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REGULATION OF NEURON-SPECIFIC ENOLASE IN NG108-15 HYBRID CELLS AND C6BU-1 GLIOMA CELLS

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Distribution of three isozymes of brain enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11) ($\alpha\alpha$, $\alpha\gamma$ and $\gamma\gamma$ forms) in clonal cell lines of neuroblastoma (NS20Y and N18TG-2), glioma (C6BU-1), and hybrid cells (NG108-15, NCB20, Nbr10A, Nbr20A, N4G-B-a and N4G-C-a) was examined with a sensitive enzyme immunoassay system, that uses a rabbit antibody to rat brain enolase $\alpha\alpha$ or $\gamma\gamma$. All cell lines tested were found to possess the enolase which contains γ subunit (a neuron-specific protein), although the $\alpha\alpha$ enolase (non-neuronal enolase) was the dominant form in these cells. A clonal rat glioma (C6BU-1) cell contained about 40, 1 and 0.07 μg/mg protein of $\alpha\alpha$, $\alpha\gamma$ and $\gamma\gamma$ enolases, respectively, at the confluent stage. Inclusion of 1 mM dibutyryl cyclic AMP or 10 μM prostaglandin E_1 plus 1 mM theophylline in the culture medium of a hybrid cell (NG108-15, mouse neuroblastoma x rat glioma) resulted in a more than 2-fold increase in the concentrations of $\alpha\gamma$ and $\gamma\gamma$ in the cell within a few days, with little change in the $\alpha\alpha$ enolase concentration. A similar increase in the concentration of γ subunit by the nucleotide (but not by prostaglandin E_1 plus theophylline) was also observed in the glioma cell (C6BU-1) line. The results suggest that the γ subunit or the neuron-specific protein can be regulated in NG108-15 and C6BU-1 cells in a cyclic AMP-dependent fashion.

Introduction

Brain extract contains three forms of enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11) activity which can be separated on DEAE-cellulose column (forms $\alpha\alpha$, $\alpha\gamma$ and $\gamma\gamma$) [1]. The most acidic form ($\gamma\gamma$ or neuron-specific enolase) has been shown to be identical to the nervous system-specific protein, 14-3-2 [2,3], and to localize in neurons [4]. The least acidic form ($\alpha\alpha$ or non-neuronal enolase), which is immunologically identical to the enolase widely distributed in other tissues, was found only in glial cells in mature brain [4].

Because of the potential usefulness of cell culture for studying the cellular localization and regulation of the specific protein, the neuron-specific enolase or 14-3-2 protein has been measured in neuroblastoma cells [5–8] and hybrid cells [9]. Only in N18 and N1E-115 cells [5] have the relative contents of enolase isozymes and their regulation been determined. Six interspecific hybrid cells derived by fusion of mouse neuroblastoma and other types of cells are now available [10], and those hybrid cells maintain many differentiated neuronal properties, such as release of acetylcholine or synapse formation. These properties are regulated by dibutyryl cyclic AMP or prostaglandin E_1 plus theophylline [10–12].

We have recently purified the three forms of rat brain enolase, raised antisera specific to each of two distinct subunits (α and γ) of the enzyme in rabbits [13], and prepared highly sensitive sandwich-type enzyme immunoassay systems for the assay of three forms of the enolase in crude extracts [14]. We describe here the determination of each of the

enolase isozymes in several clonal cell lines derived from nervous tissue and hybrid cells, together with changes in the level of the isozymes after induced differentiation of the cultured cells by dibutyryl cyclic AMP or prostaglandin E_1 plus theophylline.

Materials and Methods

Cell preparations. The following clonal cell lines obtained from Dr. M. Nirenberg, National Institutes of Health, Bethesda, were used: mouse neuroblastoma clones; NS20Y [15] and N18TG-2 [15]; rat glioma, C6BU-1 [15,16]; hybrid cell lines, NG108-15 [15], NCB20 [10], NBr10A [10], NBr20A [10], N4G-B-a [16] and N4G-C-a [16]. The origins of the hybrid cell lines are shown in Table I.

