Biochimica et Biophysica Acta 928 (1987) 235–239
Elsevier

BBA 10201

BBA Report

Identification of functional cAMP-dependent protein kinase in a 'neurite minus' mouse neuroblastoma cell line

Alice Y.-C. Liu [a], V. Kamalakannan [a] and Kuang Yu Chen [b]

Departments of [a] Biological Sciences and [b] Chemistry, Rutgers – The State University of New Jersey, Piscataway, NJ (U.S.A.)

(Received 26 September 1986)

Key words: Protein kinase, cAMP-dependent; Neuroblastoma; Differentiation

We have characterized and quantitated the level of cAMP-dependent protein kinase in the NS-20, N1E-115, N-18 and N1A-103 mouse neuroblastoma clonal cell lines, and we have correlated the occurrence of functional cAMP-dependent protein kinase with the dibutyryl cAMP-induced differentiated functions in these cells. Our results demonstrate the presence of functional cAMP-dependent protein kinase in extracts of all four cell lines examined, including the 'neurite minus' N1A-103 cell line. Dibutyryl cAMP induced neurite outgrowth and acetylcholinesterase activity in the NS-20, N1E-115 and N-18 neuroblastoma cell lines, but not in the N1A-103 cell line. However, dibutyryl cAMP caused a 2–3-fold increase in the R_I regulatory subunit protein and cAMP-phosphodiesterase activity in the 'neurite minus' N1A-103 cells in a manner similar to that of the other three 'neurite positive' cell lines. These results suggest that the biochemical lesion(s) subserving the neurite-minus phenotype of the N1A-103 cells may be distal to the activation of cAMP-dependent protein kinase and is in a biochemical pathway distinct from the induction of R_I regulatory subunit protein and cAMP-phosphodiesterase activity.

We have been interested in studying the mechanism of action of cAMP and the role of cAMP-dependent protein kinase in the control of neuroblastoma cell differentiation. In this study, we characterized and quantitated the level of cAMP-dependent protein kinase in the cholinergic NS-20, the adrenergic N1E-115, the inactive N-18 and the neurite minus N1A-103 mouse neuroblastoma clonal cell lines [1], and correlated the occurrence of cAMP-dependent protein kinase with the expression of a number of differentiated phenotypes including neurite outgrowth, induction of the R_I regulatory subunit protein, cAMP-phosphodiesterase and acetylcholinesterase activities.

Correspondence: A.Y.-C. Liu, Department of Biological Sciences, Rutgers – The State University of New Jersey, Busch Campus, Piscataway, NJ 08855, U.S.A.

Table I summarizes the quantitative results of levels of regulatory and catalytic subunits of cAMP-dependent protein kinase in extracts of the NS-20, N1E-115, N-18 and N1A-103 neuroblastoma cell lines. Experiments on the photoactivated incorporation of 8-N_3-[^{32}P]cAMP [2,3] demonstrated the presence of a 47- and a 52–54-kDa protein in extracts of all four neuroblastoma cell lines studied, with the 47-kDa species being the predominant form. The 47- and 52–54-kDa proteins have previously been identified as the regulatory subunits of the type I and type II cAMP-dependent protein kinases, respectively [2,3]. Assays of the cAMP-dependent phosphotransferase activity, using histone II AS as the substrate protein [2,4], demonstrated the presence of enzyme in extracts of all four neuroblastoma cell lines including the neurite minus N1A-103

0167-4889/87/$03.50 © 1987 Elsevier Science Publishers B.V. (Biomedical Division)

TABLE I

QUANTITATION OF THE LEVEL OF REGULATORY AND CATALYTIC SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE IN CYTOSOLS OF NS-20, N1E-115, N-18, AND N1A-103 MOUSE NEUROBLASTOMA CELLS

