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PKA and PKC Activation Induces Opposite Glial Fibrillary Acidic Protein (GFAP) Expression and Morphology Changes in a Glioblastoma Multiform Cell Line of Clonal Origin

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Possible differentiation mechanisms were investigated in a glioblastoma multiform cell line (GL15) presenting an undifferentiated phenotype with weak glial fibrillary acidic protein (GFAP) and strong vimentin (VIM) expression. Serum-free conditions induced time-dependent increases of GFAP-mRNA and GFAP protein levels, associated with a process-bearing astrocytic morphology.

Activation of protein kinase C (PKC) by tumor promoter phorbol 12-myrystate 13-acetate (PMA) induced a rapid morphological differentiation and a decrease in GFAP mRNA, whereas the GFAP level remained unchanged. Such parameters were shown to characterize a physiological differentiation stage in astroglial cultures. Treatment of process-bearing GL15 cells with dibutyryl cyclic AMP (dbcAMP), a protein kinase A (PKA) activator, induced a time-dependent decrease in the GFAP mRNA and GFAP protein levels and reverted morphological changes induced by serum-free conditions. Neither PMA nor dbcAMP influenced the VIM mRNA expression.

In GL15 cells, PKC and PKA activation have opposite effects. Understanding the role of these kinases in malignant transformation and in the in vitro differentiation process is of both basic and clinical interest.

Key words: dbcAMP, GFAP, glioblastoma cell line, PKA, PKC, PMA

INTRODUCTION

Primary astrocyte cultures express two different intermediate filament proteins, glial fibrillary acidic protein (GFAP) and vimentin (VIM; Chiu et al., 1981; Meyer et al., 1989). During central nervous system (CNS) development, the first intermediate filament protein produced by astrocyte precursors (astroblast) is VIM (Levitt and Rakic, 1980; Dahl et al., 1981). GFAP appears afterwards (Eng, 1985; Reske-Nielsen et al., 1987). In vitro, the transition from VIM to GFAP seems to be related to differentiation of the glioblasts into astrocytes (Sensenbrenner et al., 1980; Tardy et al., 1989).

In the adult CNS, GFAP is not apparently expressed by all the astroglial cells, but only in the white matter, thalamus, subpial area, and hippocampus (Kitamura and Watanabe, 1990). Instead, GFAP is always present in reactive glia (Miller et al., 1986; Roessmann and Gambetti, 1986; Miyake et al., 1988). Therefore, GFAP in vivo and in vitro is considered as a marker of differentiated and reactive astrocytes (Eng et al., 1971; Bignami et al., 1972; Lach and Weinrauder, 1978; Roessmann and Gambetti, 1986).

Several authors have reported that in neoplastic astrocytes, GFAP expression is inversely correlated to the degree of anaplasia (Jacque et al., 1979; Kaluza and Adamek, 1990). Others have not found this relation (Herpers et al., 1986). However, a high proportion of cell lines established from malignant gliomas do not or only weakly express GFAP.

Previous studies have demonstrated that in vitro GFAP expression can be affected by two important metabolic pathways, that mediated via protein kinase A (PKA) and that dependent on protein kinase C (PKC). In primary rat astrocyte cultures (Tardy et al., 1981; Pollenz

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and McCarthy, 1986; Le Prince et al., 1991) and in glioma cell lines (Raju et al., 1980; Messens and Slegers, 1992), dibutyryl cyclic AMP (dbcAMP), an activator of PKA, induced differentiated astroglial phenotype transforming flat, polygonal-shape astrocytes into stellate, process-bearing cells. DbcAMP also increased GFAP-mRNA and GFAP protein levels (Sensenbrenner et al., 1980; Shafit-Zagardo et al., 1988; Le Prince et al., 1991; Messens and Slegers, 1992). The effects of tumor promotor and PKC activator phorbol 12-myristate 13-acetate (PMA) on cell morphology and cell growth in astroglial primary cultures and glioma cell lines are rather controversial. Exposure of cultured astrocytes to PMA resulted in differentiated morphology (Mobley et al., 1986; Harrison and Mobley, 1990), inhibition of cell proliferation (Tysnes and Learum, 1993), mitogenic effects on established human glioma cell lines and on astroglial primary cultures (Baltuch et al., 1993; Yong, 1992). Such discrepancies might be due either to the heterogeneity of the PKC family or the different stage of cell maturation.

In this report we examine the mechanisms involved in the differentiation process of an anaplasic glioblastoma cell line (GL 15) (Bocchini et al., 1991) showing an undifferentiated phenotype with weak GFAP and strong VIM expression (Bocchini et al., 1993). We observed that serum deprivation and PMA addition resulted in a differentiated phenotype, whereas dbcAMP induced a regressive effect on these differentiated cells.

MATERIALS AND METHODS

Cell Cultures

Cultures of the GL15 glioblastoma multiform cell line were grown in DMEM medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin G and 100 μg/ml streptomycin. The flasks were incubated at 37°C in a 5% CO^2 humidified atmosphere. The medium was changed twice weekly and the cells were subcultivated when confluent. dbcAMP (Boehringer Mannheim, FRG) and PMA (Sigma, St Louis, MO) were added to confluent GL15 cells after 24 hr growth in serum-free medium. PMA was dissolved in dimethylsulfoxide (DMSO). DMSO controls were included at each experimental time point. The final concentration of DMSO in treated and control cells was 0.01%. GL15 control cells were grown in serum-supplemented or serum-free medium.

