cAMP-induced Astrocytic Differentiation of C6 Glioma Cells Is Mediated by Autocrine Interleukin-6*  

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Elevation in the level of intracellular cAMP is known to induce the astrocytic differentiation of C6 glioma cells by unknown mechanisms. In this report, we show that cAMP-induced autocrine interleukin 6 (IL-6) promoted astrocytic differentiation of C6 cells. Treatment of cells with N6,2′-O-dibutyryl cAMP (Bt2AMP) and theophylline caused the delayed phosphorylation of signal transducer and activator of transcription 3 (STAT3), as well as the expression of an astrocyte marker, glial fibrillary acidic protein (GFAP). Overexpression of the dominant-negative form of STAT3 leads to the suppression of GFAP promoter activity, suggesting that STAT3 activity was essential for cAMP-induced GFAP promoter activation. On the other hand, the IL-6 gene was quickly induced by Bt2AMP/theophylline, and subsequent IL-6 protein secretion was stimulated. In addition, recombinant IL-6 induced GFAP expression and STAT3 phosphorylation. Most importantly, treatment with IL-6-neutralizing antibody dramatically reduced the cAMP-induced GFAP expression and STAT3 phosphorylation and reversed the cellular morphological changes that had been caused by Bt2AMP/theophylline. Taken together, these results indicate that Bt2AMP/theophylline lead to delayed STAT3 activation via autocrine IL-6. These processes subsequently led to the induction of GFAP. IL-6 secretion is thus thought to be a key event in controlling the astrocytic differentiation of C6 cells.

Astrocytic differentiation occurs largely during the postnatal period (1), and the timing of the differentiation is regulated by various extracellular cues and cell-intrinsic programs (2). The extracellular stimuli known to promote astrocyte differentiation are fetal calf serum (3), bone morphogenetic protein 2 (4), a neuropeptide, pituitary adenylate cyclase-activating polypeptide (5), and the interleukin-6 (IL-6) family of cytokines (6). The IL-6 family of cytokines, oncostatin M and cardiotoxin-1, are known to activate a downstream transcription factor, signal transducer and activator of transcription 3 (STAT3), and then the activated STAT3 binds to the promoter region of the glial fibrillary acidic protein (GFAP) gene, an astrocyte marker, thereby inducing the expression of GFAP (7, 8). STAT3 belongs to the STAT family of transcription factors, which play crucial roles in various intracellular signaling cascades involved in proliferation and differentiation (9, 10). In response to activation of cell-surface receptors, STAT3 is phosphorylated on its Tyr705 residue by Janus kinase (JAK), and this leads to STAT3 dimerization, subsequent nuclear translocation, and the trans-activation of its target genes (11). Bone morphogenetic protein 2 synergistically acts with leukemia inhibitory factor, another IL-6 family of cytokines, to induce astrocytogenesis by promoting the complex formation of respective downstream transcription factors, Smads and STAT3, which are bridged by transcriptional coactivator p300 (12).

The above-mentioned reports have extensively elucidated the mechanisms of cytokine-induced astrocyte differentiation. However, there remain other factors that induce differentiation by unknown mechanisms. Pituitary adenylate cyclase-activating polypeptide is one of the most potent activators of adenylate cyclase in pituitary cells (13), and its receptor is expressed in the subventricular zone, an area that gives rise to both neurons and glia, around the onset of gliogenesis (14, 15). It was shown recently (5) that cAMP-elevating stimuli including pituitary adenylate cyclase-activating polypeptide can induce astrocytogenesis of rat cortical precursors. Therefore, intracellular cAMP seems to play an important role in controlling astrocyte differentiation. However, the mechanism of cAMP in astrocyte differentiation remains unclear. A C6 rat glioma cell line is known to express astrocyte markers by cAMP-elevating stimuli such as N-substituted CAMP analogues and cAMP-increasing reagents (16–18). Several protein kinase C (PKC) isoforms are induced during cAMP-dependent C6 differentiation (18, 19). These proteins are thus suggested to be involved in C6 differentiation. However, the signaling cascade in this cAMP-induced differentiation of C6 cells is not yet fully understood. We speculated that STAT3, which is essential for IL-6 family of cytokine-dependent GFAP gene expression, might be also involved in the cAMP-dependent GFAP expression in C6 cells. To examine this hypothesis, the effect of cAMP on the activation of STAT3 and the effect of STAT3 activity on cAMP-dependent GFAP induction in C6 cells were examined. In the process of conducting this study, we found that cAMP-induced autocrine IL-6 was important for STAT3 activation and subsequent GFAP expression.

