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Transglutaminase catalyzed incorporation of putrescine into surface proteins of mouse neuroblastoma cells

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Summary

Transglutaminase, purified from guinea pig liver, was used to catalyze the incorporation of [^{14}C]putrescine into exposed surface proteins of intact mouse neuroblastoma cells. This method specifically labeled two surface proteins ($Mr = 92\,000$ and 76 000) in the N-18 mouse neuroblastoma cells and three surface proteins ($Mr = 92\,000$, 76 000, and 72 000) in the NB-15 mouse neuroblastoma cells. In addition, transglutaminase also catalyzed cross-linking reactions of exposed surface proteins. In both the N-18 and NB-15 cells, differentiation was accompanied by a 2-fold increase of specific radioactivity incorporated into trichloroacetic acid insoluble cellular material, suggesting that the differentiated mouse neuroblastoma cells may possess greater amount of accessible peptide-bound glutaminyl residues on their surface than their malignant counterparts. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorographic method revealed that while the [^{14}C]putrescine-labeled protein patterns of undifferentiated and differentiated mouse neuroblastoma cells were similar, the intensity of labeling of individual bands was specifically modulated by cell differentiation.

Abbreviations

PMSF, phenylmethylsulfonyl fluoride; Bt_2cAMP, N^6,$O^{2'}$-Dibutyryl adenosine 3′:5′-cyclic monophosphate; IBMX, 3-isobutyl-1-methyl xanthine; SDS-PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis; HEPES, N-2-hydroxylethylpiperazine-N′-2-ethanesulfonic acid.

Introduction

Transglutaminases, a class of enzymes widely occurring in animal tissues and body fluids, catalyze a calcium-dependent acyl transfer reaction between the γ-carboxamide group of peptide-bound glutaminyl residue and primary amino group of various acceptor substrates (1). Depending on the acceptor substrates, either amine or peptide-bound lysine residue, transglutaminase can mediate a labeling reaction or an inter-peptide cross-linking reaction (1, 2).

Purified guinea pig liver transglutaminase has an apparent molecular weight of 85 000 and, when added to intact cells, would not penetrate cell membranes (3, 4). Since the conditions of transglutaminase catalyzed reactions are mild, purified transglutaminase has been used to identify exposed surface proteins of intact cells. For example, Dutton and Singer (4) have used guinea pig liver transglutaminase and dansylcadaverine to specifically label the Band 3 protein of mouse erythrocytes. Okumura and Jamieson (5) have shown that transglutaminase catalyzes the incorporation of [^{14}C]glycine ethyl ester specifically into the glycoprotein II of human platelet membrane.

Early investigators have demonstrated that among various primary amines, polyamines (putrescine, spermidine and spermine) serve as good acceptor substrates for transglutaminase (6). Nu-

merous reports have established that polyamines are involved in a wide spectrum of growth regulatory processes (7–9). Other studies have also provided evidence that cellular transglutaminase may play a role in growth regulation (10–13). Due to wide-occurrence of transglutaminase and polyamines in animal tissues and body fluids, it is likely that endogenous polyamines may become covalently bound to some surface proteins via transglutaminase catalyzed reaction. Studies from several laboratories have suggested that indeed polyamines may serve as physiological substrates for transglutaminase in vitro (13, 14) and in vivo (15, 16). In view of these considerations it appears likely that the transglutaminase catalyzed incorporation of polyamines into surface proteins may be used to probe the surface structure and to identify potential surface sites where polyamines can be covalently conjugated.

In this report, the cell surface proteins of the undifferentiated (malignant) and differentiated cultures of two mouse neuroblastoma cell lines, the N-18 and the NB-15 cells, were studied and compared using the transglutaminase/[^{14}C]putrescine labeling method.