Cells were grown in 90% Dulbecco's modified Eagle's minimal essential medium (GIBCO) containing 10% fetal bovine serum (GIBCO), in a humidified atmosphere of 90% air/10% CO_2 and at 37°C as described [15]. Hybrid cells were grown in the same medium supplemented with 0.1 mM hypoxanthine/1 μM aminopterine/16 μM thymidine. Cells were grown to confluency, unless otherwise specified, in Falcon flasks (75 cm^2 surface area), and then washed with Dulbecco's phosphate-buffered saline, without Ca^{2+} and Mg^{2+}. After incubation for 5 min at 37°C with buffer, dissociated cells were collected by centrifugation at 250 × *g* for 5 min at 25°C. The cell pellet was suspended in the same buffer with 0.8 mM $MgCl_2$, and aliquots were frozen and stored at −80°C. To change cells to a more differentiated state, the culture media were supplemented with 1 mM dibutyryl cyclic AMP (Boehringer), or 10 μM prostaglandin E_1 (Upjohn) plus 1 mM theophylline. Cells were counted using a haemocytometer.

TABLE I

ORIGINS OF THE CELL LINES USED

Cell types	Cell lines	Origin
Neuroblastoma	NS20Y	Mouse
	N18TG-2	Mouse
Glioma	C6BU-1	Rat
Hybrid	NG108-15	N18TG-2 × C6BU-1
	NCB20	N18TG-2 × CHBC [a]
	NBr10A	N18TG-2 × BRL-30E [b]
	NBr20A	N18TG-2 × BRL-30E [b]
	N4G-B-a [c]	N4TG-3 [d] × C6BU-1
	N4G-C-a [c]	N4TG-3 [d] × C6BU-1

[a] Fetal Chinese hamster brain cell.
[b] Buffalo rat liver cell line.
[c] New designation in Nirenberg's laboratory for N4G-B-a is 140-3; for N4G-C-a, 141-B.
[d] Mouse neuroblastoma.

The frozen cell pellet was homogenized at 0°C in 15 mM Tris-acetate buffer (pH 6.5)/5 mM $MgSO_4$/0.1 mM EDTA ($0.5–5 \cdot 10^6$ cells/ml) using a Teflon-glass homogenizer. The homogenate was centrifuged at 4°C for 1 h at 125 000 × *g*. Immunoassay of the isozymes and the enolase activity assay were carried out with the supernatant.

Antisera and antigens. Rabbit (anti-α) and (anti-γ) sera were prepared by injecting the purified $\alpha\alpha$ and $\gamma\gamma$ enolases from rat brain, as described previously [13]. The purified rat $\alpha\alpha$, $\alpha\gamma$ and $\gamma\gamma$ enolases were used as the standard for immunoassay of the isozymes [14]. Although the assay systems were composed of the antibodies to rat antigens, the antisera to rat $\alpha\alpha$ and $\gamma\gamma$ enolases cross-react with the mouse $\alpha\alpha$ and $\gamma\gamma$ enzymes, respectively [17]. We, thus, assayed the isozymes in the cell lines derived from various animal species with the above assay systems, and results are expressed as the amount of rat isozyme-equivalent.

Immunoassay of the isozymes. Three forms of the enolase were determined with the sandwich-type enzyme immunoassay systems described recently [14]. The systems are composed of the antibody-bound solid-phase and the antibody Fab′-β-D-galactosidase complex, and are sensitive with the isozymes measurable at levels less than 100 pg [14].

The cell extract was diluted 100–10 000-fold with 0.01 M sodium phosphate buffer (pH 7.0)/0.1 M NaCl/1 mM $MgCl_2$/0.1% bovine serum albumin (fraction V from Armour)/0.1% NaN_3 (buffer A), and 100 μl of each diluted sample were incubated in duplicate with the antibody-bound solid-phase at 4°C for 5 h with shaking, in a final volume of 0.15 ml buffer A. The solid-phase was washed twice with buffer A, and then incubated at 4°C overnight with 3 munits (expressed as units of β-D-galactosidase activity) of the antibody Fab′-β-D-galactosidase complex in 0.2 ml buffer A. After washing off the unbound complex, the galactosidase activity on the solid-phase was assayed with 4-methylumbelliferyl-β-D-galactoside as substrate [14].

Assay of enolase activity. Enolase activity was assayed spectrophotometrically at 340 nm by coupling the reaction with pyruvate kinase (Boehringer) and lactate dehydrogenase (Boehringer) at 30°C [13]. 1 unit enolase activity was defined as that which catalyzes the conversion of 1 μmol substrate/min.