Indirect Immunofluorescence

Cells were extensively washed with phosphate-buffered saline (PBS), immersed in cold methanol, kept at −20°C for 5 min and dried in air. The cells were then incubated for 45 min at room temperature with a rabbit anti-GFAP (Dakopatts, Denmark) polyclonal antibody (diluted 1:100 in PBS) and washed in PBS. After treatment with tetramethylrhodamin isothiocyanate (TRITC, Sigma), conjugated goat anti-rabbit IgG (diluted 1:100 in PBS) preparations were observed with a Leitz fluorescence microscope.

RNA Isolation and Northern Blot Hybridization

Total RNA was extracted from GL15 cells according to Chomczynsky and Sacchi (1987) by a single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture. RNA concentrations were evaluated by spectroscopy at 260 nm and their quality examined by minigel analysis.

RNA was fractionated on 0.8% agarose gel containing formaldehyde, as described by Lehrach et al. (1977). Transfer to nitrocellulose sheets was performed in 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7). Hybridization of the nitrocellulose sheets with a ^{32}P-labelled random primed GFAP (Rataboul et al., 1988) or VIM cDNA probe (a gift from Dr. Paulin, Inst. Pasteur) was performed according to Wahl et al. (1979). Finally blots were washed twice for 30 min at 65°C in 0.2× SSC and 0.1% SDS and exposed to Amersham MP-Hyperfilm with Dupont Lightning Plus intensifying screens. Relative amounts of GFAP-mRNA and VIM-mRNA were measured by densitometric analysis of the blot autoradiograms.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting

GL 15 cell cultures were washed with PBS and scraped with 62.5 mM Tris-HCL (pH 6.8), 2 mM EDTA, 0.5% Triton X-100 and 2 mM PMSF, 2.3% SDS and 5% mercaptoethanol and the protein content was determined by the Lowry method (Lowry et al., 1951). The proteins were separated by SDS-PAGE in 10% acrylamide gel by the Laemmli method (1970), then transferred to nitrocellulose filters according to Twobin et al. (1979). The sheets were preincubated overnight in T1 (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) buffer containing bovine serum albumin (BSA) and then incubated at room temperature for 60 min with a rabbit anti-GFAP (Dakopatts, Denmark) polyclonal antibody diluted 1:100 in T1-3% BSA buffer. The sheets were then washed in T1 and after in T1D buffer (T1 plus sodium deoxycholate, Triton X-100, SDS) and incubated at room temperature for 60 min with ^{125}I-labelled donkey anti-rabbit IG (Amersham International, England) diluted 1:160 in T1-3% BSA buffer plus 10% FCS. After washing in T1D and after in T1 buffer, the sheets were exposed to Kodak X-AR 5 X-ray film with Dupont Lightening Plus intensifying screens. Relative amount of GFAP protein was measured by densitometric analysis of the blot autoradiograms.

RESULTS

Morphology and Culture Characteristics of GL15 Cells

GL15 cells were grown in DMEM medium supplemented with 10% FCS. A confluent monolayer was obtained after 3 days. Confluent GL15 cells had a flat, bipolar shape (Fig. 1a), like glioblasts, with some giant cells and very rare clearly differentiated cells. Serum deprivation caused retraction of cytoplasm and important morphological changes, visible after 3 days (Fig. 1d), culminating in a stellate differentiated morphology with very long processes. These results were confirmed in chemically defined medium. When PMA was added (10, 100 nM) to confluent 24 hr serum-deprived cultures (Fig. 1b), rapid morphological changes appeared as soon as 3 hr after PMA addition (Fig. 1c). This effect increased until 24 hr when compared to control cells grown in serum-free medium. PMA induced a retraction of the cell body and the appearance of stellate morphology with thin processes, accelerating the morphological differentiation determined by serum deprivation. Instead, dbcAMP concentrations of 0.5, 1, 2 mM in serum-free medium and different growth phases prevented each morphological change (Fig. 1e). This effect was observed after 48 hr or chronic (9 days) treatment. When dbcAMP was added to serum-free differentiated GL15 cells, the morphology returned to the undifferentiated form.

Immunochemical Detection of GFAP

GFAP immunofluorescence was evaluated in confluent GL15 cells grown in serum-supplemented or serum-free medium and after the action of dbcAMP and PMA. In serum-supplemented medium, the percentage of GFAP-positive cells was low and the immunofluorescence localized in the centriolar and perinuclear regions with weaker positivity in the cellular body (Fig. 2a). Very rare cells showed a more intense labelling (Fig. 2a). When GL15 cells were grown in serum-free medium, there was an important time-dependent increase in the GFAP labelling, evident after 3 days. A greater number of cells were GFAP-positive with strong labelling along each process and in the cell body (Fig. 2b,d). Serum starvation induced a typical immunolabelling of differentiated astrocytes.