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1 The abbreviations used are: IL, interleukin; GFAP, glial fibrillary acidic protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Bt2AMP, N6,2′-O-dibutyryl cyclic AMP; RT-PCR, reverse transcription-PCR; CRE, cAMP response element; STAT, signal transducer and activator of transcription; ST3-WT, wild-type STAT3; ST3-DN, dominant-negative STAT3; PKA, protein kinase A; PKC, protein kinase C; JAK, Janus kinase; CREB/ATF, cAMP response element-binding protein/activating transcription factor.
**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies against GFAP, mouse IgG peroxidase conjugate, and rabbit IgG peroxidase conjugate were purchased from Sigma. Antibodies against STAT3 and the STAT3 phosphoform STAT3 Tyr705 were purchased from New England Biolabs (Beverly, MA). Anti-glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Chemicon (Temecula, CA). Anti-mouse IL-6-neutralizing antibody was purchased from R&D Systems (Minneapolis, MN). Other chemicals and materials were purchased from the following manufacturers: Dulbecco’s modified Eagle’s medium, fetal bovine serum albumin, penicillin, and streptomycin were obtained from Invitrogen; kinase inhibitors H89, SP600125, SB203580, and GF109203X were purchased from Calbiochem; a phosphodiesterase inhibitor, theophylline, was obtained from Wako Pure Chemical Industries (Osaka, Japan); cAMP analogue, N6-O-dibutyryl cyclic AMP (Bt2AMP), was purchased from Sigma; recombinant rat IL-6 was obtained from R&D Systems; and the ECL Western blot detection system was from Amersham Biosciences.

**Cell Culture**—The C6 rat glioma cell line (ATCC number CCL 107) used in this study was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The C6 cells used for Western blotting, reverse transcription-PCR (RT-PCR), and enzyme-linked immunosorbent assay were differentiated as described previously (20). Briefly, cells were thawed from a frozen stock (48–52 passages) for each experiment and were cultured for 2 days. The cells were replated on 100-mm culture plates at a density of 5 × 10⁵ cells/plate and were cultured for an additional 2 days. Cells were serum-starved for 1 h, 1 mM Bt2AMP and 0.25 mM theophylline (Bt2AMP/theophylline) were added to the medium, and then cells were incubated for the indicated periods of time in each experiment.

**Immunofluorescent Staining**—Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, treated with 0.1% Triton X-100 and 3% normal goat serum in phosphate-buffered saline (−) for 1 h, and then stained with anti-GFAP antibody (1:100). The primary antibody was detected with fluorescein isothiocyanate-conjugated secondary antibody (1:100). The cells were counterstained with Hoechst 33342 (Wako Pure Chemical Industries) to identify all nuclei.

**Plasmids**—The reporter plasmid pGF1L, which has the 2.5-kb mouse GFAP promoter connected to the firefly luciferase gene, was kindly provided by Dr. Ikenaka (21). The reporter plasmid pIL6-Luc was created by subcloning the promoter region of the rat IL-6 gene (−643 to +64) prepared by PCRam into the KpnI/HindIII site of a pTAT-Luc vector (Clontech). This promoter region of rat IL-6 bears a cAMP-response element (CRE) consensus sequence, and the highest promoter activity is indicated by stimulation with platelet-derived growth factor in C6 cells (22). The plasmid structure was verified by sequencing. pEF-BOS-STAT3 (wild-type) and pEF-BOS-STAT3 (Y705), expression vectors for wild-type and dominant-negative forms of STAT3, respectively, were kindly provided by Dr. Akira (23). The reporter plasmids bearing a variety of cis-elements (activator protein 1-binding element, glucocorticoid response element, heat shock response element, serum response element, nuclear factor of B-binding element (NFκB), and CRE) fused to the firefly luciferase gene were purchased from Clontech.

