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AN 18000-DALTON PROTEIN METABOLICALLY LABELED BY POLYAMINES IN VARIOUS MAMMALIAN CELL LINES

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The possible role of polyamines in the covalent modification of cellular protein(s) was investigated by studying the metabolic labeling of NB-15 mouse neuroblastoma cells by [^{14}C]putrescine in fresh Dulbecco's medium followed by separation of cellular proteins through sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Under such incubation conditions, a single protein band with an apparent molecular weight of 18000 was radioactively labeled. [^{14}C]Spermidine also specifically labeled this protein. The majority of the radioactivity covalently linked to the 18-kDa protein was recovered as hypusine. The radioactive labeling of this protein was stimulated 1.3-fold by 1 mM dibutyryl cAMP and 2.8-fold by 4% fetal calf serum. Fetal calf serum also stimulated the labeling of many other cellular proteins. This may be due to the conversion of putrescine to amino acids via the formation of γ-aminobutyric acid. Aminoguanidine, a potent inhibitor of diamine oxidase, completely inhibited the fetal calf serum-stimulated labeling of these cellular proteins but had no effect on the labeling of the 18-kDa protein. The specific labeling of the 18-kDa protein by [^{14}C]putrescine occurred in various mammalian cells examined including the N-18 mouse neuroblastoma cells, 3T3-L1 murine preadipocytes, and H-35 rat hepatoma cells. The specificity of labeling of the apparently ubiquitous 18-kDa protein and the stimulation of this labeling by fetal calf serum suggest that this protein may be important in mediating some of the actions of polyamines in cell growth regulation.

Introduction

Polyamines (putrescine, spermidine and spermine) are ubiquitous organic cations in mammalian tissues [1–3]. One of the earlier biochemical events during initiation of cell growth is a marked increase in the activity of ornithine decarboxylase (EC 4.1.1.17, L-ornithine carboxylyase), the rate-controlling enzyme for the biosynthesis of polyamines. Studies performed in many laboratories have indicated that polyamines may have an important role in cell growth regulation [4–6].

Despite their relatively simple chemical structures, polyamines are known to exert effects on a wide spectrum of biological events. Some of the effects of polyamines may be derived from their general cationic character and thus replaceable by inorganic cations whereas other effects may be more specific and depend on either covalent or high-affinity binding of polyamines to certain biological molecules or organelles [1,5]. To characterize the specific functions of polyamines, which may be more relevant to the essential roles of polyamines in growth regulation, it is necessary to identify the molecules or organelles to which polyamines are specifically linked. One such covalent

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linkage has been identified as γ-glutamyl polyamines in rat seminal vesicle [7,8] and in *Aplysia* giant neuron [11], catalyzed by transglutaminases. Recent studies by Folk and co-workers [12] have demonstrated that polyamines can also be covalently linked to cellular proteins via hypusine (i.e., N^{ϵ}-(4 amino-2-hydroxylbutyl)lysine) formation. In a previous study, we reported that [^{14}C]putrescine metabolically labels an 18-kDa protein in the mouse neuroblastoma cells and that the intensity of labeling is different between the undifferentiated and differentiated neuroblastoma cells [13]. In the present paper we report the characterization of the radioactive species associated with the 18-kDa protein and the identification of this protein in several other mammalian cell lines.

Materials and Methods

Materials. [1,4-^{14}C]Putrescine · 2HCl (122 mCi/mmol 1 Ci = 3.7 · 10^{10} Bq), [^{14}C]spermidine · 3HCl (122 mCi/mmol) and [1,4(n)-^{3}H]putrescine · 2HCl (19 Ci/mmol) were purchased from Amersham, Arlington Heights, IL. γ-[^{14}C(u)]Aminobutyric acid (203 mCi/mmol) was obtained from New England Nuclear, Boston, MA. Dulbecco's modified Eagle medium, fetal calf serum, Eagle's modified minimal essential medium and calf serum, were from Gibco, Grand Island, NY. Aminoguanidine, cycloheximide, γ-aminobutyric acid, dibutyryl cyclic AMP, phenylmethylsulfonyl fluoride, aminopeptidase M, carboxypeptidases A and B were purchased from Sigma Chemical Co., St. Louis, MO. Pronase was obtained from Calbiochem-Behring, La Jolla, CA.