GL15 cells grown in serum-free medium with 0.5, 1, 2 mM dbcAMP prevented every increase in GFAP immunolabelling. A time-dependent decrease in the number of GFAP-positive cells was noted, as well as a trend to induce typical GFAP labelling of immature astroglial cells (Fig. 2c,e). In serum-supplemented medium, no effect due to dbcAMP was noted. The action of dbcAMP was independent of GL15 growth-phase. The same results were obtained either at confluence or at logarithmic phase of growth.

The time-dependent differentiation induced by serum starvation was also induced by PMA (10, 100 nM). In serum-free medium, after 3 hr of PMA addition, a small increase in GFAP immunolabelling was observed and this increased until 48 hr (Fig. 3a,c), when compared to GL15 cells grown in serum-free medium (Fig. 3b,d). PMA, as serum deprivation, induced an overexpression of GFAP, but it was limited in time.

Northern Blot Analysis of the GFAP mRNA

Northern blot hybridization was performed with a ^{32}P-labelled cDNA probe that recognized a single GFAP transcript of 3 kb. GFAP-mRNA was detected in GL15 cells grown in serum-free medium and after stimulation by PMA (100 nM) and dbcAMP (1 mM). In GL15 grown in serum-supplemented medium, the GFAP-mRNA expression was very low (Bocchini et al., 1993) and was not increased by dbcAMP treatment (data not shown). Serum-free medium determined an overexpression of GFAP-mRNA and the increase was evidenced first after 27 hr of deprivation and continued until 7 days (Fig. 6a). A longer time period was not analysed.

When PMA was added to the culture medium, after 24 hr growth in serum-free medium, GL15 displayed a dramatic decrease in GFAP-mRNA, detectable at 3 hr but reaching extremely low values at 24 hr (Fig. 4a).

Regarding immunolabelling, the rise in GFAP-mRNA induced by serum deprivation was again inhibited by dbcAMP. At 3 days, the GFAP-mRNA values of dbcAMP-treated cells was 10-fold lower than the respective controls (Fig. 4b) and the decrease reached values 20-fold lower than controls at 7 days (Fig. 4b), at which time the values of dbcAMP-treated cells and GL15 cells grown in serum supplemented medium were about the same.

In GL15 cells, both PMA and dbcAMP decreased the GFAP-mRNA. PMA determined a rapid GFAP-mRNA fall, whereas the effect of dbcAMP took longer to appear.

We analysed the VIM-mRNA for internal control. Treatment with 100 nM PMA for 3, 8, 16, 24 hr and 1 mM dbcAMP for 3, 5, 7 days did not alter the VIM-mRNA expression (data not shown).

Western Blot Analysis of the GFAP

GFAP was analysed by Western blotting of total cell proteins. Blots were incubated with a rabbit anti-GFAP antibody and ^{125}I-labelled donkey antirabbit antibody. In GL15 cells grown in serum-supplemented medium, GFAP expression was weak, but in correlation with immunocytochemistry and Northern blot. Serum deprivation caused a 5 to 6-fold GFAP increase (Fig. 6b)

