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SYNTHESIS AND PHOSPHORYLATION OF CHROMATIN-ASSOCIATED PROTEINS IN cAMP-INDUCED "DIFFERENTIATED" NEUROBLASTOMA CELLS IN CULTURE

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SUMMARY

Prostaglandin E_1 and a cAMP phosphodiesterase inhibitor 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone, RO20-1724, were used to induce differentiation in mouse neuroblastoma cells in culture. The incorporation of amino acids and phosphate into nuclear proteins of control and drug-treated cells (1 h and 3 days after treatment) was examined using double radioisotopic techniques. A marked decrease in histone synthesis and H1-histone phosphorylation were observed in 'differentiated' neuroblastoma cells after 3 days of prostaglandin E_1 and RO20-1724 treatment, but only small differences were noted in the synthesis and phosphorylation of non-histone chromatin associated proteins after 3 days of drug treatment. Minimal changes were observed in the labeling of histone and non-histone nuclear proteins if the cells were treated for 1 h with prostaglandin E_1 and RO20-1724.

An elevation of the intracellular level of adenosine 3′,5′-cyclic monophosphate (cAMP) by prostaglandin E_1 (PGE_1), inhibitors of phosphodiesterase (papaverine and RO20-1724) or by analogs of cAMP (N^6,$O^{2'}$-dibutyryl cyclic AMP and 8-benzylthio cAMP) induces many differentiated functions which are characteristic of mature neurons. These include formation of long neurites [19], increase in size of soma and nucleus associated with a rise in total RNA and proteins [19, 20], increase in total mRNA content [2], increases in tyrosine hydroxylase [28, 35], choline acetyltransferase [16, 20], and acetylcholinesterase [6, 20] activities, increase in stimulation of adenylate cyclase activity in vitro by catecholamines [21] and acetylcholine [22], potentiation of PGE_1-stimulated adenylate cyclase activity in vitro by guanosine triphosphate [22], increase in cAMP phosphodiesterase activity [23] and decrease in tumorigenicity [20].

The synthesis of histones [9, 11] and the phosphorylation of H1-histone [1, 10] appear to be associated with proliferating cells and occur predominantly in the S phase of the cell cycle. Since cAMP-induced 'differentiated' neuroblastoma cells stop cell division [20], it is possible that these nuclear events are turned off in 'differentiated' neuroblastoma cells. Furthermore, qualitative and quantitative changes in non-histone chromosomal proteins (NHCP) have been observed during development in slime mold [13], the chick oviduct [32], and the sea

urchin [30]. Stage specific NHCP phosphorylation has been found in the maturation of sea urchin [18] and avian red blood cells [7]. These findings suggest that changes in the synthesis and/or phosphorylation of NHCP may be involved in the regulation of differentiated functions. We have therefore compared the synthesis and phosphorylation of histone and non-histone chromosomal proteins in control and cAMP-induced 'differentiated' mouse neuroblastoma cells in culture. We now report that the synthesis of histone and the phosphorylation of H1-histone are markedly decreased in cAMP-induced differentiated neuroblastoma cells, but there is no prominent change in the synthesis or phosphorylation of non-histone chromosomal proteins.

MATERIALS AND METHODS

L-[G-^{3}H]amino acid mixtures, L-[U-^{14}C]amino acid mixtures, $^{32}P_i$ and $^{33}P_i$ were obtained from New England Nuclear Corp. F-12 medium and Viokase (2.5%) were purchased from Grand Island Biological Co. PGE_1 was a gift from Dr J. E. Pike of Upjohn Company, and RO20-1724 was donated by Dr H. Sheppard of Hoffmann-LaRoche, Inc. Calf thymus histones were obtained from Sigma Chemical Co.

Cell culture and radiolabeling

Procedures for culturing and maintaining the mouse neuroblastoma cells were previously described [19]. Cells of clone $NBA_{2(1)}$ were used in this study. These cells contained a marker enzyme for neural tissue, tyrosine hydroxylase [25]. Differentiation was induced by PGE_1, which stimulates adenylate cyclase activity by about 2-fold [21], and by 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO20-1724) which inhibits cAMP phosphodiesterase activity by over 90% without affecting cGMP phosphodiesterase activity [26] in neuroblastoma cells.

