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Apotransferrin Induces cAMP/CREB Pathway and Cell Cycle Exit in Immature Oligodendroglial Cells

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We have demonstrated previously that a single intracranial injection of apotransferrin (aTf) in neonatal rats increases myelination and accelerates differentiation of oligodendroglial cells (OLGc). In addition, we have shown through in vitro experiments that OLGc isolated from 4-day-old rats (OLGc-4) treated with aTf were more differentiated than were controls although aTf had no effect upon OLGc isolated from 10-day-old animals (OLGc-10). In the present work, we analyzed the role of second messengers in the effect of aTf upon the maturation of OLGc at different stages of development. We isolated OLGc-4 and OLGc-10 from rat brain using a Percoll density gradient and briefly treated the cells with a pulse of aTf or kept them in culture during 2 days in the presence or absence of aTf. In OLGc-4, after a short pulse of aTf, there was an increase in the levels of cyclic AMP (cAMP), in the phosphorylation of cAMP response element-binding protein (CREB) and in the DNA-binding capacity of cAMP-responsive transcription factors. Treatment of OLGc-4 with aTf diminished bromodeoxyuridine (BrdU) incorporation and changed levels of p27 and cyclin D1. This glycoprotein seemed to act on OLGc through the cAMP pathway only at early stages of development and on a certain sensitive cell population, accelerating their differentiation, probably as a consequence of premature withdrawal from the cell cycle.

Key words: oligodendrocyte; apotransferrin; cell cycle; differentiation; cAMP; CREB

Oligodendroglial cells (OLGc), the cells that produce the myelin membrane in the central nervous system (CNS), undergo several stages of differentiation leading to the formation of myelinating cells. In the brain, they arise from progenitors located in the subventricular zone (SVZ), where they proliferate and migrate to different areas of the developing brain before differentiation (Pfeiffer et al., 1993; Miller, 1996; Goldman et al., 1997).

In rat CNS, transferrin (Tf) is produced by OLGc at early stages of development (Connor and Fine, 1987) and is essential for cell survival. We and others have demonstrated previously that a single intracranial injection of apotransferrin (aTf) in neonatal rats increases expression of some genes characteristic of mature OLGc, such as myelin basic protein (MBP), and 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNPase) and enhances myelin formation due to an accelerated differentiation of the OLGc (Escobar Cabrera et al., 1994, 1997; Espinosa de los Monteros et al., 1999; Marta et al., 2000; Espinosa-Jeffrey et al., 2002). Although some effects of aTf on the OLGc are well known, the molecular mechanisms involved in its action are not yet characterized fully. In our laboratory, we have demonstrated recently that aTf action is mediated by its receptor (TfR), by the cytoskeleton of OLGc, and by changes in the activities of several tyrosine kinases (Marta et al., 2002).

Several studies have shown that OLGc differentiation is stimulated by cAMP (McMorris et al., 1983; Pleasure et al., 1986; Raible and McMorris, 1989, 1990). This is followed by activation of cAMP-dependent protein kinase (PKA) and increased cAMP response element-binding protein (CREB) phosphorylation, which mediates cAMP-dependent stimulation of MBP expression and the outgrowth of OLGc processes (Sato-Bigbee et al., 1994, 1999; Sato-Bigbee and De Vries, 1996; Afshari et al., 2001). CREB binds to a consensus cAMP-response element sequence (TGACGTCA) present in the promoter of cAMP- and Ca^{2+}-responsive genes, such as *c-jun* and *c-fos* (Montminy et al., 1990; Sheng et al., 1991). Tran-

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scriptional activation by CREB is regulated by phosphorylation at Ser^{133} (Yamamoto et al., 1988), and this phosphorylation is attributed to cAMP-dependent protein kinase (PKA) (Yamamoto et al., 1988; Gonzalez et al., 1989), Ca^{2+}-calmodulin kinases (CamKs) (Sheng et al., 1991), protein kinase C (PKC) (Xie and Rothstein, 1995), and growth factor-induced kinases (Ginty et al., 1994; Xing et al., 1996).

Terminal cellular differentiation is accompanied generally by exit from the cell cycle. In many cell types, these two events are linked at the molecular level, the cell cycle being controlled in mammalian cells by the action of cyclins and their partner cyclin-dependent kinases (CDKs). Proliferating cells must pass at least two checkpoints in G1 to enter S phase. An earlier checkpoint is controlled by cyclin D and its partners CDK4 and CDK6, whereas a later point is regulated by the cyclin E/CDK2 complex (Elledge, 1996). Another level of regulation of cell cycle progression results from action of CDK inhibitors such as p27 (Sherr and Roberts, 1995). This CDK inhibitor has been shown to halt cells in G1 in response to external signals (Polyak et al., 1994). Cultured OLGc have also been shown to undergo withdrawal from the cell cycle in response to cAMP (Raible and McMorris, 1989, 1990).

We have demonstrated previously that aTf induces differentiation only in a certain population of OLGc isolated from 4-day-old animals (OLGc-4), having no effect in more mature cells isolated from 10-day-old rats (OLGc-10) (Garcia et al., 2003). In the present study, our interest was to obtain further insight into the intracellular mechanisms involved in this differential response. We have analyzed the role of cAMP, CREB, and D-myoinositol 1,4,5-triphosphate (IP3) in the response to aTf of OLGc isolated from rat brains at different stages of development and the participation of aTf in the withdrawal of these cells from the cell cycle. Our results show that in OLGc-4, aTf could act by increasing cAMP levels, stimulating CREB activation, and by binding to DNA specific sequences, inducing withdrawal from the cell cycle by increasing p27 levels and diminishing cyclin D1expression.