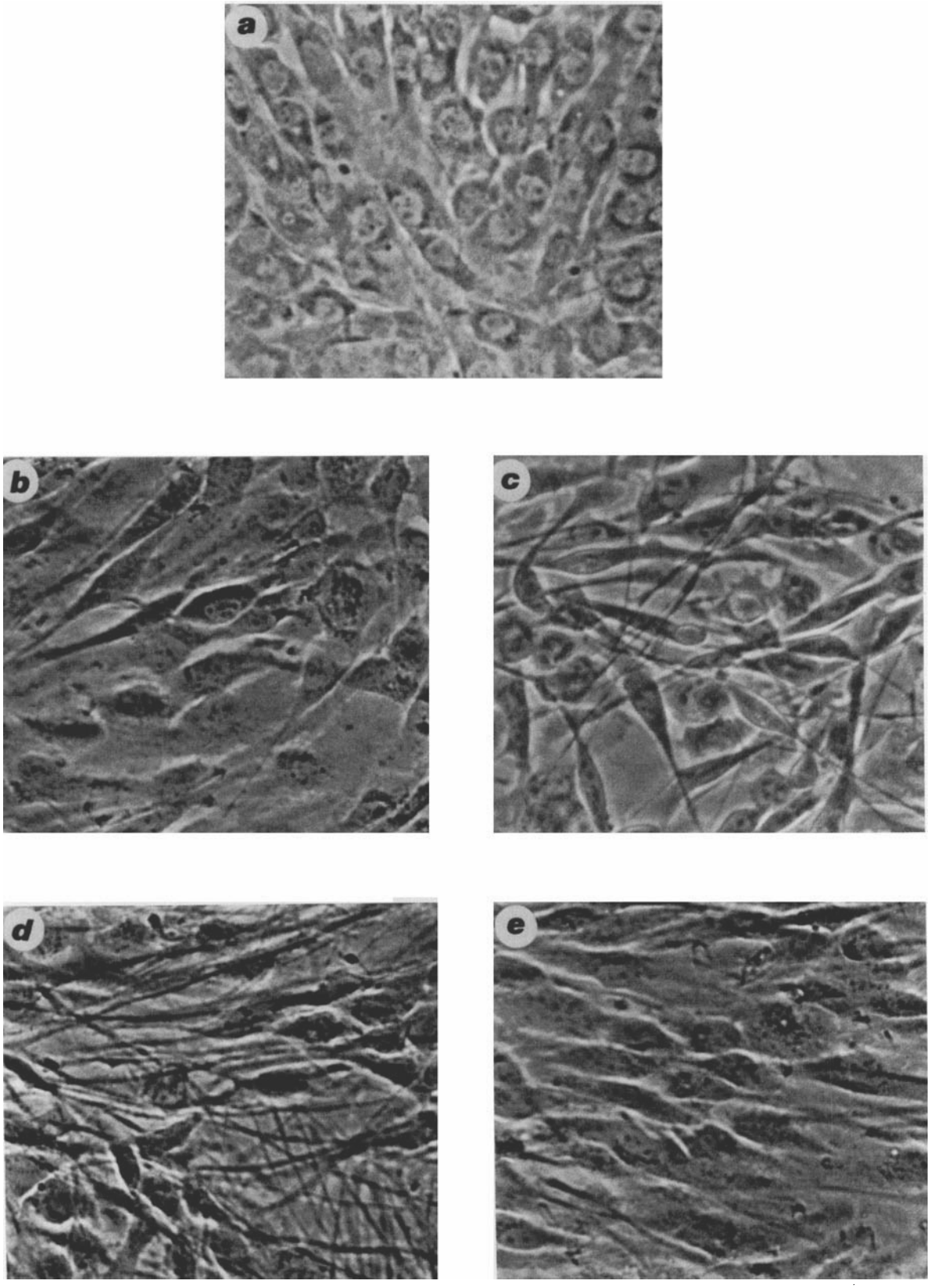
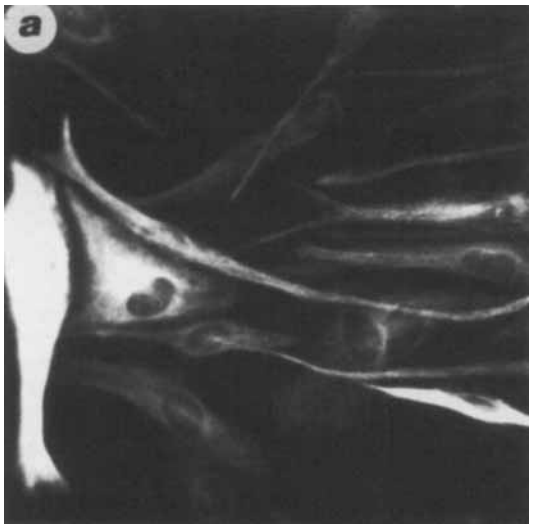
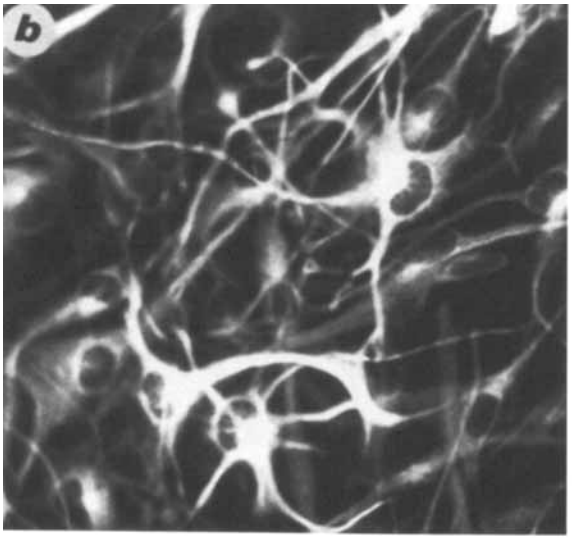
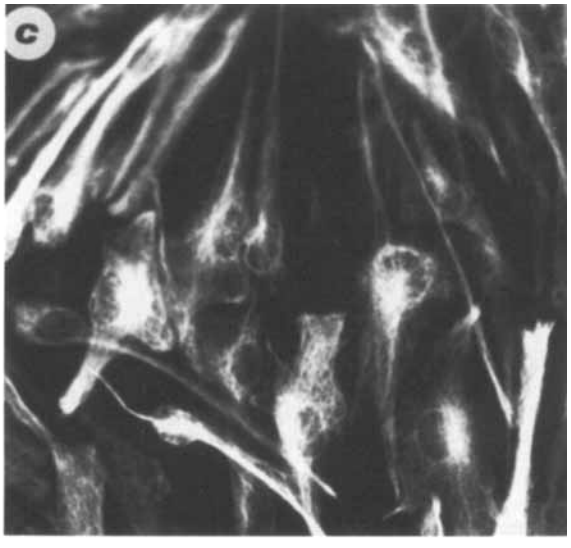
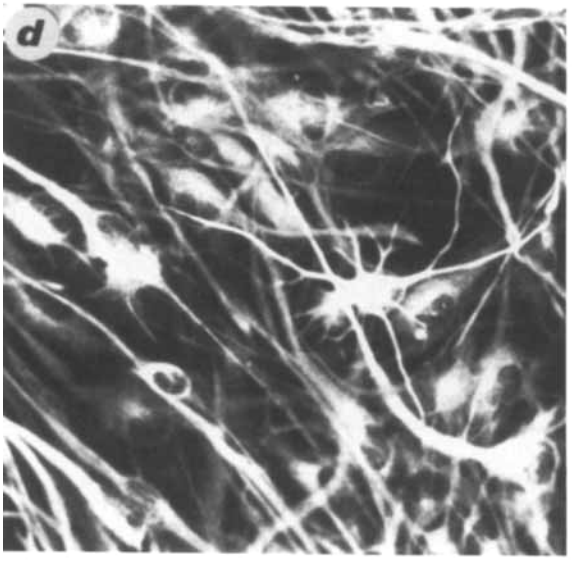


