Vol. 148, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1987 Pages 350-356

INDUCTION OF THE R_I cAMP-BINDING PROTEIN IN THE "NEURITE-MINUS" N1A-103 MOUSE NEUROBLASTOMA CELL LINE

Alice Y.-C. Liu, V. Kamalakannan, and Zheng Lin

Department of Biological Sciences, Rutgers University - Busch Campus, Piscataway, N.J. 08855

Received August 3, 1987

SUMMARY We examined the effects of agents/conditions that raise intracellular cAMP concentration in the induction of the 47,000-dalton R_I cAMP-binding protein in the "neurite-minus" N1A-103 mouse neuroblastoma cells. The amount of R_I in cell extracts was quantitated by photoaffinity labeling with 8-N_3-[^{32}P]cAMP and by ELISA and Western blot. Our results showed that treatment of the N1A-103 neuroblastoma cells with 20 μM forskolin or 1 mM dibutyryl cAMP, or reducing the serum concentration in the culture medium from 10% to 1% caused a 3-4 fold increase in the amount of R_I. Our results suggest that the induction of R_I can occur independent of morphological differentiation in the mouse neuroblastoma cells.

The addition of cAMP analogues or agents which increase intracellular cAMP concentration promotes differentiation of mouse neuroblastoma cells as characterized by neurite outgrowth, increase in enzymes involved in the synthesis and metabolism of neurotransmitters, and enhanced membrane excitability (1). We and others previously reported on the induction of a 47,000-dalton R_I cAMP-binding protein in neuroblastoma cells prompted to differentiation either by treatment with 1 mM dibutyryl cAMP or by serum withdrawal (a condition that increases cellular cAMP concentration) (2-6). To gain a better understanding of the regulation of expression and the functional role of this R_I cAMP-binding protein in neuroblastoma cell differentiation, we examined the effects of cAMP in the induction of the R_I cAMP-binding protein in a "neurite-minus" clonal derivative of the C-1300 mouse neuroblastoma tumor, the N1A-103 mouse neuroblastoma cell line (7).

MATERIALS AND METHODS

Materials. All tissue culture supplies were from Gibco Co, Grand Island, N.Y. Dibutyryl cAMP, histone II AS were from Sigma Chem. Co., St. Louis, MO. Forskolin was from Calbiochem., San Diago, CA. 8-N_3-[^{32}P]cAMP and [r^{32}P]ATP were from ICN, Irvine, CA. Goat anti-rabbit IgG-horse raddish peroxidase conjugate was from BioRad, Richmond, CA. Other chemicals were of reagent grade unless specified otherwise.

0006-291X/87 $1.50

Cell Culture. A starter culture of the N1A-103 mouse neuroblastoma cell line (7) was obtained from Dr. M. Nirenberg's laboratory at NIH. Cells were grown as monolayers in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS). To evaluate the effects of serum deprivation, cells were cultured in medium supplemented with 1% FBS. Where indicated, test agents (e.g. dibutyryl cAMP) were added to designated plates of cells approximately 4-12 hrs after seeding the cells. All cells were harvested at an early stationary phase of growth. A 100,000 X g supernatant fraction of the cell homogenate (cytosol) was used to assay for the amount of R_I by photoaffinity lebeling with 8-N_3-[^{32}P]cAMP and immuno-quantitation by ELISA and Western blot.

Quantitation of R_I by photoaffinity labeling with 8-N_3-[^{32}P]cAMP

Photoactivated incorporation of 8-N_3-[^{32}P]cAMP into cytosolic proteins was carried out according to methods previously described using 1 μM concentration of the ligand (4, 5). [In dose response studies, 1 μM 8-N_3-[^{32}P]cAMP has been shown to be sufficient to allow for maximal labeling of both the 47,000-dalton R_I and the 52-54,000-dalton R_{II} (4).]

Quantitation of R_I by ELISA and Western blot

An antiserum was raised against the 47,000-dalton regulatory subunit of the type I cAMP-dependent protein kinase, purified from rat skeletal muscle (8, 9), according to methods described (10). This antiserum was used to quantitate the amount of R_I in cell extracts in both the ELISA and Western blot methods.

