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Isolation and characterization of an 18 kDa hypusine-containing protein from cultured NB-15 mouse neuroblastoma cells

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An 18 kDa protein can be metabolically labeled by [^{3}H]putrescine or [^{3}H]spermidine in various mammalian cells. The labeling is due to a post-translational modification of one lysine residue to hypusine using the aminobutyl moiety derived from spermidine. In view of the lack of knowledge of the function of this spermidine-modified protein, we decided to use the radioactivity associated with the [^{3}H]spermidine-labeled 18 kDa protein as a tracer to develop a simple procedure for purifying this protein from cultured cells. We first screened more than 15 different affinity adsorbents for their ability to bind the labeled 18 kDa protein. This approach enabled us to develop a four-step procedure to purify the labeled 18 kDa protein from NB-15 mouse neuroblastoma cells. The procedure, including a Cibacron Blue column, an ω-aminooctyl-agarose, a Sepharose G-50, and a Mono Q column, resulted in an 800-fold purification of the labeled 18 kDa protein. Two-dimensional gel analysis of fractions enriched in the labeled 18 kDa protein revealed (i) the presence of isoforms of hypusine-containing 18 kDa protein, with p*I* values ranging from 4.7 to 5.2, and (ii) the presence of an additional labeled protein with an apparent molecular mass of 22 kDa and a p*I* value of 5.0. The labeling intensity of the 22 kDa protein, however, was less than 5% of that of the 18 kDa protein. Peptide map analysis, using the V-8 proteinase digestion method, indicated that the 18 kDa hypusine-containing protein obtained from NB-15 cells was similar to eukaryotic initiation factor 4D isolated from rabbit reticulocytes.

Introduction

Polyamines (putrescine, spermidine and spermine) are widely distributed in living organisms [1–3]. Studies from many laboratories have provided evidence that polyamines play an important role in cell growth regulation [1–6]. We have previously shown that differentiation of mouse neuroblastoma cells is accompanied by significant changes of polyamine metabolism [7–9]. To define further the physiological function of polyamines in the growth and differentiation of mouse neuroblastoma cells, we attempted to identify specific polyamine-dependent biochemical events and to correlate such events with mouse neuroblastoma differentiation. During this endeavor, we found that [^{14}C]putrescine or [^{3}H]spermidine can metabolically label an 18 kDa protein in mouse neuroblastoma cells, and that the labeling intensity was significantly reduced upon the differentiation of mouse neuroblastoma cells [9]. The labeling was found to be due to a covalent linkage between one

Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; eIF-4D, eukaryotic initiation factor 4D; hyp-18K protein, hypusine-containing 18 kDa protein; PBS, phosphate-buffered saline.

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lysine residue on the 18 kDa protein and the aminobutyl group derived from spermidine [10,11]. The free form of the modified amino acid residue, named hypusine, was thought to be an unusual amino acid when it was first discovered in bovine brain [12]. This unique post-translational modification has been demonstrated in various cultured cell lines [11,13] including lower eukaryotes such as yeast [14] and *Neurospora crassa* [15]. Based on the electrophoretic behavior on two-dimensional gel and hypusine analysis, Cooper et al. [16] reported that the hypusine-containing 18 kDa (hyp-18K) protein in human lymphocytes may be identical to the translation initiation factor eIF-4D. This finding, however, does not shed too much light on the function of the hyp-18K protein, because the role of eIF-4D in protein synthesis is ill-defined and remains to be characterized. Nevertheless, the apparent ubiquity and uniqueness of this spermidine-dependent labeling reaction, together with the recognized importance of polyamines in growth regulation suggest that hypusine-containing proteins, such as hyp-18K protein, may be fundamentally important in growth regulation. The fact that the labeling is responsive to growth stimulation [11–13] further supports this notion.

The lack of well-defined and measurable activity of the hyp-18K protein poses a problem for its purification. Nevertheless, the fact that hyp-18K protein can be metabolically labeled by radioactive polyamines in cultured cells makes it possible to monitor the purification of this protein using the radiolabel as an index. Using this approach, we screened more than 15 different affinity adsorbents in order (i) to develop a simple procedure to purify this protein from cultured mammalian cells, and (ii) to gain some insights into the physical-chemical properties of this protein through the characteristics of its binding to various media. Such screening enabled us to develop a simple procedure for isolating the hyp-18K protein from cultured mammalian cells using two general-ligand affinity columns. During the purification of the hyp-18K protein, we detected the presence of another radiolabeled protein with an apparent molecular mass of 22 kDa in NB-15 cells which co-purified with the hyp-18K protein during the initial stages of purification.

