Subtype-specific Cyclic AMP-dependent Histone H1 Phosphorylation at the Differentiation of Mouse Neuroblastoma Cells*

Kozo Ajiro‡, Kiyotaka Shibata‡, and Yasuhiro Nishikawa‡

From the Laboratory of Cell Biology, Aichi Cancer Center, Research Institute and the Department of Pharmaceutical Science, Nagoya City University, Nagoya 464, Japan

At the initial phase of cell differentiation in mouse neuroblastoma (N18) induced by dibutyrylcyclic AMP (dbcAMP), an additional site of histone H1 was extensively phosphorylated. Forskolin and various phosphodiesterase inhibitors also induced both cell differentiation and H1 phosphorylation at the identical site. The phosphorylation preferentially occurred in a single H1 subtype (H1e) among the five (H1a-e) fractionated by high performance liquid chromatography. The three H1 subtypes of N18 (H1e, H1d, and H1e) were phosphorylated in vitro, and their amino acid sequences of the phosphopeptides were identical to the known sequence of rabbit H1 peptides containing a serine 37 residue. However, the amount of H1a and H1b phosphorylations was negligible. The serine residue was replaced by threonine residue in H1a, and H1b did not have a homologous peptide. The tryptic phosphopeptides of H1 in N18 were identical to that in rat liver H1 induced by glucagon (Langan, T. A. (1969) Proc. Natl. Acad. Sci. USA 64, 1276-1283). The results indicate that 1) the response of H1 subtypes to cAMP-dependent protein kinase in vitro and in vivo is H1 subtype-specific, and 2) the H1c phosphorylation may play an important role in the restrictive area of chromatin in both cell differentiation and hormonal stimulation mediated by cAMP.

Mouse neuroblastoma cells were known to be induced in neural cells by cyclic AMP (cAMP) (1, 2), prostaglandin E1, and phosphodiesterase inhibitors (3, 4). The morphological observation of the cell differentiation indicated that the cell extruded neurites, as axon-like processes, and the nucleus enlarged at the initial phase of cell differentiation (1). Since these inducers all elevate directly or indirectly the intracellular level of cAMP, the cAMP-dependent protein kinase is activated in the cells (5, 6).

It is known that various functional proteins in cells are phosphorylated with the cAMP-dependent protein kinase (7). It was demonstrated that the catalytic subunit of cAMP-dependent protein kinase was translocated from cytoplasm into the nucleus and associated with chromatin in the nucleus (6, 8). Various chromatin proteins are also phosphorylated by cAMP-dependent protein kinase, and these phosphorylated chromatin proteins could possibly modify the chromatin structure (10).

Among the chromosomal proteins, histone H1, which is located on the linker region of nucleosome, is involved in maintaining the structural relationship between nucleosomes, DNA interacting with amino- and carboxyl-terminal ends of H1 (11, 12). H1 contains three to five different subtypes which are slightly different in size and amino acid composition, and their subtypes have possibly different biological and physiological functions (13, 14). A cAMP-dependent phosphorylation site of H1 was found in rat liver H1 at serine 37 by administration of glucagon (15, 16). It was shown that the H1 phosphorylation which accompanied the liver specific gene expressions (17-19). Most other H1 phosphorylations in mammalian cells take place cell cycle-dependently (growth-associated phosphorylation sites) (20-23) and are phosphorylated with H1 kinase which has recently been reported to be homologous with the cdc2 protein kinase in yeast (24, 25).

The present study examined the H1 phosphorylation of mouse neuroblastoma cells at the initial phase of differentiation induced by various chemicals. The results indicated that 1) the cAMP-dependent phosphorylation site in H1 was extensively phosphorylated in vivo at the initial phase of N18 cell differentiation, 2) the phosphorylation preferentially occurred in a single H1 subtype, and 3) the characteristics of the phosphorylation were quite similar to the rat liver H1 phosphorylation induced by glucagon (16).

