Multiple Routes to Astrocytic Differentiation in the CNS

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Ciliary neurotrophic factor (CNTF) acts instructively to switch multipotent stem cells of the CNS to an astrocytic fate. Here we show that CNTF causes activation of Janus kinase–signal transducers and activators of transcription and mitogen-activated protein kinase (MAPK) pathways with differential kinetics in these cells. Inhibition studies indicate that activation of the MAPK pathway is required early in the differentiation process, whereas activation of signal transducer and activator of transcription (STAT) proteins is required for commitment to an astrocytic fate. Bone morphogenetic proteins have also been shown to cause astrocytic differentiation but do not cause STAT activation or astrocytic differentiation in fibroblast growth factor 2-expanded fetal stem cells used here. These results show that there are two distinct routes to initiate astrocytic commitment in multipotent CNS precursors.

Key words: astrocytic differentiation; CNS stem cells; mammalian CNS development; intracellular signaling; CNTF; BMP4; EGF

Received Nov. 17, 1997; revised Feb. 19, 1998; accepted Feb. 20, 1998.

We thank Drs. M. Molne, L. van Grunsven, and M. Brenner for helpful discussions, E. Saphier for technical assistance, Dr. J. E. Darnell Jr (Rockefeller University) for the Stat3 plasmids, and Dr. Y. Gotoh (Kyoto University, Kyoto, Japan) for the MAPKK virus vector.

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NY), Stat1 monoclonal α-ISGF3 (anti-ISGF3) and α-pan-ERK antibody from Transduction Laboratories (Lexington, KY), and Stat3 polyclonal from Santa Cruz Biotechnology (Santa Cruz, CA). The appropriate fluorescence-tagged secondary antibodies for immunofluorescence were obtained from Jackson ImmunoResearch (West Grove, PA).

**Culture of neuroepithelial stem cells and differentiation protocol**

Cultures of stem cells were prepared according to the protocol of Johe et al. (1996). Briefly, cerebral cortices were dissected from embryonic day 14 (E14) rat embryos; cells were mechanically dissociated by trituration and were plated on dishes coated with 15 mg/ml polyornithine and 1 mg/ml fibronectin at a concentration of 1 million per 10 cm dish. Cells were maintained in 10 ng/ml FGF. Cultures were passed on approximately the fourth day. Experiments were performed on cultures either in the first or second passage.

Cultures were differentiated into astrocytes by treating with 10 ng/ml CNTF.

**Electrophoretic mobility shift assay**

The preparation of nuclear extracts from treated and untreated cells was prepared according to the protocol of Symes et al. (1994). All treatments were performed at 10 ng/ml except for EGF, which was performed at 20 ng/ml. Cells were treated with CNTF every 24 hr for time points that were >1 d. Electrophoretic mobility shift assay (EMSA) was also performed according to the protocol of Symes et al. (1994). The sequence of the SIE probe used was 5′-AGCTTCATTTCCCGTAAATCCCTA-3′ and 3′-AGTAAAGGCATTTAOGGATTGA-5′. For supershift assays, the relevant antibody was included in the binding reaction.

**Immunofluorescence**

Double-immunofluorescent staining of FLAG-Stat3 and GFAP. Cells were fixed with freshly mixed methanol and acetone in a 1:1 ratio for 2 min at room temperature. They were washed four times in Tris-buffered saline (TBS) and calcium (0.05 M Tris HCl, pH 7.4, 0.15 M NaCl, and 1 mM CaCl₂). Double immunofluorescence was performed with a biotinylated anti-FLAG monoclonal antibody at 30 μg/ml and anti-GFAP polyclonal antibody at a 100-fold dilution. Primary antibodies were left on the cells for 1 hr at room temperature or overnight at 4°C. The cells were then washed with TBS and calcium for 1 hr with four changes and incubated with streptavidin–FITC- and lissamine rhodamine-coupled goat anti-mouse antibody at a 100-fold dilution. Primary antibodies were left on the cells for 1 hr at room temperature for 2 hr before fixing in paraformaldehyde. For the 48 hr time points, cells were retreated with CNTF and PD098059 at 24 hr. The CNTF-treated cells that serve as control were treated with the relevant amount of DMSO. There is no appreciable difference in differentiation of DMSO-treated cells compared with untreated cells (data not shown).