Cells (0.5×10^6) were plated in Falcon flasks (75 cm^2). PGE_1 (10 μg/ml) and RO20-1724 (200 μg/ml) were added separately 24 h after plating. The drug and medium were changed daily. Control cultures were treated identically except that an equivalent volume of solvent without drug was added. Chromatin associated proteins were analysed (1) 1 h after drug treatment in order to identify changes which might be involved in the initiation of morphological and biochemical maturation; (2) 72 h after drug treatment in order to observe whether there were changes which could be correlated with the maintenance of a differentiated state. On the day of experiment, the growth medium was changed before the addition of radioisotopes. ^{14}C- or ^{3}H-labeled amino acid mixtures (1–2 μCi/ml) were added to control and treated cultures, respectively. In another set of experiments, ^{32}P and ^{33}P (8–12 μCi/ml) were added to control and treated cultures, respectively. Cells (5×10^7) were removed from the flask surface by Viokase solution (0.25%), washed twice with growth medium and twice with a phosphate-buffered saline (pH 6.5). Cells were 90% viable as measured by trypan blue dye exclusion at the time of harvest.

Isolation of chromatin

All procedures were carried out at 0°C. Chromatin was isolated by a modification of the procedure described by Weisenthal & Ruddon [37]. Control and treated cell pellets were combined to insure equivalent handling during the extraction procedure and suspended in a buffer (pH 6.5) containing 500 mM 2-methyl-3, 4-pentanediol, 1.0 mM $CaCl_2$, and 0.1 mM piperazine-*N-N'-bis* (2-ethanesulfonic acid) monosodium monohydrate (PIPES). After standing for 15 min, the cells were sheared with a tight pestle in a Dounce homogenizer until more than 90% of the nuclei were rendered free of cytoplasmic tags as observed by phase contrast microscopy.

The crude nuclear suspension was immediately pelleted (1 000 g, 10 min) and washed twice with 2.0 ml of a solution containing 250 mM sucrose, 10 mM Tris-HCl, 5.0 mM $NaHSO_3$, 5.0 mM $MgCl_2$, and 5.0 mM β-mercaptoethanol (pH 7.4). The washed nuclei were lysed by the addition of 2.0 ml of a solution containing 24 mM EDTA, 75 mM NaCl, and 5 mM $NaHSO_3$ (pH 7.0). After centrifugation of this suspension at 1 500 g for 10 min, the pellet was washed twice (1.0 ml) with each of the following buffered (pH 8.0) solutions to isolate chromatin: (1) 50 mM Tris-HCl; (2) 10 mM Tris-HCl; and (3) 1.0 mM Tris-HCl. After each wash the sample was centrifuged for 10 min at 1 500 g. Previous experiments (Lazo & Ruddon, unpublished) demonstrated that >95% of the total cellular DNA was found in the chromatin pellet. In order to assess the ability of the method to remove contaminating cytoplasmic proteins from chromatin, radiolabeled post-nuclear supernatant proteins were added to unlabeled purified neuroblastoma nuclei in a separate experiment. Less than 0.3% of labeled cytoplasmic proteins were found in the chromatin pellet. The protein to DNA ratio in the chromatin prepared by this method was 2.84 ± 0.4 (S.E.M.).

Extraction of nuclear proteins

Proteins were extracted from chromatin by a modification of the procedure of Weisenthal & Ruddon [37]. Two 30 min (2.0 ml) extractions with a buffer (pH 7.0) containing 300 mM NaCl, 10 mM Tris-HCl, 5.0 mM $MgCl_2$, 5.0 mM $NaHSO_3$, and 5.0 mM β-mercaptoethanol were used to remove the 0.3 M NaCl soluble non-histone chromatin-associated proteins. Following the salt extraction, histones were removed with three 1 h (1.5 ml) extractions with 0.25 N HCl. Lipids were extracted by 3 washes (2.0 ml) in 80% ethanol : 10 mM Tris-HCl, 50% chloroform : 50% methanol, and 75%

Table 1. *Distribution of total chromatin-associated proteins*

Fraction	% Total chromatin-associated protein	S.E.M.
0.3 M NaCl-soluble NHCP	7.19	±1.20
Histones	48.37	±4.49
SU-NHCP	27.27	±5.64
Residual NHCP	13.93	±7.40