Cell culture. Both mouse N-18 and NB-15 clonal neuroblastoma cells were grown in Dulbecco's modified Eagle medium (with 4500 mg glucose per liter, without pyruvate) supplemented with 10% fetal calf serum. Murine 3T3-L1 preadipocytes were grown in Dulbecco's medium with 10% newborn calf serum. Rat H-35 heptoma cells were grown in Eagle's modified minimal essential medium supplemented with 10% fetal calf serum. All cells were maintained as monolayer cultures at 37°C in a Forma water-jacketed CO_2 incubator (95% air, 5% CO_2).

Metabolic labeling with radioactive polyamines. Unless otherwise stated, cells at an early stationary phase of growth were used for all experiments. For studying the metabolic incorporation of radioactive polyamines, monolayer cells in culture dishes were washed twice with fresh Dulbecco's medium, reincubated in Dulbecco's medium (serum-free) and carrier-free radioactive polyamines were added. Various chemicals were then added to an appropriate concentration as indicated in figure legends. Metabolic labeling was carried out at 37°C in a Forma CO_2-incubator. At designated time, the incubation medium was decanted, cells were washed three times with cold phosphate-buffered saline (pH 7.4). Cells were then harvested and homogenized in a Tris buffer (20 mM, pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride. Aliquots of the cell homogenates were used to measure the incorporation of radioactivity into the trichloroacetic acid-insoluble fraction and for SDS-polyacrylamide gel electrophoresis. Protein concentration was determined by the method of Lowry et al. [14]. SDS-polyacrylamide gel electrophoresis was carried out according to the procedure of Laemmli [15]. Fluorograms were prepared according to the method of Bonner and Lasky [16]. Fluorograms were scanned with a Schoeffel SD-3000 spectromicrodensitometer and the peak areas of the optical tracings were used as a quantitative measure of the amount of radioactivity incorporated.

Identification of the radioactive species associated with the protein fraction and the 18-kDa protein. NB-15 mouse neuroblastoma cells incubated in serum-free Dulbecco's medium were metabolically labeled with [^{14}C]putrescine (1 μCi/ml) or [^{3}H]putrescine (5 μCi/ml) in the presence of 20 μM aminoguanidine. After a 20 h incubation period at 37°C, cells were harvested and washed with cold trichloroacetic acid (10%) until no radioactivity appeared in the supernatant. The trichloroacetic acid-insoluble cellular material was processed for exhaustive proteolytic digestion by sequential treatment with pronase, aminopeptidase M and carboxypeptidases A and B as described by Folk et al. [8]. Part of the proteolytic digest was analyzed directly by ion-exchange chromatography using the conditions described by Folk et al. [8]. Part of the proteolytic digest was further acid-hydrolyzed (6 M HCl, 108°C, 24 h) and then analyzed by ion-exchange liquid chromatography.

The radioactive species associated with the 18-kDa protein was determined by subjecting the 18-kDa protein band, excised from SDS-polyacrylamide slab gel, to acid hydrolysis (6 M HCl, 108°C, 24 h) and ion-exchange liquid chromatography [8].

Results

When mouse neuroblastoma cells were incubated with [^{14}C]putrescine in fresh Dulbecco's medium, radioactivity was incorporated into trichloroacetic acid-insoluble cellular material in a time-dependent manner (Fig. 1). In both the N-18 and NB-15 clonal mouse neuroblastoma cells, maximal level of incorporation was attained after a 30–40 h period of incubation. Fetal calf serum stimulated both the initial rate as well as the maximal level of incorporation in both N-18 and NB-15 mouse neuroblastoma cells. Since both fetal calf serum and dibutyryl cyclic AMP can induce ornithine decarboxylase activity in mouse neuroblastoma cells [9,10], their effects on the incorporation of [^{14}C]putrescine into trichloroacetic acid-insoluble material in the NB-15 mouse neuroblastoma cells were compared (Fig. 2). While both fetal calf serum and dibutyryl cyclic AMP stimulated the incorporation reaction, the magnitude of stimulation elicited by dibutyryl cyclic AMP was not nearly as great as that of fetal calf serum. It should be noted that after 20–30 h of incubation, fetal calf serum or dibutyryl cyclic AMP did not affect the specific radioactivity of polyamine pools (data not shown). Very little or no incorporation of [^{14}C]putrescine into the acid-insoluble fraction occurred when the labeling reaction was carried out in the presence of cycloheximide (40 μg/ml) or when cells were maintained in the old medium (data not shown).

