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METHYLATION OF 21–23 kD MEMBRANE PROTEINS BY A MEMBRANE-ASSOCIATED PROTEIN CARBOXYL METHYLTRANSFERASE IN NEUROBLASTOMA CELLS

INCREASED METHYLATION IN DIFFERENTIATED CELLS

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Abstract—Membranes of neuroblastoma N1E-115 cells contain a specific protein carboxyl methyltransferase that methylates a 70 kD protein and a group of 21–23 kD proteins which are tightly bound to the membranes. The enzyme catalyzes the transfer of [*methyl*-^{3}H] groups from [*methyl*-^{3}H]*S*-adenosyl-L-methionine (K_m = 0.22 μM) to these proteins to form base-labile carboxymethylesters. These protein methylesters are relatively stable compared to other protein methylesters, as shown by the ability of the 21–23 kD methylated proteins to retain their [*methyl*-^{3}H] groups at pH values of 7 to 8.5 for at least 12 hr at room temperature. The extent of methylation of the 21–23 kD proteins, but not that of the 70 kD protein, was increased in membranes of cells induced to differentiate by 2% dimethyl sulfoxide (from a basal level of 0.1–0.2 to 0.9–1.2 pmol [*methyl*-^{3}H] groups incorporated per mg membrane protein). This increase appeared after a lag period of 3 days of growth in the presence of the dimethyl sulfoxide and developed in parallel with the appearance of neurite-like processes in the cells. Kinetic experiments suggest that the amounts of 21–23 kD proteins available for methylation in the membranes of the undifferentiated and of the differentiated cells are limited. This and the previously observed low turnover of methylated 21–23 kD proteins in the intact cells suggest that the differentiated cells express and methylate more 21–23 kD proteins than the undifferentiated cells. These methylated proteins may be involved in differentiation or other functions of the differentiated cell membranes.

Methylations of free carboxyl groups on proteins in prokaryotic [1, 2] and eukaryotic [3, 4] cells are catalyzed by enzymes that utilize *S*-adenosyl-L-methionine (AdoMet†) as the methyl donor to form protein carboxyl methylesters. Neutralization of negative charges and increase in hydrophobicity would occur upon methylation of protein carboxyl groups. This and the reversibility of the modification led to the suggestion of a regulatory role for protein carboxyl methyltransferase [5]. Such a function was indeed demonstrated for the bacterial γ-glutamyl methyltransferase which methylates L-glutamate residues of membrane chemoreceptors to modulate their signaling functions [1, 2, 6, 7]. A regulatory role for protein carboxyl methylations in eukaryotic cells has been questioned in view of the low specificity of the methylating enzymes and the low stoichiometry of methyl group incorporation into protein molecules [8]. Evidence was also presented that reversible (rapidly hydrolyzable) protein carboxyl methylesters are formed at abnormal D-aspartyl [8] and L-isoaspartyl [9, 10] residues. It is not known, however, whether rapidly reversible protein methylations which are implicated in, for example, hormone secretion [11], cell motility [12, 13], protein synthesis [14, 15] and cell differentiation [16, 17] represent this or another type of protein carboxyl methylation in eukaryotic cells. Nonetheless, several studies have shown that, in addition to rapidly hydrolyzing protein carboxylmethylesters, other, more stable methylesters are formed in proteins of eukaryotic cells. These protein methylesters are resistant to pH values of 8.0 to 8.5 which are used in the standard Laemmli [18] gels. Examples include the cGMP phosphodiesterase [19], and other unidentified proteins in bovine rod outer segments [19, 20], the mouse nuclear lamin B [21], and unidentified 20–23 kD proteins in the membrane of a macrophage cell line [22]. The regulatory functions of this type of methylation can be inferred from the cell cycle-dependent methylation of lamin B [21] and from the GTP-dependent methylation of the 20–23 kD macrophage proteins [22]. Another example is provided by neuroblastoma N1E-115 cells. In a recent study we have shown that a group of relatively stable 21–23 kD protein carboxyl methylesters is formed by intact neuroblastoma N1E-115 cells [23].