**RESULTS**

**CNTF causes differentiation of stem cells into astrocytes**

Stem cells maintained in FGF were homogeneous in morphology and in their expression of nestin, a marker of neuroepithelial stem cells (Lendahl et al., 1990). A transient treatment with CNTF is sufficient to commit stem cells to an astrocytic fate in vitro (Johe et al., 1996). Exposure to CNTF causes these cells to express GFAP (Fig. 1). Consistent with in vivo observations, the intensity of nestin staining decreased with an increase in GFAP expression (Hockfield et al., 1985; Frederiksen et al., 1988).

**STAT proteins are activated by CNTF in neuroepithelial stem cells**

One of the major cytoplasmic signaling pathways activated by CNTF is JAK–STAT. Activation of STAT proteins involves phosphorylation of a specific tyrosine residue (Shuai et al., 1993, 1994). Activated STAT proteins can be detected by their ability to dimerize and bind specific DNA target sites. This interaction can be detected by an EMSA. Specific nucleoprotein complexes were formed when a STAT-specific DNA-binding sequence (SIE) was incubated with nuclear proteins prepared from stem cells treated with CNTF. Complexes labeled “a” and “b” were formed when the cells were treated with CNTF for times ranging from 15 min to 8 d (Fig. 2). The amount of complex b formed decreased with time, whereas complex a was activated for prolonged periods. The constitutive complex that migrates just below complex b is present even in FGF-treated cells and is not induced by CNTF treatment. Although there is some Stat3 complex in the control conditions, indicating basal levels of activation of this protein in the cell, these data clearly show that CNTF rapidly activates proteins that bind to the SIE probe.

The GFAP promoter may be used as a paradigm to study transcriptional regulation during astrocytic differentiation, because it is robustly and specifically expressed in these cells. Promoter analysis of the GFAP promoter in CG4 cells showed that regions −1857 to −1546 and −384 to −106 are important for CNTF-induced GFAP expression (Kahn et al., 1997). Another report of promoter analysis of the GFAP promoter in U251 cells showed the following regions to be necessary and sufficient for transcriptioner's instructions. All antibody dilutions were performed in the blocking solution.

**Transfection, infection, and PD098059 experiments**

Transfections were performed on cells in first passage by the calcium phosphate method. The plasmids used were the wild-type (WT) Stat3 and a DNA-binding mutant with a three-amino acid mutation at residues 462–464 (VVV→AAA). Both plasmids had an N-terminal FLAG and were described by Horvath et al. (1995). The precipitate was left on the cells for 2 hr; the cells were washed and left in FGF for 48 hr. Cells were then treated for 48 hr with 10 ng/ml CNTF. Infections and PD098059 experiments were performed on cells in first or second passage. An adenovirus vector expressing a constitutively activated form of MAPK kinase (MAPKK) was used. The activated form of the protein has a two-amino acid substitution (S218E and S222E) and its nuclear localization signal is deleted (deletion 32–51). Cells were infected at the indicated multiplicity of infection and fixed 24 or 48 hr after infection.

A 20 mm stock of PD098059 was made in DMSO. Cells were treated at a final concentration of 10 or 20 μM for 24 or 48 hr before fixing in paraformaldehyde. For the 48 hr time points, cells were retreated with CNTF and PD908059 at 24 hr. The CNTF-treated cells that serve as control were treated with the relevant amount of DMSO. There is no appreciable difference in differentiation of DMSO-treated cells compared with untreated cells (data not shown).
GFAP expression: −1757 to −1604, −1612 to −1489, and −132 to −57 (Besnard et al., 1991). On examination of the GFAP promoter we identified three putative STAT sites: −1512 to −1504, TTCCGAGAA; −1292 to −1284, TTCCAGAA; and −277 to −285, TTCCTGGAA; one of which (−1512 to −1504) is stronger in STAT complex formation by EMSA than the other two (data not shown). These observations suggest that GFAP induction by CNTF may be mediated by direct interaction of STAT complexes with the GFAP promoter.