Fig. 1. Morphology of GL15 cells grown in serum-supplemented medium and serum-free medium. **a:** Photograph of cells grown in DMEM supplemented with 10% fetal calf serum. **b:** Photograph of cells at confluence after 24 hr of growth in serum-free medium. **c:** Photograph of cells grown in serum free medium for 24 hr supplemented with 100 nM PMA for 3 hr. **d:** Photograph of cells at confluence after 4 days growth in serum-free medium. **e:** Photograph of cells grown in serum-free medium for 24 hr supplemented with 1 mM dbcAMP for 3 days. $\times 200$.









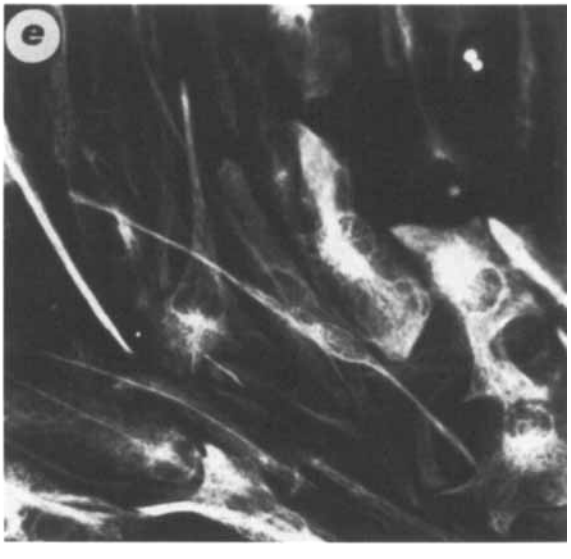


Fig. 2. Immunofluorescence staining of glial fibrillary acidic protein (GFAP). **a:** GL15 cells grown in DMEM supplemented with 10% fetal calf serum. **b,d:** GL15 cells after 3 and 5 days, respectively, growth in serum-free medium. **c,e:** GL15 cells grown in serum-free medium supplemented with 1 mM db-cAMP for 3 and 5 days, respectively. ×400.