Recent experiments have shown that several *ras* proteins [24–26], the retinal cGMP phosphodiesterase [27], and rod outer segment proteins [20], like several fungal peptidyl sex and mating factors [28, 29] contain a C-terminal carboxyl methylated cysteinyl residue. These protein carboxyl methylesters are also relatively stable. It was proposed [20] that the 20–29 kD proteins of the rod outer segments may, like the normal *ras* proteins [30], represent a group of small G proteins.

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† Abbreviations: AdoMet, *S*-adenosyl-L-methionine; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; and FCS, fetal calf serum.

Here we describe the methylating enzyme present in the membranes of neuroblastoma N1E-115 cells. This enzyme catalyzes the AdoMet-dependent methylation of membrane bound 70 kD and 21–23 kD proteins. Our results suggest that the enzyme methylates the limited amounts of 21–23 kD proteins and that the level of methyl acceptor proteins in the membranes increases in parallel with differentiation.

MATERIALS AND METHODS

Materials. [*Methyl*-^{3}H]AdoMet (11–15 Ci/mmol) was purchased from New England Nuclear. *S*-Adenosyl-L-homocysteine was from the Sigma Chemical Co. Culture medium and flasks were as detailed previously [16]. Molecular weight standards were obtained from Pharmacia.

Cell culture. Cells of mouse neuroblastoma clone N1E-115 were routinely grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37° in a humidified atmosphere of 5% CO_2/95% air as described earlier [16]. The cells were plated at a density of 2×10^4 cells/cm^2 in 75 cm^2 tissue culture flasks. Medium was replaced 1 day after plating, 3 days after plating, and daily thereafter. Control (undifferentiated cells) received only DMEM + FCS. The DMSO-treated cultures (differentiated cells) were fed with DMEM + FCS containing 2% DMSO, starting 1 day after plating. The control cells continued to multiply and reached confluence by 5–6 days in culture. The DMSO-treated cells stopped multiplication and extended processes 4–5 days after plating, in line with previous reports [16, 31, 32]. Cells were used for experiments from day 0 to day 7 in culture. They were detached from flasks, after 4 × 15 mL washes with phosphate-buffered saline (PBS), in PBS containing 5 mM EDTA. The detached cells were pelleted (1000 *g*, 5 min) and resuspended in 0.5 to 0.75 mL of 20 mM sodium-phosphate buffer containing 5 mM EDTA and 5 mM β-mercaptoethanol (buffer A). The resuspended cells were sonicated for 20 sec, and cytosolic proteins were separated from the membranes by centrifugation at 100,000 *g* for 1 hr. The cytosolic fraction was removed and saved, and the precipitated membranes were resuspended in buffer A to yield preparations containing 10–15 mg protein/mL. In cases where washed membranes were used, the membranes were resuspended in 3 vol. of the original homogenization buffer and reprecipitaed. This procedure was repeated twice.

Methylation reactions. In the standard assays membrane preparations (150–300 μg of protein) were incubated with 0.1 M sodium acetate buffer, pH 6.5, containing 25 μM [*methyl*-^{3}H]AdoMet in a total volume of 60–100 μL, for 30 min at 37°. Reactions were terminated by the addition of sodium dodecyl sulfate (SDS) gel sample buffer, and proteins were loaded into the gels. When cytosolic proteins were used, they were mixed with the membrane preparations before the methylation reactions. *S*-Adenosyl-L-homocysteine (100 μM), which was used to inhibit the methylation reactions, was added to the membranes prior to the addition of [*methyl*-^{3}H]AdoMet. Protein carboxyl methylesters were determined after separation on 12.5% polyacrylamide SDS slab gels, pH 8.5, according to Laemmli [18] as described earlier [33]. The gels were Commassie blue stained, destained, dried and subjected to the vapor phase equilibrium assay [34] as described by Chelsky *et al.* [21]. More specifically, the dried gels were cut into 2-mm sections, and each section was placed in a Beckman microfuge tube containing 100 μL of 1 N NaOH. The uncapped tubes were placed in scintillation vials containing 5 mL of scintillation liquid (Hydroluma, Lumac, The Netherlands) which were capped and allowed to equilibrate for 24 hr before counting.