MATERIALS AND METHODS

Materials

Human apotransferrin, paraformaldehyde, bovine serum albumin (BSA), anti-p27 antibody, anti-bromodeoxyuridine (BrdU) antibody, BrdU, HEPES, and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle medium/Ham's F12 (DMEM/F12) was from HyClone (Logan, UT), trypsin was from GIBCO (Grand Island, NY), and fetal calf serum (FCS) was from NATOCOR (Argentina). Anti-phosphorylated (p)CREB was obtained from Cell Signaling Technology (Beverly, MA) and Percoll was from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Antibodies against total CREB, cyclin D1, and cyclin E were from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescent secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). All other chemicals were analytical grade reagents of the highest purity available.

Isolation and Culture of Oligodendroglial Cells

OLGc were isolated from rat brain at 4 and 10 days of age, using a Percoll density gradient, according to the method described previously by Berti-Mattera et al. (1984) with slight modifications (Sato-Bigbee, personal communication). Briefly, brains were minced and dissociated in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS), 25 mM HEPES (pH 7.2), 1 mg/ml glucose, 0.01 mg/ml DNase I, and 0.1% trypsin. After incubation for 30 min at 37°C, the tissue was forced through a 74-μm pore size mesh and a 145-μm pore size mesh, and the resulting cell suspension was mixed with isosmotic Percoll (3 vol of cell suspension/2 vol isosmotic Percoll) and centrifuged at 30,000 × *g* for 15 min. The band corresponding to the OLGc was collected and washed twice with HBSS. The final cell suspension was seeded on Petri dishes and left at room temperature for 30 min to allow attachment of residual microglial and astrocyte contamination (5–10%). The dishes were then swirled gently for approximately 10 sec and nonadherent cells were recovered and plated on poly-L-lysine coated coverslips placed in multiwell dishes (4×10^4 cells/well) or on Petri dishes ($2–3 \times 10^6$ cells/dish) and cultured in a chemically defined medium (Sato-Bigbee et al., 1999) (DMEM/F-12 medium [1:1 vol/vol] supplemented with 1 mg/ml BSA, 5 μg/ml insulin, 30 nM sodium selenite, 0.11 mg/ml sodium pyruvate, 10 nM biotin, 2 μM hydrocortisone, 15 nM triiodothyronine, 1% FCS, 20 U/ml penicillin, and 20 μg/ml streptomycin) at 37°C in 5% CO_2. When specified, aTf (50 μg/ml) was added to the incubation medium and cells were kept in culture for a further 2 days.

Immunocytochemical Studies

Multiwells containing 4×10^4 OLGc were kept for 2 days in defined medium alone or defined medium containing aTf (50 μg/ml). Cells were fixed with 4% paraformaldehyde and 0.12 M sucrose in phosphate buffer for 1 hr at room temperature and used for immunocytochemical analysis. Samples were blocked with 5% BSA in phosphate buffer for 2 hr and incubated overnight at 4°C with two different primary antibodies: anti-MBP (a gift from Dr. A. Campagnoni, UCLA; 1/100) or anti-p27 (1/100). Coverslips were rinsed and incubated with appropriate secondary antibodies.

After immunocytochemical assay of the above-mentioned antigens, nuclei were stained with the fluorescent dye Hoechst 33342 (5 μg/ml in 1% dimethylsulfoxide [DMSO]) according to the method of Oberhammer et al. (1993). The cells were mounted and analyzed by epifluorescence using an Olympus BX50 microscope. Antigen-positive cells and Hoechst-positive cells were counted in 20 randomly selected fields, which resulted in counts of >3,000 cells for each condition. Counts of antigen-positive cells were normalized to the counts of total Hoechst-positive cells for each condition.

BrdU Incorporation

To label the cells in S phase, OLGc from 4-day-old animals were treated at different time points with a pulse of 10 μM BrdU in the presence or absence of 100 μg/ml aTf.

Cells were fixed in 4% paraformaldehyde and incubated in 6 N HCl and 1% Triton X-100 to denature nuclear DNA, and then incubated in 0.1 M sodium borate (in PBS and 1% Triton X-100) for 10 min. Cells were then blocked and treated as mentioned for immunocytochemistry using the anti-BrdU antibody (1/1,000) and the corresponding fluorescent secondary antibody. Finally, cell nuclei were labeled with Hoechst 33342 as described above. The percentage of positive cells was measured in 15 randomly selected fields, which resulted in counts of >1,000 cells for each condition. Quantitative evaluation was done using Image Pro Plus v.4.5 software.

Assay of cAMP

After 2 days in culture, Petri dishes containing 2×10^6 OLGc were washed thoroughly with PBS and incubated for 3 min at 37°C in HBSS containing 1 mM of 3-isobutyl-1-methylxantine (IBMX) followed by treatment during 9 min with different concentrations of aTf. Cultures were then washed with PBS and extracted with 1 ml of ethanol. Cells were carefully scraped off the Petri dish and the suspension was centrifuged at 3,000 × *g* for 5 min. The supernatants were air-dried and resuspended in 50 mM Tris-HCl buffer (pH 7.4) to determine cAMP content by competition with [^{3}H] cAMP (New England Nuclear, Boston, MA) for PKA as described previously (Davio et al., 1995). Results were expressed as percentage of stimulation relative to the basal response. Dose–response studies were carried out using aTf concentrations between 0.01–40 μg/ml.