Other methods. Protein concentrations were estimated with Bio-Rad Protein Assay, which utilizes the principle of protein-dye binding [18]. Whole brains of rat or mouse were homogenized in 4 vol. 15 mM Tris-acetate buffer (pH 6.5)/5 mM $MgSO_4$/0.1 mM EDTA, and the soluble fractions were obtained as described above.

Results

Existence of enolase isozymes containing γ subunit in various clonal cell lines

Enolase activities and levels of the isozymes determined with the immunoassay in various cell lines and in adult brains of Wistar rat and CF1 mouse are summarized in Table II. Enolase activity in the cell lines ranged from 1.2 to 3.0 units/mg protein, showing similar values to those of adult rat and mouse brains.

Values of the isozymes concentration obtained with the immunoassay were quite different between the cell lines, although the dominant form of the isozyme was αα in all cells. The present immunoassay systems involve two steps of the specific immunoreaction, and are composed of antibodies to rat isozymes and rat isozymes as the standard. Antisera to rat αα and rat γγ cross-react with the corresponding subunit (α and γ) of mouse enolase [5,17]. However, the reactivities of the antisera to mouse enolase are lower than those to the rat enzyme [5]. Apparently low values of the isozymes in mouse brain compared to those in rat brain (Table II) were probably due to the above reasons. Low values of the isozymes in mouse neuroblastoma (NS20Y and N18TG-2) and a hybrid cell line (NCB20) may have resulted for the same reasons, because these cell lines were derived from non-rat origins. Hybrid cell lines containing rat origin showed relatively high values of the isozymes. Hybrid cell lines may possess the two species of each subunit derived from the parental cells.

Because of different reactivities of the immunoassay systems to the isozymes from various animal species, and the possible existence of the two parental proteins in each subunit in hybrid cell lines, it is hard to compare the level of each isozyme between the cell lines. However, it can be concluded that all cell lines possess the isozymes containing γ subunit, which has been reported as a neuron-specific protein [4].

Induction of γ subunit-possessing isozymes by dibutyryl cyclic AMP or prostaglandin E_1 in hybrid cell (NG108-15) and glioma cell (C6BU-1).

The γ subunit of enolase (or neuron-specific pro-

TABLE II

DETERMINATION OF ENOLASE ISOZYMES IN CLONAL CELL LINES

ng are of rat enolase-equivalent. Measurements are the mean ± S.D.

Cell lines	No. of assay	Enolase activity (units/mg protein)	Enolase isozyme (ng/mg protein)		
			αα	αγ	γγ
NS20Y	3	1.74 ± 0.01	458 ± 43	43.7 ± 21.1	7.5 ± 3.2
N18TG-2	3	2.96 ± 0.15	537 ± 38	66.0 ± 4.2	10.6 ± 1.2
NCB20	5	1.76 ± 0.23	1 580 ± 668	13.6 ± 11.6	3.1 ± 3.0
N4G-B-a	4	2.03 ± 0.29	9 690 ± 2 640	43.5 ± 16.2	3.1 ± 2.2
N4G-C-a	3	1.22 ± 0.11	5 890 ± 1 220	144 ± 51	50.6 ± 9.3
NBr10A	3	2.12 ± 0.35	21 600 ± 10 300	433 ± 113	147 ± 33
NBr20A	3	1.39 ± 0.10	4 530 ± 1 120	27.9 ± 6.0	9.3 ± 0.7
NG108-15	16	1.95 ± 0.72	15 500 ± 7 510	157 ± 94	23.8 ± 31.2
C6BU-1	6	1.42 ± 0.31	39 600 ± 15 300	1 160 ± 490	68.2 ± 10.3
Mouse brain	3	2.86 ± 0.46	752 ± 60.2	186 ± 35.4	1 290 ± 165
Rat brain	4	2.03 ± 0.13	29 600 ± 4 400	13 800 ± 1 200	11 300 ± 2 400