Materials and methods

[1,4-^{14}C]putrescine · 2HCl (60 mCi/mmol) and [^{14}C]spermidine · 3HCl (122 mCi/mmol) were purchased from Amersham, Arlington Hgts., Ill. Dulbecco's modified Eagle medium, fetal calf serum and Earle's balanced salt solution were obtained from Gibco, Grand Island, N.Y. Phenylmethylsulfonyl fluoride (PMSF), N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate (Bt_2cAMP) and dithiothreitol were purchased from Sigma Chem. Co., St. Louis, Mo. 3-Isobutyl-1-methyl xanthine (IBMX), an inhibitor of phosphodiesterase, was from Aldrich Chem. Co., Milwaukee, WI. N,N-dimethylcasein was obtained from Calbiochem-Behring Corp., San Diego, CA. QAE-Sephadex ion exchanger was purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Hydroxyapatite was purchased from Bio-Rad Lab., Richmond, CA.

Purification of guinea pig liver transglutaminase

Guinea pig liver transglutaminase was purified by a modified version of the method of Connellan et al. (17). Transglutaminase activity was determined by the method of Lorand et al. (18). Briefly, a 100 000 **g** supernatant from guinea pig liver homogenate was chromatographed twice over a QAE-Sephadex column. Fractions containing transglutaminase activity were pooled and further purified by hydroxyapatite column chromatography.

Cell culture and differentiation

Mouse N-18 and NB-15 neuroblastoma cells were grown as monolayer cultures in Dulbecco's modified Eagle medium (with 4 500 mg glucose per liter, without sodium pyruvate) supplemented with 10% fetal calf serum. Cells were plated at a density of 2×10^4 cells/cm^2 and were maintained at 37 °C in a Forma water jacketed CO_2 incubator (95% air, 5% CO_2). Differentiation of neuroblastoma cells was induced by adding Bt_2cAMP (1 mM) and isobutylmethylxanthine (0.5 mM) to sparse culture 15 h after plating (19). The undifferentiated and differentiated neuroblastoma cells at the early stationary phase of growth (approximately 4–5 days after subculture) were used for the labeling experiments.

Transglutaminase/[^{14}C]putrescine labeling of surface proteins

Cells grown in monolayer cultures (100 mm tissue culture dish) at the early stationary phase of growth were rinsed three times with phosphate-buffered saline (0.15 M NaCl, 10 mM sodium phosphate; pH 7.4) and removed from the substratum by gentle flushing with a stream of Earle's balanced salt solution. Cells were then sedimented by centrifugation at 1 000 **g** for 2 min. The cell pellet was resuspended in Earle's balanced salt solution (pH 7.6), containing 20 mM Tris-HCl, 9.5 mM dithiothreitol, and 4.5 mM $CaCl_2$. The cell concentration was kept at 1.5×10^7 cells/ml. [1,4-^{14}C]putrescine (5 ~ 10 μCi/ml) was added to the cell suspension, and the labeling reaction was initiated by the addition of purified transglutaminase (60 μg/ml) to the cell suspension. The mixture was incubated at 35 °C for 1 h with occasional gentle agitation. The cell suspension was then diluted with Earle's balanced salt solution containing 5 mM cold putrescine, and washed three times by centrifugation at 1 000 **g**. The final cell pellet was suspended in a 10 mM Tris-HCl (pH 7.2) buffer containing 1 mM

PMSF. Samples were then prepared for SDS-PAGE according to methods described below. Alternatively, after labeling, the cell pellet was resuspended and homogenized in 10 mM HEPES (pH 7.2), containing 1 mM $MgCl_2$, 2 mM CaCl, and 0.5 mM PMSF. The plasma membrane enriched fraction was prepared by discontinuous sucrose gradient (15%, 30% and 50%) centrifugation at 23 300 **g** for 30 min as previously described (20). The incorporation of [^{14}C]putrescine into acid-insoluble cellular material was determined by a filter-paper assay method (21). Protein concentrations were measured by the procedure of Lowry et al. (22) using bovine serum albumin as the standard.