Cells were grown as monolayer cultures in Dulbecco's modified Eagle medium (with 4500 mg glucose per liter without sodium pyruvate) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. For each of the cell lines studied, groups of six 100-mm plates of cells were used. Cells were harvested at an early stationary phase of growth, and a $100{,}000 \times g$ supernatant fraction was prepared according to methods described [2,3]. Regulatory subunit of cAMP-dependent protein kinase present in cell extracts was quantitated by the covalent incorporation of 8-N_3-[^{32}P]cAMP into the 47-kDa R_I and the 52–54-kDa R_{II}. Assays were carried out under standard conditions according to methods previously described [2,3] using 100–200 μg cytosol protein. The catalytic subunit activity was determined by assaying for histone kinase activity in the absence and presence of 5 μM cAMP according to the method of Witt and Roskoski [4]. An inhibitor of the catalytic subunit of the kinase, partially purified from bovine brain according to published methods [5–7], was used to distinguish the activity attributable to cAMP-dependent protein kinase from that of other kinases present in cell extracts. 1 unit of histone kinase activity is defined as the transfer of 1 pmol of ^{32}P from [γ^{32}P]ATP to histone II AS under the conditions described. Results represent the means of three independent measurements ± S.D. of the means.

Cell line	Regulatory subunit (pmol/mg)		Catalytic subunit (units/min)	
	R_I	R_{II}	−cAMP	+cAMP
NS-20	8.87 ± 1.0	0.62 ± 0.2	65.0 ± 29	207 ± 26
N1E-115	2.25 ± 0.6	1.25 ± 0.4	71.2 ± 26	121 ± 8
N-18	2.75 ± 0.4	0.78 ± 0.4	49.5 ± 12	117 ± 16
N1A-103	3.27 ± 0.8	1.27 ± 0.4	52.8 ± 7.7	138 ± 28

cells; this activity was maximally stimulated by 5 μM cAMP and inhibited by a partially purified inhibitor of the catalytic activity of cAMP-dependent protein kinase [5–7]. DEAE-Sephacel column chromatography confirmed the results on quantitation of levels of regulatory and catalytic subunits of cAMP-dependent protein kinase in crude cell extracts (data not shown).

The presence of cAMP-dependent protein kinase in extracts of the 'neurite minus' N1A-103 cells suggests that the biochemical lesion(s) subserving the neurite minus phenotype may be distal to the activation of cAMP-dependent protein kinase, and that, possibly, other cAMP-regulated events may be operational in these neurite minus cells. In our previous studies, we have demonstrated the induction of R_I regulatory subunit protein, cAMP-phosphodiesterase, and acetylcholinesterase activities by dibutyryl cAMP, coincident with neurite extension, in the N-18 neuroblastoma cell cultures [2,3]. A variety of evidence suggests that these parameters may serve as biochemical indices of neuroblastoma cell differentiation [2,3]. We, therefore, examined and quantitated the effects of dibutyryl cAMP on induction of the R_I regulatory subunit protein, cAMP-phosphodiesterase, and acetylcholinesterase activities in the four neuroblastoma cell lines with the specific intention of determining (a) whether dibutyryl cAMP, through activation of cAMP-dependent protein kinase, can induce the expression of at least one of the differentiation phenotypes in the neurite minus N1A-103 cells, and (b) whether the dibutyryl cAMP-induced increase in R_I regulatory subunit protein is observed in the cholinergic NS-20 or the adrenergic N1E-115 cells, in addition to the N-18 cell line, as we have previously established [2,3].

