, involving the action of multiple potential soluble and insoluble factors, the current study strongly indicates that TGF-β is an important soluble component that contributes to the neuroprotective efficacy of astrocytes. It further implicates activation of MKK4/JNK/c-Jun/AP-1 signaling as an important signal transduction pathway for elaboration of ACM/TGF-β protective effects.

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