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Carboxyl methylation of 21–23 kDa membrane proteins in intact neuroblastoma cells is increased with differentiation

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Evidence is presented for specific enzymatic methylation of 21–23 kDa membrane proteins in intact neuroblastoma N1E 115 cells, which is increased in dimethylsulfoxide-induced differentiated cells. Methylation of these proteins has characteristics typical of enzymatic reactions in which base labile volatile methyl groups are incorporated into proteins, consistent with the formation of protein carboxyl methylesters. However, these methylesters of the 21–23 kDa proteins are relatively stable compared to other protein carboxyl methylesters. The 3-fold increase in methylated 21–23 kDa proteins in the differentiated cells suggest biological significance in differentiation of the cell membranes.

Protein methylation; Methylester; Neuroblastoma; Differentiation

1. INTRODUCTION

Methylation of protein carboxyl groups is one type of post-translational modification by which structure and function of proteins can be regulated. Enzymes that catalyze these reactions utilize *S*-adenosyl-L-methionine as a methyl donor to form protein carboxyl methylesters [1,2]. One of these enzymes is a bacterial methyltransferase which methylates *L*-glutamate residues of membrane chemoreceptors to modulate their signaling functions [3–5]. A second methyltransferase, which is abundant in eukaryotic cells, methylates abnormal D-aspartyl or L-isoaspartyl residues and may function in the repair of age-damaged proteins [6,7]. The products of this enzyme are highly unstable, rapidly hydrolyzed, protein carboxyl methylesters. It is not known whether the rapidly reversible protein methylations, which were implicated in several cellular functions, e.g. hormone secretion [8], cell motility [9] and differentiation [10,11], reflect this type of protein methylation in eukaryotic cells. A third type of protein methyltransferase appears to be an enzyme which forms the C-terminal cysteinyl α-carboxymethylesters of some fungal peptidyl sex factors [12], several ras proteins [13,14] and certain unidentified 23–29 kDa proteins of bovine rod outer segments [15].

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; DMSO, dimethylsulfoxide; PBS, phosphate buffered saline; EDTA, ethylenediaminetetra-acetic acid; SDS, sodium dodecyl sulfate

The cGMP phosphodiesterase in bovine rod outer segments [16] and the nuclear lamin B [17] as well as certain membrane proteins of mouse macrophage RAW 264 cells [18], are also carboxyl methylated proteins whose methyl esters show the relatively high stability seen with ras proteins. Here we report on another group of relatively stable 21–23 kDa protein methylesters, which are formed in the membranes of mouse neuroblastoma N1E 115 cells, and show that these protein methylesters are increased in differentiated cells.

2. MATERIALS AND METHODS

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°C in a humidified atmosphere of 5% CO_2/95% air, as described previously [11]. The cells were plated at a density of $2 \cdot 10^4$ cells/cm^2 in 75 cm^2 tissue culture flasks. Media were replaced one day after plating, three days after plating and daily thereafter. Control (undifferentiated cells) received only DMEM + FCS. The dimethylsulfoxide (DMSO)-treated cultures (differentiated cells) were fed with DMEM + FCS containing 2% DMSO, starting one 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 several days (4–5) after plating, in line with previous reports [11,19]. Cells were incubated at 37°C in 2.5 ml DMEM (without methionine) containing 250 μCi [methyl-^{3}H]methionine (15 Ci/mmol, New England Nuclear). After 60 min the medium was discarded, cells were washed 4 × with 15 ml of phosphate buffered saline (PBS) and detached in PBS containing 5 mM ethylenediaminetetra-acetic acid (EDTA). The detached cells were collected by centrifugation (1000 × *g* for 5 min), and the pelleted cells were resuspended (in 500 μl of 20 mM phosphate buffer, pH 7.5, containing 5 mM EDTA and 5 mM β-mercaptoethanol) and sonicated for 20 s. The homogenates contained 0.7–1 mg protein per ml. Particulate and cytosolic fractions were separated by centrifugation (100 000 × *g* × 1 h). In each experiment 50-70 μl of sample containing the same amount of protein from control cell homogenates or

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DMSO-treated cell homogenates, or similar amounts of proteins from subcellular fractions, were loaded onto slab gels.

Protein carboxyl methylesters were determined after electrophoresis on 12.5% polyacrylamide sodium dodecylsulfate (SDS) slab gels (pH 8.5) according to Laemmli [20]. The gels were Coomassie blue stained, dried, sectioned (2 mm sections) and subjected to the vapor phase equilibrium assay as described by Chelsky et al. [17].