SDS-polyacrylamide gel electrophoresis and fluorographic procedure

Protein samples obtained after labeling experiments were mixed with one-fifth volume of SDS-stop solution containing 12% SDS, 0.5 M Tris-HCl (pH 8.0) 10% β-mercaptoethanol, 5 mM EDTA, 25% glycerol and 0.005% pyronin Y and heated at 100 °C for 5 min. The protein samples were then subjected to SDS-PAGE on a linear gradient slab gel (7.5–15% acrylamide) as previously described (20). Gels were stained for protein with Coomassie blue. Fluorographs were prepared according to the procedure of Bonner and Lasky (23). Apparent molecular weights of the radioactive protein bands were estimated by the method of Fairbanks et al. (24). Standard proteins (and their molecular weights) used were cytochrome C (12 000), chymotrypsinogen (25 000), aldolase (40 000), ovalbumin (45 000), catalase (57 000), bovine serum albumin (67 000), phosphorylase a (94 000) and β-galactosidase (130 000). Fluorographs were scanned with a Schoeffel SD-3 000 spectromicrodensitometer, and the peak areas of the optical tracings were used as a quantitative measure of the incorporation of radioactivity.

Results

When intact mouse neuroblastoma cells were incubated with [^{14}C]putrescine in the presence of purified guinea pig liver transglutaminase, several surface proteins were specifically labeled. Figure 1 shows the specific labeling of two protein bands with apparent molecular weight of 92 000 and 76 000 of the N-18 cells and three protein bands with apparent molecular weight of 92 000, 76 000 and 72 000 of the NB-15 cells by [^{14}C]putrescine. In addition to these labeled proteins, a band of radioactivity was also apparent at the top of the gradient gel in both N-18 and NB-15 cells. It seems likely that this very high molecular weight radioactive band may represent cross-linked proteins using [^{14}C]putrescine as a cross-linking agent. This notion is supported by the finding that this band did not penetrate appreciably into the gel after electrophoresis even when acrylamide concentration was reduced to 5% (for example see Fig. 3B).

The amount of [^{14}C]putrescine incorporated into

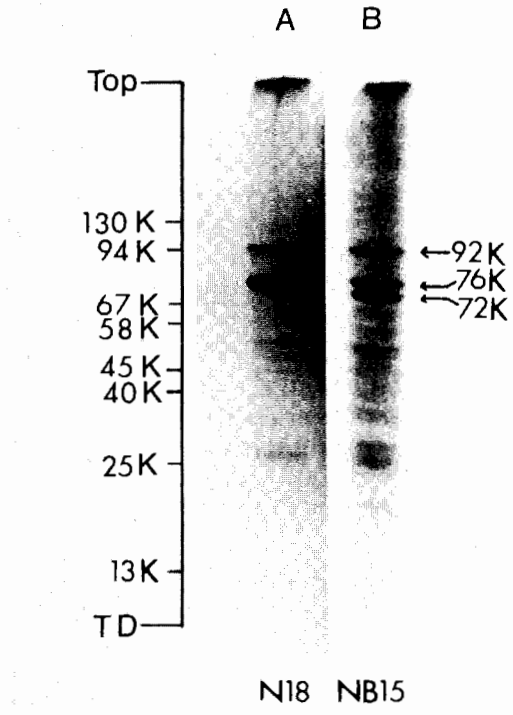

Fig. 1. Fluorograph illustrating the transglutaminase catalyzed incorporation of [1,4-^{14}C]putrescine into surface proteins of the N-18 and NB-15 mouse neuroblastoma cells. Both N-18 and NB-15 cells were labeled under identical conditions and plasma membrane fractions of each cell line were prepared (see Methods). The plasma membrane preparations used in this experiment were 9-fold purified judging by the increase in specific activity of 5'-nucleotidase (see Methods). The protein samples were then solubilized in SDS-Stop solution and subjected to analysis by SDS-PAGE as described in Methods. Each lane contained 50 μg protein. T.D. refers to the dye front of tracking dye, pyronin Y.

the major labeled protein bands in N-18 and NB-15 cells was calculated from the densitometric tracings of the fluorographs and was expressed as percentage of radioactivity incorporated (Table 1). While the three labeled surface proteins (Mr = 92 000, 76 000 and 72 000) were of approximately equal intensity in NB-15 cells, the 76 000-dalton band of N-18 cells alone accounted for about 50% of the total radioactivity incorporated.