Materials and Methods

Materials. Dulbecco's modified Eagle medium, fetal bovine serum and gentamycin were purchased from Gibco, Grand Island, N.Y. [2,3-^{3}H(n)] Putrescine dihydrochloride (30 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Reactive Blue-2-Sepharose CL-6B, ω-aminooctyl-agarose, Sepharose G-50 (superfine), spermine and various affinity column resins were obtained from Sigma Chemical Co., St. Louis, MO. Mono Q column and the FPLC system are products of Pharmacia, Piscataway, N.J.

Cell culture and labeling. NB-15 mouse neuroblastoma cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum as previously described [7]. Cells at the early stationary phase of growth were serum-deprived for 12 h and then replenished with fresh Dulbecco's medium containing 10% fetal bovine serum and 0.2 mM aminoguanidine. The metabolic labeling was initiated by adding [^{3}H]putrescine at 3 μCi/ml to the cultures which were incubated at 37 ° C in a Forma water-jacketed CO_2 incubator (95% air/5% CO_2). After an incubation of 24 h, cells were washed with cold phosphate buffered saline (PBS: 135 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) and suspended in the same buffer. The final volume of the cell suspension was adjusted to give a protein concentration of approx. 2.2 mg per ml. Cells were disrupted by sonication and the cell homogenate was separated into supernatant and pellet fractions by a centrifugation at 27000 $\times$ g for 30 min. The supernatant fraction was used for subsequent protein purification.

Binding affinity of labeled hyp-18K protein with various affinity adsorbents. The supernatant fraction (0.2 ml) was mixed with an equal volume of various affinity media for a period upto 8 h at 4 ° C. The mixture was centrifuged and the amount of unbound hyp-18K protein was determined by minigel electrophoresis and radioactive counting of the excised gel from the 18 kDa band. The relative binding affinity of hyp-18K protein with various affinity media was determined by measuring the percentage of hyp-18K protein released from the adsorbents by washing with buffer A (20 mM Tris-HCl, 0.1 mM EDTA, pH 7.5).

Purification of the labeled hyp-18K protein. For a typical purification procedure, approx. $3 \cdot 10^8$ cells were employed. The supernatant fraction was loaded directly onto the Reactive Blue-2-Sepharose CL-6B (Cibacron blue) column (1×20 cm) that had been equilibrated first with buffer A. The column was then washed with buffer A and eluted subsequently with buffer A containing 6 mM spermine. The spermine-eluted fractions contained all the labeled hyp-18K protein. Fractions containing hyp-18K protein were combined (15 ml) and applied to an ω-aminooctyl-agarose (C-8) column (0.7×15 cm). The column was first washed with buffer A and then eluted sequentially with 0.4 M KCl and 1 M KCl in buffer A. The fractions eluted by 0.4 M KCl were combined, dialyzed against buffer A, lyophilized, redissolved in 1 ml of PBS concentrated 2.5 times and loaded onto a Sepharose G-50 column. The G-50 column was eluted with 2.5 concentrated PBS, fractions obtained after the void volume were combined, dialyzed against buffer A, and further chromatographed on a Mono Q column attached to a Pharmacia FPLC system. The chromatogram was developed by a salt gradient of 100 mM to 400 mM KCl in buffer A. The labeled hyp-18K protein was eluted out at 200 mM KCl. Fig. 1 illustrates the representative chromatograms obtained for various steps used in the above purification scheme.

Gel electrophoresis and other methods. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the procedure of Laemmli using a 7.5–15% acrylamide gradient [17]. To quantify the amount of labeled hyp-18K protein in each fraction after column chromatography, samples were analyzed by SDS-PAGE using a mini gel apparatus (Bio-Rad) and the radioactivity recovered from the 18 kDa band, excised from the gel, was determined. For two-dimensional gel analysis, the method of O'Farrel [18] was employed. Fluorograms were prepared according to the procedure of Bonner and Laskey [19] except that Enhancer (New England Nuclear) was used. Protein amount was determined by the procedure of Lowry [20]. V8 proteinase digestion and peptide map analysis were carried out as described by Cleveland et al. [21].