MATERIALS AND METHODS

Cell Culture and Induction of Neuroblastoma Cell Differentiation—Mouse neuroblastoma cells (N18) were grown at 37 °C in Dulbecco’s modification Eagle’s medium (GIBCO) supplemented with 10% fetal calf serum. The cells were cultured in dishes (13-cm diameter) at 10% CO2 gas. For the induction of cell differentiation, the cells (approximately 5 × 10^6/dish) were exposed to various chemicals such as 1 mM dbcAMP, 90 μM forskolin, or 1 mM isobutylmethylxantine (IBMX). In most cases, the cells started to extend the neurites around 4 h after the treatment of the chemicals and differentiated into neural cells within 3 days.

Isolation and Purification of 32P-Labeled Histone H1—For the 32P-labeling of H1, the culture medium was replaced with a warm phosphate-free medium (22) containing [32P]orthophosphate (30–40 μCi/mI) and chemicals for the induction of cell differentiation, then the cultures were incubated further for 3 h at 37 °C. In some experiments, the cells were pretreated with the chemicals for 4-18 h. The cells were harvested and washed with saline three times. The H1 was extracted twice with 5% perchloric acid (PCA) solution. The PCA soluble fractions were combined and the H1 was precipitated with cold 20% trichloroacetic acid by adding 1% volume of 120% trichloroacetic acid. The H1 pellet was washed with acetone containing 0.3% HCl and twice with acetone.

Preparation of 32P-Labeled Histone H1 from Rat Liver in Which Glucagon Was Administered—A Wistar rat (1 ~ 2 months old, ~200 g) was administered 500 μg of glucagon (Sigma) dissolved in 1 mL of saline with 1 mL of [32P]orthophosphate. The second animal was

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also administered the same solution as above without glucagon. One hour later, the animals were decapitated. The livers were dissected out and frozen immediately with acetone-dry ice. Each liver was minced and H1 was extracted with 5% PCA.

H1 Fractionation with HPLC—The 32P-labeled H1 (200 µg) was applied on HPLC (Varian 5000) with a reverse-phase column (RP-8, Beckmann). The H1 was fractionated by an acetonitrile gradient (55-45%) containing 0.1 M sodium perchlorate (26). The flow rate was 1 ml/min, and fractions were collected every 1.5 ml. The protein was monitored at 208 nm with a densitometer (UV-50, Varian). The fractions of each protein were pooled and vacuum-dried. The protein pellets were washed twice with acetone. The H1 protein in the fractions was confirmed with SDS-gel electrophoresis (27). The gel (15 x 30 cm, 0.75 mm in thickness) was run at 60 V for 72 h. The proteins were stained with 0.2% Comassie Brilliant Blue.

Histone H1 Phosphorylation in Vitro—H1 phosphorylation in vitro was carried out in a reaction mixture containing 20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.1 mM dithiothreitol, cell extract (10 µg of protein), 10 µg of H1 from N18 cells, 0.01 mM [γ-32P]ATP (500 - 800 cpm), and 2 µM of cAMP. As an enzyme source, cell extract of N18 cells prepared by the method of Adlakha et al. (28), or purified catalytic subunit of cAMP-dependent protein kinase (10 units for each reaction, kindly given by Dr. Inagaki, Aichi Cancer Center Research Institute) were used. The reaction was incubated at 30 °C and terminated by adding 1/2 volume of 75% trichloroacetic acid. The 32P-labeled H1 was recovered by trichloroacetic acid precipitation.