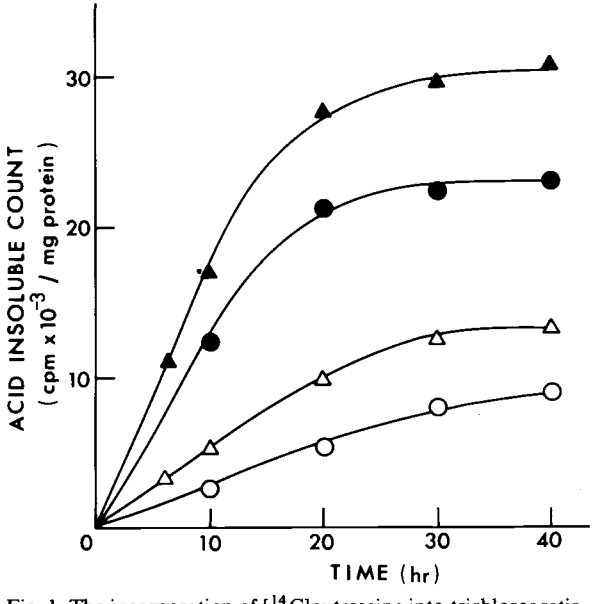

Fig. 1. The incorporation of [^{14}C]putrescine into trichloroacetic acid-insoluble material in N-18 and NB-15 mouse neuroblastoma cells. Both N-18 and NB-15 cells at an early stationary phase of growth were used. N-18 cells in the absence (△) and in the presence (▲) of 10% fetal calf serum, and NB-15 cells in the absence (○) and in the presence (●) of 10% fetal calf serum were incubated with [^{14}C]putrescine (0.75 μCi/ml) at 37°C in a CO_2-incubator. At designated times, cell samples were harvested and trichloroacetic acid-insoluble radioactivity was determined. Each point represents the average of duplicate samples.

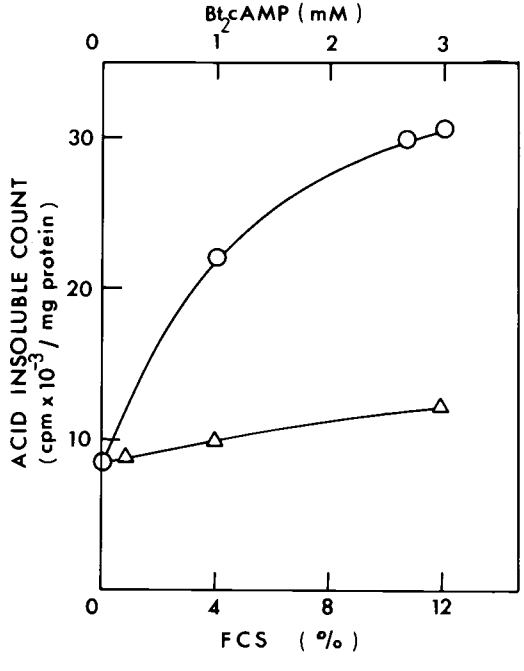

Fig. 2. Dose-response curve of the effects of fetal calf serum (FCS) and dibutyryl (Bt_2) cyclic AMP on the incorporation of [^{14}C]putrescine into trichloroacetic acid-insoluble proteins in the NB-15 mouse neuroblastoma cells. NB-15 cells at an early stationary phase of growth were incubated with [^{14}C]putrescine (1 μCi/ml) various concentrations of fetal calf serum (○) or dibutyryl cyclic AMP (△) in fresh Dulbecco's medium, for 20 h at 37°C. Trichloroacetic acid-insoluble radioactivity in the cell samples was determined.