Results

In order to search for a suitable affinity adsorbent for purifying the hyp-18K protein, we examined the binding affinity of hyp-18K protein with various adsorbents. The labeled hyp-18K protein could bind to both dye affinity columns and ω-aminoalkyl-agarose media with varying degrees of affinity as shown in Table I. The labeled hyp-18K protein, however, did not bind to either nucleotide (GMP, GDP, ATP) or polynucleotide (e.g., poly(A), poly(C), and oligo(dT)) affinity media (Table I). Both the dye affinity adsorbents and ω-aminoalkyl-agarose are general-ligand affinity media in which a wide range of complementary proteins can bind [22]. The dye affinity column, particularly the Cibacron blue dye column, are known to bind proteins which possess the dinucleotide fold or adenine-containing nucleo-

TABLE I

BINDING AFFINITY OF THE hyp-18K PROTEIN TO VARIOUS AFFINITY CHROMATOGRAPHY MEDIA

The affinity chromatography adsorbents examined did not bind the labeled hyp-18K protein: poly(A)-agarose, poly(C)-agarose, oligo dT-cellulose, guanosine 5′-monophosphate-agarose, guanosine 5′-diphosphate-agarose, and L-methionine-agarose. Binding affinity was expressed as follows: +++, strong binding, the bound hyp-18K protein could not be eluted by buffer A; ++, 20–30% of bound hyp-18K protein was released by buffer A; +, more than 60% of bound hyp-18K protein was eluted by buffer A. Conditions of spermine elution were as follows: 5 mM spermine in buffer A (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA); 0.4 M KCl in buffer A completely removed hyp-18K protein from the ω-aminooctyl-agarose column. n.d., not determined.

Affinity resin	Binding affinity	Percent of hyp-18K protein eluted by spermine
Spermine-agarose	+++	100
L-Lysine-agarose	+++	100
ω-Aminooctyl-agarose	+++	0
ω-Aminopentyl-agarose	++	0
ω-Aminobutyl-agarose	+	0
Cibacron blue 2	+++	100
Reactive green	++	100
Reactive brown	++	100
Reactive red	+	n.d.
Reactive blue 4	++	n.d.
Reactive yellow	+	100
Heparin-agarose	+++	50

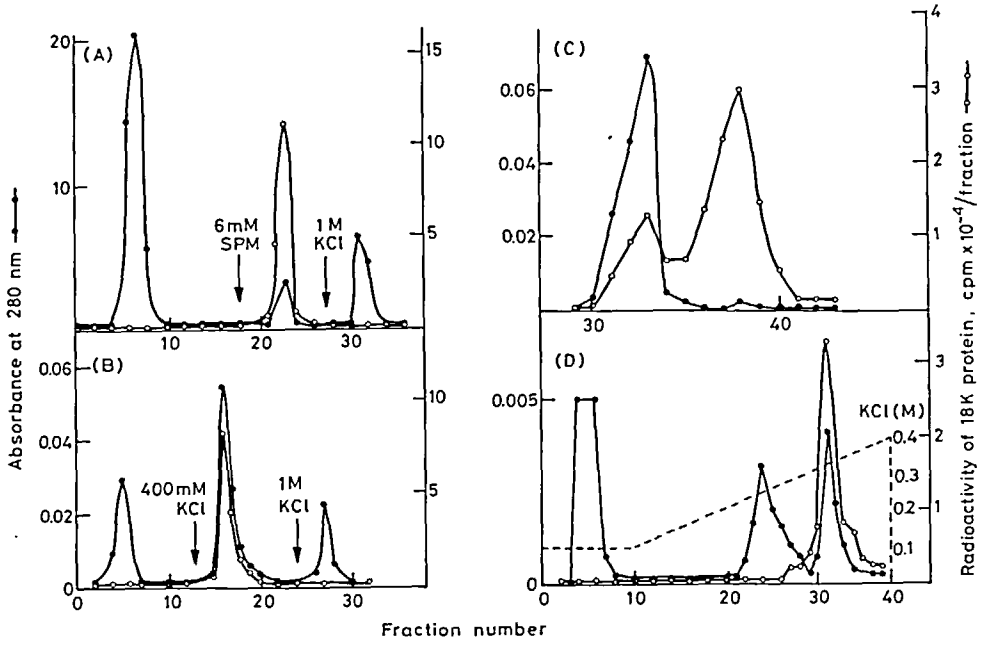

Fig. 1. Chromatography of radiolabeled hyp-18K protein on (A) Cibacron blue, (B) aminooctyl-agarose, (C) Sepharose G-50 and (D) Mono Q columns, Conditions for chromatography are described in Materials and Methods.