Resolution of Phosphopeptides on Cellulose TLC Plates—The procedures for trypsin digestion and tryptic peptide mapping of phosphorylated H1 were followed by the methods described previously (22). The H1 and H1 subtypes were digested with 2% trypsin in 0.05 M ammonium bicarbonate for 4 h at room temperature. The H1 peptides were mapped on thin layer plates (TLC, cellulose 20 x 20 cm, 0.1 mm in thickness, Merck). In the first dimension, the peptides were electrophoresed with a buffer (butanol/acetic acid/water/pyridine, 50:25:900:25, pH 4.7) at 500 V for 50 min. For the analysis of two-dimensional peptide maps, the plates were dried after the electrophoresis, and chromatography was performed in the second dimension with a solution (butanol/acetic acid/water/pyridine, 48.5:15:2:800:475,6) as described previously (23). The plates were exposed to x-ray films (X-Omat, Kodak) for 1 week.

Amino Acid Sequence Analysis of Phosphopeptides—Individual phosphorylated H1 subtypes were digested with trypsin and the peptides were applied on an HPLC as described above. The peptides were fractionated with a gradient of acetonitrile (0-30%) containing 0.1% trifluoroacetic acid.

Fig. 1. Tryptic phosphopeptide maps of histone H1 extracted in early phase of differentiated mouse neuroblastoma cells. A, N18 cells (approximately 5 x 10⁵ cells/15-cm dish) were treated with 1 mM dbcAMP for 18 h after 2 days of plating. -, control N18 cells; + dbcAMP, N18 cells cultured in 1 mM dbcAMP. B, autoradiographs of ³³P-labeled histone H1 phosphopeptides. 50 µg of ³³P-labeled H1 protein (~3000 cpm) extracted from the above cells was digested with trypsin and the phosphopeptides were mapped on TLC plates. Electrophoresis and chromatography were conducted as described under "Materials and Methods." The plates were exposed for 4 days to x-ray films. An additional spot in the + dbcAMP indicates a spot which was induced in dbcAMP-treated N18 cells with neurite formations.
RESULTS

Mouse Neuroblastoma Cell Differentiation and an Intramolecular Site of cAMP-dependent Histone H1 Phosphorylation—N18 cells were treated with 1 mM dbcAMP for 18 h. Neurite extrusions were observed in more than 75% of the cells in the culture, whereas no change appeared in the control cells (Fig. 1A). The N18 cells were labeled with [32P]phosphate for 3 h at the initial phase of the differentiation (18 h after pretreatment of 1 mM dbcAMP). Histone H1 was extracted and trypsinized. The tryptic phosphopeptides of H1 were analyzed on two-dimensional TLC plates and autoradiographed (Fig. 1B). In the H1 from the control cells, there were 9 major and about 10 minor spots of phosphopeptides (Fig. 1B). In the phosphopeptide map, most of the spots were due to growth-associated H1 phosphorylation, as observed in our previous data on the H1 phosphopeptide pattern of HeLa cells (23). However, an additional spot was observed in the H1 of N18 cells which induced cell differentiation by dbcAMP (Fig. 1B, +dbcAMP). The phosphopeptide was the most hydrophobic peptide among others, judging from the position by chromatography. From densitometric quantitation on the autoradiography of one-dimensional electrophoresis (TLC plates), the intensity of the spot was approximately 5~8% of the total H1 phosphopeptides. The site of phosphorylation was designated as A-site phosphorylation by Langan (30).

Both cell differentiation and the identical spot of A-site phosphorylation were observed in the cells treated with inhibitors for phosphodiesterase (IBMX, theophylline, and caffeine) and an activator of adenylcyclase (forskolin). Table I shows the relationship between the cell differentiation and the A-site phosphorylation under the various chemicals in the same method as above. The data indicate that the chemicals which induced the cell differentiation induced the A-site phosphorylation. Moreover, it is evident that a higher rate of N18 cell differentiation approximately correlated to a higher intensity of A-site autoradiography. The chemicals, especially dbcAMP, IBMX, and forskolin enhanced the rate in both cell differentiation and A-site phosphorylation. In the cases of caffeine and theophylline, the rate of cell differentiation was lower than those of the above three chemicals. Papaverine and prostaglandin E1 had intermediate effects. Since the rate of N18 cell proliferation was reduced by treatment with these chemicals, it might be considered that the inhibition of the cell proliferation causes the A-site phosphorylation. However, hydroxyurea, an inhibitor of DNA synthesis, neither induces cell differentiation nor enhances A-site phosphorylation. Hexamethylene-bisacetamide, which is known to induce the differentiation of murine erythroleukemia cells (31), had no effect either. Consequently, it is quite likely that the A-site phosphorylation was caused by an elevated phosphorylation in the A-site level.