The difference in the labeling pattern between N-18 and NB-15 cells is unexpected in view of the relatedness of these two clonal lines. Both the N-18 and NB-15 mouse neuroblastoma cells are clonal lines derived from the original tumor line C-1300 mouse neuroblastoma cells (25). Both cell lines can be induced to undergo differentiation by the addition of cyclic AMP analogs and/or phosphodiesterase inhibitors (19, 26). The significance of such small difference in the membrane structure revealed by the [^{14}C]putrescine labeling pattern between these two closely related clonal lines is not clear. However, it is interesting to note that, despite many similarities shared by these two cell lines, they differed slightly in their doubling time; being ~21 h for N-18 cells and ~25 hrs for NB-15 cells.

The fact that [^{14}C]putrescine can selectively label exposed surface proteins suggests that this method may be used to monitor possible changes of plasma membrane during the differentiation of mouse neuroblastoma cells. Other surface protein labeling methods such as lactoperoxidase catalyzed iodination method and galactose oxidase catalyzed reductive tridiation method have been used to study the changes of plasma membranes in cell differentiation and malignant transformation (20, 27).

In both the N-18 and NB-15 neuroblastoma cells, the amounts of radioactivity incorporated into the trichloroacetic acid-insoluble material of the differentiated cells were approximately 2-fold higher than that of their undifferentiated (malignant) counterparts (Table 2).

The electrophoretic pattern of [^{14}C]putrescine-labeled surface proteins of the undifferentiated and differentiated N-18 neuroblastoma cells is shown in Fig. 2. The intensities of the gel top protein band and the 76 000-dalton band of the differentiated N-18 cells were greater than that of the undifferentiated cells by 2.1-fold and 2.3-fold, respectively. However, the intensity of the 92 000-dalton band of the differentiated N-18 cells was diminished when compared to that of the undifferentiated cells. The reciprocal relationship of the intensities of the gel top band the 92 000-dalton band in the differentiated N-18 cells could be due to a more preferential cross-linking of the 92 000-dalton band in the differentiated N-18 cells than that in the undifferentiated counterparts.

The labeling patterns of the undifferentiated and differentiated NB-15 cells are shown in Fig. 3. In contrast to results obtained with the N-18 cells, the intensity of all the labeled protein bands of NB-15 cells increased during cell differentiation. In addition, differentiation of NB-15 cells, was also accompanied by increases of labeling of some low

Table 1. Transglutaminase catalyzed incorporation of [^{14}C]putrescine into various surface proteins of the N-18 and NB-15 mouse neuroblastoma cells.

	Percentage of the total amount of radioactivity incorporated into individual protein bands	
	N-18	NB-15
gel top band	20 ± 5[a]	17 ± 3
92 000	15 ± 3	20 ± 5
76 000	48 ± 6	16 ± 3
72 000	0	18 ± 4
others	~15	~25

[a] Mean ± S.D. of three separate experiments

The percentage of the total amount of radioactivity incorporated into individual protein bands was estimated from the densitometric tracings of the fluorographs.

Table 2. Transglutaminase catalyzed incorporation of [^{14}C]putrescine into acid-insoluble material of mouse neuroblastoma cells.