RESULTS

Confluent and 2% DMSO-induced differentiated cells (6 days in culture) were lysed, and the membranes of the cells were separated from the cytosol. The membranes were then incubated for 30 min at 37° with 25 μM [*methyl*-^{3}H]AdoMet, and proteins were fractionated by SDS polyacrylamide gel electrophoresis at pH 8.5. Incorporation of [*methyl*-^{3}H] groups into proteins was determined by the vapor phase equilibrium assay for the dried gel sections which were immersed in 1 N NaOH. This assay specifically detects the [^{3}H]methanol that was formed by the alkaline hydrolysis of the protein carboxyl methylesters [21, 34]. In a typical experiment we observed in gels of both confluent (Fig. 1A) and DMSO-induced differentiated (Fig. 1B) cell membranes two major radioactive peaks. Significant radioactivity was present just behind the tracking dye. The two major radioactive peaks correspond to a ~70 kD protein and to 21–23 kD proteins. The latter peak was relatively broad and probably contains more than one protein (Fig. 1). The amounts of radioactivity found in the 70 kD protein of membranes from confluent cells were similar to those found in membranes from differentiated cells. Radioactivity in the 21–23 kD peak, however, was higher in the membranes from differentiated cells (Fig. 1). In either case, incorporation of [*methyl*-^{3}H] groups into the proteins was inhibited strongly by the potent methylation inhibitor *S*-adenosyl-L-homocysteine (Fig. 1).

Additional experiments confirmed the base lability and subsequent volatility of the [*methyl*-^{3}H]groups that were incorporated into the proteins: (1) exposure of the gel sections to 1 N NaOH for 12 hr at room temperature and drying down volatilized more than 90% of the counts; (2) when the gel sections were exposed to 50 mM sodium-phosphate buffer, pH 7.0 (12 hr), instead of base in the vapor phase equilibrium assay, no radioactive peaks were detected, and subsequent addition of 1 N NaOH resulted in the appearance of volatile radioactivity corresponding to the 70 kD and 21–23 kD proteins peaks. These results are consistent with the formation of carboxyl methylesters of 70 kD and 21–23 kD proteins.

The methylesters in the proteins which were selectively increased in the membranes of the differentiated cells (21–23 kD proteins) were relatively stable. They could resist prolonged exposure to