SU-NHCP, SDS, urea soluble non-histone chromatin-associated proteins. Protein determinations are based on the method of Lowry et al. [14].

ethanol : 25% ether, respectively. The pellet resulting from the final 1500 g, 10 min centrifugation was suspended in a buffer solution (pH 6.9) containing 1% sodium dodecyl sulfate (SDS), 4.0 M urea, 10 mM NaH_2PO_4 and 5.0 mM β-mercaptoethanol. This suspension was disrupted by three 15 sec treatments with a Tekmar Tissuemizer, boiled for 3 min, and stirred for 1 h at 25°C to remove most of the proteins associated with DNA. The proteins which remained in the supernatant after a 100000 g, 16 h centrifugation were termed SDS-urea soluble non-histone chromatin-associated proteins (SU-NHCP), and the proteins found in the pellet were termed residual non-histone chromatin-associated proteins.

To insure complete removal of phospholipids and nucleic acids in the experiment designed to study the phosphorylation of nuclear proteins 1 h after addition of PGE_1 and RO20-1724, the dehistonized lipid-free chromatin was extracted by the phenol procedure of Teng et al. [34]. DNA and RNA were removed in the aqueous phase while a majority of the NHCP entered the phenol phase.

Gel electrophoresis

Electrophoresis was conducted on 0.1% SDS, 10% polyacrylamide gels (8 mm diameter × 10 cm length) for 18–21 h as described by Weisenthal & Ruddon [37]. The slices were placed in counting vials with 6% Protosol in toluene scintillator (8 g, 2,5-diphenyloxazole/l), incubated for 3 days at 37°C and counted on a Packard-Tri Carb liquid scintillation spectrometer. The data were corrected to dpm and plotted as percent of total dpm for each isotope utilizing computer programs designed by P. M. Schwartz & J. Drach (personal communication). The average counting efficiency for each isotope was as follows: ^3H (26%), ^{14}C (52%), ^{33}P (67%), ^{32}P (89%). Molecular weight determinations were made using the method of Weber & Osborne [36].

Protein determination

Protein determinations were made with a modification of the method of Lowry et al. [14], using bovine serum albumin as a standard.

Protein banding patterns obtained by SDS gel electrophoresis

Each fraction exhibited a typical, reproducible pattern of polypeptides on SDS-polyacrylamide gels. The 0.25 N HCl fraction contained a polypeptide band with an apparent molecular weight of 22000 D which co-electrophoresed with calf thymus H1-histone. Low molecular weight histones (H3, H2*b*, H2*a*, and H4) migrated together as a single dense band with an apparent molecular range of 11000 to 14000 D. Although negligible amounts of histones were found in the other nuclear protein fractions, the 0.25 N HCl extract (histone fraction) contained small amounts of high molecular weight proteins which represented contamination of this fraction by non-histone proteins. The SU-NHCP and residual NHCP obtained by extraction of dehistonized chromatin with 1% SDS–4 M urea were characterized on SDS gels as a heterogeneous group of polypeptides. The molecular weight of most of these polypeptides ranged from 30000 to 150000 D. Similar observations were made with the non-histone protein extracts which were obtained from dehistonized chromatin by treatment with phenol; that is, NHCP present in both the phenol and aqueous phases were free of histones and contained primarily high molecular weight polypeptides. Recently, Suria & Liew [33] have reported that the phenol and SDS methods extract similar classes of NHCP.

RESULTS

Distribution of chromatin-associated proteins

Table 1 illustrates the protein content of the chromatin extracts from the pooled control and treated cell samples. The 0.3 M NaCl soluble NHCP fraction contained the lowest amount of chromatin-associated protein, and the histone fraction contained the highest amount of protein. About 70% of the proteins which remained associated with dehistonized chromatin were removed with 1% SDS–4 M urea, whereas 55% of these proteins were extracted into phenol.

Incorporation of amino acids into histone and non-histone chromosomal proteins (NHCP)

A marked decrease in the amino acid incorporation into histones (fig. 1*A*) was observed after 3 days of treatment of neuroblastoma cells with PGE_1 and RO20-1724.