**Western Blot Analysis**—Cell extracts were prepared by ultrasonic treatment as described previously (20) using extraction buffer (20 mM TrisCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). To detect the phosphoform of STAT3 by Western blotting, cells were lysed using an extraction buffer containing tyrosine phosphatase inhibitor mixture set II (Calbiochem). Representative Western blots from at least three independent experiments are shown in all of the figures.

**Reporter Gene Assay**—C6 cells were plated at 6 × 10⁵ cells/well in 6-well plates. Sixteen hours after the passage, cells were transfected with 1.0 μg of reporter plasmid and 0.04 μg of Renilla luciferase-expressing plasmid (as an internal transfection efficiency control) by the LipofectAMINE method following the manufacturer’s instructions (Invitrogen). When STAT3 constructs were cotransfected with 1.0 μg of pGF1L and 0.04 μg of Renilla luciferase-expressing plasmid, the total amount of DNA was kept constant at 3.04 μg by supplementing with expression plasmids encoding wild-type STAT3 (ST3-WT) or the dominant-negative form of STAT3 (ST3-DN) in the presence of the reporter plasmid pGF1L. For transfection, the total amount of plasmid DNA was kept constant by supplementing with an empty vector as described under “Experimental Procedures.” Cells were then either left unstimulated (−) or were stimulated (+) with Bt2AMP/theophylline (cAMP) for 24 h. Each observation of luciferase activity was performed at least three independent experiments, with each experiment run in triplicate. Firefly luciferase activity was normalized to Renilla luciferase activity and was expressed as fold luciferase activity. The values represent the mean ± S.D. (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001, compared with the values of the Bt2AMP/theophylline-treated (cAMP+) and empty vector alone transfected group (second left group).

![Fig. 1. STAT3 phosphorylation during cAMP-dependent differentiation of C6 cells.](image1)

C6 cells treated with Bt2AMP/theophylline (cAMP) were collected at the indicated time points, and the cell lysates were immunoblotted to detect GFAP, STAT3, Tyr(P)705 (pSTAT3), STAT3, and GAPDH.

![Fig. 2. STAT3 activity was important for cAMP-dependent GFAP promoter activation.](image2)

C6 cells were cotransfected with expression plasmids encoding wild-type STAT3 (ST3-WT) or the dominant-negative form of STAT3 (ST3-DN) in the presence of the reporter plasmid pGF1L. For transfection, the total amount of plasmid DNA was kept constant by supplementing with an empty vector as described under “Experimental Procedures.” Cells were then either left unstimulated (−) or were stimulated (+) with Bt2AMP/theophylline (cAMP) for 24 h. Each observation of luciferase activity was performed at least three independent experiments, with each experiment run in triplicate. Firefly luciferase activity was normalized to Renilla luciferase activity and was expressed as fold luciferase activity.
empty vector. Twenty-four hours after transfection, the cells were serum-starved and stimulated with Bt$_2$AMP/theophylline. The transfected cells were examined for firefly and Renilla luciferase activities using the Dual-Luciferase Reporter Assay system (Promega). 