Assay of IP3

After 2 days in culture, Petri dishes containing 2×10^6 OLGc were washed thoroughly with PBS and incubated for 10 min at 37°C in HBSS containing 10 mM LiCl to avoid recycling of IP3 formed during the assay, thus increasing the sensitivity of the method. OLGc were then incubated during 20 min in the same solution containing different aTf concentrations. After incubation, the medium was removed and the reaction was terminated by adding ice-cold 10% (vol/vol) $HClO_4$. Petri dishes were kept on an ice bath for a further 10 min. The cells were gently scraped off the plate, sedimented by centrifugation at 1,500 × g rpm for 15 min at 4°C and the supernatant was decanted into a siliconized tube and neutralized to pH 7.5 by titrating with ice-cold 10 M KOH. To sediment $KClO_4$, tubes were centrifuged at 2,000 × *g* for 15 min at 4°C and the supernatant was decanted into a siliconized tube. IP3 levels were determined using the [^{3}H]-labeled IP3 assay kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Dose–response studies were carried out using aTf concentrations between 0.01–40 μg/ml.

Western Blot Analysis

Petri dishes containing 2×10^6 OLGc cultured for 2 days in the absence or presence of 50 μg/ml aTf were used for SDS-PAGE of proteins according to Laemmli (1970). Electroblotting onto polyvinylidene (PVDF) membranes was carried out for 90 min at 400 mA. Nonspecific sites were blocked by incubation with 5% BSA in PBS (pH 7.4) plus 0.05% Tween 20 for 1 hr at 37°C. The membranes were then treated with the specific anti-pCREB (1/1,500), anti-p27 (1/1,500), anti-cyclin D1 (1/1,500), and anti-cyclin E (1/1,500) antibodies. After washing three times with PBS, antibody binding was visualized with horseradish peroxidase-conjugated secondary antibodies (1/20,000) and the ECL Plus substrate kit (Amersham Pharmacia Biotech). In the experiments in which pCREB and tCREB levels were analyzed, cells were cultured during 2 days without aTf, and then treated with 50 μg/ml aTf for different times. To normalize levels of pCREB, blots were de-hybridized after incubation with 0.1 M urea, 7 mM SDS, 0.5 M NaCl, 8 mM Tris, pH 4, for 3 hr at 65°C. After washing three times with PBS-Tween 20, Western blot analysis was carried out onto the pCREB membranes as described, using total anti-CREB (1/1,500) as primary antibody. Evaluation was done with a STORM 840 Plus PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and quantitation was carried out using the GelPro System.

Electrophoretic Mobility Shift Assay

To study the DNA-binding capacity of activated CREB (pCREB), we carried out an electrophoretic mobility shift assay (EMSA). At the corresponding time points, the medium was discarded and cells were rinsed with PBS and scraped off the plates. After centrifugation at 800 × *g* for 10 min, the pellet ($2–3 \times 10^6$ cells) was resuspended in 200 μl of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], and 0.1% Igepal), incubated for 30 min at 4°C, and centrifuged for 15 min at 10,000 × *g*. The pellet was resuspended in 100 μl of buffer B (10 mM HEPES, pH 7.9, 15 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride [PMSF], and 25% glycerol), incubated for 20 min at 4°C, and centrifuged for 15 min at 10,000 × *g*. The supernatant containing the nuclear fraction was transferred to a new tube and diluted in buffer C (20 mM HEPES, pH 7.9, 50 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, and 0.5 mM PMSF) to carry out the assay. Protein concentration was determined by the method of Bradford (1976) and samples were stored at −80°C.

For the EMSA, the oligonucleotide containing the consensus sequence for CREB was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified using Chroma Spin-10 columns (Clontech). Samples were incubated with the labeled oligonucleotide (20,000–30,000 cpm) for 20 min at room temperature in 50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, and 0.25 mg/ml poly(dI-dC). The products were separated by electrophoresis in 4% nondenaturing polyacrylamide gels using 0.5 × TBE (45 mM Tris borate, 1 mM EDTA) as the running buffer. The gels were dried and the radioactivity quantitated using a STORM 840 Plus PhosphorImager.

Statistical Analysis

Statistical analysis was carried out using Student's paired *t*-test, in which $P < 0.05$ was defined as statistically significant.

RESULTS

Only the OLGc-4 Population Responds to ATf Activating the cAMP-CREB-Mediated Pathway

We showed previously that under similar experimental conditions, immunocytochemical reactivity of