The level of the R_I regulatory subunit protein was quantitated by photoaffinity labeling with 8-N_3-[^{32}P]cAMP and by immunoblot analysis [8,9], results of these experiments are shown in Table II and Fig. 1. In each of the cell lines studies, there was a 2-3-fold increase in the amount of 8-N_3-[^{32}P]cAMP incorporated into the 47-kDa R_I in the dibutyryl cAMP-induced cell cultures over that of the controls (Table II). This increased incorporation of radioactivity represents an increase in the amount of R_I, as indicated by the results shown on the immunoblot (Fig. 1) quantitation of the R_I protein using an antibody directed against the purified type I kinase regulatory

TABLE II

CORRELATION OF NEURITE OUTGROWTH, THE EXPRESSION OF ACETYLCHOLINESTERASE, R_I REGULATORY SUBUNIT PROTEIN AND cAMP-PHOSPHODIESTERASE ACTIVITIES IN CONTROL AND DIBUTYRYL cAMP-INDUCED NS-20, N1E-115, N-18, AND N1A-103 MOUSE NEUROBLASTOMA CELLS

Conditions for cell culturing are identical to those described in the legend of Table I. To induce differentiation of the neuroblastoma cells, 1 mM dibutyryl cAMP was added to the cell cultures 8 h after plating. Cells were allowed to grow (in the absence or presence of 1 mM dibutyryl cAMP) to an early stationary phase of growth, at which time, representative fields of the cell cultures were photographed and the number of cells with neurites scored. The remaining plates of cells were harvested and fractionated to yield a cytosol fraction used to assay for acetylcholinesterase, the photoactivated incorporation of 8-N_3-[^{32}P]cAMP into the 47-kDa R_I protein, and cAMP-phosphodiesterase activity according to methods described [2,3]. Results represent means ± S.D. of the means of three separate experiments.

Cell line	Condition	Morp. Diff. (% cells with neurites)	Acetylcholinesterase (nmol/min per mg)	R_I (pmol/mg)	Phosphodiesterase (pmol/min per mg)
NS-20	control	5 ± 1	6.8 ± 2.6	8.5 ± 0.9	52.5 ± 12.2
	Bt_2 cAMP	80 ± 10	25.3 ± 10.2	14.0 ± 2.4	86.0 ± 22.3
N1E-115	control	15 ± 5	18.8 ± 5.8	2.2 ± 0.7	45.0 ± 9.5
	Bt_2 cAMP	98 ± 5	55.8 ± 12.7	5.6 ± 1.8	71.0 ± 14.0
N-18	control	4 ± 1	10.0 ± 3.1	2.7 ± 0.3	24.0 ± 8.1
	Bt_2 cAMP	90 ± 14	23.1 ± 7.5	7.2 ± 1.3	45.5 ± 16.2
N1A-103	control	n.d.	3.2 ± 1.0	3.3 ± 0.9	35.0 ± 7.1
	Bt_2 cAMP	n.d.	4.8 ± 1.4	6.3 ± 1.4	76.5 ± 19.5

n.d.: non-detectable.

subunit isolated from bovine skeletal muscle [3]. While the relative magnitude of the increase in R_I regulatory subunit protein in the dibutyryl cAMP-treated cells over that of the control was similar for the NS-20 and N-18 cell lines, the concentration of R_I present in extracts of the NS-20 cells was substantially greater than that present in equivalent samples of the N-18 cells, a result consistent with the pattern of photoaffinity labeling of R_I (Table II). From the immunoblot experiment (Fig. 1), we determined that the amount of R_I present in extracts of the control and dibutyryl cAMP-induced NS-20 cells was 0.113% and 0.23% of total cytosolic protein, respectively, equivalent to values of 24 and 49 pmol/mg protein. It may be noteworthy that the level of R_I quantitated by the immunoblot method was approx. 3-times higher than that obtained using the photoaffinity labeling technique (Table II), suggesting that the photoactivated incorporation of 8-N_3-[^{32}P]cAMP, while useful in giving a relative measure of the amount of R_I in cell extracts, may not be strictly stoichiometric.

Results in Table II further demonstrate that dibutyryl cAMP induced cAMP-phosphodiesterase activity in all neuroblastoma cell lines examined including the neurite minus N1A-103 cells. Dibutyryl cAMP also induced acetylcholinesterase activity in the NS-20 and N1E-115 cells in addition to the N-18 cells; however, dibutyryl cAMP failed to induce acetylcholinesterase activity in the neurite-minus N1A-103 cells.