**RT-PCR**—Poly(A)$^+$ RNA was prepared using a QuickPrep Micro mRNA purification kit (Amersham Biosciences). Briefly, 1.5 μg of partially purified poly(A)$^+$ RNA was reverse transcribed using the SuperScript$^{TM}$ First-Strand Synthesis System for RT-PCR (Invitrogen). The PCR was then carried out in a 25-μl reaction mixture that contained 1 μl of the cDNA template, 0.6 μM specific oligonucleotide primer pair, and 1.5 units of Taq DNA polymerase (Roche Diagnostics). The following oligonucleotides were used as DNA primers: IL-6, 5’-CAGTTCGCTTCTTGGAACGT-3’ (forward) and 5’-ACAGTCGACATCGCTGTTTC-3’ (reverse); GAPDH, 5’-ACCCACGTTCATGCGCATAC-3’ (forward) and 5’-CACCACCGCTGGTACC-3’ (reverse). In addition, the following PCR programs were employed: for IL-6, 25 cycles of 30 s at 94 °C, 1 min at 61 °C, and 1 min at 72 °C; and for GAPDH, 16 cycles of 30 s at 94 °C, 1 min at 62 °C, and 1 min at 72 °C. The number of cycles employed for each program had been determined by preliminary experiments, which ensured that the PCR would terminate during the log stage of amplification.

**Enzyme-linked Immunosorbent Assay**—Culture medium from C6 cells treated with Bt$_2$AMP/theophylline was harvested at the indicated time points. Rat IL-6 protein levels in the culture medium were measured by enzyme-linked immunosorbent assay using Quantikine M (R&D Systems) according to the manufacturer’s protocol.

**Quantitative Analysis of Astrocytic Processes**—Cells were fixed with 1% glutaraldehyde for 15 min at room temperature, stained with Coomasie solution (0.1% Coomassie Brilliant Blue R-250, 10% acetic acid, and 50% methanol) for 20 min at room temperature and then washed three times with water. Quantitative analysis of astrocytic processes was performed using NeuroZoom software (The Scripps Research Institute, La Jolla, CA and Mount Sinai School of Medicine, New York). For quantitative analysis, isolated cells were selected so that individual processes could be traced. Process lengths were obtained by analyzing at least 100 cells/treatment group from three independent experiments and calculating in each cell body.

**Statistical Analysis**—Differences in the means between the treatment groups were statistically assessed using an analysis of variance followed by the post hoc Tukey test. The differences were considered to be statistically significant at the $p < 0.05$ level.

**RESULTS**

cAMP-dependent STAT3 Activation in C6 Cells—Treatment of C6 cells with Bt$_2$AMP/theophylline greatly elevated GFAP protein expression within 24 h of stimulation (Fig. 1, upper
Activation of CRE in Early Stage of C6 Differentiation—To screen transcription factor(s) activated during C6 differentiation, we examined the influence of Bt2AMP/theophylline on the activity of a variety of transcription factors by using reporter plasmids bearing six different cis-elements linked to the luciferase gene (described under “Experimental Procedures”). Among these cis-elements, only CRE-mediated transcription was significantly activated by Bt2AMP/theophylline (data not shown). Then the time course of CRE- and GFAP promoter-mediated transcriptional activities were investigated (Fig. 3). Significant activation of CRE-mediated transcription was detected within 1 h after Bt2AMP/theophylline treatment, and activity reached a plateau 12 h after treatment (Fig. 3A). On the other hand, cAMP-dependent GFAP promoter activity was moderately activated for 12 h after the treatment. However, this activity was accelerated between 12 and 24 h after the treatment (Fig. 3B). From these results, it appears likely that CRE-mediated gene expression occurs first and then the translated protein probably leads to activation of the GFAP promoter.

Protein Kinase A (PKA) Was Involved in CRE- and GFAP Promoter-mediated Transcription—The transcription factors that bind to the CRE consensus sequence are those of the CREB/ATF family (24). The activation of CREB/ATF family factors is regulated by several kinases including PKA, c-Jun amino-terminal kinase, and p38 (24, 25). Among the inhibitors