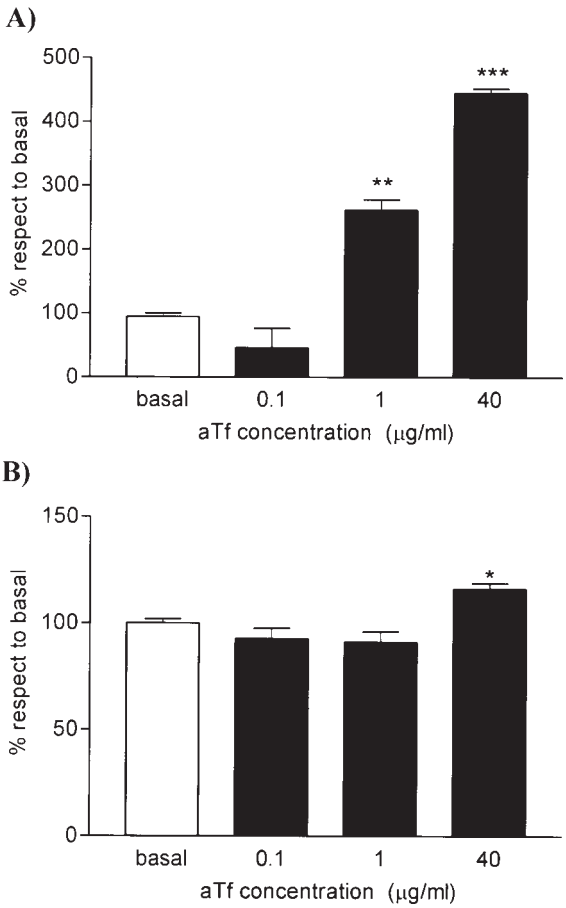

Fig. 1. Levels of cAMP in cultures of OLGc-4 (**A**) and OLGc-10 (**B**) after the addition of aTf. cAMP content was determined by the competitive protein binding assay. Results are the means ± SEM of three independent experiments and are expressed as percentage with reference to basal levels. $\star P < 0.05$, $\star\star P < 0.001$, $\star\star\star P < 0.0001$ vs. basal levels.

OLGc-4 was 82% positive for A2B5 and 27% for MBP. On the other hand, OLGc-10 displayed a more mature phenotype (40% A2B5 positive; 29% MBP positive). These data were confirmed by morphologic analysis (Garcia et al., 2003).

We analyzed the behavior of second messengers after treatment of OLGc cultures with aTf. Figure 1 shows that aTf treatment of OLGc-4 induced an increase in cAMP levels compared to basal values and this response was dose dependent (Fig. 1A). Cyclic AMP levels in OLGc-10 treated with similar low aTf concentrations did not change with reference to control values. Only at high concentrations of aTf (10 μg/ml and above) was there a slight increase in the levels of cAMP (Fig. 1B). We found that 40 μg/ml was the concentration at which maximal accumulation of cAMP was obtained. The response was the same beyond this concentration, so we used a concentration close to that resulting in the maximal response (50 μg/ml).

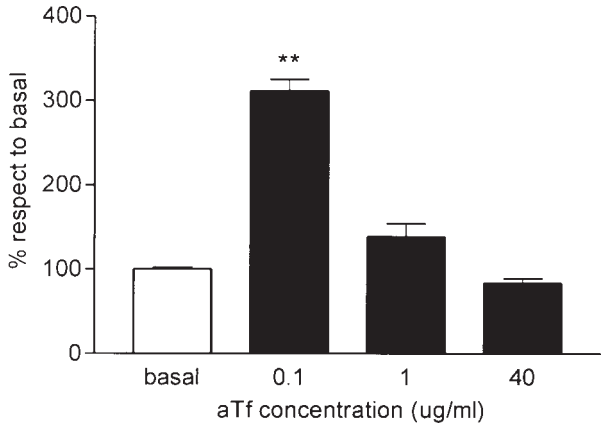

Fig. 2. Levels of IP3 in cultures of OLGc-4 after the addition of aTf. IP3 levels were detected using a [^{3}H]-labeled IP3 assay kit. Results are means ± SEM of three independent experiments and are expressed as percentage with reference to basal levels. $\star\star P < 0.001$ vs. basal level.

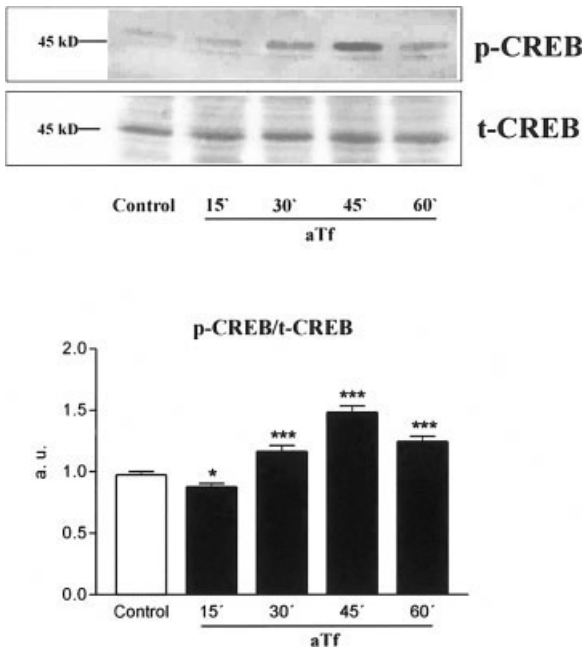

Fig. 3. After 2 days in culture, Western blot analysis of phosphorylated CREB (pCREB) and total CREB (tCREB) was carried out in OLGc-4 incubated with aTf (50 μg/ml) for different times. Levels of pCREB and tCREB were determined and expressed in arbitrary units (a.u.). The result of one of three different experiments is shown. $\star P < 0.05$, $\star\star\star P < 0.0001$ vs. control.