The activated STAT proteins are Stat1 and Stat3

Supershifting of nucleoprotein complexes with antibodies against specific transcription factors indicate the involvement of these proteins in the formation of that complex. The identity of the
STAT proteins activated by CNTF was established by supershift assays (Fig. 3). Complex b was supershifted by the Stat1 antibody, forming complex c, indicating that Stat1 is a part of complex b (Fig. 3a, lane 4). An α-Stat3 antibody caused the disappearance of both complexes a and b, forming the supershifted complex d (Fig. 3b, lane 8). Thus, complex a comprises homodimers of Stat3 complexed with DNA, whereas complex b comprises heterodimers of Stat1 and Stat3 with DNA.

The specificity of the complexes formed was determined by competition with 20-fold excess of unlabeled SIE oligomer (Fig. 3c, lanes 11, 12). Both the induced (complexes a and b) and the constitutive complexes were competed with the unlabeled oligo, indicating that both are specific. However, the constitutive complex does not contain either Stat1 or Stat3, as shown by the supershift assays. There was no competition with an unrelated probe of similar length (data not shown).

CNTF induces the formation of three complexes in several other systems studied (Bonni et al., 1993; Symes et al., 1994; Rajan et al., 1995, 1996). The largest is composed of Stat3 homodimers, the middle of Stat1 and Stat3 heterodimers, and the fastest-migrating one of Stat1 homodimers. From these previous observations it is probable that a third complex comprising homodimers of Stat1 migrates at the same position as the constitutive complex that forms just below complex b. It is of interest to note that there are two distinct complexes supershifted by the Stat1 antibody, indicating that there are two species of Stat1 activated by CNTF treatment in these cells. These results show that both Stat1 and Stat3 are activated by CNTF in the stem cells.

Blocking of Stat3 function prevents astrocytic differentiation by CNTF

The requirement of STAT activation in CNTF-mediated astrocytic differentiation was determined by assaying the effect of blocking Stat3 transcriptional function. Stat3 was targeted because EMSA indicated that it was the major protein that was activated. Plasmids expressing FLAG-tagged WT and mutant forms of the Stat3 protein (Horvath et al., 1995) were transfected into cells. The mutant Stat3 has a three-amino acid substitution in the DNA-binding domain, thus functioning as a dominant negative because of loss of DNA-binding function. The effect of the overexpressed transfected proteins on astrocytic differentiation was determined by double immunofluorescence with antibodies against GFAP and the FLAG epitope (Fig. 4a). Transfection of WT Stat3 allows astrocytic differentiation in 80% of the cells, whereas differentiation takes place in only ~25% of cells transfected with the mutant Stat3 (Fig. 4b), indicating that Stat3 function is required for astrocytic differentiation. The fact that differentiation is not 100% in the WT transfectants and 0% in the mutant one could be attributable to the fact that perturbations in this important signaling pathway cause responses of the cells to be skewed or, in the case of the mutant transfections, that the levels of dominant negative protein expressed are not sufficient to override the effect of the endogenous WT protein.

CNTF treatment causes activation of MAPK in neuroepithelial stem cells

Activated Jak2 can cause the activation of MAPK, possibly through a ras-raf pathway (Winston and Hunter, 1998). To determine whether CNTF activates the MAPK pathway in the stem cells, immunoblots were performed on cell extracts treated with CNTF for time points ranging from 15 min to 24 hr. CNTF causes activation of p44 MAPK in the neuroepithelial stem cells (Fig. 5). A time course shows that this activation is transient and peaks at 15–30 min. The levels of activation return to baseline by 4 hr after CNTF treatment (Fig. 5, compare lanes 6, 7). FGF-treated cells were used as a positive control because FGF is a known activator of MAPK.