**Fig. 5.** Induction of IL-6 in C6 cells. A, induction of the IL-6 gene during the early period of C6 differentiation. C6 cells were stimulated with (+) or without (−) Bt2AMP/theophylline (cAMP) for 1 h. RT-PCR analysis was used to amplify the IL-6 or GAPDH gene. B, activation of the IL-6 promoter. C6 cells transfected with pIL6-Luc vector were left unstimulated (−) or were stimulated (+) with Bt2AMP/theophylline. Each of the kinase inhibitors was added simultaneously with Bt2AMP/theophylline. Luciferase activity was measured 3 h after stimulation. The values represent the mean ± S.D. (n = 3). **, p < 0.01 and ***, p < 0.001, compared with the values of Bt2AMP/theophylline (cAMP (+)) and vehicle-treated control group (0.1% dimethyl sulfoxide (DMSO)). C, secretion of IL-6 protein. Culture medium was collected after Bt2AMP/theophylline incubation for the indicated periods, and IL-6 levels were assayed by enzyme-linked immunosorbent assay. The values represent the mean ± S.D. (n = 3). *, p < 0.05, compared with the values of the unstimulated (cAMP−) control group harvested at 24 h.

**Fig. 6.** Recombinant IL-6-induced GFAP expression and STAT3 phosphorylation. C6 cells were treated with Bt2AMP/theophylline (cAMP, lanes 2–7) or 100 ng/ml IL-6 (lanes 8–13) for the indicated durations. The cell lysates were immunoblotted to detect GFAP, STAT3 Tyr705 (pSTAT3), STAT3, and GAPDH. Control indicates the unstimulated control (lane 1).

**Fig. 7.** IL-6-neutralizing antibody inhibited cAMP-dependent GFAP expression and STAT3 phosphorylation. C6 cells were treated with control IgG or IL-6-neutralizing antibody (IL6 ab.) together with Bt2AMP/theophylline (cAMP) for 24 h. The cell lysates were immunoblotted to detect GFAP, STAT3 Tyr705 (pSTAT3), STAT3, and GAPDH.
of these kinases, a PKA inhibitor, H89, suppressed both CRE- and GFAP promoter-mediated transcriptional activity (Fig. 4). Although the p38 inhibitor, SB203580, had a moderate effect on CRE-mediated transcriptional activity, it had no effect on GFAP promoter activity. The c-Jun amino-terminal kinase inhibitor, SP600125, had no effect on either CRE- or GFAP promoter-mediated transcription. Therefore, PKA is likely to be involved in the cAMP-dependent activation of both CRE- and GFAP promoter-mediated gene expression. We also examined the effects of PKC inhibitors on cAMP-dependent GFAP expression because some PKC isoforms, such as PKCβ1, -β, and -δ, have been reported to increase during differentiation (18). A PKC inhibitor, GF109203X, was shown to dose dependently attenuate cAMP-dependent GFAP promoter activity (Fig. 4B). Therefore, cAMP-dependent GFAP promoter activity also appeared to be regulated by certain PKC isoforms.

**IL-6 Induction during C6 Differentiation**—What was the gene induced by Bt2AMP/theophylline treatment, and the translated protein-promoted STAT3 phosphorylation and subsequent GFAP induction? One candidate gene was the IL-6 gene, the transcription of which by cAMP requires a CRE sequence in its promoter region (26). Moreover, the binding of IL-6 to its receptor activates the JAK/STAT pathway, thereby stimulating STAT3-mediated gene expression. As expected, the IL-6 gene was induced 1 h after Bt2AMP/theophylline treatment (Fig. 5A), and the rat IL-6 promoter was activated by this treatment (Fig. 5B). In addition, promoter activity was suppressed by H89, suggesting the involvement of PKA in IL-6 induction. The pattern of IL-6 promoter activity inhibited by kinase inhibitors was similar to that of CRE-mediated transcriptional activity inhibited by kinase inhibitors, suggesting that cAMP-dependent IL-6 promoter activation is mediated by the CRE sequence in its promoter. To further investigate the secretion of IL-6, we performed an enzyme-linked immunosorbent assay to measure the protein levels in a cultured medium. We found that IL-6 levels were greatly enhanced from 12 to 24 h after stimulation (Fig. 5C). This IL-6 secretion pattern correlated with the activation of the GFAP promoter (Fig. 3B).