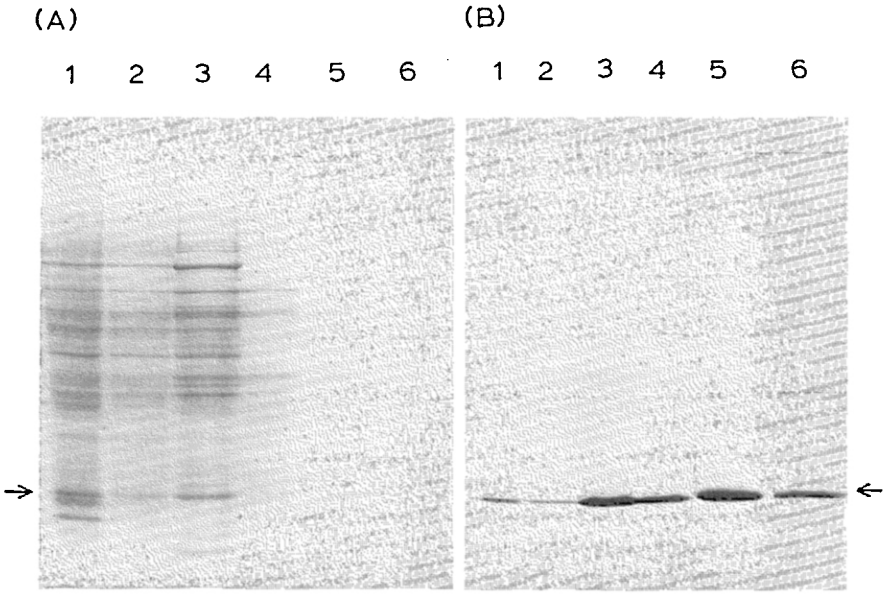

Fig. 2. SDS-polyacrylamide gel analysis of protein fractions during various stages of purification. (A) Coomassie blue-stained gel pattern. (B) Fluorogram of the same gel. Lane 1, cell homogenate, 50 μg proteins applied; lane 2, supernatant, 25 μg proteins applied; lane 3, spermine fractions from Cibacron blue column 35 μg proteins applied; lane 4, KCl fraction from C-8 column, 10 μg proteins applied; lane 5, G-50 fractions, 1 μg applied; lane 6, Mono Q fractions, 0.2 μg purified hyp-18K protein. Arrows indicate the position of hyp-18K protein.

tides [23]. We reasoned that proper application of principles of affinity elution chromatography [22] may result in specific elution of the hyp-18K protein. We therefore examined the ability of various ligands in eluting the hyp-18K protein from the Cibacron blue column. The ligands that we tested included putrescine (20 mm), spermidine (10 mM), spermine (10 mM), puromycin (1 mM), NAD^+ (20 mM), ATP (20 mM), GTP (20 mM), 2,4-diaminobutyric acid (10 mM) and KCl (250 and 400 mM). We included puromycin in our study because of a previous report that eIF-4D can stimulate the ApUpGp-dependent formation of methionylpuromycin [24]. In addition, in view of the similar chemical structure between spermine and the side chain of hypusine residue, and the speculation that the long hydrocarbon chain of hypusine residue may be involved in affinity binding, we also included polyamines as possible ligands in this study. We found that all the nucleotides tested, except puromycin, failed to elute the hyp-18K protein from the Cibacron blue column, suggesting that the interaction between the dye column and the hyp-18K protein may be at sites other than nucleotide pocket. The labeled hyp-18K protein can be eluted quantitatively from the Cibacron blue column by spermine at 5 mM. Putrescine or spermidine at even higher concentrations (20 mM) was not as effective. This finding prompted us to use spermine elution of the Cibacron blue column as the first step in isolation of the hyp-18K protein.