Inhibition of activation of MAPK causes a delay in astrocytic differentiation by CNTF

To determine the relevance of MAPK activation in astrocytic differentiation, activation of MAPK was blocked by inhibiting the
Figure 4. Effect of blocking Stat3 function on astrocytic differentiation. 

a. Cells were transiently transfected by the calcium phosphate method with plasmids expressing either wild-type (WT) or dominant negative mutant of Stat3 defective for DNA binding (mutant), both proteins expressing an N-terminal FLAG epitope. Forty-eight hours after transfection cells were treated with CNTF for 48 hr and fixed with freshly mixed methanol and acetone in a 1:1 ratio for 2 min at room temperature, and double-immunofluorescent staining was performed for FLAG and GFAP. A–C, Representative experiment of cells transfected with the mutant Stat3. D–F, Representative experiment of cells transfected with WT Stat3. A, D show staining specific for the FLAG epitope on the transfected proteins, B, E for GFAP, and C, F for both antigens. Based on experiments performed as described in Figure 3a, the percentage of transfected cells expressing GFAP was plotted for each of the two plasmids. Blocking of Stat3 function with a dominant negative protein inhibits CNTF-mediated astrocytic differentiation.

b

<table>
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<th>% transfected cells expressing GFAP</th>
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<tr>
<td>WT Stat3</td>
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<td>mutant Stat3</td>
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activation of its upstream activator, MAPKK. PD098059 is a specific inhibitor of MAPKK at concentrations of ≤50 μM (Alessi et al., 1995). The effect of adding PD098059 to cells differentiating in response to CNTF was determined, as shown in Figure 6, a and b. Addition of CNTF to cells in which activation of MAPKK has been inhibited leads to a complete lack of differentiation at 24 hr after the addition of CNTF (Fig. 6a, compare C, D with B). However, by 48 hr the extent of differentiation in CNTF-treated cells is comparable both in the presence and absence of PD098059 (Fig. 6a, compare K, L with J). Thus there is a delay in the onset of differentiation by CNTF in the absence of normal levels of MAPK activation.

The CNTF-mediated activation of MAPK in these cells peaks at 15 min, whereas the effect of its inhibition is seen at 24 hr, indicating that activation of MAPK leads to events downstream that regulate differentiation. The eventual differentiation at 48 hr may be a consequence of the residual activation of MAPK in the cell despite the inhibitor (see Fig. 7a), which is sufficient for differentiation to occur in a delayed manner. These results indicate that one of the earliest steps in CNTF-mediated astrocytic differentiation includes the activation of MAPK.

Overexpression of constitutively active MAPKK causes transient GFAP expression

Since the previous set of experiments established that MAPK is required in the initiation of astrocytic differentiation, we investigated the effects of aberrant activation of MAPKK in the absence of CNTF treatment. FGF-expanded stem cells were infected with an adenovirus vector expressing a constitutively activated form of MAPKK. A representative set of experiments is shown in Table 1. The result is the inverse of that seen in Figure 6. There was a transient expression of GFAP at 24 hr after infection that was lost at 48 hr. Thus MAPK activation in the absence of other CNTF-activated pathways leads to a transient appearance of a differentiated astrocytic phenotype.

Treatment of stem cells with PD098059 blocks activation of MAPK but does not affect STAT activation

The activation of MAPK by CNTF was appreciably inhibited by PD098059, as seen in Figure 7a. The intensity of the heavier bands denoting activation are reduced in lanes 3 and 5 (+PD098059) when compared with lanes 2 and 4 (control). Because MAPK and STAT activation both lead to astrocytic differentiation, it is possible that there is an interaction between the two pathways, and the inhibition of differentiation seen in Figure 6 could be attributed to a block of STAT activation by PD098059, via the inhibition of MAPK activity. As detected by EMSA, PD098059 did not affect STAT activation in response to CNTF (Fig. 7b). The amounts of complexes a and b formed are unchanged in the presence and absence of PD098059 at early (1 hr, lanes 1, 2) and late (48 hr, lanes 7, 8) time points. It appears that there is no interaction between the MAPK and JAK–STAT pathways at the level of STAT activation in the FGF-expanded stem cells.