Cell line	Morphology	Trichloroacetic acid insoluble radioactivity (cpm per mg protein)
N-18	Undifferentiated	55 000 ± 800[a]
	Differentiated	114 000 ± 2 000
NB-15	Undifferentiated	77 000 ± 900
	Differentiated	153 000 ± 3 000

[a] Mean ± S.D. of three separate experiments

Undifferentiated and differentiated mouse neuroblastoma cells were labeled by the transglutaminase/[^{14}C]putrescine method under identical conditions. The specific radioactivity incorporated into acid insoluble cellular material was determined by filter-disc assay method (21).

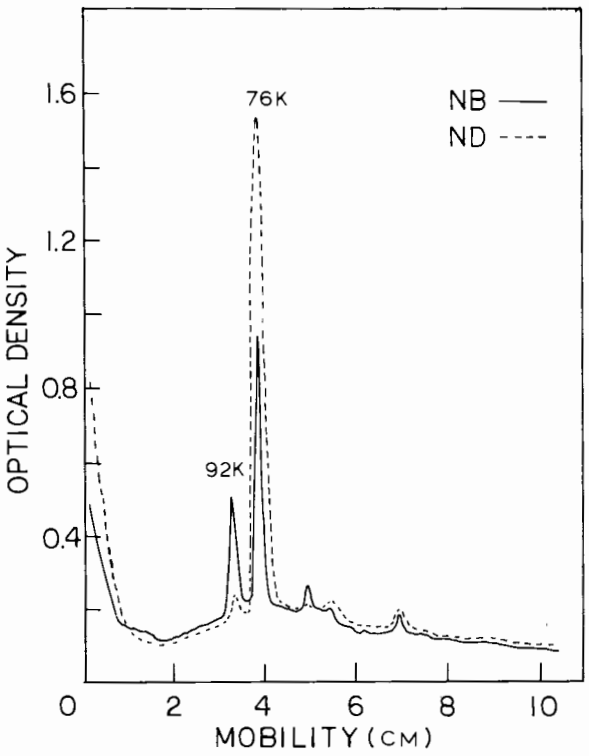

Fig. 2. Densitometric tracing of fluorograph illustrating the pattern of transglutaminase/[^{14}C]putrescine labeled surface proteins of the undifferentiated (NB) and differentiated (ND) N-18 mouse neuroblastoma cells. Undifferentiated and differentiated N-18 cells were labeled under identical conditions. Labeled proteins were separated on a linear gradient (7.5–15%) of SDS-polyacrylamide slab gel. The fluorograph was made on a SB-5 X-ray film (Eastman Kodak). Densitometric tracings were obtained by scanning the fluorographs. For both the NB and ND cells, 50 μg of cellular protein were used for gel electrophoresis.

molecular weight protein bands (Fig. 3A). Fig. 3B emphasizes the fact that the very high molecular weight band in both undifferentiated and differentiated NB-15 cells did not penetrate appreciably into the 5% gel suggesting that this gel top band is a cross-linked band.

The results illustrated in Fig. 2 and Fig. 3 also demonstrated that while the patterns of proteins labeled by the transglutaminase catalyzed incorporation of [^{14}C]putrescine of the undifferentiated and differentiated mouse neuroblastoma cells were similar, the intensity of labeling of the individual bands was modulated by cell differentiation.

Discussion

In this study, the transglutaminase/[^{14}C]putrescine labeling method was used to compare and probe the possible differences in surface architectures of undifferentiated and differentiated neuroblastoma cells.

A significantly greater amount of radioactive putrescine was incorporated into the surface proteins of the differentiated N-18 and NB-15 cells than that of their undifferentiated counter parts (Figs. 2 and 3). These results indicated that the differentiation of mouse neuroblastoma cells was accompanied by an increase of available acyl donor groups (i.e., glutaminyl residues) at cell surface proteins. Such an increase may be due to any one or a combination of the following mechanisms: (a) both the undifferentiated and differentiated neuroblastoma cells have the same number of peptide-bound glutaminyl residues, but in the undifferentiated cells these residues are partially occupied by endogenous polyamines; (b) the conformation of surface proteins of the differentiated cells is such that they are more susceptible to the transglutaminase catalyzed transamidation reaction than their undifferentiated counterparts; (c) there are more copies per cell of those surface proteins which can be labeled by [^{14}C]-putrescine in the differentiated cells than that in the undifferentiated cells.