The hyp-18K protein also bound tightly to the ω-aminooctyl-agarose (C-8) column. The binding affinity decreased as the length of hydrocarbon chain decreased (Table I), suggesting that the interaction is a hydrophobic one. The hyp-18K protein bound to the C-8 column could be eluted by 0.4 M KCl quantitatively. Polyamines at 6 mM or higher concentrations could not elute this protein from the C-8 column. The data presented above enabled us to develop a relatively simple purification procedure, using Cibacron blue column and C-8 column as initial steps. The representative chromatograms are shown in Fig. 1. Fig. 2 shows the Coomassie blue-stained SDS-PAGE pattern of protein samples obtained at various stages of purification (Fig. 2A) and the fluorogram of the corresponding gel (Fig. 2B). Based on the radioactivities recovered from the labeled 18 kDa bands on the gel, we calculated the degree of purification for each column chromatographic step (Table II). The combination of the Cibacron blue and C-8 columns removed more than 90% of other proteins with very little loss of the hyp-18K protein. The inclusion of the C-8 column is advantageous, since it not only removed spermine used as eluent in the previous step but also separated the hyp-18K protein from other proteins with similar apparent molecular mass (18 kDa) as judged by two-dimensional gel analysis (data not shown). Gel filtration using a Sepharose G-50 column removed almost all the high molecular mass proteins (above 30 kDa) in the void volume. However, about 10% of labeled hyp-18K protein was recovered in the void volume despite the high ionic strength (PBS 2.5 ×) of the elution buffer (Fig. 1C), suggesting that the hyp-18K protein may either self-associate or associate with other proteins. The preparation obtained by Sepharose G-50 column chromatography was rather pure judging from the SDS-PAGE analysis, it gave a prominently Coomassie blue-stained band at the position of 18 kDa which corresponded to the radioactive label (Fig. 2A). When the protein sample, obtained either after the C-8 column or G-50 column, was analyzed by two-dimensional gel electrophoresis, we noticed the presence of another labeled protein with an apparent molecular mass of 22 kDa and a p*I*

TABLE II

PURIFICATION OF THE RADIOLABELED hyp-18K PROTEIN FROM NB-15 MOUSE NEUROBLASTOMA CELLS

Specific radioactivity was determined as the radioactivity associated with the 18 kDa band on the minigel per mg protein applied to the gel.

Purification step	Total protein (mg)	Total cpm ($\times 10^{-4}$)	Spec. radioact. (cpm/mg)	Yield (%)
Homogenate	121	46.1	2.3	–
Supernatant	81	37.5	2.4	–
Cibacron blue column	19	16.9	9.1	99
C-8 column	7	14.9	23	87
Sepharose G-50 column	0.12	8.3	712	49
Mono Q	0.04	7.2	1850	41

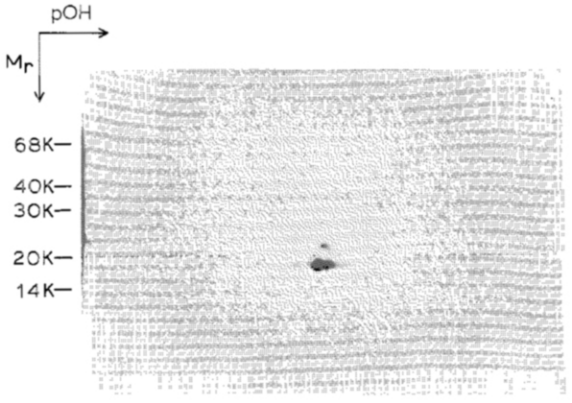

Fig. 3. Two-dimensional gel electrophoresis of the [^{3}H] spermidine labeled proteins in the 0.4 M KCl fractions eluted from the ω-aminooctyl-agarose column. A total of 200 μg proteins were applied onto the isoelectric focusing gel.

value of 5.0 in these fractions (Fig. 3). The radioactivity associated with the 22 kDa protein was less than 5% of that of the hyp-18K protein, makes it difficult to characterize the chemical nature of the labeled species on this protein. The relatively weak labeling of the 22 kDa protein also explains why this protein escaped our detection in cell homogenate in our previous study [9,11]. The 22 kDa protein, however, can be separated from the hyp-18K protein by using a Mono Q column eluted with a salt gradient (Fig. 1). The fluorogram shown in Fig. 3 also indicates that the labeled hyp-18K protein exhibited isoforms with p*I* values in the range 4.7–5.2. The cause for the differences in p*I* values is currently under investigation.

Both the purified hyp-18K protein from NB-15 cells and eIF-4D from rabbit reticulocytes exhibited identical electrophoretical mobility on two-dimensional gel (data not shown). When the spots corresponding to hyp-18K and eIF-4D on two-dimensional gel were excised and digested with V8 proteinase, a similar peptide map for these two proteins was obtained (Fig. 4), indicating that hyp-18K protein from mouse neuroblastoma cells is similar, if not identical, to rabbit reticulocyte eIF-4D.