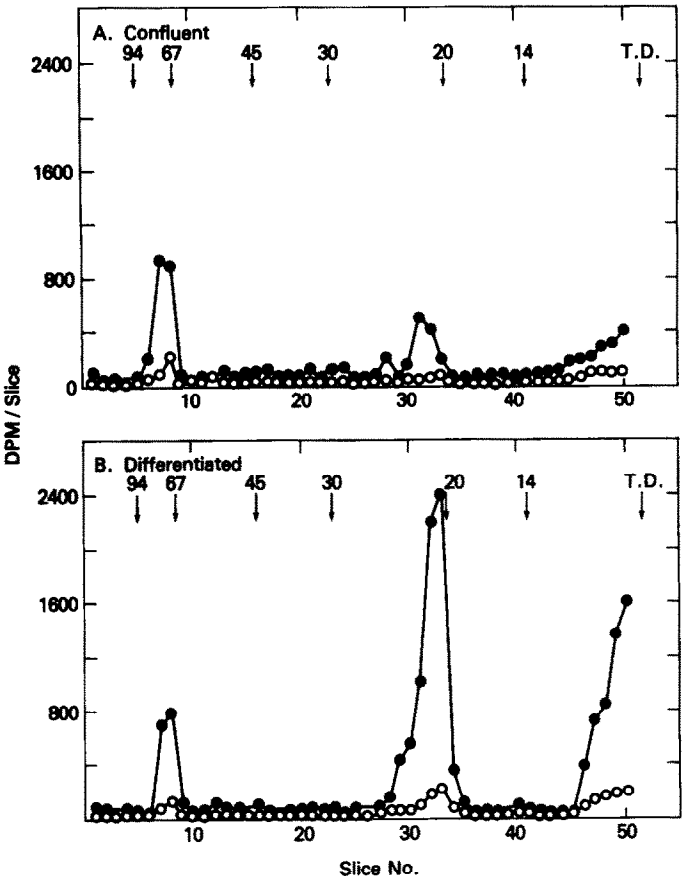

Fig. 1. Methylation of proteins in membranes of confluent and differentiated neuroblastoma N1E-115 cells. Membranes (200 μg) of 6-day confluent cells (A) and of 6-day 2% DMSO-treated cells (B) were incubated for 30 min at 37° with 25 μM [*methyl*-^{3}H]AdoMet in the absence (●) and in the presence (○) of 100 μM *S*-adenosyl-L-homocysteine. Proteins were then separated on SDS polyacrylamide slab gels which were dried, sliced, and subjected to the vapor phase equilibrium assay as described in Materials and Methods. Molecular weight stanards (kD) were electrophoresed in parallel lanes, and their migration distance is indicated by arrows. T.D. = tracking dye. Data are presented as [*methyl*-^{3}H] groups (in dpm) detected in each gel section. The results are from one experiment out of four that gave similar results.

pH 8.5 at room temperature, as well as 20-min exposure to 1 M sodium-borate, pH 11 (Table 1). These experiments emphasize the distinction between these carboxyl methylesters in the 21–23 kD proteins and the readily hydrolyzable protein carboxyl methylesters which are formed also in membranes of neuroblastoma cells [16]. The latter methylesters are not stable at pH 8.5 and are degraded completely within 10 min in 1 M sodium-borate [16]. Another important property of the 21–23 kD proteins is their association with the cell membrane: 1 M NaCl did not remove them from the membranes, but 0.1% Nonidet NP40 did solubilize most of these methylated proteins (Table 1). The observations that methylations proceeded with membranes alone, as well as with extensively washed membranes (Table 2), and that the addition of cytosol did not increase methylation of the 21–23 kD proteins (Table 2), suggest that the methylating enzyme itself is also associated with the membranes. The reaction was blocked completely by heating the membranes to 100° (Table 2), indicating that either the enzyme or the substrate is denatured by heat.

Since the 21–23 kD proteins have not yet been purified, no attempts were made at this point to separate between the enzyme and its substrates. Analysis of methylation as a function of individual protein concentrations cannot yet be performed. Kinetic analysis of methylation of the 21–23 kD proteins in the intact membranes, however, showed typical characteristics of enzymatic reactions. The reactions were time and temperature dependent (Fig. 2) though they leveled-off after 5–10 min at 37° or somewhat later (10–30 min) at 25°. Initial methylation of the 21–23 kD proteins (1 min at 25°) showed typical Michaelis–Menten kinetics with respect to [*methyl*-^{3}H]AdoMet (Fig. 3); the estimated K_m was 0.22 μM. The slowing rate of the reaction (Fig. 2) could have been due to product inhibition by *S*-adenosyl-L-homocysteine, or to depletion of unmethylated substrate. We have estimated the concentration of *S*-adenosyl-L-homocysteine that would be formed under the conditions

Table 1. Methylated 21–23 kD membrane proteins of neuroblastoma cells are stable and tightly bound to the membrane

Treatment of membranes after methylation		[*Methyl*-^{3}H] groups in 21–23 kD proteins (pmol/mg membrane protein)
None		1.12 ± 0.11
1 M Sodium-borate, pH 11, 20 min, 25°		1.21 ± 0.12
0.05 M Tris buffer, pH 8.5, 24 hr, 4°		0.98 ± 0.10
1 M NaCl extract:	supernatant	0.07 ± 0.04
	pellet	0.95 ± 0.21
0.1% NP 40 extract:	supernatant	1.06 ± 0.15
	pellet	0.10 ± 0.03