In the ELISA protocol, aliquots of cytosol preparations or the purified R_I protein (in a volume of 100 μl) were incubated with 100 μl of a protein A-purified, 1:1600 diluted antiserum against the R_I protein for overnight at room temperature. The mixture was then carefully transferred to individual wells of a 96-well microtiter plate, each well pre-coated with 5 ng of the purified R_I protein, and incubated at 37°C for 2 hrs. The reaction mixture was then decanted and the microtiter plate was rinsed 5X with a buffer containing 125 mM borate (pH 8.2), 140 mM NaCl, 0.05% Tween 20, and 0.05% Triton X-100 (BTT). A 100 μl aliquot of a 1:1000 diluted goat anti-rabbit IgG-horse raddish peroxidase conjugate (BioRad) was added to individual wells and incubated at 37°C for 1 hr. After pouring off the supernate and rinsing the wells with borate buffer, the peroxidase activity on the microtiter well was determined spectrophotometrically ($O.D._{490}$) using O-phenylenediamine as the chromagen (11). The amount of cytosol protein needed to give a 50% inhibition of the binding of antibody to microtiter wells pre-coated with R_I was used to estimate the concentration of R_I in that sample; a competition curve generated with known amounts of purified R_I in solution against R_I coated on the microtiter well was used as the standard.

A modified version of the Western blot technique (6, 12) was also used to quantitate the amount of R_I in cell extracts. Aliquots of cytosol preparation, containing 200-300 μg protein, and known amounts of the purified R_I from rat skeletal muscle were applied to sample wells of an SDS-polyacrylamide slab gel (4, 5). Upon completion of the electrophoresis procedure, proteins were electrophoretically transferred to a sheet of nitrocellulose membrane. The membrane was then incubated in Tris-buffered saline (TBS; 20 mM Tris, pH 7.5, 500 mM NaCl) containing 10% non-fat dry milk and 1% bovine serum albumin (from here on referred to as the blocking medium) for 30 min at room temperature to saturate non-specific binding sites. The membrane was rinsed once with TBS and then probed with a 1:100 dilution of the rabbit antiserum aganist R_I in the blocking medium that contained 0.05% Tween 20 for 15 hrs at 4°C. The membrane was extensively washed and incubated with a 1:1000 dilution of goat anti-rabbit IgG-horse raddish peroxidase conjugate (BioRad) in the blocking

medium containing 0.05% Tween 20 and 20% goat serum for 1 hr at room temperature. The peroxidase activity on the nitrocellulose membrane was then developed using 4-chloro-1-napthol as the color indicator, according to procedures described in the BioRad Immun-Blot assay manual.

Histone kinase assay

Cytosol from neuroblastoma cells was dialyzed overnight against 1,000 X volume of a buffer containing 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, and 50 µg/ml phenylmethylsulfonyl fluoride. The dialyzed cytosol was used to assay for cAMP-dependent phosphotransferase activity according to the methods of Witt and Roskoski (13) as previously described (5).

RESULTS AND DISCUSSION

Results in Figure 1 on the photoaffinity labeling of cAMP-binding proteins by 8-N_3-[^{32}P]cAMP demonstrated the presence of the 47,000-dalton R_I and 54,000-dalton R_{II} cAMP-binding proteins in extracts of the N1A-103 mouse neuroblastoma cells, with R_I being the predominant species. Treatment of the cells with 20 µM forskolin or 1 mM dibutyryl cAMP, or depriving the cells of serum (DMEM with 1%, not 10%, FBS) caused a 3-4 fold increase in the incorporation of 8-N_3-[^{32}P]cAMP into the 47,000-dalton R_I protein. The amounts of 8-N_3-[^{32}P]cAMP incorporated into R_I of control, 20 µM forskolin-, 1% FBS-, and 0.2 and 1 mM dibutyryl cAMP-treated N1A-103 neuroblastoma cells were 2.8,

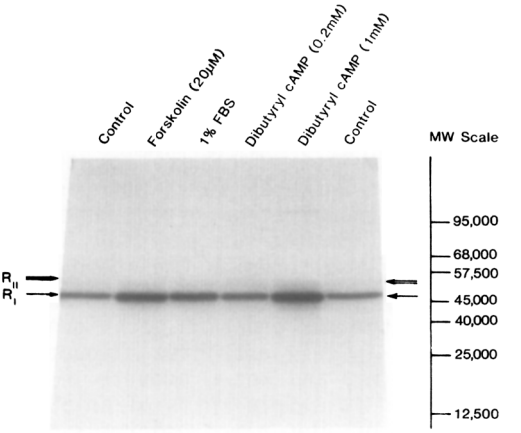

Figure 1. Autoradiogram illustrating the incorporation of 8-N_3-[^{32}P]cAMP into cytosol proteins of the control, 20 µM forskolin-, 1% FBS-, and 0.2 and 1 mM dibutyryl cAMP-treated N1A-103 mouse neuroblastoma cells. Cells were plated in 100 mm dishes and the test agests were added 4 hrs after seeding the cells. Groups of 5 X 100 mm plates of cells were used for each of the culture conditions. The incorporation of 8-N_3-[^{32}P]cAMP into cytosol protein was done under standard conditions (4, 5) using 200 µg cytosol protein and 1 µM 8-N_3-[^{32}P]cAMP (specific activity 5 Ci/mmol). The arrows identify the positions of the 47,000-dalton R_I and the 54,000-dalton R_{II} on the gel.