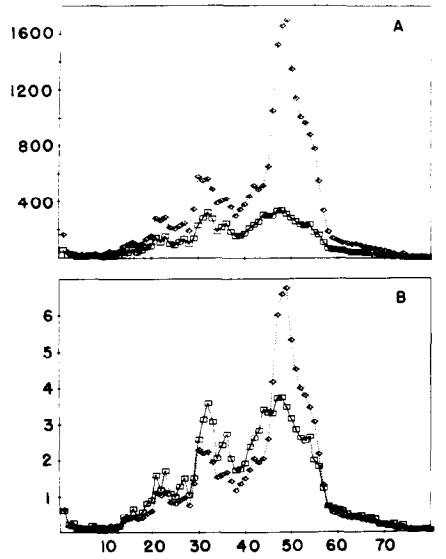

Fig. 1. Abscissa: mm; *ordinate* (*A*) dpm; (*B*) % total dpm.

Distribution of radiolabeled amino acids in SDS polyacrylamide gels of histone fractions from control and differentiated cells. Seventy-two hours after the addition of PGE_1 and RO20-1724 or vehicle, control and treated cells (5×10^7) were labeled with [^{14}C] or [^{3}H]amino acids for 2 h. Gels were cut into 1 mm slices and the radioactivity in control (◇---◇) and treated (□—□) was determined. (*A*) 0.25 N HCl fraction; (*B*) 0.25 N HCl fraction, total dpm, ^{14}C-25 133, ^{3}H-8975. Gels were run 17.5 h at 6 mA/gel. Direction of migration is from left to right. H histone slices 29–35.

In the treated cells there was a greater decrease in amino acid incorporation into the lower molecular weight histones than into H1 histone. This accounted for the apparent increase in the H1 histone contribution to the treated cell histone fraction when the data are plotted as percent total dpm (fig. 1*B*). There was no significant difference in the amino acid incorporation into histones between control and neuroblastoma cells which were treated with PGE_1 and RO20-1724 for only 1 h (data not shown).

The incorporation of amino acids into NHCP did not significantly change in neuroblastoma cells treated with PGE_1 and RO20-1724 for 1 h. There was, however, a small decrease in the incorporation of amino acids into a 40 000 mol. wt peak in both the SU-NHCP and residual NHCP fractions of cells treated for 3 days (fig. 2).

Incorporation of phosphate into histone and non-histone chromosomal proteins (NHCP)

The phosphorylation of H1-histone was markedly decreased in neuroblastoma cells 3 days after treatment with PGE_1 and RO20-1724 (fig. 3*A*); however, no significant change was observed if the neuroblastoma cells were treated with drugs for only 1 h (data not shown). There was no significant difference in the incorporation of radioactive phosphate into the 0.3 M NaCl soluble fractions of neuroblastoma cells when they were treated with PGE_1 and RO20-1724 for 1 h or 3 days. However, a small increase in incorporation of phosphate into a

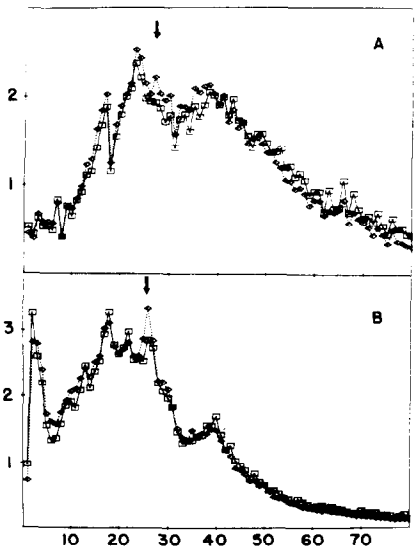

Fig. 2. Abscissa: mm; *ordinate:* % total dpm.

Distribution of radiolabeled amino acids in SDS polyacrylamide gels of various fractions from control and 72 h treated cells. Procedure and symbols same as fig. 1. (*A*) SU-NHCP, total dpm ^{14}C-24 577, ^{3}H-12 413; (*B*) residual-NHCP, total dpm, ^{14}C-50 658, ^{3}H-25 588. Gels were run for 20 h at 6 mA/gel. Arrows mark 40 000 D peaks.