The pattern of covalent incorporation of [^{14}C]putrescine into cellular proteins was examined by SDS-polyacrylamide gel electrophoresis and fluorography. The fluorogram shown in Fig. 3 indicated that only one protein band, with an apparent molecular weight of 18000, was radioactively labeled in the NB-15 cells by [^{14}C]putrescine (Fig. 3, lane A). The radioactivity associated with the 18-kDa protein band only accounted for 15–20% of the total trichloroacetic acid-insoluble radioactivity. This suggested that most of the acid-insoluble radioactivity may bind tightly but not necessarily covalently to cellular macromolecules. The presence of fetal calf serum not only stimulated the labeling of the 18-kDa protein but also caused the labeling of many other cellular proteins (Fig. 3, lanes B and C vs. lane A). In comparison, dibutyryl cyclic AMP only stimulated the labeling of the 18-kDa band (Fig. 3, lanes D and E vs. lane A). It may be noted that the presence of 20 μM aminoguanidine completely inhibited the labeling of all other cellular proteins by [^{14}C]putrescine except the 18-kDa protein band [13]. We have previously shown that such aminoguanidine-sensitive labeling was due to the conversion of putrescine to amino acids via γ-aminobutyric acid formation [13]. The formation of γ-aminobutyric acid from putrescine is catalyzed sequentially by diamine oxidase present in fetal calf serum and cellular aldehyde dehydrogenase [17,18].

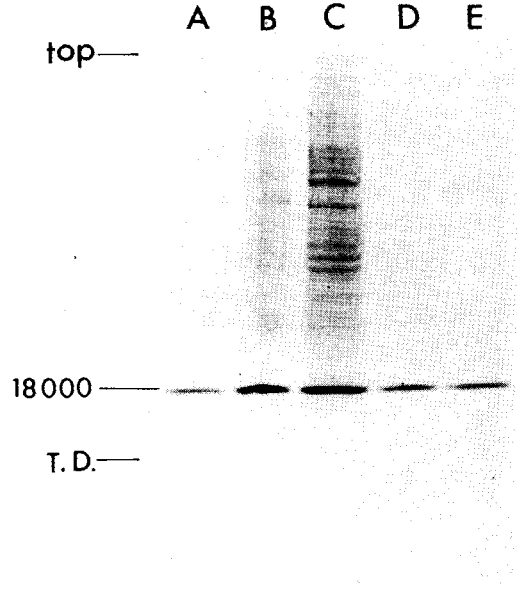

Fig. 3. Fluorogram illustrating the pattern of radioactively labeled proteins of NB-15 mouse neuroblastoma cells exposed to [^{14}C]putrescine. Cell samples obtained from the experiment illustrated in Fig. 2 were subjected to SDS-polyacrylamide gradient gel electrophoresis (7.5–15% acrylamide). Radioactive bands were detected by the fluorographic method of Bonner and Lasky [16]. Standard proteins used for molecular weight determination were cytochrome *c* (M_r 12500), chymotrypsinogen (M_r 25000), catalase (M_r 57500), bovine serum albumin (M_r 68000), phosphorylase *a* (M_r 94000) and β-galactosidase (M_r 130000). Lane A, NB-15 cells in Dulbecco's medium. Lane B, NB-15 cells in Dulbecco's medium supplemented with 4% fetal calf serum. Lance C, NB-15 cells in Dulbecco's medium supplemented with 12% fetal calf serum. Lane D, NB-15 cells in Dulbecco's medium with 1 mM dibutyryl cyclic AMP added. Lane E, NB-15 cells in Dulbecco's medium with 3 mM dibutyryl cyclic AMP added. Protein samples were prepared for gel electrophoresis as described in Experimental Procedures. The amount of proteins applied to each lane was 50 μg. Exposure time of the fluorogram was 28 days.

To identify the radioactive species associated with the labeled 18-kDa protein band, the radioactive band was excised from the SDS-polyacrylamide gel, hydrolyzed in 6 M HCl at 108°C for 24 h and then analyzed by ion-exchange liquid chromatography (Fig. 4). More than 75% of the radioactivity recovered was eluted in a region corresponding to the hypusine standard. Two minor peaks also appeared, one was identified as sper-