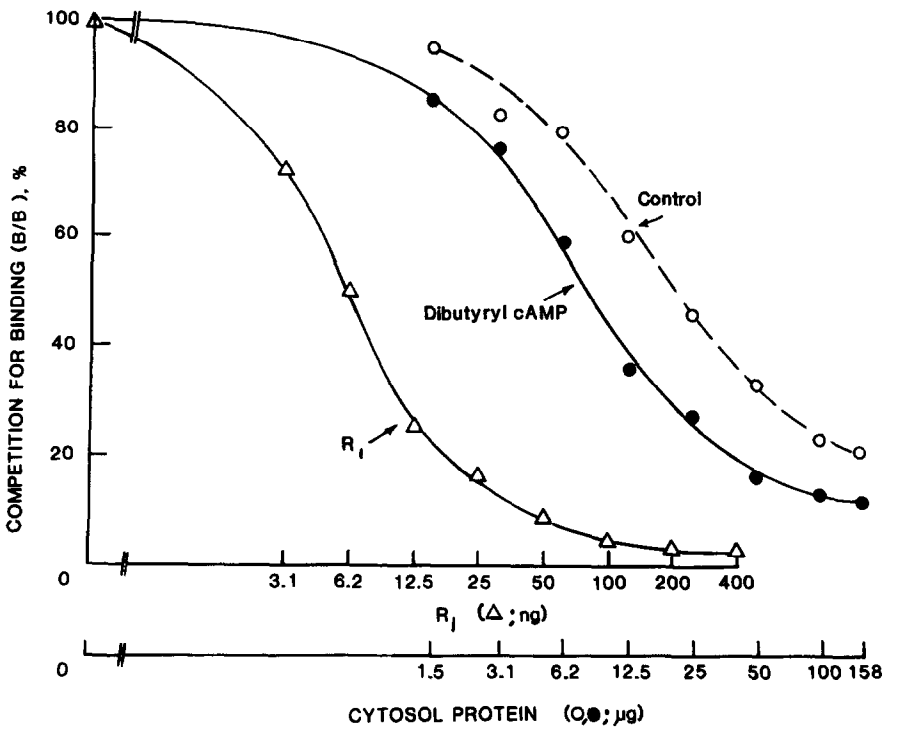

Figure 2. ELISA of R_I in cytosols of control and 1 mM dibutyryl cAMP-treated N1A-103 mouse neuroblastoma cells. The procedures are described in detail in the text. Results illustrate the inhibition of binding of anti-R_I antiserum to microtiter wells pre-coated with 5 ng of R_I by either the purified R_I protein (△) or R_I in cell extracts (○,●). The amounts of purified R_I and cytosol protein needed to give 50% inhibition of binding of the antibody were used to calculate the amount of R_I in cell extracts. Using this method, we determined that the amount of R_I in control and dibutyryl cAMP-treated N1A-103 mouse neuroblastoma cells were 7 pmol/mg (0.0337%) and 18.5 pmol/mg (0.088%), respectively. Bo is the OD_{490} obtained when the R_I antiserum was preincubated with buffer alone; B is the OD_{490} when the antibody was preincubated with either purified R_I or cell extracts.

7.7, 6.2, 3.9, and 8.7 pmol/mg protein, respectively. There was no significant difference in the amount of 8-N_3-[^{32}P]cAMP incorporated into R_{II}.

To affirm results obtained by labeling R_I with 8-N_3-[^{32}P]cAMP, we quantitated the amount of R_I in cell extracts by ELISA and Western blot using an antibody against the 47,000-dalton regulatory subunit of the type I cAMP-dependent protein kinase purified from rat skeletal muscle. The ELISA method (Figure 2), based on inhibition of binding of the antibody to microtiter wells pre-coated with purified R_I by R_I present in cell extracts, gave estimates of 7 and 18.5 pmol of R_I per mg cytosol protein for the control and dibutyryl cAMP-treated N1A-103 cells, respectively; i.e. dibutyryl cAMP caused a 2.6 fold increase in the amount of R_I.