Membranes from 2% DMSO-induced differentiated cells were methylated under the conditions described in Fig. 1, and then treated as indicated. The 1 M NaCl and 0.1% Nonidet NP 40 treatments were for 30 min at 4° and supernatant fractions were separated from non-soluble material by a spin at 100,000 *g*. In each case, proteins equivalent to the amount of 250 μg of non-treated membranes were separated on gels and the bands corresponding to the 21–23 kD proteins were subjected to the vapor phase equilibrium assay. Data are means ± SD of three separate determinations.

Table 2. Methylation of 21–23 kD membrane proteins of neuroblastoma cells by a membrane associated methyltransferase

Cells	Preparation	[*Methyl*-^{3}H] groups incorporated into 21–23 kD proteins (pmol/mg membrane protein)
Confluent	Membranes	0.17 ± 0.03
Confluent	Washed membranes	0.16 ± 0.04
Confluent	Boiled membranes	0.03 ± 0.01
Confluent	Membranes + cytosol	0.19 ± 0.03
Differentiated	Membranes	0.91 ± 0.21
Differentiated	Washed membranes	0.88 ± 0.13
Differentiated	Boiled membranes	0.08 ± 0.02
Differentiated	Membranes + cytosol	1.06 ± 0.12

Membranes (300 μg protein) were either boiled for 15 min or mixed with 300 μg of cytosolic proteins, and then subjected to the methylation reactions along with non-treated membranes or washed (see Materials and Methods) membranes. Methylation reactions and experimental procedures were as described in Fig. 1. Data are the means ± SD of three separate determinations. Confluent and differentiated (2% DMSO) cells were 6 days in culture.

used here to be 0.2 to 0.4 μM. This is inadequate to account for the data in view of the low K_m of the enzyme for [*methyl*-^{3}H]AdoMet, and the high concentration of the methyl donor used (25 μM). It appears, therefore, that the methylation of the 21–23 kD proteins leveled-off (Fig. 2) due to substrate limitations.

In the experiments described thus far we have used membranes of confluent or DMSO-induced differentiated cells that were 6 days in culture. Results shown in Fig. 4 demonstrate that the increase in methylation of the 21–23 kD proteins developed with time of treatment of the cells with 2% DMSO. After a lag period of 3 days in culture, DMSO induced a marked increase in the ability of membranes to methylate 21–23 kD proteins. Methylation of the 70 kD proteins was not changed in either the confluent or the DMSO-induced differentiated cells (Fig. 4).

DISCUSSION

This study demonstrates enzymatic carboxyl methylation of proteins in membranes of neuroblastoma N1E-115 cells. These membranes contain the methylating enzyme and two major methyl acceptor proteins, a ~70 kD protein and a group of 21–23 kD proteins. Thus, the addition of [*methyl*-^{3}H]AdoMet to the membranes was sufficient to allow the rapid incorporation of [*methyl*-^{3}H] groups into the 70 kD and the 21–23 kD proteins. The reaction was inhibited strongly by a potent methylation inhibitor, was time and temperature dependent, and was saturable with respect to [*methyl*-^{3}H]AdoMet. The incorporated [*methyl*-^{3}H] groups appear to be in the form of methylesters with relatively high stability. Methylation of these 21–23 kD membrane proteins and increased methylation in the DMSO-induced differentiated cells have also been observed in intact