The rates of [32P]phosphate incorporated into each H1 subtype were shown in Fig. 3. In the N18 cells which were treated with 1 mM IBMX, the rates were decreased approximately 10% compared with control cells. It could not be determined whether the reduction of phosphorylation was caused directly by the effect of IBMX, or it simply represented a lower level of H1 phosphorylation which was usually observed in various kinds of differentiated cells. The individual H1 subtypes were trypsinized and fingerprinted on TLC plates and autoradiographed. The phosphopeptide maps of H1 subtypes (a–c, and e in Fig. 4) were different from one subtype to another. The maps of H1a and H1b showed no difference between the control and IBMX-treated cells. The induced spot of A-site phosphorylation was clearly observed in H1c but was very faint in H1e (Fig. 4c and e, indicated by arrows).

The spot of A-site phosphorylation also occurred in H1c when cells were induced by dbcAMP, forskolin, and theophylline (data not shown). H1d had the faint spot in the identical position and may have been derived from a trace amount of H1c because of an incomplete resolution between H1c and H1d (see Fig. 2A). The intensity of the spot in H1c was approximately 8% of total H1c radioactivity estimated by a scanning of one-dimensional electrophoresis.
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**Fig. 2.** Fractionation of H1 from N18 cells with HPLC. A, \(^{32}P\)-labeled H1 (~300 μg) from the N18 was loaded on a reverse-phase HPLC column. H1 was fractionated with a gradient of acetonitrile (30-45%) containing 0.1 M sodium perchlorate. The protein was monitored by UV at 208 nm, and the fractions were collected every 1.5 min. The H1 had five peaks: H1a, H1b, H1c, H1d, and H1e, in the order of HPLC elution. B, the 5% PCA-soluble H1 fractions were recovered and run on SDS-gel electrophoresis at 60 V for 72 h. T, total H1; 1, H1a; 2, H1b; 3, X, unidentified protein; 4, H1b; 5, H1b a HMG protein; 6, H1c; 7, H1d; 8 and 9, H1e.

**Fig. 3.** Rate of \(^{32}P\)-incorporation into H1 subtypes of N18 cells. N18 cells were preincubated with 1 mM IBMX for 18 h, then labeled with \(^{32}P\) for 3 h with IBMX, and H1 was extracted. H1 was fractionated by HPLC as shown in Fig. 2, and the fractions were pooled and their \(^{32}P\) radioactivities were estimated. The value was represented as cpm/μg H1 protein. Open column, H1 from control cells; hatched column, H1 from dbcAMP-treated N18 cells.

H1 Phosphorylation of N18 Cells in Vitro—To confirm that the site was phosphorylated cAMP-dependently, a phosphorylation in vitro was conducted. Both H1 and the cell extract were prepared from N18 cells and used in the cell-free reaction mixture. After the reaction, phosphorylated H1 was digested with trypsin and mapped on TLC plates. Fig. 6 indicates that a single major spot appeared in the reaction mixture with 2 μM cAMP (Fig. 5B), whereas no corresponding spot was seen in the control (Fig. 5A). The position of the major spot was identical to the spot of A-site phosphorylation which was induced in vivo by dbcAMP or IBMX (Fig. 1 or 4). Several faint spots of growth-associated phosphorylation were also observed.