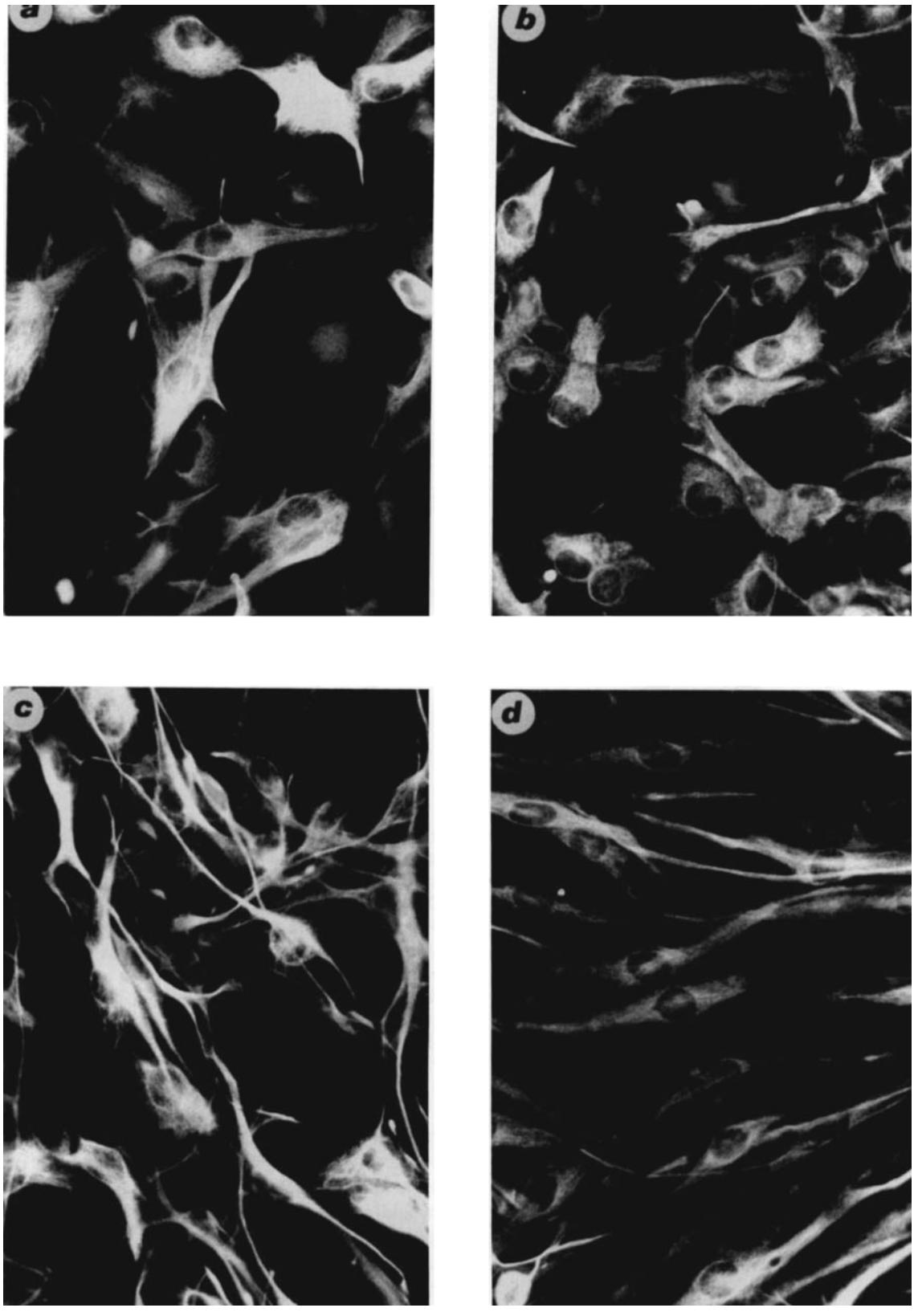


Fig. 3. Immunofluorescence staining of glial fibrillary acidic protein (GFAP). **a,c:** GL15 cells grown in serum-free medium for 24 hr supplemented with 100 nM PMA for 3 hr and 16 hr, respectively. **b,d:** GL15 control cells grown in serum-free medium. ×400.

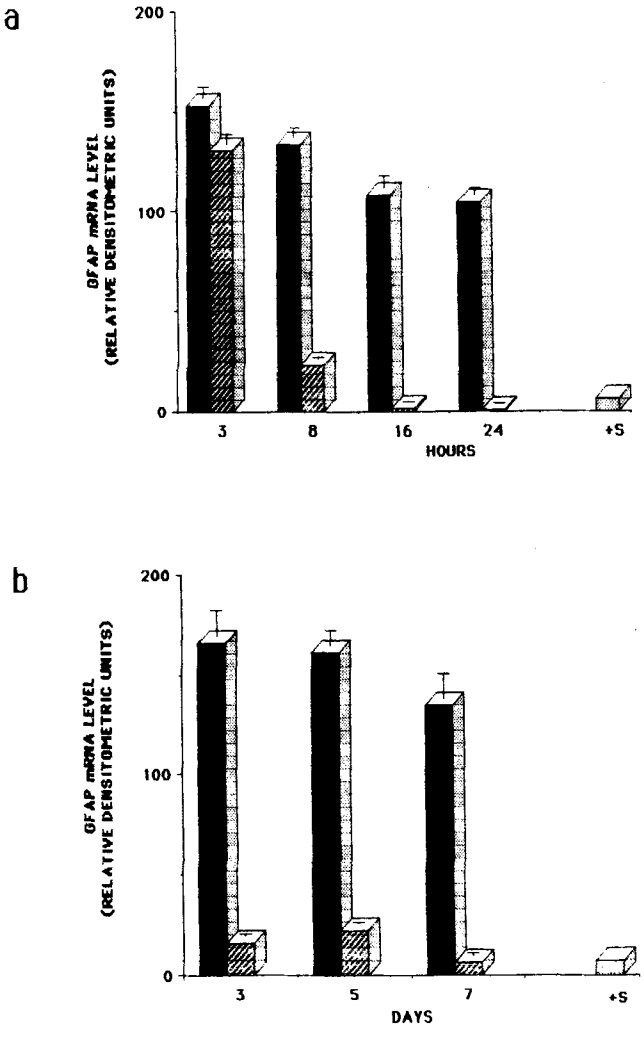


Fig. 4. Expression of GFAP-mRNA in GL15 cells after PMA (**a**) and dbcAMP (**b**) treatment. Results were expressed as means ± SEM. of three different experiments, with samples containing 30 μg total RNA. **a:** GL15 cells cultured without (solid bars) fetal calf serum (control) and supplemented with 100 nM PMA (hatched bars). **b:** GL15 cells cultured without (solid bars) fetal calf serum (control) and supplemented with 1 mM dbcAMP (hatched bars).