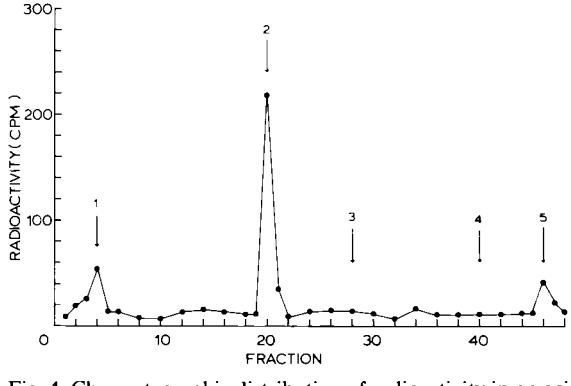

Fig. 4. Chromatographic distribution of radioactivity in an acid hydrolysate of the 18-kDa protein band. Acid hydrolysate was prepared from the 18-kDa protein band excised from a SDS-polyacrylamide gel and analyzed on a Dionex D-400 analyzer by using DC-6A resin and a three buffer elution scheme, as described by Folk et al. [8]. Arrows indicated: 1, unidentified species; 2, hypusine; 3, putrescine; 4, spermidine and 5, spermine.

mine, the other, eluted at the beginning of the chromatogram, was not further characterized. The presence of spermine in the acid hydrolysate of the 18-kDa protein suggested possible existence of γ-glutamyl spermine linkage. Taking advantage of the fact that γ-glutamyl polyamine linkage is resistant to proteolytic digestion, we examined the possible existence of γ-glutamyl polyamines in the proteolytic digest of cells radiolabeled by [^{3}H]putrescine. The chromatogram (Fig. 5a) showed that a significant amount of radioactivity in the proteolytic digest was recovered as hypusine, spermidine and spermine. Since hypusine, the unusual amino acid that behaves like the γ-glutamyl spermidine on HPLC [11,12,19], is acid-resistant whereas γ-glutamyl spermidine is not, the proteolytic digest was further treated with 6 M HCl at 108°C for 24 h and the acid hydrolysate thus obtained was analyzed by ion-exchange chromatography. The chromatogram (Fig. 5b) indicated a slight increase of spermidine and spermine peaks with a concomittant decrease of the hypusine peak. This might indicate that there existed a small percentage of γ-glutamyl polyamine linkages in the [^{14}C]putrescine-labeled cell extracts. Altogether, these data (Fig. 4 and Fig. 5) indicated that (i) the major radioactive species associated with the 18-kDa protein was acid-resistant hypusine, (ii) more than 50% of acid-insoluble radioactivity present in [^{3}H]putrescine-labeled mouse neuroblastoma cell extracts was non-covalently bound spermidine and spermine, (iii) hypusine recovered in the acid-insoluble protein faction was probably derived solely from the 18-kDa protein.

Recent studies by Folk and co-workers [10] suggested that spermidine is the immediate precursor of hypusine. When [^{14}C]spermidine was used to label NB-15 mouse neuroblastoma cells, two protein bands were predominantly labeled (Fig. 6), one had an apparent molecular weight of 18 000 and co-migrated with the 18-kDa protein band metabolically labeled by [^{14}C]putrescine, the other radioactively labeled band appeared at the top of the gel. The possibility that this high molecular weight protein band may represent cross-lin-

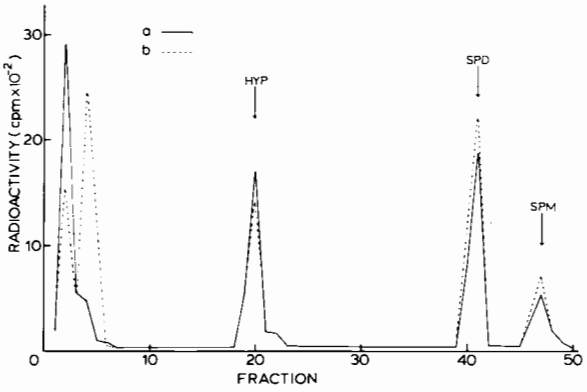

Fig. 5. Chromatographic distribution of radioactivity in a protease digest (a, ———) and an acid hydrolysate of the protease digest (b, - - - - - -) of the protein fraction from NB-15 mouse neuroblastoma cells. NB-15 mouse neuroblastoma cells were metabolically labeled with [^{3}H]putrescine (2 μCi/ml) for 25 hours at 37°C. The protein fraction was prepared for protease digestion. One-half of the digest was then analyzed directly by ion-exchange chromatography (a, ———), the other half was treated with 6 M HCl at 108°C for 24 h and analyzed by ion-exchange chromatography (b, - - - - - -).