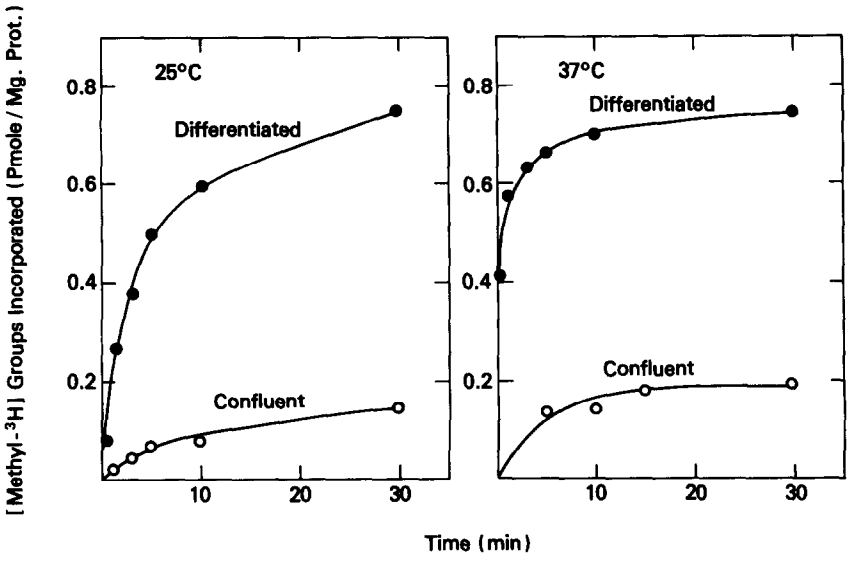

Fig. 2. Time and temperature dependence of methylation of 21–23 kD proteins in membranes of confluent and differentiated neuroblastoma cells. Membranes (200 μg) were of 6-day cultures grown in the absence (confluent) or in the presence (differentiated) of 2% DMSO. They were incubated at 25° or at 37° as detailed in Fig. 1, except that incubation times were varied as indicated in the figure. Proteins were then separated on gels, and carboxyl methylesters were determined in bands corresponding to the 21–23 kD proteins as described in Fig. 1. Data, expressed in terms of [*methyl*-^{3}H] groups incorporated per mg membrane proteins, are the means of duplicate determinations that varied by less than 25%.

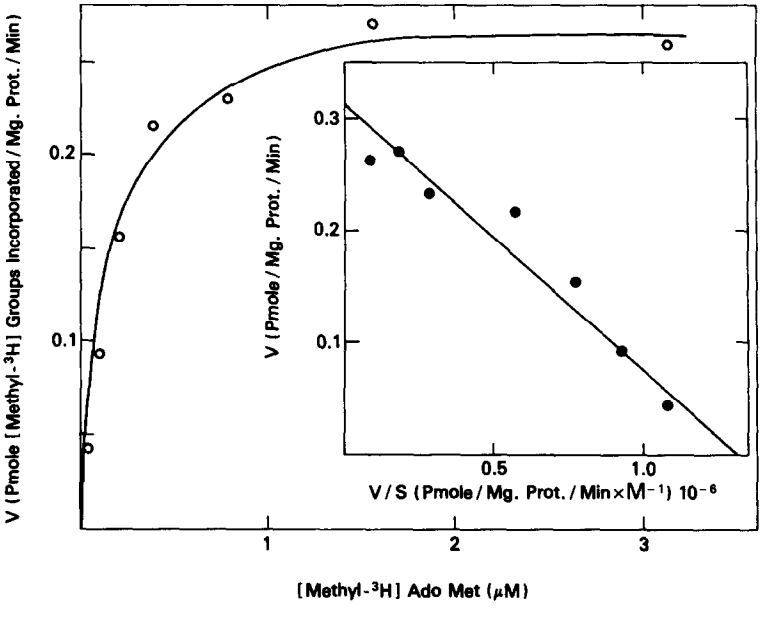

Fig. 3. Methylation of the 21–23 kD protein as a function of [*methyl*-^{3}H]AdoMet concentration. Membranes (150 μg) of differentiated cells (6 days in culture) were incubated at 25° for 10 min in the presence of various concentrations of [*methyl*-^{3}H]AdoMet. Proteins were then separated and the amounts of [*methyl*-^{3}H] groups incorporated into the 21–23 kD proteins was determined as detailed in Fig. 1. Data, expressed in terms of [*methyl*-^{3}H] groups incorporated per mg membrane protein per min, are the means of duplicate determinations that varied by less than 25%. Inset: Eadie–Hofstee plot of the same data.