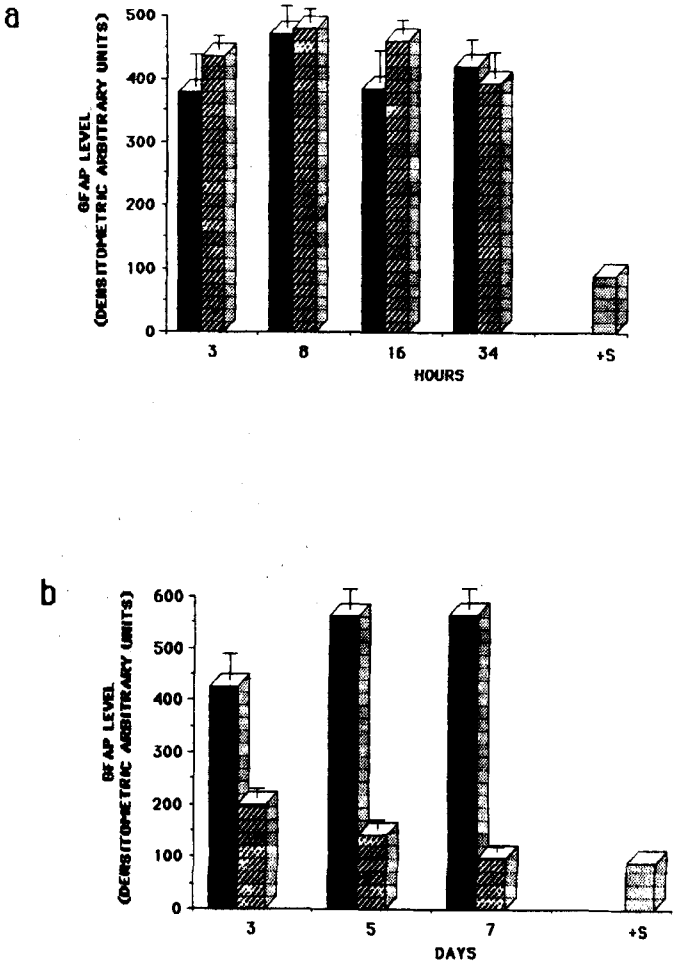


Fig. 5. Densitometric analysis of Western blot autoradiographs of GFAP expression in GL15 cells after PMA (**a**) and dbcAMP (**b**) treatment. Results were expressed as means ± SEM. of three different experiments, with samples containing 7.5 μg of protein. **a:** GL15 cells grown in serum-free medium (solid bars; control) and supplemented with 100 nM PMA (hatched bars). **b:** GL15 cells grown in serum free medium (solid bars; control) and supplemented with 1 mM dbcAMP (hatched bars).

which remained high up to 8 days. PMA and dbcAMP influenced the GFAP protein differently. PMA (100 nM) did not significantly alter the GFAP level during 48 hr, (Fig. 5a), whereas dbcAMP (1 mM) induced a time-dependent decrease in GFAP. During the 7 days of dbcAMP treatment, the GFAP values returned to those determined in GL15 cells grown in serum-supplemented medium (Fig. 5b).

DISCUSSION

In glial tumors of different anaplasia stages, the number of GFAP-positive cells is inversely related to the degree of malignancy (Kaluza and Adamek, 1992). In agreement with these data, GL15 glioblastoma cells display a weak GFAP positivity with an immature phenotype and a high undifferentiated morphology. The presence of GFAP-positive and negative cells can be related to different growth rates (Kajiwara et al., 1992). This variability in antigen expression and cell proliferation capacity has been proposed as a manifestation of tumor heterogeneity. Such heterogeneity is expressed in the GL15 cell line of clonal origin which displays a mosaic expression of GFAP-positive cells (Bocchini et al.,