Similar results were obtained by the Western blot technique (Figure 3). The amount of R_I in extracts of the control, forskolin- and dibuturyl

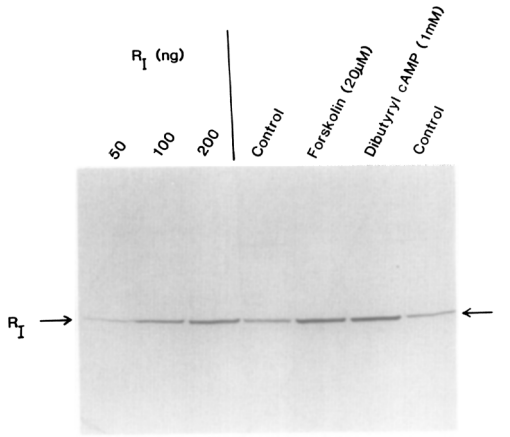

Figure 3. Quantitation of R_I by Western blot. 50, 100, and 200 ng of the purified R_I protein and aliquots of cytosol preparations, containing 300 μg protein, from control, 20 μM-forskolin-, and 1 mM dibuturyl cAMP-treated N1A-103 neuroblastoma cells were subjected to Western blot analysis according to methods described in the text. The position of the 47,000-dalton R_I is indicated by an arrow. The amount of R_I in cytosol of the control, forskolin-, and dibutyryl cAMP-treated cells were 80, 280, and 290 ng per 300 μg cytosol protein, respectively; translating to 5.6, 19, and 20.6 pmol/mg protein, respectively.

cAMP-treated N1A-103 neuroblastoma cells were 5.6, 19, and 20.6 pmol/mg protein, respectively. The Western blot technique also detected a high molecular weight protein in the control cell extract; the identity of this protein is not known.

Together, these results provide firm evidence of an increase in the R_I cAMP-binding protein in the "neurite-minus" N1A-103 cells treated with agents that elevate cellular cAMP concentration. It should be noted that the amount of R_I quantitated by the ELISA and Western blot methods was approximately 2-3 times higher that that obtained by photoaffinity labeling of R_I with $8\text{-}N_3\text{-}[^{32}P]$cAMP.

In our previous studies, we have demonstrated identity of the 47,000-dalton R_I protein in mouse neuroblastoma cells and the 47,000-dalton regulatory subunit of the type I cAMP-dependent protein kinase (5). The results in Figure 4 demonstrated that the increase in R_I in extracts of the dibutyryl cAMP-treated N1A-103 cells was not associated with an increase in the cAMP-dependent phosphotransferase activity; in fact, the activity of the dibutyryl cAMP-treated cells was lower than that of the control. The result is consistent with that of our previous observations in the N-18 mouse neuroblastoma cells; together, they suggest that the expression of the