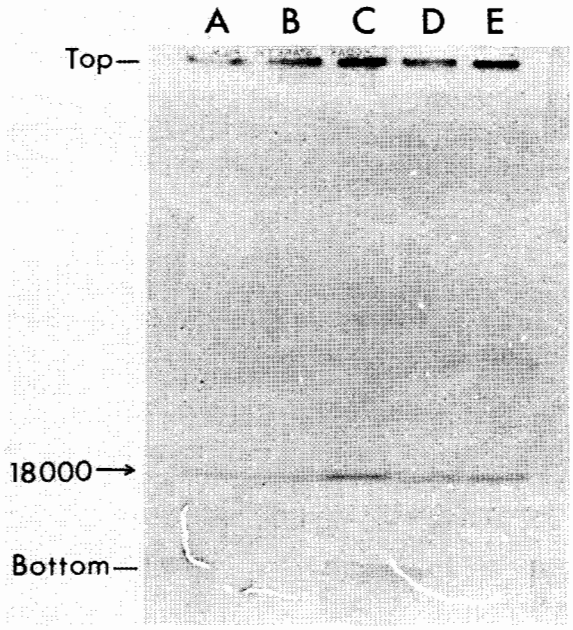

Fig. 6. Fluorogram of the radioactively labeled proteins in mouse neuroblastoma cells exposed to [^{14}C]spermidine for various time intervals. NB-15 cells were incubated with [^{14}C]spermidine at 1 μCi/ml for 15 h (lanes B and C) or for 30 h (lanes D and E) in the absence (lanes B and D) and in the presence (lanes C and E) of 10% fetal calf serum. Lane A was cell sample from mouse N-18 neuroblastoma cells exposed to [^{14}C]spermidine for 15 h in the absence of fetal calf serum. The amount of proteins applied to each lane was 50 μg.

ked proteins using spermidine as a cross-linking agent is under investigation. The labeling of both the 18-kDa band and the high molecular weight band was stimulated by fetal calf serum (Fig. 6). Interestingly, fetal calf serum did not cause the labeling of other cellular proteins indicating that γ-aminobutyric acid formation from spermidine was not significant under the present experimental condition.

The metabolic labeling of the 18-kDa protein by putrescine appeared to be a general phenomenon. It occurred in all of the mammalian cell lines that I have examined, including the mouse neuroblastoma cells (NB-15, N-18, N2a and NS-20 clones), human skin fibroblasts (HF-24, HF-40 clones), mouse melanoma cells (Cloudman S91), H-35 rat hepatoma cells, 3T3-L1 preadipocytes and PC-12 pheochromocytoma cells. Fig. 7 illustrates the pattern of labeling N-18, H-35 and 3T3-L1 cells by [^{14}C]putrescine. The 18-kDa protein band was the most prominently-labeled protein in these three cell lines. Since the labeling experiment was carried out in the presence of fetal calf serum the labeling of other cellular proteins due to putrescine to γ-aminobutyric acid conversion also occurred in these cells. Interestingly enough, such labeling was significant in mouse neuroblastoma cells and 3T3-L1 preadipocytes but not in H-35 hepatoma cells. This could be due to lack of cellular aldehyde dehydrogenase or alternatively, a defect of the γ-aminobutyric shunt enzymes in the H-35 hepatoma cells.