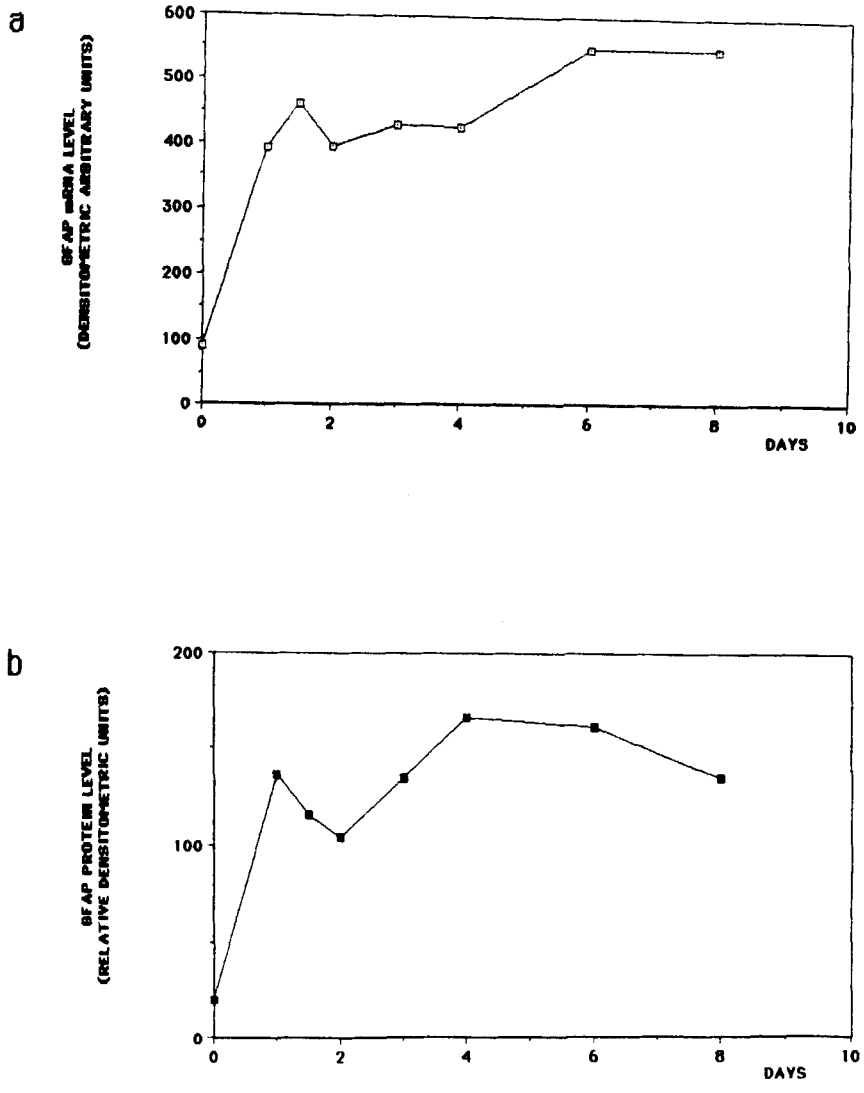


Fig. 6. Densitometric analysis of Northern and Western blot autoradiographs. Effect of serum starvation on GFAP-mRNA (**a**) and GFAP protein (**b**) levels as a function of culture time. Results were expressed as means ± SEM. of three experiments. At time zero, the cells were changed in serum-free medium. No other change was done. These results were confirmed in chemically defined medium.

1993), about 30% of the cell population, and mimics the heterogeneity of the tumors themselves.

GL15 grown in serum-free medium undergoes evident morphological changes. Flat polygonal cells transform into process-bearing cells with a retraction of their cytoplasm and the formation of thin, numerous processes. These morphologically differentiated cells were evident 3 days after the serum deprivation and the processes were more numerous in the following days. The mature astrocytic morphology, highly and homogeneously GFAP-immunolabelled, was associated with high levels of GFAP-mRNA and GFAP protein. The complex network of labelled astroglial processes, obtained by serum deprivation, indicates that serum-responsive elements are involved in inhibiting this spectacular morphological change and associated GFAP expression.

In GL15 cells grown in serum-free medium, the correlation between GFAP-mRNA and GFAP protein levels are in good agreement with a transcriptional control of GFAP gene expression.

In the presence of PMA, a rapid, time-dependent change in GL15 cell morphology was observed. A more differentiated morphology was visible as early as 3 hr after PMA addition when the cells had been cultured in the absence of serum for 24 hours. PKC activation accelerated the process of morphological differentiation in-

duced by serum deprivation. However, these very differentiated cells were able to survive only for few days.

These results indicate that even highly undifferentiated glioma cells maintain, at least in vitro, the capacity to switch to a differentiated state in response to both serum deprivation and PKC activation. Such a concomitant rise in GFAP-mRNA and GFAP protein has been described in astrocyte primary cultures and in mouse brain during the postnatal proliferation stage followed by a progressive decline of the GFAP-mRNA and a stabilized GFAP level (Tardy et al., 1989). Serum deprivation might push GL15 cells towards transition from proliferation to differentiation and PMA to the maturation stage, observed both in astroglial primary cultures and mouse brain tissue.

Previous reports demonstrated that dbcAMP treatment of astrocytes in primary cultures changed their polygonal flat morphology into process-bearing cells. This morphological differentiation is associated either with a phosphorylation of the GFAP protein (Neary et al., 1987; Harrison and Mobley, 1991) or transcriptional regulation of its expression (Le Prince et al., 1991). Rat C6 glioma cells are sensitive to PKA activation by dbcAMP. They change their morphology (Raju et al., 1980) and upregulate their GFAP expression (Messens and Slegers, 1992). Transacting factors as well as cis regulatory elements have been proposed as regulators of the GFAP gene expression. One of these transacting factors is a cAMP-responsive element (CRE) which might be directly responsible for the rise in this GFAP transcriptional rate (Miura et al., 1990).

When dbcAMP was added to GL15 cells grown in serum-supplemented medium, GFAP did not increase labelling or expression, nor was there a clearly differentiated phenotype even after 8 days of treatment. Short and chronic dbcAMP treatment was also ineffective in increasing GFAP expression in adult human (Osborn et al., 1981) and rodent (Pilkington et al., 1983) glial cultures and immortalized astrocyte cells lines (Groves et al., 1993).

Thus, in GL15 cell line, some transacting factors (Miura et al., 1990) might either be absent or do not act in the right way.

dbcAMP added to the serum-deprived or PMA-treated cells induced regression in both the morphological phenotype and the GFAP expression. This is the first evidence, in the astrocytic lineage, that demonstrates dbcAMP behaving as a dedifferentiation factor. Moreover, dbcAMP treatment of GL15 cells determined a decrease in the glutamine synthetase (GS) expression. GS is considered a biochemical marker of mature astrocytes and it has been shown that dbcAMP increases GS expression in rodent astrocyte primary cultures and C6 glioma cells.

The dedifferentiation action of dbcAMP may be explained by a common transcriptional factor (Foulknes et al., 1991) which could block the expression of GFAP and GS induced by cAMP in normal astrocytes and some cell lines.

Finally, in GL15 cells, serum deprivation first and then PKC activation induced a differentiation mechanism parallel to that observed in astrocyte primary cultures with a change in the GFAP gene regulation. The serum deprivation increased the transcriptional rate of GFAP gene, and PKC activation determined a translational and posttranslational control of GFAP expression as observed during mouse brain maturation (Riol et al., 1992). The PKA activation induced a biochemical and morphological regression mechanism.

Therefore, in GL15 cells these kinases act through different and opposite mechanisms. Alterations in the PKA signalling system are evident. Further investigations are necessary to define these abnormalities and to determine their role in the transformation mechanisms.

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