EGF and BMP4 do not activate STAT proteins

EGF treatment of FGF-expanded stem cells leads to astrocytic differentiation (Johe et al., 1996). In addition, EGF-treated aggregates from the fetal subventricular zone show enhanced astrocytic differentiation in response to BMPs 2, 4, and 7 (Gross et al., 1996). These observations raise the possibility that EGF and BMPs might activate the STAT signaling system in CNS stem cells. Treatment of cells with EGF and BMP4 does not cause activation of STAT proteins (Fig. 8, compare lanes 2, 3 with lane 1). Because BMP4 causes astrocytic differentiation in EGF-expanded stem cells, activation of STATs by combinations of these factors was determined. There was no enhancement of STAT activation caused by BMP4 or CNTF in the presence of EGF (Fig. 8). These data suggest that the astrocytic differentiation effects of EGF and BMP4 are not mediated through JAK–STAT activation.

DISCUSSION

FGF-expanded stem cells maintained in monolayers are multipotent and give rise to astrocytes, oligodendrocytes, and neurons on withdrawal of the mitogen (McKay, 1997). All the experiments described here were performed on cultures that were in first or second passage. The cells in these cultures are homogeneous by morphological criteria and nestin immunohistochemistry. It is thus an excellent primary cell culture system in which to study the biochemical mechanisms of differentiation of CNS cells and, particularly, for defining the biochemical basis for the transition from a multipotent stem cell to a committed glial progenitor.

CNTF treatment leads to a prolonged activation of the JAK–STAT pathway and transient activation of the MAPK pathway. The requirement of these pathways in astrocytic differentiation was shown by blocking activation. A dominant negative Stat3 that prevents DNA binding of activated Stat3 homodimers and heterodimers completely blocks astrocytic differentiation, whereas blocking activation of MAPKK, the upstream activator of MAPK, causes a delay in astrocytic differentiation by CNTF. Overexpression of MAPKK causes the converse effect, a transient appearance of GFAP. Thus, the MAPK pathway functions in the initiation of astrocytic differentiation, whereas activation of Stat3 is required for complete differentiation. Stat3 activation as seen by EMSA occurs within 15 min of CNTF treatment. It is likely to be transcriptionally active at this time, thus indicating a function for this protein early in the differentiation process.
Figure 6. Blocking MAPK activation causes a delay in astrocytic differentiation. a, MAPK inhibitor PD098059 was used to block MAPK activation. Cells were treated with CNTF for 24 or 48 hr in the presence or absence of 10 or 20 μM PD098059, fixed with 4% paraformaldehyde, and stained for GFAP. For the 48 hr time points, cells were retreated with CNTF and PD098059 at 24 hr. A representative experiment of the data plotted in b is shown. A–D, I–L, Immunofluorescent staining of GFAP. E–H, M–P, Corresponding bright-field (phase) images of these fields, E being the same field as A and so on. A–H, Images of cells fixed 24 hr after treatment and I–P at 48 hr after treatment. Scale bar: P, 100 μm. b, Average percentage of GFAP-positive cells was plotted for each condition. Squares, 24 hr time points; diamonds, 48 hr time points. Astrocytic differentiation is prevented by PD098059 only at the 24 hr time point.
Our data suggest that there is interaction between the MAPK and JAK–STAT pathways during CNTF-mediated differentiation. The MAPK pathway has been shown to have an effect on STAT activation (Zhang et al., 1995; Chung et al., 1997) and on transactivation by STATs (Wen et al., 1995). The experiments shown here indicate that the MAPK pathway is not involved in CNTF-mediated STAT activation in FGF-expanded stem cells. Perhaps interaction between the two pathways occurs at the level of the GFAP promoter through an MAPK-sensitive transcriptional regulatory site. In the MAPKK overexpression experiment it appears that activation of MAPK causes a measure of astrocytic differentiation. FGF also causes MAPK activation but does not cause astrocytic differentiation. This may be a consequence of different levels of MAPK activation in FGF-treated and virus-infected cells. However, it may also be the case that FGF stimulates other pathways that inhibit astrocytic differentiation. The different response of the cells to FGF and CNTF is probably the result of the activation of distinct pathways in response to the two factors.