The N1A-103 clonal cell line, originally isolated and characterized in Dr. M. Nirenberg's laboratory, has an impairment in the cAMP-evoked neurite outgrowth response [1], which we have independently confirmed in our laboratory. In this study, we further established that dibutyryl cAMP failed to induce acetylcholinesterase activity in this neurite minus cell line. The effects of dibutyryl cAMP on the R_I regulatory subunit protein and cAMP-phosphodiesterase activity in the neurite minus N1A-103 cells, however, were qualitatively different from the effects on neurite outgrowth and induction of acetylcholinesterase activity. Dibutyryl cAMP caused a 2–3-fold increase in the R_I regulatory subunit protein and cAMP-phosphodiesterase activity in the neurite minus N1A-103 cells in a manner similar to that observed in the other neuroblastoma cell lines. These results, together with the knowledge of the presence of functional cAMP-dependent protein

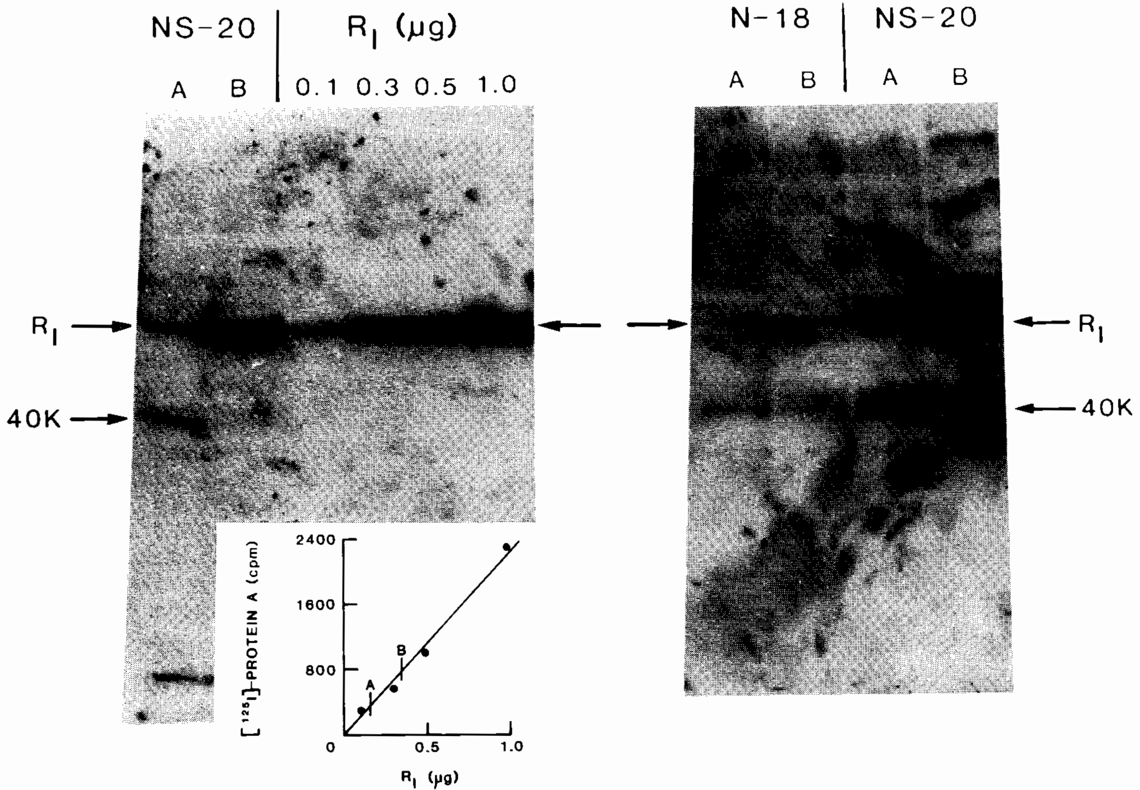

Fig. 1. Autoradiogram of an immunoblot of the 47 kDa R_I protein using a monospecific antibody directed against the type I kinase regulatory subunit protein purified from bovine skeletal muscle [3] following by probing with ^{125}I-protein A. Aliquots of cytosol preparations from control (lane A) and dibutyryl cAMP-induced (lane B) NS-20 and N-18 mouse neuroblastoma cells containing 150 μg protein, and various amounts (as indicated) of the purified R_I protein were subjected to analysis according to methods described [8,9]. Briefly, samples were subjected to SDS-polyacrylamide gel electrophoresis and the resolved proteins were electrophoretically transferred to nitrocellulose membrane (0.22 μ pore size). The membranes were then incubated in phosphate-buffered saline containing 5% bovine serum albumin, 0.24% gelatin and 0.01% NaN_3 (blocking media) overnight at 4°C to saturate non-specific protein binding sites. Membranes were then probed with a 1:50 dilution of the antiserum against R_I in blocking media that contained 0.05% Tween 20 and 0.3% Triton X-100 overnight at 4°C. Membranes were then washed overnight with several changes of phosphate-buffered saline containing 0.05% Tween 20 and 0.3% Triton X-100. Antigen-antibody complex on the nitrocellulose membranes was probed and quantitated by