IP3 levels in OLGc-4 are known to be affected by the addition of aTf. At low concentrations of aTf, IP3 increased threefold compared to basal levels. In contrast, at high concentrations of aTf, there were no changes in this second messenger (Fig. 2). Interestingly, levels of cAMP and IP3 in cells treated with 50 μg/ml aTf show opposite patterns of behavior (compare Fig. 1A and Fig. 2), in

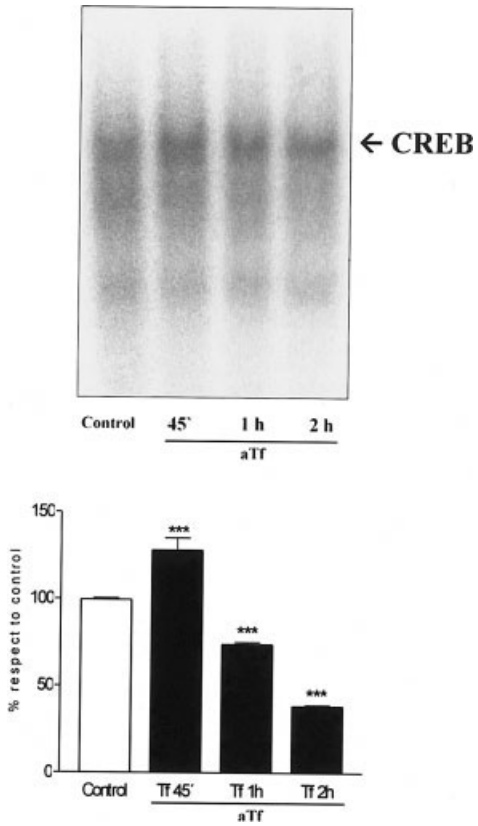

Fig. 4. Electrophoretic mobility shift assays (EMSA) with CREB were carried out using a radiolabeled oligonucleotide probe containing a consensus CREB-binding sequence with 5 μg of protein for each condition of cell extracts prepared from cultured OLGc-4 in the absence (C; lane 1) and the presence of aTf (aTf) at different times (lanes 2–4). Sequence specificity of binding was verified by the addition of a 100-fold excess of either CREB or a heterologous DNA fragment (Octamer transcription factor 1) of similar length (not shown). ***$P < 0.0001$ vs. control.

agreement with previous data from our laboratory (Marta et al., 2002).

CREB phosphorylation in developing OLGc could be regulated by both the cAMP-and Ca^{2+}-dependent signaling pathways. To investigate whether cAMP upregulation leads to an increase in CREB phosphorylation, we evaluated by Western blot the expression of total CREB and its activated form (pCREB) in control and aTf-treated cultures. As shown in Figure 3, in OLGc-4, aTf treatment significantly increased expression of this activated transcription factor in a time-dependent manner up to 45 min. After this reaction time, pCREB levels returned to control values. To determine if the increase in pCREB observed in OLGc-4 correlated with increased DNA-binding capacity, an EMSA was carried out using a consensus sequence for the CREB family of transcription factors. Treatment with aTf during 45 min resulted in a 30% increase in the DNA-binding capacity compared to that in controls (Fig. 4).

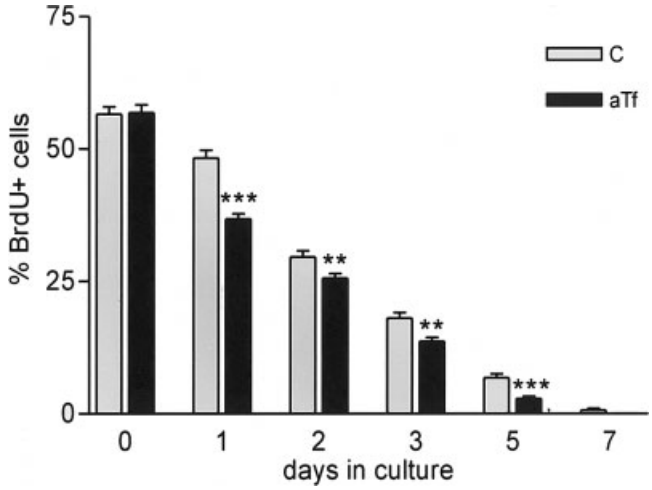

Fig. 5. OLGc-4 were cultured for various times in the presence of aTf 50 μg/ml (aTf) or in its absence (C). Cells were pulsed with BrdU for the times indicated, and the percentage of BrdU+ cells was determined by immunofluorescence. Results are expressed as mean ± SEM of two independent experiments, each with duplicates cultures for each time point. The addition of aTf significantly diminished the percentage of BrdU+ cells compared to that in controls after 1 day in culture. **$P < 0.01$, ***$P < 0.001$.

ATf Decreases the Percentage of BrdU+ Cells and Changes Expression of Some Key Cell Cycle Proteins

To analyze if the increase in aTf-induced OLGc-4 differentiation was accompanied by a change in their proliferation rate, we carried out BrdU labeling experiments. As shown in Figure 5, treatment with aTf significantly decreased the percentage of BrdU+ cells after 1 day in culture compared to control cells.

The levels of p27, one of the most important CDK inhibitors, were also evaluated. Figure 6 shows the results obtained in OLGc-4 and OLGc-10 cultured in the absence or presence of 50 μg/ml aTf. The number of p27+ cells in OLGc-4 cultures treated with aTf increased with reference to controls (Fig. 6A), whereas there was no change in the number of p27+ cells in treated OLGc-10 (Fig. 6B). Similar results were obtained when the levels of p27 were evaluated by Western blot in both experimental conditions (Fig. 6C,D).

Cyclin D1 and cyclin E expression of in cultures of OLGc isolated at 4 and 10 days of age changed in the presence of aTf (Fig. 7). Western blots show that in both types of aTf treated cultures there is a decrease in cyclin D1 of around 25–30% compared to that in controls (Fig. 7A,B). On the other hand, the cyclin E expression in OLGc-4 showed a slight increase in the presence of aTf, whereas in OLGc-10 there were no changes (Fig. 7A,B).