Table 1. Activated MAPKK causes a transient expression of GFAP in the absence of CNTF treatment

<table>
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<tr>
<th>Condition</th>
<th>MOI</th>
<th>24 hr</th>
<th>48 hr</th>
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<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mock</td>
<td></td>
<td>0.67</td>
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<tr>
<td>WT</td>
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<tr>
<td>MAPKK</td>
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<tr>
<td>Experiment 2</td>
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<tr>
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<td>0</td>
</tr>
<tr>
<td>WT</td>
<td>50</td>
<td>2.3</td>
<td>0</td>
</tr>
<tr>
<td>MAPKK</td>
<td>50</td>
<td>8.75</td>
<td>0.05</td>
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</table>

Cells were infected with either WT adenovirus or an adenovirus vector expressing a constitutively active form of MAPKK at a multiplicity of infection (MOI) of ~30. Cells were fixed with 4% paraformaldehyde 24 or 48 hr after infection and stained for GFAP. The number of GFAP-positive cells per 40× field is shown. There is an 11.3-fold increase in GFAP expression at 24 hr after infection in experiment 1, and a 3.8-fold increase in experiment 2. When compared with a WT infection, there is no increase in GFAP expression at 48 hr after infection in an MAPKK infection.

Figure 7. PD098059 inhibits MAPK activation but does not affect STAT activation. a, Cells were treated with CNTF in the presence or absence of 20 μM PD098059 for the indicated time points, and activation of MAPK was analyzed as described in Figure 5. At 20 μM, PD098059 blocks most of the MAPK activation caused by CNTF (compare lanes 3, 2 and 5, 4). b, Nuclear extracts prepared from stem cells treated with CNTF for the time points indicated, in the presence or absence of PD098059. EMSA was performed on these extracts as described in Figure 2. The specific complexes formed as a result of CNTF treatment are marked a and b. There is no inhibition of STAT complex formation seen at early (1h, lanes 1, 2) or late (48h, lanes 7, 8) time points in the presence of 20 μM PD098059.

Figure 8. EGF and BMP4 do not activate STAT proteins. Stem cell cultures were treated with the mentioned factors for 15 min, and nuclear extracts were prepared. EMSA assays were performed to determine the extent of activation of STAT proteins as described in Materials and Methods and the legend to Figure 2. STAT complexes are marked a and b. EGF and BMP4 do not activate STAT proteins.
CNTF and LIF have been implicated in astrocytic differentiation in two experimental models. CNTF causes O2A progenitor cells to form astrocytes in vitro (Hughes et al., 1988; Lillien et al., 1990). The number of astrocytes was greatly reduced in E18.5 LIFRβ null animals when compared with a wild-type littermate, implicating LIF in either the survival or differentiation of astrocytes (Ware et al., 1995). In a related model, LIF may be involved in the appearance of astrocytes after CNS injury, because LIF expression is upregulated 30-fold within 24 hr after injury to the CNS (Bannerman et al., 1997). It is interesting to speculate that the mechanisms by which astrocytic differentiation occurs during embryonic development may be reactivated in an adult cell during injury.

Our results contrast with that of Bonni et al. (1997), who have recently suggested that the JAK–STAT pathway alone is instrumental in causing CNTF-mediated astrocytic differentiation. In a transfection–luciferase assay, inhibition of MAPK activation enhances activation from a transfected GFAP promoter. An explanation for this discrepancy is that the cultures being used in the two studies are different. The data presented here were obtained on a homogeneous population of cells that consistently gave 50–60% astrocytic differentiation after 2 d and ~98% after 4–6 d of CNTF treatment. Because the cultures of Bonni et al. (1997) are 60% neuronal, the results of any biochemical assay in which the entire population of cells is used may reflect neuronal rather than stem cell responses.