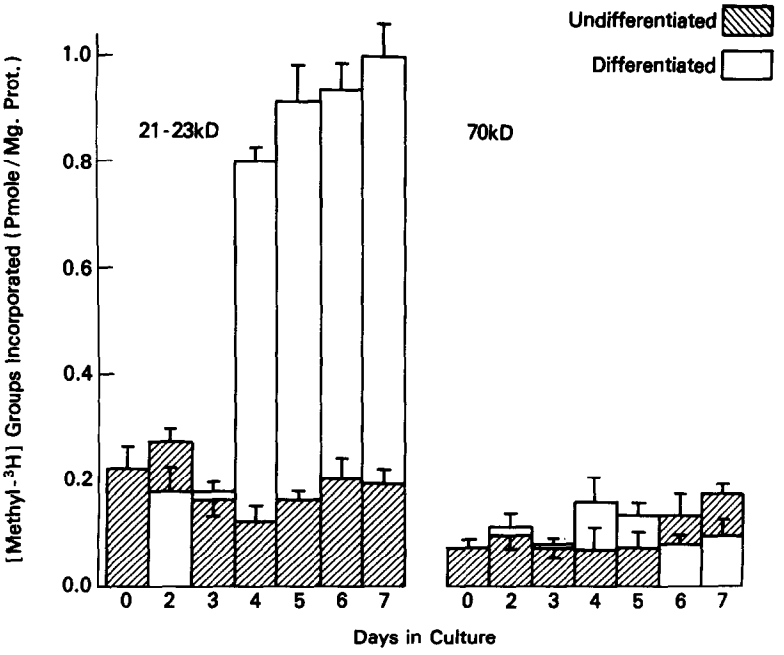

Fig. 4. Methylation of the 70 kD and the 21–23 kD proteins of neuroblastoma cells as a function of days cultured in the absence (undifferentiated) and in the presence (differentiated) of 2% DMSO. Experimental procedures were as described in Fig. 1, except that membranes were prepared from cells cultured for the indicated periods of time. Data, expressed in terms of pmol [*methyl*-^{3}H] groups incorporated per mg membrane proteins, are the means ± SD of three to five separate determinations.

neuroblastoma cells [23]. Increased methylation appeared after a lag period of 3 days of growth in the presence of 2% DMSO; thus it developed in parallel with the appearance of neurite-like processes [16, 31, 32]. The results suggest that the methylated 21–23 kD proteins are involved either in the DMSO-induced differentiation mechanism or in other functions of the differentiated cells. It is also possible that these proteins are associated with cessation of cell division, since DMSO-treated cells do not multiply [31, 32].