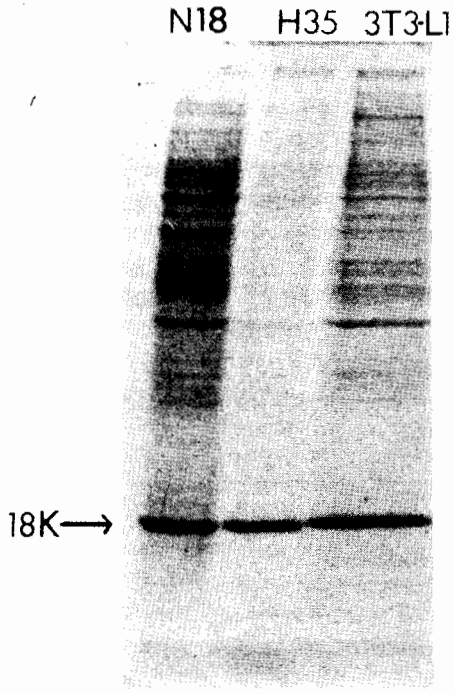

Fig. 7. Fluorogram of radioactively labeled proteins in three different cell lines exposed to [^{14}C]putrescine. Mouse N-18 neuroblastoma cells, rat H-35 hepatoma cells, and mouse 3T3-L1 fibroblasts were incubated with [^{14}C]putrescine (1.5 μCi/ml) in fresh growth medium in the presence of 10% fetal calf serum for 20 h. The amount of proteins applied to each lane was 50 μg. Fluorogram was obtained after 30 days exposure.

Discussion

The results presented in this study indicate the existence of an 18000-kDa protein which can be metabolically labeled by putrescine or spermidine in various mammalian cell lines. The majority of radioactivity associated with the 18-kDa protein in mouse neuroblastoma cells appeared to be hypusine. During the preparation of this manuscript, Folk and co-workers [19,20] reported the specific labeling of an 18-kDa protein by [^{3}H]spermidine in Chinese hamster ovary cells, human lymphocytes, lymphoma cells and 3T3 fibroblasts. Taking these data together, the metabolic labeling of the 18-kDa protein in living cells may represent a ubiquitous phenomenon.

The amount of hypusine recovered from the 18-kDa protein band could account for more than 90% of the hypusine present in the acid hydrolysate of the acid-insoluble protein fraction. This suggests that the 18-kDa protein may be the only cellular protein that contained radioactive hypusine. Hypusine was first isolated from homogenates of bovine brain [21] and subsequently discovered in other tissues [22,23] as a constituent of animal proteins [24]. However, specific protein(s) containing this amino acid were not identified and the function of such hypusine-containing proteins was completely unknown. The finding of a specific hypusine-containing protein (M_r 18000) in various mammalian cell lines suggests that similar protein may exist in various animal tissues. The present studies showed that labeling of this protein could be stimulated by fetal calf serum, dibutyryl cyclic AMP (Fig. 3) but

was inhibited by cycloheximide. Our previous studies indicated that the labeling intensity of this protein was different between undifferentiated and differentiated mouse neuroblastoma cells [13]. Folk et al. [8] have shown that phytohemagglutinin stimulated the labeling of the 18-kDa protein in human lymphocytes. In light of these studies it appears that the labeling of the 18-kDa protein may be growth-related. It would be interesting to see whether some of the growth-stimulatory effect of polyamines is mediated by it's covalent incorporation into this 18-kDa protein.

Although hypusine and γ-glutamyl spermidine have close chromatographic behavior [11,12,19], they can be distinguished by the fact that hypusine is acid-resistant whereas γ-glutamylspermidine is not [24]. By this criterion, the majority of radioactivity associated with the 18-kDa protein was identified as hypusine instead of γ-glutamyl spermidine. However, the possible existence of small quantity of γ-glutamylpolyamine linkages in the 18-kDa protein or other cellular proteins cannot be ruled out. In this regard, it is interesting to note that Ambron and Kremzer [11] have identified γ-glutamyl polyamines in *Aplysia* neuron injected with [^{14}C]putrescine.

More than 50% of the acid-insoluble radioactivity was recovered in the proteolytic digest of NB-15 mouse neuroblastoma cells as spermidine and spermine (Fig. 5) indicating that substantial amount of polyamines formed tight high-affinity but non-covalent binding to certain cellular proteins. Similar findings have also been reported in the *Aplysia* neurons [11]. While the physiological significance of such non-covalent binding remained to be studied we noticed that such high affinity binding was also inhibited by cycloheximide (data not shown).

At present, the functional significance of the 18-kDa protein is not known. It is conceivable that metabolic labeling of this protein by polyamines can change its net charge, conformation and thus biological activities. In view of the abundant literature evidence that indicates the importance of post-translational modification of proteins as a biological control mechanism [25–27], it may be relevent to speculate that some of the growth-regulatory actions of polyamines may be related to their ability to covalently modify cellular proteins, either through γ-glutamylpolyamine linkage or hypusine formation. The fact that the 18-kDa protein was the only cellular protein prominently labeled by putrescine and spermidine in various mammalian cell lines further underscores the potential significance of this protein.

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