Since GFAP is robustly and specifically induced in astrocytes, it may be used as a paradigm for transcriptional regulation. It remains to be seen whether the STAT sites in the GFAP promoter are functional, and if so, whether they are equivalent. Also, it remains to be determined whether sites corresponding to transcription factors activated by MAPK-related pathways such as AP1 and CREB, which are present in this promoter, are functional (Besnard et al., 1991; Kahn et al., 1997). In other systems, interaction between the MAPK-related and STAT pathways has been shown to occur through integrators such as the p300 and CREB-binding proteins (Horvai et al., 1997). It will also be important to determine whether SMAD proteins interact with the GFAP promoter either directly or indirectly. The prolonged activation of STAT for 8 d in the stem cells is in contrast to the brief activation usually seen in vitro (Symes et al., 1994; Rajan et al., 1997) but is reminiscent of LIF-induced in vivo STAT activation in injured peripheral neurons (Rajan et al., 1995). In the experiment shown in Figure 2, cells were treated every 24 hr for time points >24 hr. However, Stat1 and Stat3 activation was seen even 72 hr after one dose of CNTF (data not shown).

EGF promotes astrocytic differentiation, but it is not identical in its effects to CNTF. In a clonal assay done with FGF-expanded cells, mitogen withdrawal causes ~10% of cells to differentiate into astrocytes (Johe et al., 1996). A similar experiment done with EGF-expanded cells causes the number of astrocytes to increase to 50% (Johe et al., 1996). In another experiment performed along the same lines, FGF-expanded cells were treated with EGF for the final passage, and both mitogens were withdrawn. This scenario also yields 50% astrocytes (K. Johe, T. Hazel, and R. D. G. McKay, unpublished results). Thus, exposure to EGF just before mitogen withdrawal causes the cells to have a greater propensity to differentiate into astrocytes. However, it is not instructive in its action like CNTF. Although EGF has been reported to activate STATs (Fu and Zhang, 1993; Schindler and Darnell, 1995), in the experiments reported here there is no evidence for STAT activation after EGF treatment. Although we have not investigated the possibility of STAT activation after EGF withdrawal, it seems highly unlikely. We conclude that EGF promotes astrocytic differentiation by mechanisms that do not include activation of STATs. BMP4 treatment causes astrocytic differentiation in EGF-expanded precursors isolated from the murine E17 subventricular zone grown in neurosphere cultures (Gross et al., 1996). This effect is not seen in our cells, which are isolated from E14 cortex and maintained in monolayers. Instead they respond to BMP4 by differentiating into neural crest progeny (Hazel et al., 1997). BMP4 also does not cause activation of STAT proteins in our system. Thus, both EGF and BMP4 cause astrocytic differentiation in different paradigms. Both of these factors signal through SMAD proteins. BMP4 causes direct activation of the SMAD1 protein by receptor-mediated phosphorylation (Lagna et al., 1996; Liu et al., 1996), whereas EGF causes inhibition of transcriptional activation by the SMADs via the MAPK pathway (Kretzschmar et al., 1997). Astrocytic differentiation in both the EGF withdrawal and BMP4 treatment paradigms may be regulated positively by the SMAD proteins. In the case of BMP4 this would occur through direct activation, whereas withdrawal of EGF may cause an alleviation of SMAD inhibition, leading to astrocytic differentiation. These results are consistent with a model in which the involvement of either STAT or SMAD signaling is necessary for astrocytic differentiation depending on the type of stem cell undergoing differentiation and the ligand causing it. In the experimental models discussed here, CNTF causes astrocytic differentiation of FGF-expanded CNS stem cells by STAT activation, whereas EGF and BMP may cause astrocytic differentiation in their respective models via the SMAD pathway. It is possible that FGF primes the cells for the differentiation effects of CNTF in the model studied in this report, whereas EGF exposure is required in the model of Gross et al. (1996) for astrocytic differentiation in response to BMPs. Thus different growth factors may activate distinct groups of transcription factors, providing a method for plasticity in the timing, quantity, and quality of astrocytes generated in the adult brain.

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