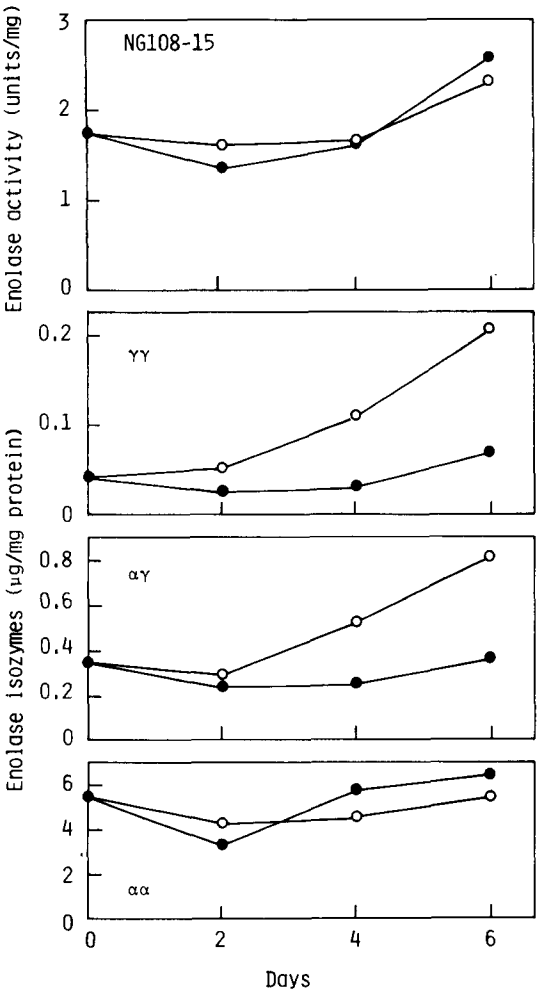

Fig. 1. Induction of $\alpha\gamma$ and $\gamma\gamma$ enolases in the hybrid NG108-15 cells by dibutyryl cyclic AMP. NG108-15 cells in each flask were grown as described in Table III with (○———○) or without (●———●) 1 mM dibutyryl cyclic AMP. On the indicated days, the cells were collected, and the enolase activity and isozyme concentrations were determined.

tein, 14-3-2) has been shown to increase in concentration during differentiation in nervous tissue [17,19] or in neuroblastoma cell lines, N18 and N1E-115 [5]. In order to find the effect of cell differentiation on the level of γ subunit in a hybrid cell, NG108-15 cells were cultured with or without 1 mM dibutyryl cyclic AMP (Fig. 1). After the indicated period of culture, cells were collected. Enolase activity and the concentration of each isozyme were determined with the soluble fraction of the cells, and results were expressed as units enolase activity or ng rat isozyme-equivalents/mg protein. As shown in Fig. 1, a supplement of 1 mM dibutyryl cyclic AMP in culture medium resulted in a more than 2-fold increase in the levels of $\alpha\gamma$ and $\gamma\gamma$ enolases in a few days, whereas the control culture showed little change in these forms of enolase. Enolase activity and the level of $\alpha\alpha$ enolase were not affected by supplement of the nucleotide in the medium. A longer period of culture with dibutyryl cyclic AMP (>7 days) usually brought about a decrease in the levels of $\alpha\gamma$ and $\gamma\gamma$ enolase to the control levels (data not shown).

The NG108-15 cells are reported to have an adenylate cyclase sensitive to prostaglandin E_1 [20], and morphological differentiation of the cell is also induced by prostaglandin E_1 plus theophylline. Effect of the supplement of prostaglandin E_1 (10 μM) with theophylline (1 mM) in the medium on the levels of enolase isozymes was examined in NG108-15 cells (Table III). After 4 days of culture with or without the supplement, cells were collected. The isozyme concentration was assayed and expressed as ng rat isozyme-equivalents/10^6 cells. On the fourth day, soluble protein contents/cell cultured with dibutyryl cyclic AMP, and prostaglandin E_1 plus theophylline were about 1.8-fold and 1.3-fold, respectively, compared with that of the control. Similar degrees of increase in $\alpha\alpha$ enolase/cell were seen in the cyclic AMP-treated and prostaglandin E_1-treated cells, indicating that the level of $\alpha\alpha$ enolase was increased in a nonspecific manner. However, the levels of $\alpha\gamma$ and $\gamma\gamma$ enolases/cell were increased by more than 3.7-fold with dibutyryl cyclic AMP, and 2.6-fold with prostaglandin E_1 plus theophylline, when compared with the control culture. These results indicate that the γ subunit of enolase was specifically induced by prostaglandin E_1 plus theophylline as well as by dibutyryl cyclic AMP in hybrid NG108-15 cells.