The H1 and the subtypes of N18 cells were also phosphorylated in vitro by purified cAMP-dependent protein kinase (Fig. 5, lower panel). The phosphorylations of total H1 (Fig. 5, T) and three H1 subtypes (Fig. 5, a, c, and e) are shown. The A-site of H1a was phosphorylated in a negligible amount (Fig. 5a), whereas those of the two H1 subtypes (H1c and H1e) were extensive (Fig. 5, c and e). The phosphorylation rates of individual H1 subtypes were shown in Fig. 6. The A-site of H1c was extensively phosphorylated both in vivo and in vitro, whereas that of H1a was not phosphorylated in vivo and very little in vitro. In H1e, it was phosphorylated extensively in vitro but not much in vivo (Figs. 4 and 6). The results indicate clearly that the A-site in H1 was phosphorylated cAMP-
Amino Acid Sequence of Phosphopeptide of Individual H1 Subtypes—The phosphopeptide of individual H1 subtypes was purified by HPLC and their amino acid sequences were determined (Table II). The sequences in the three subtypes (H1c, H1d, and H1e) were identical to that of rabbit thymus H1 as determined by Cole (32). There were 2 serine residues in the peptides. One was in a consensus sequence for cAMP-dependent protein kinase and corresponded to 37th serine of rabbit thymus H1. The other serine residue at 42 was not in a consensus sequence for the protein kinase. Therefore, the site of phosphorylation in N18 cells was tentatively determined at serine 37 in the three H1 subtypes. However, H1a had a sequence where the serine residue was replaced by threonine at the homologous site. The H1a had very little incorporation of [32P]phosphate in vitro by cAMP-dependent protein kinase. It is possible the minor incorporation of [32P]phosphate was the phosphorylation at this threonine residue. On the other hand, H1b had no incorporation of [32P]phosphate by the protein kinase and no corresponding peptide was found by HPLC analysis (data not shown). This indicates that the distribution of lysine or arginine residues in H1b is different from that of other H1 subtypes, and a different peptide(s) may be produced by trypsin.

DISCUSSION

It has been shown that the cAMP-dependent H1 phosphorylation which occurs in vivo is quite limited in extent (18), suggesting that there may be some selectivity in the phosphorylation which takes place in the cell. The present data indicated limited phosphorylation also in the neuroblastoma H1 and that there is some mechanism in which H1c was primarily phosphorylated among the H1 subtypes. It is possible that the difference might derive from the different accessibilities to the protein kinase or the different rates of protein turnover among the three H1 subtypes (H1c, H1d, and H1e) (33). It was reported that H1c subtypes were non-uniformly distributed on chromatin (34). The fact that one of the H1 components phosphorylated extensively in vivo suggests that the effect of cAMP-dependent H1c phosphorylation may exist primarily in a restrictive chromatin and may facilitate the administration of the structure or activity of a large segment of the genome.

The characteristics of the cAMP-dependent H1 phosphorylation in mouse neuroblastoma cell differentiation and hormonal stimulation in rat liver were quite similar. 1) The phosphorylation site occurred in a homologous serine site in both cases. 2) It occurred mainly in a specific H1 subtype (H1c). The result correspond to Ohba et al. (35) which showed that phosphorylation of one subtype in rat liver H1 was preferentially stimulated by glucagon. 3) The maximum phosphorylation occurred around 40 ~ 60 min after treatment of the chemicals, and cyclohexamide and actinomycin D did not block the phosphorylation (data not shown). Therefore, the H1 phosphorylation of N18 cells takes place on intact histone molecules and is a primary effect of the protein kinase as observed by Langan (16) in rat liver H1 phosphorylation. Consequently, it is quite likely that the cAMP-dependent H1 phosphorylation in the two different systems occurs by way of the same mechanism. When phosphorylation of rat liver H1 by cAMP-dependent protein kinase was demonstrated in vivo, the H1 phosphorylation might provide the primary step in the mechanism for the induction of certain enzymes whose synthesis has been shown to be increased by cAMP (16). It has been reported that cAMP increases the amount of poly(A) containing cytoplasmic RNA in cultured neuroblastoma cells...
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TABLE II