It has been shown that transglutaminase can cross-link proteins through two mechanisms, one is the direct cross-linking of two proteins via γ-glutaminyl-lysine linkage, (2, 4) the other is using diamine or polyamine as a bridge to cross-link proteins via the γ-glutaminyl-diamine (polyamine)-γ-glutaminyl linkage (28). The incorporation of radioactive putrescine into protein band at gel top of both N-18 and NB-15 cells suggests that [^{14}C]putrescine may be used as a cross-linking agent under the present experimental conditions. If so, the increased cross-linking of surface proteins in the differentiated N-18 and NB-15 cells suggest that membrane structure of the differentiated cells may be different from that of the undifferentiated cells.

Polyamine-conjugated proteins have been described in human plasma (15), human amniotic fluid (16), and possibly human lymphocytes (29). Since transglutaminase and polyamines exist both in tissues and tissue fluids, transglutaminase may catalyze the incorporation of extracellular polyamines

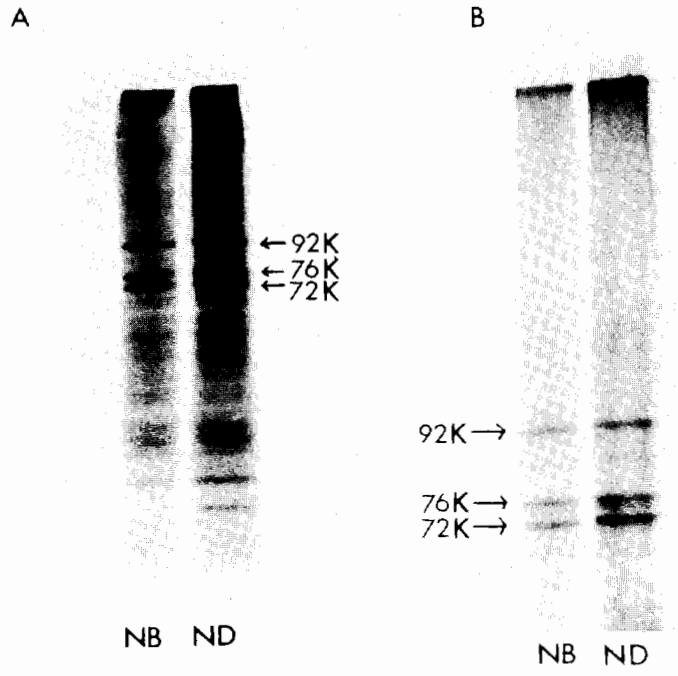

Fig. 3. Fluorograph illustrating the transglutaminase/[^{14}C]putrescine labeled surface proteins of undifferentiated (NB) and differentiated (ND) NB-15 mouse neuroblastoma cells.
Panel A: labeled proteins were separated on a 7.5–15% linear gradient SDS-polyacrylamide slab gel.
Panel B: labeled proteins were separated on a 5% SDS-polyacrylamide gel. Each lane contained 50 μg protein.

into cell surface proteins in vivo. In light of this, it is of interest to note that Quash et al. have shown that anti-polyamine antibody binds tightly to the surface of chick embryo cells (30) and is able to lyse L cells and BHK Py cells (31) in the presence of complement (31). Their results suggest that tightly-bound polyamines do exist at cell surface, however, the chemical properties of such binding remain to be identified. In the present study, I have shown that putrescine can covalently bind to certain specific surface proteins of mouse neuroblastoma cells. If indeed there are tightly bound polyamines at cell surface, these labeled surface proteins (92 000- and 72 000-dalton proteins for N-18 cells and 92 000-, 76 000- and 72 000-dalton proteins for NB-15 cells) may represent potential binding sites for polyamines in vitro and in vivo.

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