To see whether or not this specific protein in the glioma cell could be induced by dibutyryl cyclic AMP or prostaglandin E_1 plus theophylline, similar experiments to those described above were carried out with C6BU-1 cells. As shown in Table IV, when the cells were cultured in the presence of dibutyryl cyclic AMP (1 mM) for 4 days, the concentration of γ subunit was elevated to more than 2-fold that of the control with little change in $\alpha\alpha$ enolase concentra-

TABLE III

EFFECT OF DIBUTYRYL CYCLIC AMP OR PROSTAGLANDIN E_1 ON THE LEVELS OF ENOLASE ISOZYMES IN HYBRID NG108-15 CELLS

About $27 \cdot 10^3$ and $54 \cdot 10^3$ NG108-15 cells were plated in each flask (25 cm^2 surface area) for control and treated cultures, respectively, and maintained in the medium with or without the supplement for 4 days, ng are of rat enolase-equivalent. Measurements are mean ± S.D.

Culture media	No. of assay	Soluble protein (mg/10^6 cells)	Enolase isozyme (ng/10^6 cells)		
			αα	αγ	γγ
Control	3	0.163 ± 0.029	455 ± 66	24.4 ± 2.7	10.4 ± 2.0
+Dibutyryl cyclic AMP	3	0.292 ± 0.091	787 ± 105	99.8 ± 23.8	38.3 ± 8.3
+Prostaglandin E_1 with theophylline	3	0.210 ± 0.017	646 ± 230	67.8 ± 14.8	27.5 ± 9.9

TABLE IV

EFFECT OF DIBUTYRYL CYCLIC AMP OR PROSTAGRANDIN E_1 ON THE LEVELS OF ENOLASE ISOZYMES IN RAT GLIOMA (C6BU-1) CELLS

About $1.6 \cdot 10^6$ C6BU-1 cells for control and $3.1 \cdot 10^6$ cells for the treated cultures were plated in each flask (25 cm^2 surface area), and cultured in the medium with or without the supplement for 4 days. Measurements are mean ± S.D.

Culture media	No. of assay	Enolase activity (units/mg protein)	Enolase isozyme (ng/mg protein)		
			αα	αγ	γγ
Control	7	1.49 ± 0.30	24 900 ± 3 600	1 120 ± 80	55.9 ± 25.5
+Dibutyryl cyclic AMP	4	1.51 ± 0.07	24 000 ± 3 400	2 500 ± 790	186 ± 90
+Prostaglandin E_1 with theophylline	5	1.57 ± 0.36	22 300 ± 2 100	839 ± 91	48.6 ± 25.8

tion, as observed in hybrid NG108-15 cells. However, the supplement of prostaglandin E_1 plus theophylline to the culture medium did not induce the γ subunit of enolase in C6BU-1 cells. Ineffectiveness of prostaglandin E_1 on the induction of the γ subunit may be attributed to the fact that the adenylate cyclase system in C6BU-1 cells is much less sensitive to prostaglandin E_1 than that of NG108-15 cells [20].

Discussion

Developmental increase in the γ subunit-containing enolase in nervous tissue [17,19] and in the culture of neuroblastoma cell lines [5], and the immunocytochemical demonstration of the localization of this protein in neurons suggested some roles of this specific protein on the highly integrated functions of the nervous system. To confirm these findings, we have examined the distribution of three forms of brain enolase in three neuronal clones and six hybrid cells with a sensitive enzyme immunoassay method.

All cell lines tested were found to possess the γ subunit-containing enolase, and the concentration of the specific enolase was elevated along with the cell-differentiation induced by dibutyryl cyclic AMP, not only in the hybrid cells (NG108-15, mouse neuroblastoma x rat glioma), but also in the clonal rat glioma cells (C6BU-1). Induction of the γ subunit of the enolase by prostaglandin E_1 plus theophylline, observed in NG108-15 cells, is probably due to the accumulation of cyclic AMP in the cell, since this clonal cell has an adenylate cyclase system sensitive to prostaglandin E_1 [20]. The C6BU-1 cell, which has the less prostaglandin E_1-sensitive adenylate cyclase, did not respond to prostaglandin E_1.

It is unknown, however, which parental gene (mouse neuroblastoma or rat glioma) caused the induction of neuron-specific enolase by cyclic AMP in the hybrid cells.

Increase in the concentration of neuron-specific enolase with the induced cell-differentiation of glioma cell line is an unexpected phenomenom, since this form of enolase has been considered not to localize in glial cells in nervous tissue [4]. It is under investigation whether or not the neuron-specific enolase localizes strictly in nerve cells in nervous tissue, with an improved immunoassay system using purified antibodies at the level of single cells in our laboratory.

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