**Effect of Recombinant IL-6 on GFAP Expression**—To confirm the effects of IL-6 on GFAP expression, C6 cells were treated with recombinant rat IL-6. This treatment resulted in STAT3 phosphorylation within 15 min (Fig. 6, upper middle panel, lane 8) and GFAP induction within 12 h (Fig. 6, top panel, lane 12). These data suggest that secreted IL-6 is able to activate STAT3, and this might lead to subsequent GFAP expression.

**Effect of IL-6-neutralizing Antibody on the Differentiation of C6 Cells**—To examine whether cAMP-dependently secreted IL-6 is critical for GFAP expression, IL-6-neutralizing antibody was added to the medium together with the Bt2AMP/theophylline treatment, and then the medium was incubated for 24 h. Treatment with an anti-murine IL-6-neutralizing antibody significantly inhibited GFAP expression (Figs. 7, top panel, and 8, D–F), STAT3 phosphorylation (Fig. 7, upper middle panel), and GFAP promoter activity (data not shown). These data clearly indicate that the secretion of IL-6 plays a pivotal role in cAMP-induced GFAP expression in C6 cells. IL-6-neutralizing antibody also affected the morphology of these C6 cells (Fig. 8, A–C). Treatment of the cells with Bt2AMP/theophylline changed the cell morphology; cells were no longer flat (Fig. 8A) but rather were spindle-shaped with processes (Fig. 8B). The extension of processes of spindle-shaped cells was significantly attenuated by the addition of IL-6-neutralizing antibody (Figs. 8C and 9A). In addition, spindle-shaped cells decreased, whereas flat-shaped cells increased after treatment with IL-6-neutralizing antibody (Fig. 8C). Treatment of cells with H89 also attenuated the extension of the processes, whereas SP600125 did not (Fig. 9B), validating the involvement of PKA in the differentiation of C6 cells. These results suggested that the IL-6 autocrine mechanism was essential not only for GFAP induction but also for the morphological changes observed in the cells.

**DISCUSSION**

IL-6 exerts a variety of functions in the central nervous system. For example, IL-6 carries out trophic functions such as ensuring neuronal survival and astrocyte proliferation, and it can also function as a mediator of inflammation and astrogliosis (27). Accumulating evidence has suggested that an essential role is played by IL-6 in the development and differentiation of neural cells (6, 27). The expression of both IL-6 and its receptor mRNA in the rat central nervous system is increased in the striatum, hippocampus, and the neocortex during postnatal development (28). The major sites of IL-6 synthesis in the central nervous system are neurons and glial cells (27). In this report, we demonstrated that the elevation of intracellular cAMP enables C6 cells to provide their own astrocytic differentiation-inducing factor, IL-6.

Mature astrocytes are known to secrete IL-6 in response to a variety of signals; these signals include IL-1, lipopo-
lysaccharide, tumor necrosis factor α, transforming growth factor β, neurotransmitters, and various second messengers (29).

Such ligands bring about the activation of intracellular signal-transducing factors, including PKA, PKC, NFκB, and activator protein 1-binding element. In undifferentiated C6 cells, PKA activity is thought to be involved in cAMP-dependent IL-6 induction (Fig. 5). Therefore, PKA seems to play an important part in C6 cell differentiation. However, there remains the possibility that other factors are involved in cAMP-dependent GFAP induction. Although recombinant rat IL-6 has been shown to induce GFAP expression in C6 cells 12 h earlier than in Bt₂AMP/theophylline-treated cells (Fig. 6, top panel, compare lane 6 with 12), the level of GFAP protein expression induced by recombinant IL-6 was much lower than that of Bt₂AMP/theophylline-induced expression at 24 h after treatment (Fig. 6, top panel, compare lane 7 with 13). This finding implies that recombinant IL-6 alone is not sufficient to elevate GFAP to a level that can be induced by Bt₂AMP/theophylline treatment. In addition, the introduction of a dominant-negative form of STAT3 did not completely suppress cAMP-dependent GFAP promoter activity (Fig. 2). Moreover, treatment with IL-6-neutralizing antibody did not completely suppress GFAP protein induction (Fig. 7). Here, we demonstrated that PKC might be involved in GFAP promoter activity (Fig. 4B). Therefore, the induction of cAMP-dependent GFAP appears to be regulated by both autocrine secretion of IL-6 and some PKC isoforms.