To determine whether the aTf-induced increase in cAMP levels enhances cell differentiation and thus induces cells to exit the cell cycle, or if elevation of cAMP inhibits cell proliferation, resulting in the generation of more ma-

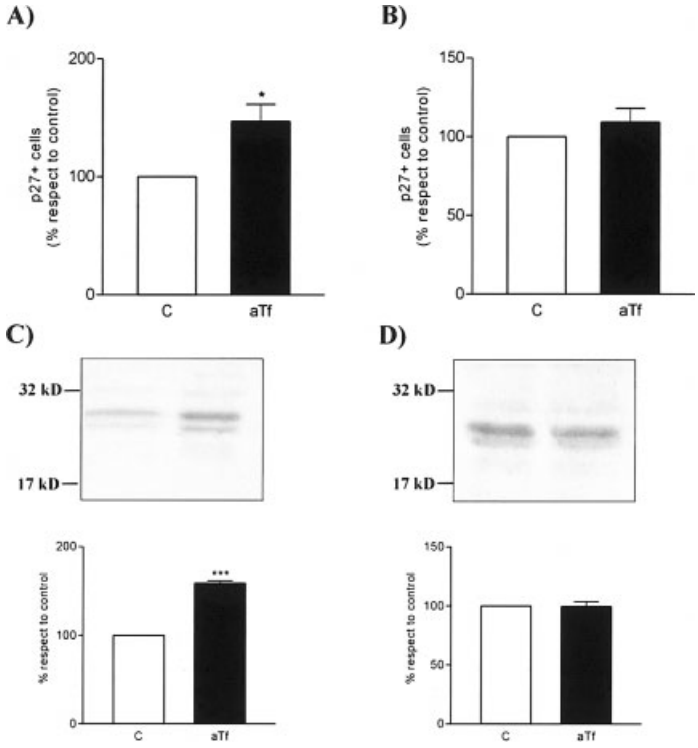

Fig. 6. OLGc-4 (**A**) and OLGc-10 (**B**) were cultured on coverslips for 2 days without aTf (C) or with 50 μg/ml aTf (aTf) added to the medium. The cells were fixed and immunostained with anti-p27 antibody. The total number of cells was determined by staining with Hoechst 33342. The percentage of cells positive for p27 was determined. Values are expressed as means ± SEM. $\star P < 0.05$ vs. control. Western blot analysis of p27 in OLGc-4 (**C**) and OLGc-10 (**D**) incubated with 50 μg/ml of aTf (aTf) or without aTf (C). Levels of p27 were determined and results of one of three different experiments for each condition are shown. $\star\star\star P < 0.0001$ vs. control.

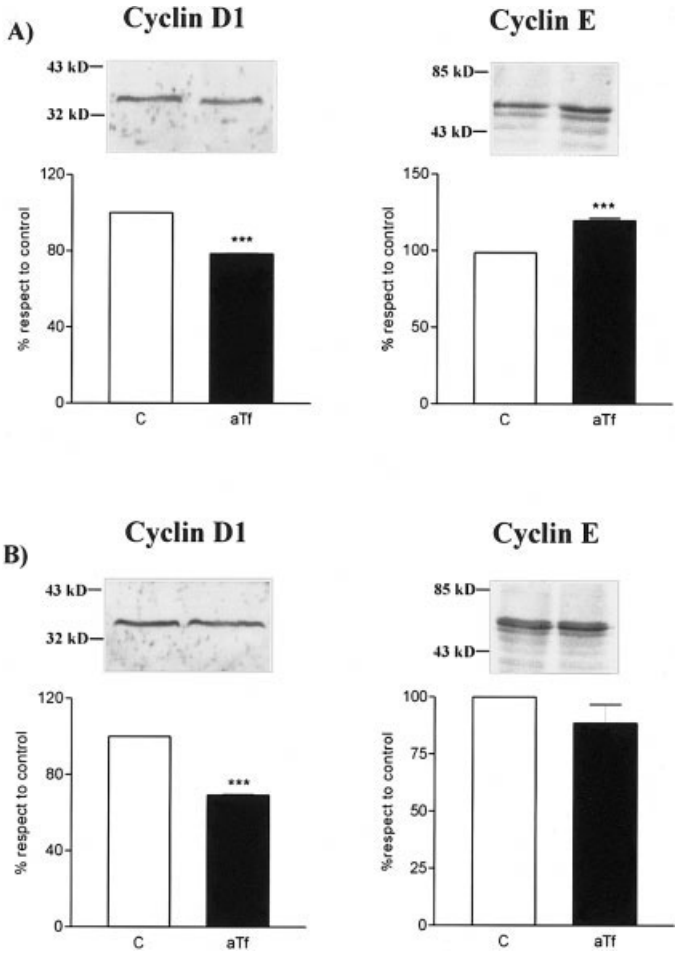

Fig. 7. Western blot analysis of cyclin D1 and cyclin E in OLGc-4 (**A**) and OLGc-10 (**B**) incubated with 50 μg/ml aTf (aTf) or control cells (C) during 2 days. Levels of both cyclins were determined and results of one of three different experiments for each condition are shown. $\star\star\star P < 0.0001$ vs. control.

ture OLGc, we studied the cAMP response to aTf in cells with different degrees of proliferation and differentiation. Figure 8A shows the changes in BrdU incorporation and MBP expression in a culture of OLGc-4. We studied the cAMP response to aTf at three differentiation stages: (1) in immature OLGc (BrdU+/MBP−); (2) in an intermediate population; and (3) in a more mature population of OLGc (BrdU−/MBP+). We found that the most immature population containing a high percentage of BrdU+ cells was responsive to aTf, displaying the highest increase in cAMP levels (Fig. 8B).