3. RESULTS

Confluent neuroblastoma cells (7 days in culture) were incubated in a methionine free DMEM for 1 h with [methyl-^{3}H]methionine, the precursor for the methyl donor *S*-adenosyl-L-methionine. Incorporation of [methyl-^{3}H] groups was assayed by the vapor-phase equilibrium assay after separating the proteins by SDS polyacrylamide gels, pH 8.5. This assay specifically detects protein carboxyl methylesters that are degraded by base (1 N NaOH) to yield volatile methanol [17]. A typical experiment with the confluent NIE 115 cells (fig.1A) detected a major group of peaks containing base labile volatile radioactivity at M_r 21–23 kDa and minor peaks at M_r 33–36 kDa. Incorporation of [methyl-^{3}H] groups into the proteins was strongly inhibited by the known methylation inhibitors 3-deazaadenosine and homocysteine thiolactone (fig.1A), indicating that they were methylated by a specific transmethylation reaction. The major group of the neuroblastoma methylated proteins, viz. the 21–23 kDa proteins, were found to be associated with the cell membrane; more than 90% of these methyl-labeled proteins were detected in the particulate fraction (fig.1B). Methylester radioactivity in the membranes was not removed by extensive washings or treatment with 1 M NaCl (table 1). Treatment with the nonionic detergent nonidet NP-40 (1%) did, however, release the 21–23 kDa methylated proteins from the membranes (table 1). Other experiments confirmed the base lability and volatility of the [methyl-^{3}H] groups in the 21–23 kDa proteins, yet also demonstrated their relatively high stability: (i) exposure of the dried gel bands containing the 21–23 kDa proteins to 1 N NaOH for 24 h and drying down at room temperature prior to the base/vapor phase equilibrium assay resulted in a total loss of the radioactivity (from 2760 dpm to 30 dpm); (ii) in a similar experiment with exposure to Tris buffer, pH 8.5, no loss of radioactivity was observed (2680 dpm); (iii) exposure of the [methyl-^{3}H] labeled cell membranes to 1 M sodium borate, pH 11, for 10 and 20 min (which

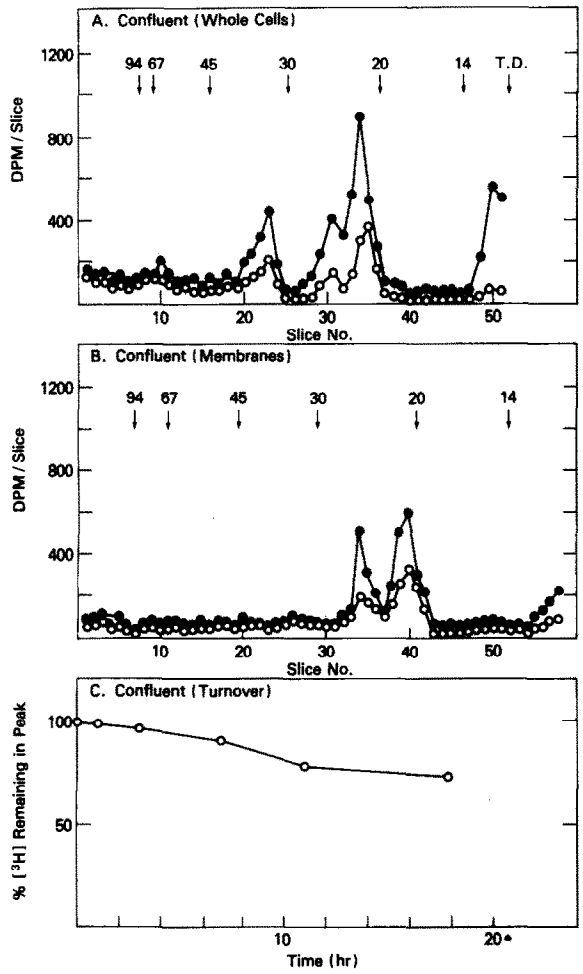

Fig.1. Methylation of confluent neuroblastoma N1E 115 cells' proteins. Cells were labeled with [methyl-^{3}H] methionine for 1 h in the absence (●) or in the presence (○) of 100 μM 3-deaza-adenosine and 100 μM homocysteine thiolactone (A and B) or for 1 h with [methyl-^{3}H]methionine which was followed by a chase with DMEM for the indicated periods of time (C). Proteins from whole cells (A) or from their membrane fraction (B) were separated on SDS polyacrylamide slab gels and protein methylesters determined as described in section 2. Molecular mass standards (kDa) were electrophoresed in separate lanes in each experiment and their migration distance is indicated by arrows. T.D., tracking dye. Turnover of the major peak of the 21–23 kDa proteins is shown in C; data are expressed as percentage of the methylation at zero time.