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Amino Acid Sequence</th>
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<tbody>
<tr>
<td>H1a</td>
<td>Lys-Ala-Thr-Gly-Pro-Pro-Val-Ser-Glu-Leu-Ile-Thr-Lys</td>
</tr>
<tr>
<td>H1b</td>
<td>No corresponding peptide</td>
</tr>
<tr>
<td>H1c</td>
<td>Lys-Ala-Ser-Gly-Pro-Pro-Val-Ser-Glu-Leu-Ile-Thr-Lys (P)</td>
</tr>
<tr>
<td>H1d</td>
<td>Lys-Ala-Ser-Gly-Pro-Pro-Val-Ser-Glu-Leu-Ile-Thr-Lys (P)</td>
</tr>
<tr>
<td>H1e</td>
<td>Lys-Ala-Ser-Gly-Pro-Pro-Val-Ser-Glu-Leu-Ile-Thr-Lys (P)</td>
</tr>
</tbody>
</table>

(36). However, at present, the relationship between the cAMP-dependent H1 phosphorylation and the RNA synthesis is still not clear. Certain alterations in transcription of a class of diverse genes would be affected through the modification of the chromatin structure, possibly mediated by protein phosphorylation. It is conceivable that cAMP-dependent phosphorylation at the amino-terminal end of H1 could be involved in the alterations of nucleosome structure or repeated lengths along the chromatin (37).

Our data indicate that 1) the serine residue of H1a was replaced by threonine, 2) no corresponding peptide was observed in H1b, and 3) the other three subtypes (H1c, H1d, and H1e) had serine at the identical position. The findings also indicate that H1 subtypes vary in both site and level of phosphorylation. A distinct functional difference may well exist among the five different H1 components. It is notable that serine 37 (analogous to calf thymus serine 38) is a site of amino acid heterogeneity in H1 molecules of certain species. H1 fraction 3 from rabbit thymus has alanine in place of serine (38). The replacement of serine by threonine was also observed recently in a human H1 subtype (H1a) (39). The heterogeneity in H1b is more diversified than our current evidence among the five H1 subtypes fractionated with HPLC.

There was little evidence to indicate that cAMP-mediated histone phosphorylation occurred in cell differentiation. It is reasonable to speculate that the chromatin structure can be changed to some extent from replication state to differentiation state. In fact, Prasad and Hse (1) observed decreasing cell growth and enlargement of nuclei at the beginning of the neuroblastoma cell differentiation induced by dbcAMP. It has been shown that the H1 phosphorylation by cAMP-dependent protein kinase markedly alters the interaction of the histone with DNA as measured by changes in circular dichroic spectra (40, 41). The evidence suggests the H1 phosphorylations may relate to the initiation of the cell differentiation, at least in cells which are induced the differentiation by cAMP or the related chemicals. Then, the solid change of chromatin structure from proliferation state to differentiation may be associated with compositional changes of H1 subtypes in dividing and non-dividing mouse neuroblastoma (42, 43).

The present data indicate that H1 phosphorylation at serine 37 occurred in rapidly growing cells as observed in rat H35 hepatic cells (44). There appear to be two quantitatively distinct types of H1 phosphorylation in N18 cells, growth-associated and cAMP-dependent. This does not imply that the reactions are completely independent. It is therefore important to determine whether the H1 phosphorylation at certain specific sites influences either the rate or extent of phosphorylation at other sites. During mitosis and premature chromosome condensation, H1 and H3 are extensively phosphorylated (45-48). It is of interest whether the cAMP-dependent protein kinase is active and the H1 site is phosphorylated during mitosis, since the mitotic-specific phosphorylation site in H3 is phosphorylated in vitro by cAMP-dependent protein kinase (49). The N18 cells may provide an excellent system for the study of the relationship between growth-associated H1 phosphorylations and cAMP-dependent H1 phosphorylations.

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