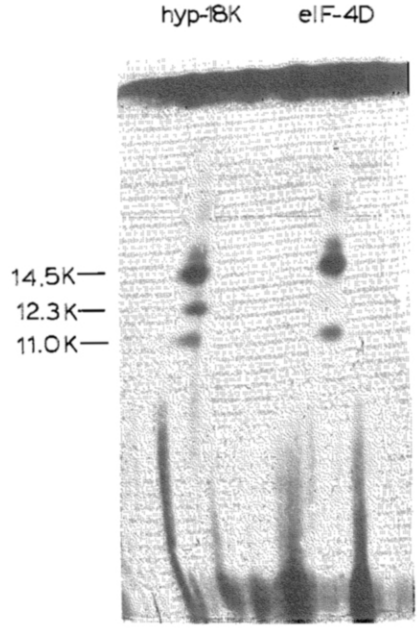

Fig. 4. V8 proteinase-digested peptide map for the hyp-18K protein and eIF-4D. The spots corresponding to the hyp-18K protein and eIF-4D were excised from a urea-SDS-polyacrylamide two-dimensional gel, treated with *Staphylococcus aureus* V8 proteinase, and then processed for analysis on a 15% SDS-polyacrylamide gel as described by Cleveland et al. [21].

Discussion

The procedure described in this paper was designed to isolate the hypusine-containing 18 kDa (hyp-18K) protein from cultured mouse neuroblastoma cells. The radioactive label on the hyp-18K protein, derived from [^{3}H]putrescine or spermidine was used as a convenient marker for monitoring the degree of purification. The four-steps procedure, including a Cibacron Blue column, an ω-aminooctyl-agarose column, a Sepharose G-50 and a Mono Q column, gave an 800-fold purification of the labeled 18 kDa protein from mouse neuroblastoma cells (Table II).

Recently, Sano [25] has purified 1500-fold the 18 kDa hypusine-containing protein from rat liver. Park et al. [26] have reported a purification of a 17000 kDa hypusine-containing protein from human red blood cells. Our study differs from theirs in two aspects: firstly, we used culture cells, which can be metabolically radiolabeled under various physiological conditions, as our starting material. This not only allowed us to study the metabolism of this protein in a more defined system, but also enabled us to detect additional proteins which are similarly modified by polyamines in vivo. This

point is emphasized by our finding of a 22 kDa protein in NB-15 neuroblastoma cells that was also labeled by [^{3}H]putrescine (Fig. 3). Secondly, our procedure included two general ligand affinity columns, a Cibacron blue and a C-8 column. The strong affinity between eIF-4D and Cibacron blue dye has also been previously noted by Van der Mast and Voorma [27]. Since our initial screening indicated that spermine, but not ATP, GTP or NAD^+, could elute hyp-18K protein quantitatively from the Cibacron blue column, we decided to use spermine as the eluent despite the fact that Cibacron blue adsorbents are known to have a high affinity for enzymes containing nucleotide or binucleotide binding sites [22,23]. It is possible that we have not fully exploited the potential of the Cibacron blue column as an affinity column. Further testing of various combinations of ligands may result in the discovery of a more specific eluent. Nevertheless, the combination of the Cibacron blue column and the C-8 column has already offered us a fast and convenient method for purifying the hyp-18K protein. Since both Cibacron blue and C-8 column chromatography can be carried out by batch-wise elution, our procedure can be easily adopted for large scale purification of the hyp-18K protein from other sources [28].

The post-translational modification of the 18 kDa protein by spermidine involves at least two enzyme systems: one catalyzes the transfer of the butylamino moiety from spermidine onto the lysine residue of the protein and the other hydroxylation [10]. We recently found that the first step of hypusine formation can be stimulated by NAD^+ [29]. Thus far, the hyp-18K protein is the only reported hypusine-containing protein modified by these enzymes [12,13]. Our finding that the 22 kDa protein co-purified with the hyp-18K protein during the first three steps and that the 22 kDa protein was also metabolically labeled, albeit faintly, raise the possibility that hypusine formation may not be limited only to the 18 kDa protein. In this regard, it is interesting to note that we recently found two proteins (18 and 20 kDa) metabolically labeled by [^{3}H]putrescine to the similar degree in chick embryo fibroblasts, and in both proteins the label was due to hypusine formation [28]. However, it should also be noted that polyamines may label proteins via transglutaminase-catalyzed post-translational modification [30–32]. Thus, the radioactivity associated with the 22 kDa protein in NB-15 cells could be due to the hypusine formation or due to glutamyl spermidine linkage or both.

Acknowledgements

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