incubation with 1 μCi of ^{125}I-protein A in the blocking media containing 0.05% Tween 20 and 0.3% Triton X-100 for 5 h at room temperature followed by extensive washing of the membrane. The nitrocellulose membrane was then processed for autoradiography. The positions of the 47-kDa R_I and a 40-kDa degradative product of R_I are indicated. The inset illustrates quantitation of the amount of R_I in control (A) and dibutyryl cAMP-induced (B) NS-20 cell extracts using the purified R_I from bovine skeletal muscle as standard.

kinase in the N1A-103 cells, suggest that the failure of dibutyryl cAMP to induce neurite outgrowth and acetylcholinesterase activity in the N1A-103 cells is probably not a consequence of impaired cAMP-dependent protein kinase activity. The results further suggest that the pathways of cAMP action on (a) neurite outgrowth and increased acetylcholinesterase activity and (b) induction of R_I regulatory subunit protein and cAMP-phosphodiesterase activity diverge after activation of cAMP-dependent protein kinase. The simplest interpretation is that the N1A-103 cell line is deficient or defective in a gene product that is distal to the activation of cAMP-dependent protein kinase and in a biochemical pathway distinct from the induction of R_I protein and cAMP-phosphodiesterase activity.

The authors are indebted to Jennifer J.-H. Kim

for her assistance with this work. This work was supported by grant number R0-1 CA 39667 awarded by the National Cancer Institute, DHHS, and by grant number DCB 84-17775 awarded by the National Science Foundation.

References

1 Amano, T., Richelson, E. and Nirenberg, M. (1972) Proc. Natl. Acad. Sci. USA 69, 258–263

2 Liu, A.Y.-C., Fiske, W.W. and Chen, K.Y. (1980) Cancer Research 40, 4100–4108.

3 Liu, A.Y.-C., Chan, T. and Chen, K.Y. (1981) Cancer Research 41, 4579–4587.

4 Witt, J.J. and Roskoski, R. (1975) Anal. Biochem. 66, 253–256

5 Ashby, C.A. and Walsh, D.A. (1972) J. Biol. Chem. 247, 6637–6642

6 Ashby, C.A. and Walsh, D.A. (1973) J. Biol. Chem. 248, 1255–1261

7 Walsh, D.A., Ashby, C.D., Gonzalez, C., Calkins, D., Fischer, E.H. and Krebs, E.G. (1971) J. Biol. Chem. 246, 1977–1985

8 Burnette, W.N. (1981) Anal. Biochem. 112, 195–203

9 Lohmann, S.M., Schwoch, G., Reiser, G., Port, R. and Walter, U. (1983) EMB0 2, 153–159