DISCUSSION

Apotransferrin added to cultures of OLGc isolated from 4-day-old rats has marked effects on their differentiation. It produced an increase in MBP+ cells and a decrease in A2B5+ OLGc, coincident with the appearance of a more complex and mature cell morphology (Garcia et al., 2003). These results agree with those described previously in in vivo studies (Marta et al., 2000) and in primary OLGc cultures (Paez et al., 2002).

Raible and McMorris (1989) have shown that dibutyryl cAMP (dbcAMP) only stimulates OLGc differentiation at a specific developmental period. They demonstrated that treatment with this cAMP analog does not increase the number of OLGc expressing MBP until just before the appearance of positive cells for this antigen in control cultures. Cells were no longer responsive to db-cAMP after cultures showed the presence of more mature OLGc. In mixed glial cell cultures established from 1-day-old rat cerebrum, treatment with cAMP analogs during the first week of culture accelerated the onset of expression of myelin components after the stage at which precursors become committed to develop into OLGc (Raible and McMorris, 1989, 1990). We observed that in immature OLGc-4, aTf induced an increase in cAMP levels compared to that in controls, whereas in the more mature OLGc-10 group, it induced no changes. We suggest that the increased maturation of OLGc observed in the pres-

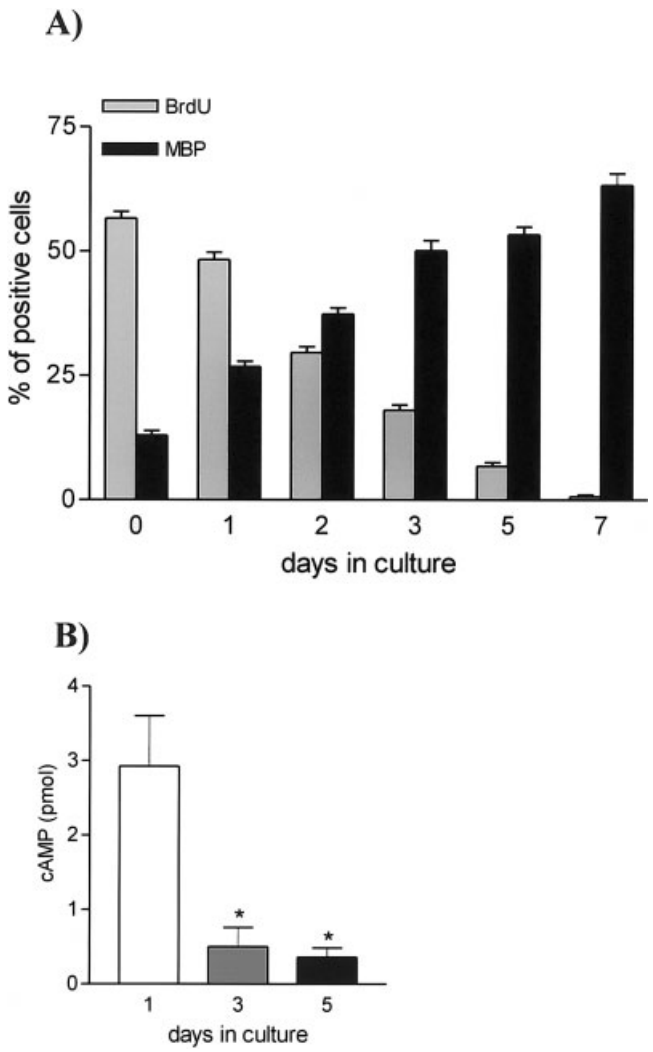

Fig. 8. **A:** OLGc-4 were cultured on coverslips for various periods of time. The cells were fixed and immunostained with anti-BrdU and anti-MBP antibodies. The total number of cells was determined by staining with Hoechst 33342. The percentage of positive cells for each antigen was determined; values are expressed as means ± SEM. **B:** Levels of cAMP in cultures of OLGc-4 at different points after the addition of aTf. cAMP content was determined by competitive protein binding assay. Results are means ± SEM of three independent experiments. $\star P < 0.05$ vs. 1 day in culture.

ence of aTf could be mediated by processes involving cAMP and during a specific developmental stage of these cells. The pattern of CREB expression in OLGc suggests a role for this compound in the developmental period that precedes the peak of myelination in rat brain (Sato-Bigbee and Yu, 1993; Sato-Bigbee et al., 1994, 1999). Moreover, studies in which CREB synthesis was inhibited support the idea that this transcription factor is one mediator of cAMP-dependent stimulation of OLGc differentiation (Sato-Bigbee and De Vries, 1996; Afshari et al., 2001). As mentioned before, CREB phosphorylation could be stimulated by increasing the intracellular levels of cAMP. The rapid and transient increase in CREB phosphorylation found in aTf-treated OLGc-4 suggest that aTf may act, at least in part, through activation of this transcription factor. Contrariwise, changes in cAMP levels or in CREB phosphorylation were not observed in OLGcs-10, which as described previously displayed a more mature phenotype than did OLGcs-4 (Garcia et al., 2003). In addition to these data, we found that in the presence of aTf and using a CRE sequence, there was increased DNA-binding capacity. Taking into consideration the results of the Western blots, it is tempting to assume that this increase in DNA-binding capacity could be mediated by this factor. Because CREB belongs to a large family, however, including several activating transcription factors (ATFs) and cAMP-responsive element modulator (CREM) proteins that bind to a common DNA sequence (CRE), we cannot discard the possibility that the increase mentioned before could be due totally or partially to binding of different transcription factors of this family. The results mentioned above are in close agreement with the findings of Raible and McMorris (1990) indicating that OLGc expressing MBP become unresponsive to cAMP stimulation. The fact that the transferrin receptor (TfR) is no longer present in rat white matter after 7 days of age could be an alternative explanation of why aTf is effective in OLGc-4 but not in OLGc-10. Our previous results clearly indicate that the effects of aTf on the expression of MBP, CNPase, and tubulin are mediated by TfRs, because treatment with an antibody against the TfR blocks increased gene expression induced by treatment with aTf (Marta et al., 2002).