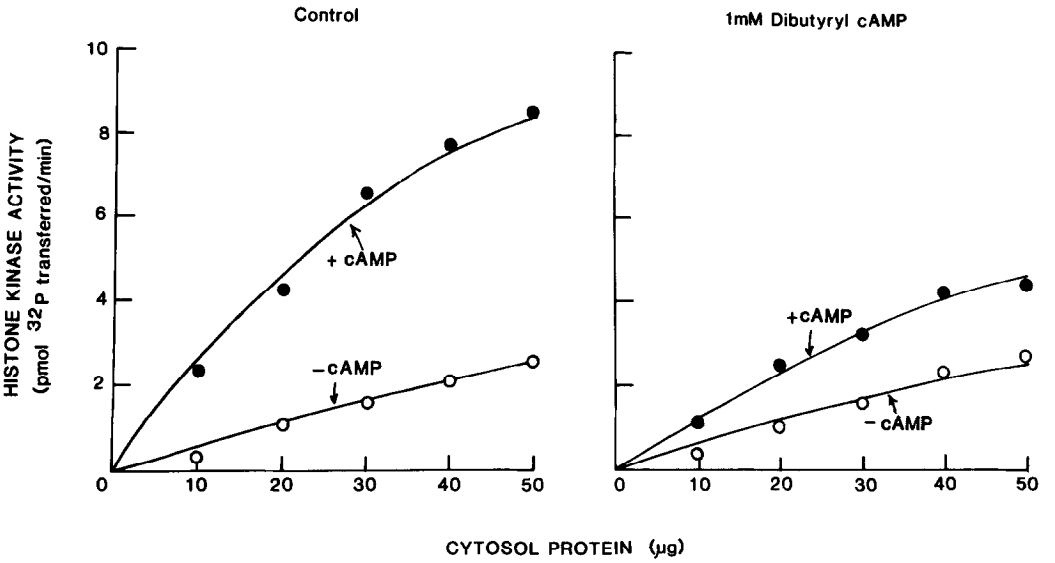

Figure 4. cAMP-Dependent protein kinase activity present in cytosol of the control and 1 mM dibutyryl cAMP-treated N1A-103 neuroblastoma cells. The assay mixture (final volume 100 µl) contained 50 mM 2(N-morpholino)ethane sulfonate (pH 6.2), 10 mM $MgCl_2$, 40 µg histone II AS, 40 µM [r^{32}P]ATP (specific activity 1-5 X 10^5 cpm/nmol), and various amounts of cytosol protein as indicated. The reaction was carried out for 10 min at 30°C in the absence (open circles) and presence (closed circles) of 5 µM cAMP. The amount of radioactivity incorporated into histone II AS was determined by the method of Witt and Roskoski (13) as previously described (5).

regulatory and catalytic subunit proteins of cAMP-dependent protein kinase may be differentially regulated, and that the R_I protein may serve a function distinct from that of the regulation of catalytic subunit activity of cAMP-dependent protein kinase.

The neurite-minus phenotype of the N1A-103 cells was absolute. We have not been able to evoke neurite extension in the cells using a range of concentration and various combinations of cAMP-analogues, activators of adenyl cyclase, and inhibitors of phosphodiesterase (e.g. 1 mM dibuityryl cAMP plus 0.5 mM 3-isobutyl-1-methyl xanthine; 20 µM forskolin plus 0.5 mM IBMX). In some cases, the cells flattened somewhat; but in no case did we observed neurite outgrowth (defined as processes with a length that is at least twice the diameter of the neuroblastoma soma, i.e. > 40µm) from the N1A-103 cells.

The observation that analogues of cAMP or agents that increase intracellular cAMP concentration can induce R_I in the neurite-minus N1A-103 mouse neuroblastoma cells in a manner similar to that observed in the neurite-positive cell lines (4, 5) suggests that the induction of R_I can occur independent of neurite extension; that the increase in expression of R_I is not a simple consequence of morphological differentiation in the mouse

neuroblastoma cells. In other experiments, we showed that neurite extension can occur without a concomitant increase in the R_I cAMP-binding protein in NS-20 and N-18 mouse neuroblastoma cells (Liu et al., manuscript in preparation). These results, together with the differential inhibition of neurite outgrowth and induction of R_I cAMP-binding protein by phorbol-12-myristate-13-acetate suggest divergent pathways of cAMP action in evoking neurite outgrowth and induction of the R_I cAMP-binding protein.

ACKNOWLEDGEMENTS

This work was supported by grants CA 39667 from the National Cancer Institute, DHHS, and 84-17775 from National Science Foundation. Z. Lin is a Busch Post-doctoral Fellow.

REFERENCES

1. Prasad, K. N. (1977) In Cell, Tissue, and Organ Cultures in Neurobiology. (S. Fedoroff and L. Hertz, eds), pp. 447-483, Acad. Press, N.Y.
2. Prashad, N., Rosenberg, R. N., Wischmeyer, B., Ulrich, C. and Sparkman, D. (1979) Biochem. 18, 2717-2725.
3. Walter, U., Costa, M. R. C., Breakefield, X. O. and Greengard, P. (1979) Proc. Natl. Acad. Sci., U.S.A. 76, 3251-3255.
4. Liu, A. Y.-C., Fiske, W. W. and Chen, K. Y. (1980) Cancer Res. 40, 4100-4108.
5. Liu, A. Y.-C., Chan, T. and Chen, K. Y. (1981) Cancer Res. 41, 4579-4587.
6. Lohmann, S. M., Schwoch, G., Reiser, G., Port, R. and Walter, U. (1983) EMBO Journal 2, 153-159.
7. Amano, T., Richelson, E. and Nirenberg, M. (1972) Proc. Natl. Acad. Sci., U.S.A. 69, 258-263.
8. Potter, R. L., Stafford, P. H. and Taylor, S. (1978) Arch. Biochem. Biophys. 190, 174-180.
9. Zoller, M. J., Kerlavage, A. R. and Taylor, S. S. (1979) J. Biol. Chem. 254, 2408-2412.
10. Campbell, D. H., Garvey, J. S., Cremer, N. E. and Sussforf, D. H. (1970) Immunology: A laboratory text for instruction and research. N. A. Benjamin Inc., MA.
11. Engvall, E. (1980) Methods Enzymol. 70, 419-439.
12. Burnette, W. N. (1981) Anal. biochem. 112, 195-203.
13. Witt, J. J. and Roskoski, R. (1975) Anal. Biochem. 66, 253-258.
14. Liu, A. Y.-C. and Chen, K. Y. (1985) J. Cell. Physiol. 125: 387-392.