Table 1

Methylated 21–23 kDa proteins of N1E 115 cells are membrane bound proteins

Cellular fraction and treatment	[methyl-^{3}H] in 21–23 kDa proteins (dpm)
Homogenate	2920
Membranes	2840
Washed membranes	2760
Membranes treated with 1 M NaCl:	
Supernatant	160
Pellet	2510
Membranes treated with 1% NP-40:	
Supernatant	2770
Pellet	30

Confluent cells were labeled and fractionated as described in section 2. Membranes were either used directly (membranes) or: (i) washed three times with 6 ml of the homogenization buffer; (ii) incubated for 30 min at 4°C with either 1 M NaCl or 1% Nonidet NP-40, followed by a 100000 × *g*, 1 h spin to separate supernatants and pellet. Whole homogenate (460 μg protein) or the equivalent amount of proteins in the various fractions were separated on gels and the amount of [methyl-^{3}H] labeled 21–23 kDa proteins was determined in the vapor-phase equilibrium assay.

has been used to hydrolyze protein carboxyl methyl esters in many systems [8–11], failed to release the [methyl-^{3}H] groups incorporated into the 21–23 kDa proteins. In a pulse-chase experiment, a very small loss of [methyl-^{3}H] groups was detected even after 18 h (fig.1C).

DMSO-induced differentiated cells (7 days in culture) were used for measurements of the incorporation of [methyl-^{3}H] groups into proteins under the same conditions used for the confluent cells. The observed methylation pattern in these cells (fig.2A) and membranes (fig.2B) was similar to that observed in the confluent cells. Comparison between the extent of [methyl-^{3}H] incorporation into the 21–23 kDa proteins of the confluent cells and of the DMSO-induced differentiated cells, revealed that it was higher in the latter (respectively 10650 ± 2080 dpm/mg membrane protein, n = 8 and 31800 ± 2800 dpm/mg membrane protein, n = 6; see also figs 1B and 2B). In pulse chase experiments it was found that the [methyl-^{3}H] labeled 21–23 kDa proteins in the DMSO-induced differentiated cells had a significant, though slow (half time of 8–9 h) turnover (fig.2C).

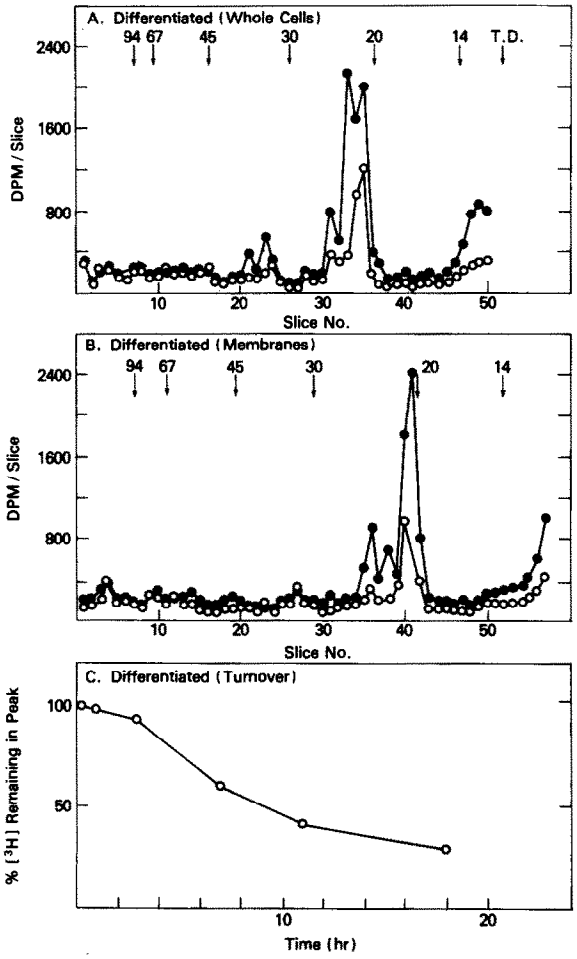

Fig.2. Methylation of DMSO-induced differentiated N1E 115 cells' proteins. Cells were labeled with [methyl-^{3}H]methionine for 1 h in the absence (●) or in the presence (○) of 3-deaza-adenosine and homocysteine thiolactone (A,B) or for 1 h with [methyl-^{3}H]methionine which was followed by chase with DMEM (C). Experimental procedures and definitions are as in fig.1. (A) Methylation pattern of whole cells proteins. (B) Methylation patterns of membrane proteins. (C) Turnover of the major peak of the 21–23 kDa methylated proteins.