We have considered carefully the possibility that aTf could bind iron present in the incubation medium and that the effects that we observed could be due actually to the in situ generated holoTf. Determination of the iron content in the culture medium used for our studies showed that only ~5% of the apoTf could be saturated with iron, a minute amount that cannot explain our results. In addition, previous in vivo studies from our laboratory have shown that in the presence of deferroxamine, aTf effects do not differ from those obtained with aTf alone. This indicates that effects we observed do not depend on the presence of iron bound to the transferrin molecule (Escobar Cabrera et al., 2000). It has been argued that in reticulocytes and at 4°C, holoTf competes more effectively for TfR than aTf (Young et al., 1984). Our results could therefore be due to minute amounts of holoTf with high binding capacity for the TfR. This is not supported, however, by the findings of Mash et al. (1990), who analyzed the behavior of both compounds in rat brain membranes and showed that their affinity for the TfR is almost identical.

As demonstrated by Pende et al. (1997), Ca^{2+} ions could influence OLGc maturation, because Ca^{2+} is able to act on calmodulin-dependent adenylate cyclase, which in turn may activate cAMP-dependent protein kinase (PKA). All these kinases are responsible for phosphorylation of CREB (Sheng et al., 1991). The pattern of behavior of IP3 in OLGc-4 shows an increase at low aTf concentra-

tions. At high concentrations, aTf was effective in stimulating cAMP, CREB, and OLGc maturation but did not produce changes in IP3. We do not have a reasonable explanation for the response observed in the IP3 levels and further studies should be carried out to clarify this point.

The possible link between OLGc differentiation and cessation of proliferation has been postulated for many years (Casaccia-Bonnefil and Liu, 2003). In agreement with this hypothesis, we have found that in the presence of aTf, there is a drop in proliferation rate in OLG-4, accompanying the appearance of a more mature phenotype.

Mammalian cell cycle progression is regulated by the activity of several cyclin-dependent kinases (CDKs) and their inhibitors (CDKis) (Ohnuma et al., 2001). The involvement of p27, a CDKi acting at the beginning of the G1 phase of the cell cycle, in the response of OLGc to aTf treatment is supported by the present results, which show that aTf induces an increase in the level of p27. The increases in this protein may be a common mechanism controlling the withdrawal from the cell cycle of actively proliferating cells, in response to extracellular signals that lead to an increase in intracellular cAMP levels (Friessen et al., 1997). Apotransferrin could then act as one of the extracellular signals able to promote OLGc maturation through regulation of certain proteins that control the cell cycle.

Although p27 is an important factor participating in cell cycle arrest, it is not an absolute requirement and there are other proteins involved in the exit of OLGc from the cell cycle and in their terminal differentiation. In oligodendrocyte progenitors, arrest of proliferation is regulated mainly at the level of CDK2 (Ghiani and Gallo, 2001; Belachew et al., 2002). This decrease in enzymatic activity of cyclinE/CDK2 could be due to decreased levels of the CDK itself (Casaccia-Bonnefil et al., 1997; Ghiani and Gallo, 2001), decreased cyclin levels (Casaccia-Bonnefil et al., 1997; Tokumoto et al., 2001), or to progressive accumulation of CDK inhibitors such as p27 and p21 (Casaccia-Bonnefil et al., 1997; Durand et al., 1997). Within this context, our observations show that the addition of aTf produces a decrease in cyclin D1 levels in both OLGc-4 and OLGc-10, suggesting that other steps of the cell cycle of OLGc are influenced by aTf. On the other hand, we found that cylin E levels were higher in aTf-treated OLGc-4 whereas no changes occurred in OLGc-10 with respect to the respective controls. Ghiani and Gallo (2001) have shown that early G1 molecules (cyclin D/CDK4) play a key role in permanent cell cycle withdrawal of OLGc. In contrast, cyclin E/CDK2 seems to be involved in both reversible cell cycle arrest and the quiescent state associated with terminal differentiation. This would explain in part our results and it is quite probable that longer exposure times to aTf would decrease cyclin E levels.

We have shown here that cells with a high proliferation rate are more sensitive to aTf. Another possible interpretation of our results could be that as the cells mature (MBP+ cells), they become less responsive to aTf. Previous results from our lab using immunopanning techniques showed that an A2B5-selected OLGc-10 population does not respond to the addition of aTf (Garcia et al., 2003), thus immaturity clearly is not the only condition for the cells to be sensitive to the differentiating effects of aTf. Together with our previous data, the results presented in this work would suggest that cells must be cycling to be responsive to aTf.

The data presented here suggest that the effect of aTf on OLGc-4 could be mediated by an increase in cAMP levels, leading to a decrease in proliferation associated with a rise in p27 and a fall in cyclin D1, which is followed by exit from the cell cycle. Concomitantly, activation of the transcriptional factor CREB could be involved in onset of the differentiation program.

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