From previous studies we know that protein carboxyl methylations are increased in differentiated cells [16, 17]. In particular, DMSO treatment of neuroblastoma N1E-115 cells increases these reactions [16]. The formerly described protein carboxyl methylations, however, differed from that described here since they were related to the rapidly hydrolyzing carboxyl methylesters. These are degraded at pH 8.5 and would not survive the gel conditions used here. We now show (Table 1) that the more stable protein carboxyl methylesters would not have been detected in the former assays that used 1 M sodium-borate (10 min) to hydrolyze the methylesters. Recently Barten and O'Dea [35] reported changes in protein carboxyl methylation in N1E-115 cells. Several differentiating agents induced an increase in sodium-borate-labile methylesters formed upon incubation of cells with [^{3}H]methionine. They also examined the protein carboxyl methylesters formed by [*methyl*-^{3}H]AdoMet in intact cells using acidic SDS gels to preserve the base-labile methylesters. Under these conditions, there was a general increase in methylation observed on the gels. However, this increase was limited to cells differentiated by lowering serum concentration. The protein carboxyl methylation that we have described here represents a small fraction of the labile and relatively stable methylesters measured by Barten and O'Dea [35], and changes in this group of proteins would be obscured by the relatively large fraction of [*methyl*-^{3}H] groups incorporated into the more base-labile protein methylesters. Nonetheless, it is clear that both types of protein carboxyl methylesters, the rapidly hydrolyzing [16] and the more stable ones, are increased in the membranes of DMSO-induced differentiated neuroblastoma cells. If the latter are D-aspartyl or L-isoaspartyl protein methylesters [8–10], their increase could be related to aging of the cultures, in view of the proposed function for the non-selective D-aspartyl/L-isoaspartyl methyltransferase in the repair of age damaged proteins [8–10]. This possibility would be in line with the increase in substrates for the non-selective human red blood cell methyltransferase observed in differentiated neuroblastoma cells [16]. However, other possibilities cannot be ruled out yet, since the large amounts of non-selective methylations may mask a more selective and rapidly hydrolyzable protein methylester. We emphasize this in view of the precedent of the bacterial chemoreceptors which are reversibly methylated [6, 7]. An analogous methylation may also occur in eukaryotic cells. As discussed below, we do not yet know whether the relatively stable methylesters are reversible under physiological conditions.

The previous studies with intact neuroblastoma

cells [23] could not tell whether the 21–23 kD proteins were methylated by a cytoplasmic or a membrane-bound enzyme, or whether the site of methylation was in the membranes or in the cytosol, followed by transfer of product to the membranes. The experiment with the washed membranes described here showed that the enzyme, the substrates and the reaction occur in the membranes. We did not find evidence for cytosolic 21–23 kD proteins; they should have showed up in the experiments where cytosol was added to the membranes (Table 2). Since we also did not detect methylated 21–23 kD proteins in the cytoplasm of intact cells [23], it appears that these proteins are first inserted into the membranes and only then methylated.

Although the 70 kD protein could accept methyl groups *in vitro*, we did not observe a [*methyl*-^{3}H]-labeled 70 kD protein in the intact neuroblastoma cells [23]. We suppose that the 70 kD protein is the previously described nuclear lamin B, since it was shown that this protein can be methylated *in vitro* but not in intact cells which are fully differentiated, or in intact cultures which are not synchronized [21]. Chelsky *et al.* [21] proposed that the apparent lack of methyl group turnover in lamin B of the mouse brain could be related to the inability of brain cells to divide. The case of the 21–23 kD proteins of the neuroblastoma cell membranes is clearly different, since their methylation is increased in the non-dividing cells both *in vitro* (Fig. 4) and in the intact cells [23]. While they are readily available for methylation in the intact cells, turnover of their methyl groups was slow (half times of 7–8 hr and >18 hr in differentiated and confluent cells respectively).

The identities of the 21–23 kD proteins of neuroblastoma cell membranes and of their methylated amino acid(s) are not yet known. The similarities in molecular weights, stabilities, and membrane localization between the neuroblastoma methylated proteins and those detected in bovine rod outer segments [20] and in macrophage RAW 264 cells [22] raise the possibility that all of these proteins represent a family of gene products and that, like several *ras* proteins [25, 26] and the rod outer segments proteins [20], they are methylated on the C-terminal cysteinyl residue. Ota and Clarke [20] proposed that the 23–29 kD methylated proteins of rod outer segments may be, like *ras* proteins, a group of GTP binding proteins.

Purification of the protein carboxyl methyltransferase and the various 20–29 kD protein substrates will permit investigation of the role of guanine nucleotides in the function of the enzyme or products. The cell cycle dependency of lamin B methylation [21], the increase in rapidly hydrolyzing protein methylesters [16], and the selective increase in 21–23 kD carboxyl methylated proteins in DMSO-induced differentiated neuroblastoma cells, all demonstrate the variety of protein carboxyl methylations that may be altered during growth, differentiation, and aging of eukaryotic cells. They emphasize the importance of these post-translational modifications of proteins.

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