4. DISCUSSION

Intact neuroblastoma N1E 115 cells methylate a group of 21–23 kDa membrane bound proteins. A 3-fold increase was observed in DMSO-induced differentiated cells. Methylation of the 21–23 kDa proteins has characteristics typical of enzymatic reactions in which [methyl-^{3}H] groups are incorporated into base-labile methylesters at free carboxyl groups. However, these protein methylesters show a relatively high degree of chemical and biological stability; unlike certain other protein carboxyl methylesters [6,7], they are not degraded at mild alkaline pH's. The pulse-chase experiments also suggested biological stability of the 21–23 kDa protein methylesters. After 18 h, there was only a small loss of methylated protein (~ 25%) in the confluent cells and a slow turnover in the differentiated cells (~7.5%). The differences in turnover, however, may be associated with the increased methylation observed in the differentiated cells. We do not think that they are related to methylesterase activity because, under the conditions used here, less than 5% of the radioactivity incorporated in 1 h was lost after chase for 3 h (fig.1C and 2C). In this regard the methylated proteins of the neuroblastoma resemble the C-terminal α-carboxymethylated cysteinyl p 21^{ras} protein, in which no methyl group turnover was detected [14]. The 21–23 kDa methylated proteins of the neuroblastoma cells share similarities in molecular weights, membrane localization and stabilities with the methylated ras proteins [13,14], 23–29 kDa proteins of bovine rod outer segments [15] and 20–23 kDa proteins of macrophage RAW 264 cells [18]. Based on the common C-terminal cysteinyl methylation of the rod outer segments proteins and the ras proteins, Ota and Clarke [15] suggested that the former, like the normal ras proteins, would be functional G-proteins. This notion would be in line with the GTP-dependent methylation of the 20–23 kDa macrophage proteins [18], if all of these proteins represent a family of closely related gene products. Perhaps the neuroblastoma methylated membrane proteins are also small G-proteins. The results with the neuroblastoma also raise an important question regarding the reversibility of methylation of the 21–23 kDa proteins under physiological conditions. We suspect that these highly stable protein methylesters may either be demethylated by a methylesterase that might be activated by a specific stimulus, or that their metabolic fate includes cleavage which releases a methylated peptide.

REFERENCES

[1] Axelrod, J. and Daly, J. (1965) Science 150, 892–893.
[2] Kim, S. and Paik, W.K. (1970) J. Biol. Chem. 245, 1806–1813.
[3] Kleene, S.T., Toews, M.L. and Adler, J. (1977) J. Biol. Chem. 252, 3214–3218.
[4] Van Der Werf, P. and Koshland, D.E. Jr. (1977) J. Biol. Chem. 252, 2793–2795.
[5] Simms, S.A., Stock, A.M. and Stock, J.B. (1987) J. Biol. Chem. 262, 8537–8543.
[6] McFadden, P.N. and Clarke, S. (1982) Proc. Natl. Acad. Sci. USA 79, 2460–2464.
[7] Aswad, D.W. (1984) J. Biol. Chem. 259, 10714–10721.
[8] O'Dea, R.F., Viveros, O.H. and Diliberto, E.J. Jr. (1981) Biochem. Pharmacol. 30, 1163–1168.
[9] O'Dea, R.F., Viveros, O.H. and Axelrod, J. (1978) Nature (London) 272, 462–464.
[10] Zukerman, S.H., O'Dea, R.F., Olson, J.M. and Douglas, D.S. (1982) Mol. Immunol. 19, 281–286.
[11] Kloog, Y., Axelrod, J. and Spector, I. (1983) J. Neurochem. 40, 522–529.
[12] Sakagami, Y., Oshida, M., Isogai, A. and Suzuki, A. (1981) Science 212, 1525–1527.
[13] Deschenes, R.J., Stimmel, J.B., Clarke, S., Stock, J. and Broach, J.R. (1989) J. Biol. Chem. 264, 11865–11873.
[14] Gutierrez, L., Magee, A.I., Marshall, C.J. and Hancock, J.F. (1989) EMBO J. 8, 1093–1098.
[15] Ota, I.M. and Clarke, S. (1989) J. Biol. Chem. 264, 12879–12884.
[16] Swanson, R.J. and Applebury, M.L. (1983) J. Biol. Chem. 258, 10599–10605.
[17] Chelsky, D., Olson, J.F. and Koshland, D.E. Jr. (1987) J. Biol. Chem. 262, 4303–4309.
[18] Backlund, P.S. and Aksamit, R.R. (1989) J. Biol. Chem. 263, 15864–15867.
[19] Kimchi, Y., Palfrey, C., Spector, I., Barak, Y. and Littauer, U.Z. (1976) Proc. Natl. Acad. Sci. USA 73, 462–466.
[20] Laemmli, U.K. (1970) Nature (Lond.) 227, 680–683.