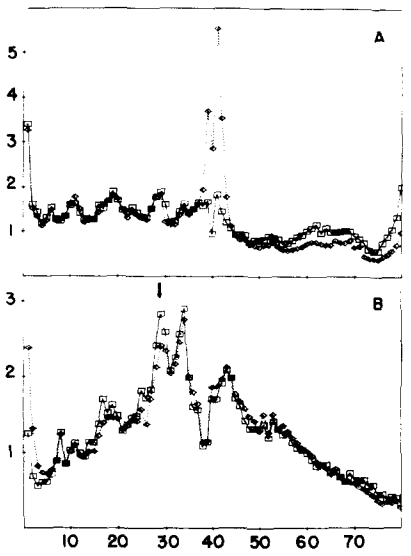

Fig. 3. Abscissa: mm; *ordinate:* % total dpm.
Distribution of radiolabeled phosphate in SDS polyacrylamide gels of various fractions from control and differentiated cells. Seventy-two hours after the addition of PGE_1 and RO20-1724 or vehicle, control and treated cells (5×10^7) were labeled with ^{32}P or ^{33}P for 2 h. Gels were cut into 1 mm slices and the radioactivity in control (◇---◇) and treated (□—□) was determined. (*A*) 0.25 N HCl fraction, total dpm, ^{32}P-110414, ^{33}P-49931; (*B*) SU-NHCP, total dpm, ^{32}P-298079, ^{33}P-108670. Gels were run for 21 h at 6 mA/gel. Arrow marks 40000 D peak. H, histone slices 38–44.

40000 D polypeptide of SU-NHCP fractions was observed in 'differentiated' cells (fig. 3*B*).

DISCUSSION

The results of this study demonstrate that a marked decrease in histone synthesis and H1-histone phosphorylation occurs in cAMP-induced 'differentiated' neuroblastoma cells. Studies with a number of inhibitors of cell division show that the synthesis of histones is correlated with the proliferative state of a cell [1, 9–11]. Thus, the decrease in histone synthesis most likely reflects the G1 phase arrest of the differentiated cells. It is of interest that H1-histone phosphorylation is markedly decreased in the differentiated cells at a time when H1 synthesis is relatively less decreased than the other histones. The reason for this is not clear but it may indicate that H1-histone phosphorylation is more tightly coupled to cell proliferation than is its synthesis.

A change in H1-histone phosphorylation is not observed in dividing neuroblastoma cells 1 h after treatment with PGE_1 and RO20-1724 even though these cells have an increased cAMP content [24]. H1-histone phosphorylation is controlled by at least two kinase activities; one that is cAMP-dependent and unassociated with cell division, and a second that is most active during DNA synthesis and does not appear to be regulated by cAMP [15]. A reduction in H1-histone phosphorylation is observed only in cells treated for 72 h. Since the cAMP levels are elevated in both proliferating (1 h drug-treated) [24] and non-proliferating (72 h drug-treated) neuroblastoma cells [29], it appears that phosphorylation of neuroblastoma H1-histone is mediated by a proliferation-dependent protein kinase. The possibility that neuroblastoma cells also contain a cAMP-dependent H1-histone kinase activity is not ruled out by these data, since only a small percentage of H1-histone phosphorylation is mediated by a cAMP-dependent kinase [12]. Neither do these data preclude the possible importance of cAMP-mediated histone phosphorylation in the differentiation process [31].

In contrast to histone synthesis and H1-histone phosphorylation, no significant change occurs in either the synthesis or phosphorylation of non-histone chromosomal proteins in cAMP-induced differentiated neuroblastoma cells. Other in-

vestigators [3, 5, 17] have been unable to demonstrate any significant changes in NHCP during development of rat brains. The significance of the small decrease in the synthesis and the small increase in the phosphorylation of 40000 D peptides in differentiated neuroblastoma cells, in which DNA synthesis is markedly reduced [27], is not presently known. It has been suggested that a non-histone protein of the 40000–45000 D range may be involved in DNA replication in eukaryotic cells [4]. Thus, the observed changes in the synthesis and phosphorylation of chromatin-associated proteins in differentiated neuroblastoma cells seem to reflect primarily the shift from a proliferating to a non-proliferating cell population. Whether any of these effects are involved in switching on specific differentiated functions is not evident. A correlation of these effects with the transcription of a specific gene product, for example, with the appearance of significant amounts of mRNA coding for tubulin [8], might provide such evidence.

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