Anciaux et al. (30) reported previously that cAMP-mediated induction of GFAP was independent of PKA activation in C6 cells. They found that the presence of 1 μM H89 did not sup-
press the level of Bt2AMP-induced GFAP mRNA 48 h after stimulation. In addition, pretreatment with H89 potentiated Bt2AMP-mediated GFAP protein synthesis. In the present study, the presence of 1 μM H89 in the culture medium suppressed both IL-6 and GFAP promoter activity at 3 and 24 h after stimulation with Bt2AMP/theophylline, respectively (Figs. 5B and 4B, respectively). Taken together, these findings suggest that 1 μM H89 might inhibit cAMP-mediated GFAP induction after a relatively short period of treatment (up to 24 h), whereas 1 μM H89 might promote GFAP expression after longer periods of treatment (after 24 h). Hence, the transcriptional regulation of some genes that are possibly involved in GFAP expression might take place during longer periods of H89 treatment in C6 cells.

The treatment of C6 cells with IL-6-neutralizing antibody inhibited GFAP induction (Figs. 7 and 8F) and attenuated the extension of astrocytic processes (Figs. 8C and 9A). This effect of IL-6-neutralizing antibody was similar to that of 2,3,7,8-tetrachlorodibenzop-dioxin, which significantly inhibited cAMP-dependent GFAP induction and attenuated the extension of processes in C6 cells (20). These observations suggest the importance of GFAP accumulation for the induction of cellular morphological changes. Thus, the present findings support those of a previous report demonstrating that the formation of astrocyte-lineage cells. Furthermore, the present findings indicated that such cAMP-dependent morphological changes might require additional regulation by other signaling cascades.

To date, the experimental observations suggest that the following signaling cascade is operative in cAMP-dependent C6 differentiation (Fig. 10). First, Bt2AMP/theophylline treatment elevates intracellular cAMP levels. The cAMP molecules then activate PKA, which stimulates CRE-binding protein to bind to the IL-6 gene promoter and activate its expression. IL-6 protein is secreted and binds to its receptor on the cell surface, which triggers the intrinsic tyrosine kinase activity that leads to the activation of the tyrosine kinase, JAK. Then activated JAK phosphorylates STAT3, which subsequently translocates into the nucleus and binds to its specific DNA sequence in the GFAP promoter. This in turn leads to the induction of the GFAP gene.

The second messenger cAMP regulates multiple signaling cascades and plays a large variety of roles in the living body. Every type of cell in the central nervous system shows peculiar reactions to elevations in intracellular CAMP. Therefore, the cAMP-elevating stimuli that are required for astrocyte differentiation must be strictly regulated during neural development. One such candidate is a neuropeptide, pituitary adenylate cyclase-activating polypeptide, which promotes astrocytogenesis in vitro; the pattern of expression of its receptor appears to indicate its relationship to gliogenesis. C6 cells are known to display astrocytic oligodendrocytic, and neuronal characteristics (32, 33). Therefore, it remains unknown whether or not the secretion of IL-6 from undifferentiated C6 cells is characteristic of astrocyte-lineage cells. Thus, further study will be required to identify the type(s) of neural cells that provide IL-6 during astrocytogenesis. Based on the results of present study, we propose that an important function of cAMP in the developing central nervous system is to induce IL-6 secretion from neural cells; this process is thought to play an essential role in the promotion of astrocytogenesis.

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